Bacterial Toxigenesis

Food Toxicology

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Learning Objectives

- Define bacterial toxigenesis.
- Explore bacterial toxins, their background and nomenclature.
- Differentiate exotoxins and endotoxins.
- Explore the toxicity, properties, and mode of action of exotoxins.
- Understand the toxicity, properties, and mode of action of endotoxins (pyrogens).
- Understand the origins of sepsis.
- Review the detection of endotoxins.

Bacterial Toxigenesis

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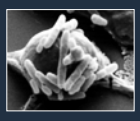
- The ability to produce toxins: a mechanism of bacterial disease.
- Cell-associated lipopolysaccharide (LPS) toxins are referred to as endotoxins.
- Extracellular diffusible toxins are referred to as exotoxins.



Escherichia coli (Gram - bacteria)

Micrococcus luteus (Gram + bacteria)

Bacterial Toxins

- '[Substances] that are toxic to eukaryotic cells as measured in a variety of ways', Orndorff 1992
- 'A microbial substance able to induce host damage', Madigan *et al*. 1997
- 'Any organic microbial product or substance that is harmful or lethal to cells, tissue cultures, or organisms', Atlas 1995

Bacterial Toxins

- '[Toxins are] a common and series cause of tissue damage, especially in bacterial infection', Mims *et al*. 1993;
- 'Disease is frequently determined by production of microbial toxins', Murray *et al*. 1994
- 'Bacterial [toxins] are thus important determinants of bacterial virulence' Poxton and Arbuthnott 1990

Bacterial Toxins

- 'Microbial toxins are components or products of microorganisms which, when extracted and introduced into host animals, can reproduce disease symptoms normally associated with infection without infestation by those microorganisms', Williams and Clarke, 1998

Bacterial Toxins

- Terms
  - Exotoxin = extracellular protein toxin
  - Endotoxin = lipid A portion of Gram-neg outer membrane
  - Enterotoxin = toxin that acts on gastrointestinal tract, producing typical food poisoning symptoms
- Nomenclature
  - Named for host cell attacked: cytotoxin, neurotoxin
  - Named for producer or disease: cholera, Shiga
  - Named for activity: lecithinase, adenylate cyclase
  - Letter designation: exotoxin A

Endotoxins and Exotoxins

- Endotoxins are cell-associated substances that are structural components of the outer membrane of Gram-negative bacteria.
  - Released from growing bacterial cells
  - Released from cells which are lysed from effective host defense (e.g. lysozyme)
  - Released from activities of certain antibiotics (e.g. penicillins)
- Exotoxins usually secreted by bacteria but in some cases they are released by lysis of the bacterial cell.

Exotoxins

- Most well-characterized family of toxins.
- Secreted by bacterium as soluble proteins.
- Enter eukaryotic cells primarily through receptor-mediated endocytosis.
- Exotoxin-mediated infections:
  - Involve local colonization of host
  - Exotoxin-mediated systemic disease, which often occurs distal to the site of infection.

Types of Exotoxins

1. Membrane-acting toxins
2. Toxins with cytosolic targets

Mode of Action

- Damage membranes
  - Forms pores
- Inhibit protein synthesis
  - N-glycosidase
- Activate 2nd messenger pathways
  - ADP-ribosyltransferase
- Activate immune response
  - Superantigen
- Protease
  - Zinc-metalloprotease

Exotoxins: Bacterial Protein Toxins

- Exotoxins are typically soluble proteins secreted by living bacteria.
  - Both Gram-positive and Gram-negative bacteria produce soluble protein toxins.
- A specific toxin is generally specific to a particular bacterial species
  - e.g. only *Clostridium tetani* produces tetanus toxin;
  - Only *Corynebacterium diphtheriae* produces the diphtheria toxin.

Exotoxins: Virulence

- Usually, virulent strains of the bacterium produce the toxin while non-virulent strains do not
  - The toxin is the major determinant of virulence.

Exotoxin Lethal Toxicity Comparison

Toxin	Toxic Dose (mg)	Host	Strychnine Ratio	Endotoxin Ratio	Snake Venom Ratio
Botulism	0.8×10^{-8}	Mouse	**3×10^{6}**	**3×10^{7}**	**3×10^{5}**
Tetanus	4×10^{-8}	Mouse	**1×10^{6}**	**1×10^{7}**	**1×10^{5}**
Shigella Neurotox.	2.3×10^{-6}	Rabbit	**1×10^{6}**	**1×10^{7}**	**1×10^{5}**
Diphtheria	6×10^{-5}	Guinea Pig	**2×10^{3}**	**2×10^{4}**	**2×10^{2}**

Bacterial protein toxins are the most powerful human poisons known and retain high activity at very high dilutions.

Protein Toxins: Resemble Enzymes

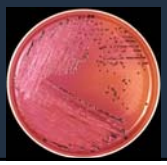
- Denatured by heat, acid, and proteolytic enzymes
- High biological activity
- Most act catalytically
- Highly specific in the substrate utilized
  - Tissue cells, organs, or body fluid
- Highly specific mode of action.
- Site of damage caused by the toxin indicates the location of the substrate.
  - Enterotoxin, neurotoxin, leukocidin, or hemolysin.

Protein Toxins: Cytotoxic Activity

- Certain protein toxins have very specific cytotoxic activity
  - Attack specific types of cells.
  - e.g. tetanus or botulinum toxins attack only neurons.
- Some toxins have fairly broad cytotoxic activity and cause nonspecific death of all sorts of cells and tissues, eventually resulting in necrosis.
  - Staphylococci, streptococci, clostridia, etc.
- Some broadly lethal but with unknown specifics
  - e.g. anthrax toxin LF.

Protein Toxins: Strongly Antigenic

- *In vivo* antibody (antitoxin) neutralizes the toxicity of bacterial proteins.
- Protein toxins are inherently unstable
  - In time they lose their toxic properties but retain their antigenic properties.
- Toxoids are detoxified toxins which retain their antigenicity and their immunizing capacity.



Protein Toxins: Toxoids

- The formation of toxoids can be accelerated by treating toxins with a variety of reagents
  - Formalin, iodine, pepsin, ascorbic acid, ketones, etc.
  - 37ºC, pH 6-9 for several weeks.
- Resulting toxoids can be use for artificial immunization where the primary determinant of bacterial virulence is toxin production.
  - e.g. diphtheria and tetanus.
- Toxoids can be genetically engineered.

Gram-Negative Bacteria: Endotoxins

- An important part of the toxicity of these organisms is conferred through the release of endotoxins, as occurs in septicemia, toxic shock syndrome, and sometimes in food poisoning.
- Endotoxins = lipopolysaccharides.
  - A part of cell membrane envelopes.
- Killed bacteria can release endotoxins as they decay.
  - *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other pathogens.

Endotoxins (Pyrogens)

- Cause a wide variety of serious reactions such as fever, shock, changes in blood pressure, and in other circulatory functions.

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Endotoxins

- Endotoxins are toxic to most mammals
- 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.

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Endotoxins

- The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions.
- Includes: fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death.

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Endotoxins

- 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.

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Exotoxins and Endotoxins

- 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 min does not destabilize endotoxin)
  - Certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, have been reported to neutralize them.
- Endotoxins, although antigenic, cannot be converted to toxoids.

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Characteristics of Endotoxins/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	Weakly	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

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Role of Toxins in Foodborne Disease

- Consumed as pre-formed → self-limiting
- Produced by colonized bacteria → local or distal effect
- Produced by infecting bacteria to aid invasion
- Autoimmune response to superantigens
- Endotoxin elicits immune response → shock

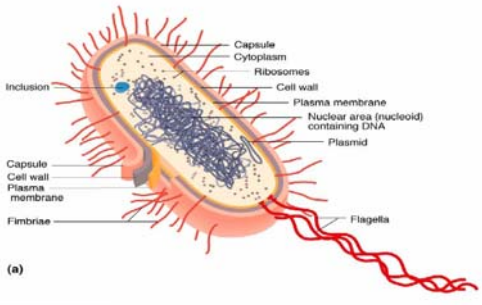
Organisms Recognized for Foodborne Intoxications

- *Staphylococcus aureus*
  - Enterotoxins
- *Clostridium botulinum*
  - Neurotoxin
- *Clostridium perfringens*
  - Enterotoxin, other toxins
- *Bacillus cereus*
  - Diarrheagenic and emetic toxins
- *Vibrio cholerae*
  - Cholera toxin
- enterotoxigenic *E. coli*
  - Heat-stable & heat-labile toxins

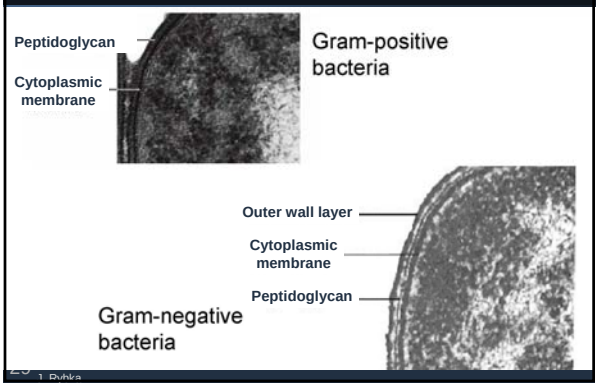
Agents of Foodborne Infections That Also Produce Toxins

- Shigella sp.
  - Shiga toxin
- *Listeria monocytogenes*
  - Listeriolysin
- Salmonella sp.
  - Enterotoxin, cytotoxin
- enterohemorrhagic E. coli
  - Shiga-like toxin
- *Vibrio parahaemolyticus*
  - Hemolysin
- *Yersinia enterocolitica*
  - Enterotoxin
- *Campylobacter jejuni*
  - Enterotoxin
- *Aeromonas hydrophila*
  - Multifunction toxin
- *Plesiomonas shigelloides*
  - Enterotoxin

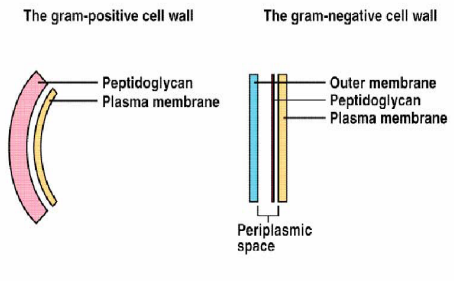
Bacterial Cell Structures



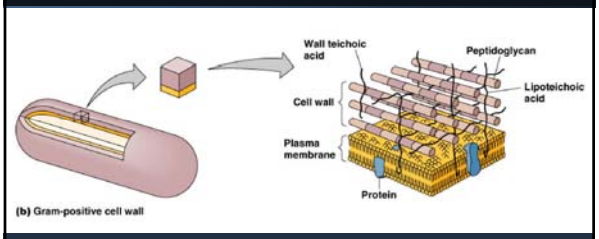
Bacterial Cell Wall



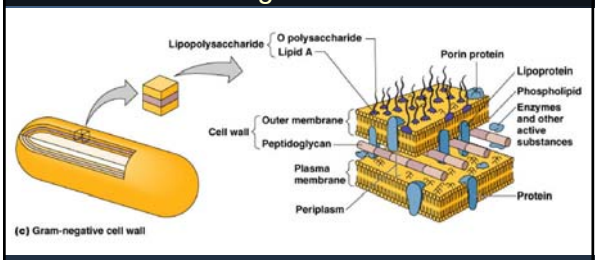
Bacterial Cell Wall



Gram-Positive Cell Wall



Gram-Negative Cell Wall



Lipoplysaccharide (LPS) GNB

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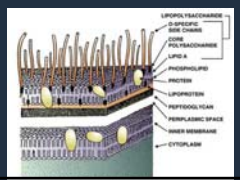
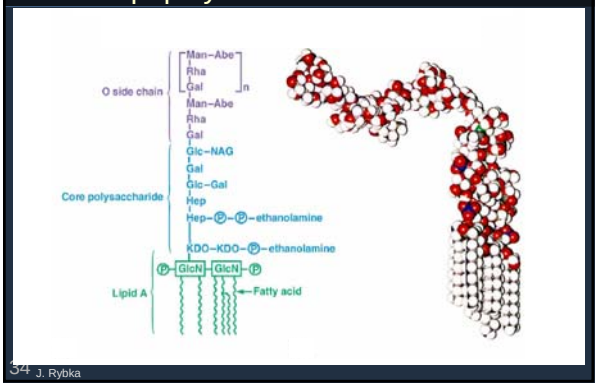
- Major constituent of outer surface of the outer membrane
- Covers ~75% of the OM
- Gives colonies a smooth appearance
- ~3-10% of the total dry cell weight
- 3-4 million LPS molecules per cell



Lipopolysaccharide Structure



LPS Lipid A

- Lipid A is a powerful biological response modifier that can stimulate the mammalian immune system.
  - During infectious disease caused by Gram - 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.

Lipopolysaccharide Lipid A

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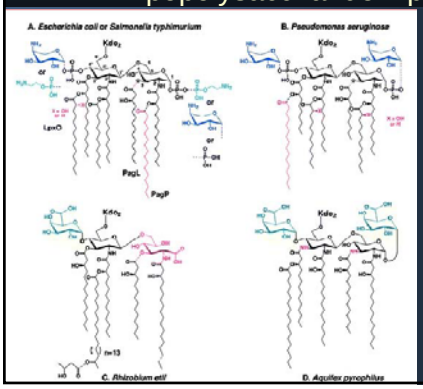


LPS Mode of Action

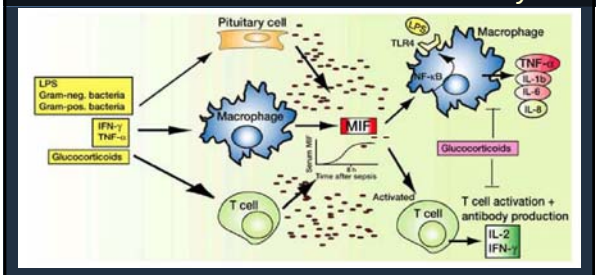
- Bound by plasma proteins LPS-binding proteins (LBP).
- LBP interacts with receptors on monocytes and macrophages and other types of receptors on endothelial cells.
- In monocytes and macrophages three types of events are triggered during their interaction with LPS:

1) Production of cytokines, (IL, TNF, etc.) which stimulate production of prostaglandins and leukotrienes (powerful mediators of inflammation and septic shock).
2) Activation of the complement cascade. (cause histamine release leading to vasodilation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.
3) Activation of the coagulation cascade:
   - a. Coagulation → fibrinolysis and hemorrhaging → blood clotting cascade → bradykinins and other vasoactive peptides → coagulation, thrombosis → acute disseminated intravascular coagulation → depleted platelets and clotting factors → internal bleeding.
   - b. Inflammation
   - c. hypotension.

Inflammation → Sepsis

- The LPS net effect is to induce inflammation, intravascular coagulation, hemorrhage, and shock.
- LPS also acts as a immune system B cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins (e.g. IgG).

Infection/Intoxication: Inflammation Cycle



- Macrophage migration inhibitory factor (MIF) is a pro-inflammatory pituitary and immune cell cytokine and a critical mediator of septic shock.

Inflammation Cycle: Toll Receptor

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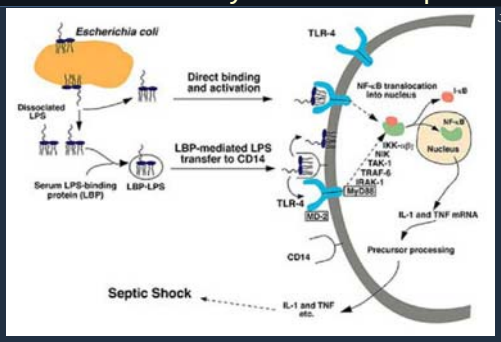
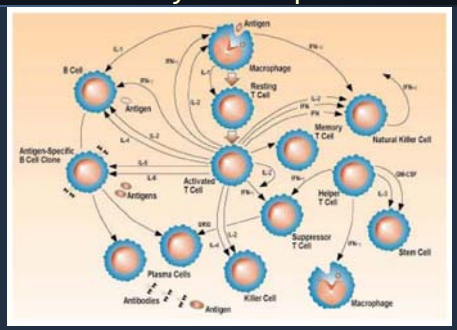


- Activation of the toll receptors causes the release of antimicrobial peptides (TNF) and inflammatory cytokines (IL)

Inflammation Cycle: Adaptive Immunity

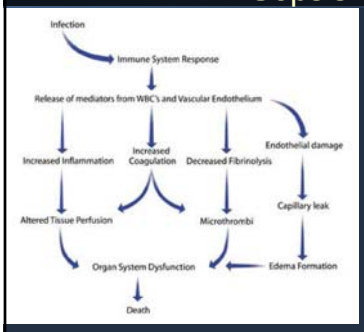


- Cell-mediated immunity and humoral immunity.

Immunological Response

- Amounts of endotoxin which trigger immunological response are very low.
- They range from pg/ml in humans to ng/ml in rats.

Sepsis



Facts About Severe Sepsis

- US ~ 1 million cases per year
- Mortality rates range from 28% to 50% or more
  - Sepsis kills 215,000 people/year in the US (10th rank)
- $17B/year health costs

Detection of Endotoxins (Pyrogens)

- Pharmaceutical industry:
  - Intravenous and parenteral drugs, medical devices
- Biomedical and pharmaceutical industry:
  - Tracking the bacterial content during processing
- Environmental monitoring:
  - Indoor and outdoor detection of air, water or dust contamination
- Medicine:
  - Detection of Gram-negative bacterial infection, diagnosis of sepsis

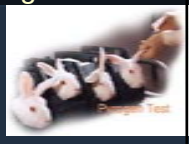
Pyrogen Limits: Pharmacological Products

- Amoxicillinum natricum 0.25EU/mg
- Clindamycini hydrochloridium 0.58EU/mg
- Water for intravenous infusion 0.25EU/ml
- Therapeutic devices for cerebrospinal contact 0.06EU/ml
  - 1EU = 0.2 ng LPS

Detection of Endotoxin

- Biological tests:
  - Rabbit Pyrogen test**
  - Limulus Amebocyte Lysate test**
  - Neutrophil Chemiluminescence test
- Non-biological endotoxin detection:
  - Chemical markers (3-OH fatty acids, Kdo)
  - Detection by molecules specifically recognizing LPS

Detection of Endotoxin – Biological Tests



- For most of the 20th Century, the Rabbit Pyrogen Test was the standard method of testing for pyrogenicity. This test, which took approximately 4 hrs, is accomplished by injecting the substance being analyzed into a rabbit's ear.
- If the animal developed a fever, it confirmed the presence of pyrogens.

Detection of Endotoxin – Biological Tests

- **L**imulus **A**mebocyte **L**ysate test (LAL)

- The Atlantic horseshoe crab *Limulus polyphemus*

Detection of Endotoxin – Biological Tests

- The LAL Test was commercially introduced during the 1970s.
  - In 1977, the FDA described conditions for the use of LAL as an end-product test for endotoxin in human biological products and medical devices.
- To obtain the lysate required for the LAL test, a small amount of horseshoe crabs' blood is drawn.
- Next, blood cells (amebocytes) are separated and lysed to obtain the cellular proteins.

Limulus Amebocyte Lysate test (LAL)

- Gel clot LAL provides a simple +/- result
- Chromogenic end-point LAL offers a quantitative result and exhibits less product interference than LAL methods utilizing the clotting protein.
- Kinetic turbidimetric LAL gives quantitative results but its use of the clotting protein limits its sample compatibility.
- Kinetic chromogenic LAL provides automation and greater sensitivity to 0.005 EU/ml (1pg of LPS)

Limits of Rabbit Pyrogen Test and LAL

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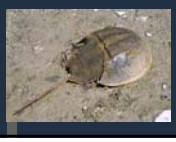
- Cannot be used for:
  - Diagnostic testing of blood and other body fluid for endotoxin content
  - Testing of concentrated salts solutions
  - Testing of chemicals
  - Solutions of various proteins
- Non-biological endotoxin detection used.



Endotoxin Levels in Ground Beef (LAL)

Log Anaerobic Plate Count (APC)	Mean Endotoxin (ng/g)
<5.50	51
5.50-5.99	100
6.00-6.49	1,100
6.50-7.00	5,100
>7.00	7,500

Applied and Environmental Microbiology (1979) 38:5, 885-890