MECHANISMS OF BACTERIAL PATHOGENICITY: ENDOTOXINS

BACTERIAL ENDOTOXINS

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxins are invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the **lipopolysaccharide** complex associated with the outer membrane of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). **Toxicity** is associated with the lipid component (**Lipid A**) and **immunogenicity** is associated with the **polysaccharide** components. The cell wall antigens (**O antigens**) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal. Because it activates complement by the alternative (properdin) pathway, it is often part of the pathology of Gram-negative bacterial infections.

The relationship of endotoxins to the bacterial cell surface is illustrated in Figure 1 below.

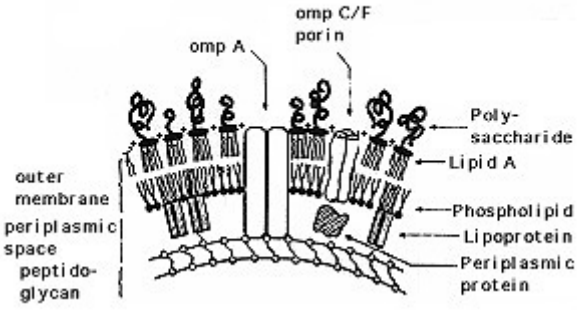

Figure 1. Structure of the cell surface of a Gram-negative bacterium

In vivo, Gram-negative bacteria probably release minute amounts of endotoxin while growing. It is known, that small amounts of endotoxin may be released in a soluble form, especially by young cultures. However, for the most part, endotoxins remain associated with the cell wall until disintegration of the bacteria. In vivo , this results from autolysis of the bacteria, external lysis mediated by complement and lysozyme, and phagocytic digestion of bacterial cells.

Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, degrade them. Endotoxins, although antigenic, cannot be converted to toxoids. A comparison of the properties of bacterial endotoxins and classic exotoxins is shown in Table 1.

Table 1. Characteristics of bacterial endotoxins and classic exotoxins

PROPERTY	ENDOTOXIN	EXOTOXIN
CHEMICAL NATURE	Lipopolysaccharide(mw = 10kDa)	Protein (mw = 50-1000kDa)
RELATIONSHIP TO CELL	Part of outer membrane	Extracellular, diffusible
DENATURED BY BOILING	No	Usually
ANTIGENIC	Yes	Yes
FORM TOXOID	No	Yes
POTENCY	Relatively low (>100ug)	Relatively high (1 ug)
SPECIFICITY	Low degree	High degree
ENZYMATIC ACTIVITY	No	Usually
PYROGENICITY	Yes	Occasionally

The Role of LPS in the Outer Membrane of Gram-negative Bacteria

The function of the outer membrane of Gram-negative bacteria is to act as permeability barrier. The outer membrane is impermeable to large molecules and hydrophobic compounds from the environment. Endotoxin (LPS) is located on the outer face of the membrane, where it mediates contact with the environment. LPS is essential to the function of the outer membrane, and as a structural component of the cell, it may play several roles in the pathogenesis of Gram-negative bacterial infections. First, it is a permeability

barrier that is permeable only to low molecular weight, hydrophilic molecules. In the *E. coli*the ompF and ompC porins exclude passage of all hydrophobic molecules and any hydrophilic molecules greater than a molecular weight of about 700 daltons. This prevents penetration of the bacteria by bile salts and other toxic molecules from the GI tract. It also a barrier to lysozyme and many antimicrobial agents. Second, it impedes destruction of the bacterial cells by serum components and phagocytic cells. Third, LPS plays an important role as a surface structure in the interaction of the pathogen with its host. For example, LPS may be involved in adherence (colonization), or resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection.

Chemical Nature of Endotoxin

Most of the work on the chemical structure of endotoxin has been done with species of *Salmonella* and *E. coli*. LPS can be extracted from whole cells by treatment with 45% phenol at 90°. Mild hydrolysis of LPS yields Lipid A plus polysaccharide.

Lipopolysaccharides are complex amphiphilic molecules with a mw of about 10kDa, that vary widely in chemical composition both between and among bacterial species The general architecture of LPS is shown in Figure 2. The general structure of *Salmonella* LPS is shown in Figure 3 and the complete structure of *Salmonella* lipid A is illustrated in Figure 4.

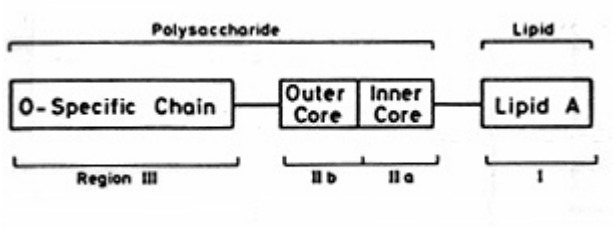

Figure 2. General architecture of Lipopolysaccharide

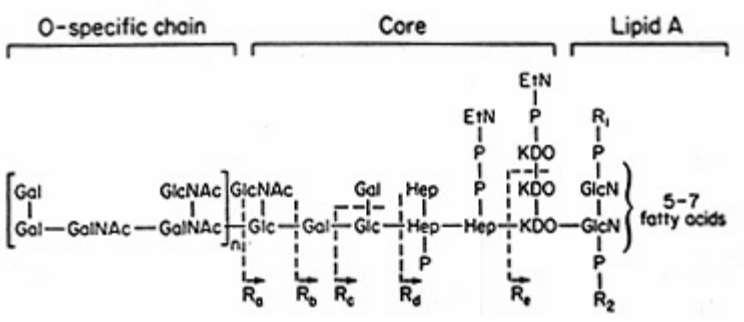

Figure 3. General Structure of *Salmonella* LPS

Glc = glucose; GlcNac = N-acetyl- glucosamine; Gal = galactose; Hep = heptose; P = phosphate; Etn = ethanolamine; R1 and R2 = phoshoethanolamine or aminoarabinose. Ra to Re indicate incomplete forms of LPS. The Rd2 phenotype (not shown) would have only a single heptose unit. The Rc, Rd2, and Rd1 mutants lack the phosphate group attached to Hep.

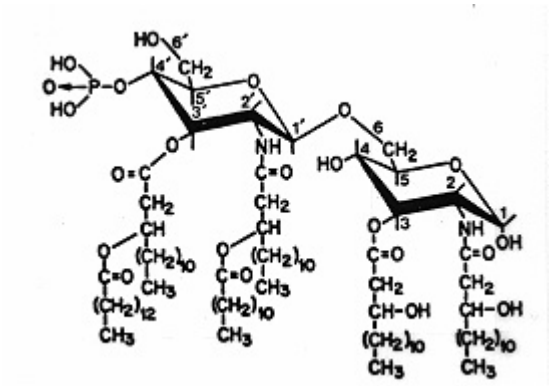

Figure 4. Complete structure of the Lipid A Moiety of LPS of *S. typhimurium*, *S. minnesota*, and *E. coli*

LPS consists of three components or regions**, Lipid A**, an **R polysaccharide** and an **O polysaccharide**.

Region I. Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among *Enterobacteriaceae* Lipid A is virtually constant.

Region II. Core (R) antigen or R polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO - Hep - Hep - Glu - Gal - Glu - GluNAc -

Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin).

With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. *Salmonella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

Region III. Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3 - 5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide.

Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses, which occur in nature only in Gram-negative cell walls. Variations in sugar content of the O polysaccharide contribute to the wide variety of antigenic types of *Salmonella* and *E. coli* and presumably other strains of Gram-negative species. Particular sugars in the structure, especially the terminal ones, confer immunological specificity of the O antigen, in addition to "smoothness" (colony morphology) of the strain. Loss of the O specific region by mutation results in the strain becoming a "rough" (colony morphology) or R strain.

The elucidation of the structure of LPS (Figure 3) relied heavily on the availability of mutants each blocked at a particular step in LPS synthesis. The biosynthesis of LPS is strictly sequential. The core sugars are added sequentially to Lipid A by successive additions, and the O side chain is added last, one preassembled subunit at a time. The properties of mutants producing incomplete LPS molecules suggests the nature and biological functions performed by various parts of the LPS molecule.

Loss of the O antigen results in loss of virulence suggesting that this portion is important during a host-parasite interaction. It is known that such "rough" mutants are more susceptible to phagocytosis and serum bactericidal reactions.

Loss of the more proximal parts of the core, as in "deep rough" mutants (i.e. in Rd1, Rd2, and Re mutants in Figure 3) makes the strains sensitive to a range of hydrophobic compounds, including antibiotics, detergents, bile salts and mutagens. This area contains a large number of charged groups and is thought to be important in maintaining the permeability properties of the outer membrane.

Mutants in the assembly of Lipid A cannot be isolated except as conditional lethal mutants and this region must therefore be essential for cell viability. The innermost region of LPS, consisting of Lipid A and three residues of KDO,

appears to be essential for viability, presumably for assembling the outer membrane.

LPS and virulence of Gram-negative bacteria

Both Lipid A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria.

The O polysaccharide and virulence

Virulence and the property of "smoothness", associated with an intact **O polysaccharide**, are regularly associated in many bacterial infections. The involvement of the polysaccharide chain in virulence is shown by the fact that small changes in the sugar sequences in the side chains of LPS result in major changes in virulence. How are the polysaccharide side chains involved in the expression of virulence? There are a number of possibilities:

1. Smooth antigens could allow organisms to **adhere** specifically to certain tissues, especially epithelial tissues.

2. Smooth antigens probably allow **resistance to phagocytes**, since rough mutants are more readily engulfed and destroyed by phagocytes.

3. The hydrophilic O polysaccharides could act as water-solubilizing **carriers for toxic Lipid A**. It is known that the exact structure of the polysaccharide can greatly influence water binding capacity at the cell surface.

4. The O antigens could provide **protection from damaging reactions with antibody and complement**. Rough strains of Gram-negative bacteria derived from virulent strains are generally non virulent. Smooth strains have polysaccharide "whiskers" which bear O antigens projecting from the cell surface. The O antigens are the key targets for the action of host antibody and complement, but when the reaction takes place at the tips of the polysaccharide chains, a significant distance external to the general bacterial cell surface, complement fails to have its normal lytic effect. Such bacteria are virulent because of this resistance to immune forces of the host. If the projecting polysaccharide chains are shortened or removed, antibody reacts with antigens on the general bacterial surface, or very close to it, and complement can lyse the bacteria. This contributes to the loss of virulence in "rough" colonial strains.

5. The O-polysaccharide or **O antigen** is the basis of **antigenic variation** among many important Gram-negative pathogens including *E. coli, Salmonella* and *Vibrio cholerae*. Antigenic variation guarantees the existence of multiple serotypes of the bacterium, so that it is afforded multiple opportunities to infect its host if it can bypass the immune response against a different serotype. Furthermore, even though the O polysaccharides are

strong antigens, they seldom elicit immune responses which give full protection to the host against secondary challenge with specific endotoxin.

Lipid A and virulence

Endotoxins are toxic to most mammals, and regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host. Most of our knowledge of the biological activities of endotoxins derives not from the study of natural disease but by challenge of experimental animals.

The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: **fever**, **changes in white blood cell counts**, **disseminated intravascular coagulation**, **hypotension**, **shock** and **death**. Injection of fairly small doses of endotoxin results in death in most mammals. The sequence of events follows a regular pattern: (1) latent period; (2) physiological distress (diarrhea, prostration, shock); (3) death. How soon death occurs varies on the dose of the endotoxin, route of administration, and species of animal. Animals vary in their susceptibility to endotoxin.

The physiological effects of endotoxin are thought to be mediated by **Lipid A**. Since Lipid A is embedded in the outer membrane of bacterial cells, it probably only exerts its toxic effects when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex (MAC), ingestion and killing by phagocytes, or killing with certain types of antibiotics.

It is thought that LPS released into the bloodstream by lysing Gram-negative bacteria is first bound by certain plasma proteins identified as **LPS-binding proteins**. The LPS-binding protein complex interacts with CD14 receptors on monocytes and macrophages and other types of receptors on endothelial cells. In **monocytes and macrophages** three types of events are triggered during their interaction with LPS:

1. **Production of cytokines**, including IL-1, IL-6, IL-8, tumor necrosis factor (TNF) and platelet-activating factor. These in turn stimulate production of prostaglandins and leukotrienes. These are powerful mediators of inflammation and septic shock that accompanies endotoxin toxemia. LPS activates macrophages to enhanced phagocytosis and cytotoxicity. Macrophages are stimulated to produce and release lysosomal enzymes, IL-1 ("endogenous pyrogen"), and tumor necrosis factor (TNFalpha), as well as other cytokines and mediators.

2. **Activation of the complement cascade**. C3a and C5a cause histamine release (leading to vasodilation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.

3. **Activation of the coagulation cascade**. Initial activation of Hageman factor (blood-clotting Factor XII) can activate several humoral systems resulting in

a. coagulation: a blood clotting cascade that leads to coagulation, thrombosis, acute disseminated intravascular coagulation, which depletes platelets and various clotting factors resulting in internal bleeding.

b. activation of the complement alternative pathway (as above, which leads to inflammation)

c. plasmin activation which leads to fibrinolysis and hemorrhaging.

d. kinin activation releases bradykinins and other vasoactive peptides which causes hypotension.

The net effect is to induce inflammation, intravascular coagulation, hemorrhage and shock.

LPS also acts as a **B cell mitogen** stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM.

The physiological activities of LPS are mediated mainly by the Lipid A component of LPS. Lipid A is a powerful biological response modifier that can stimulate the mammalian immune system. During infectious disease caused by Gram-negative bacteria, endotoxins released from, or part of, multiplying cells have similar effects on animals and significantly contribute to the symptoms and pathology of the disease encountered.

The primary structure of Lipid A has been elucidated and Lipid A has been chemically synthesized. Its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO_4 groups, the acyl chains, and also the KDO-containing inner core.

ENDOTOXIN INACTIVATION.

Endotoxin inactivation was found to be proportional to UV fluence between 100 and 600 mJ/cm^2. Figure 3 shows the results of two experiments with a regression line and equation for each experiment. The linear regression equations show that endotoxin from *E. coli* strain O55:B5 is removed at a rate of approximately 0.55 (EU/ml)/(mJ/cm^2) of medium-pressure UV fluence. UV disinfection treatment of drinking water will likely vary from 40 to 100 mJ/cm^2. Therefore, in practical terms, an inactivation rate of 0.55 (EU/ml)/(mJ/cm^2) has the potential to completely remove or substantially reduce endotoxin levels (Table 1) when the initial concentration is in the lower end of the range (1 to 50 EU/ml) typically found in untreated water. Removal ranging from 11 to 55% can be expected in the range from 50 to 200 EU/ml. An OH^- scavenger (2-methylpropan-2-ol) was added in one series of tests to confirm that hydrogen peroxide-mediated reactions were not affecting the inactivation rate, and it was found that they were not (data not shown). This research suggests that typical endotoxin concentrations in drinking water could be effectively completely inactivated by applying UV fluences of up to 500 mJ/cm^2 (assuming that all endotoxins are inactivated equally). This previously unreported finding is significant because of the rapidly increasing use of UV for drinking water disinfection, although the economic feasibility of such high doses for a given plant would need to be considered. To provide a greater quantitative database, this research should be repeated for endotoxin isolated from other strains of bacteria and/or cyanobacteria and for different water types (with different transmittance and/or turbidity values). Additional confirmation could be obtained by evaluating some full-scale drinking water treatment plant units when they become accessible for testing. The present work may also have implications with regard to dialysis issues (e.g., point-of-use applications) and the potential for advances in the treatment of liquids and equipment for routine medical applications involving both healthy and immunosuppressed patients.

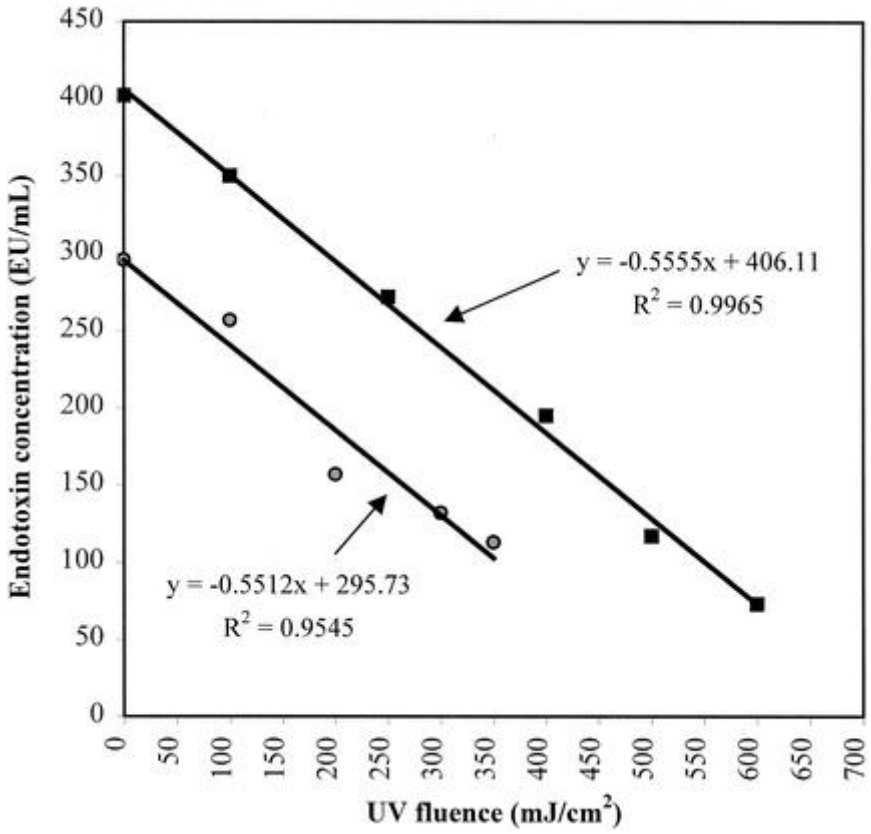

FIG. 3.

UV fluence versus endotoxin remaining in spiked deionized water samples. Results from two separate experiments with differing initial endotoxin concentrations (multiply the millijoule per square centimeter value by 10 to convert to joules per square meter).

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