DETECTION OF ENDOTOXINS FROM GRAM-NEGATIVE BACTERIA

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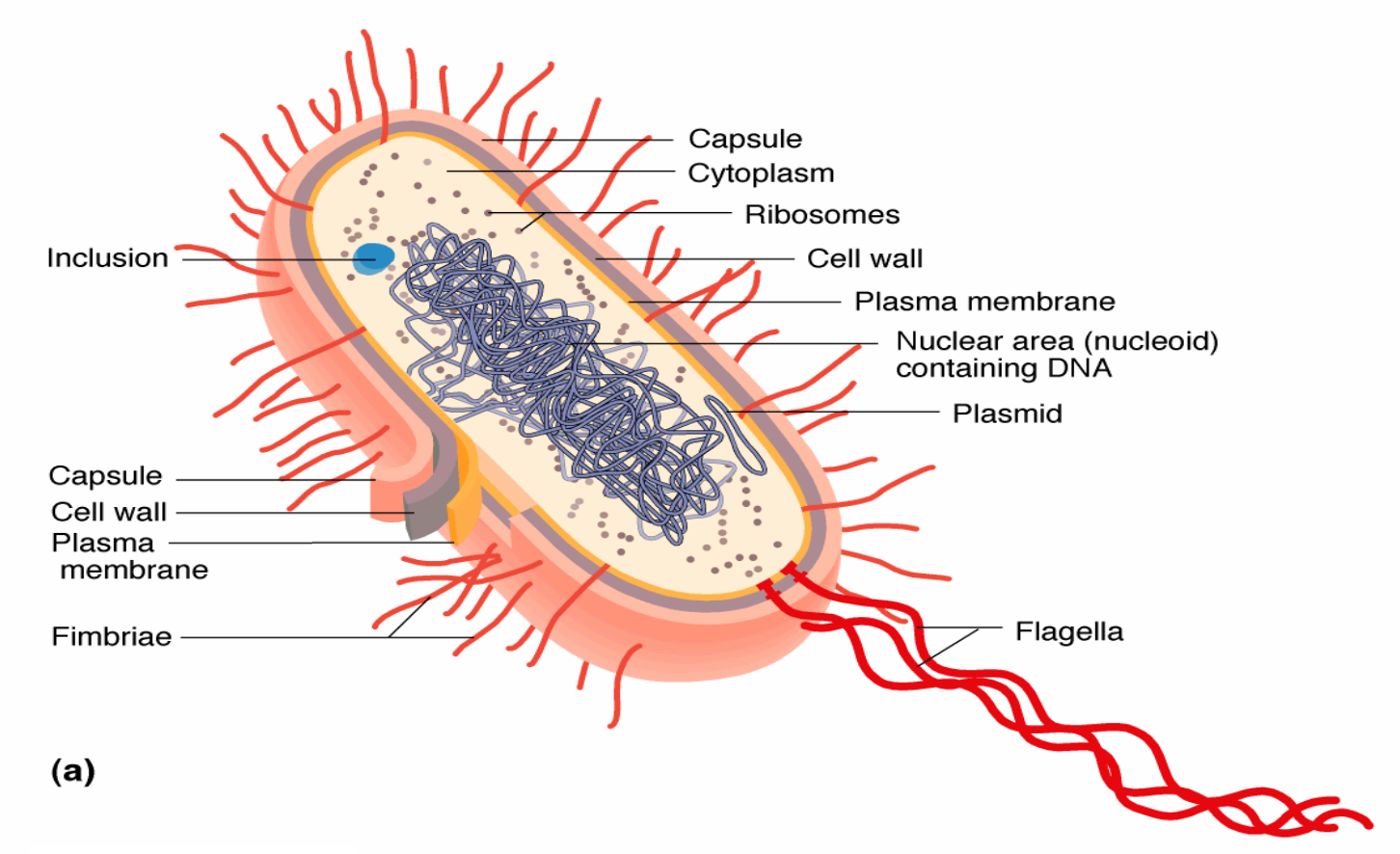
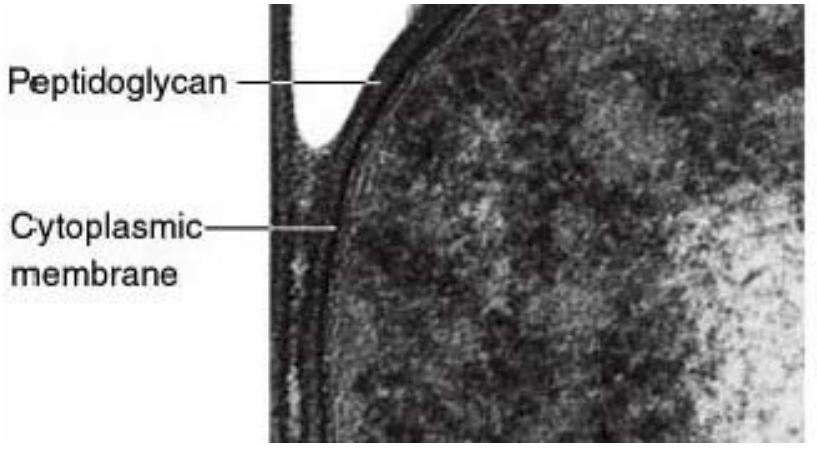
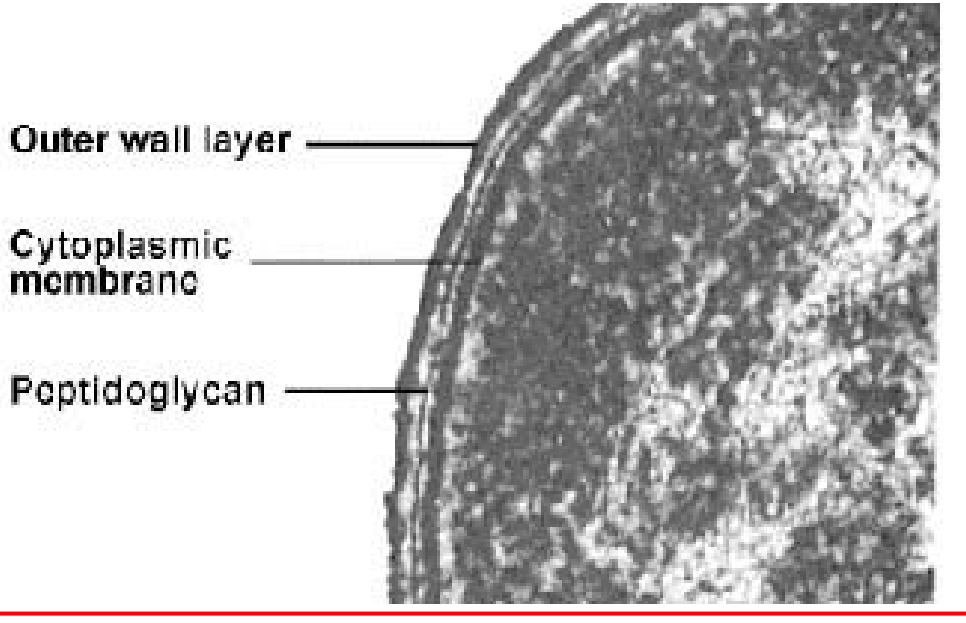
Bacterial Cell Structures



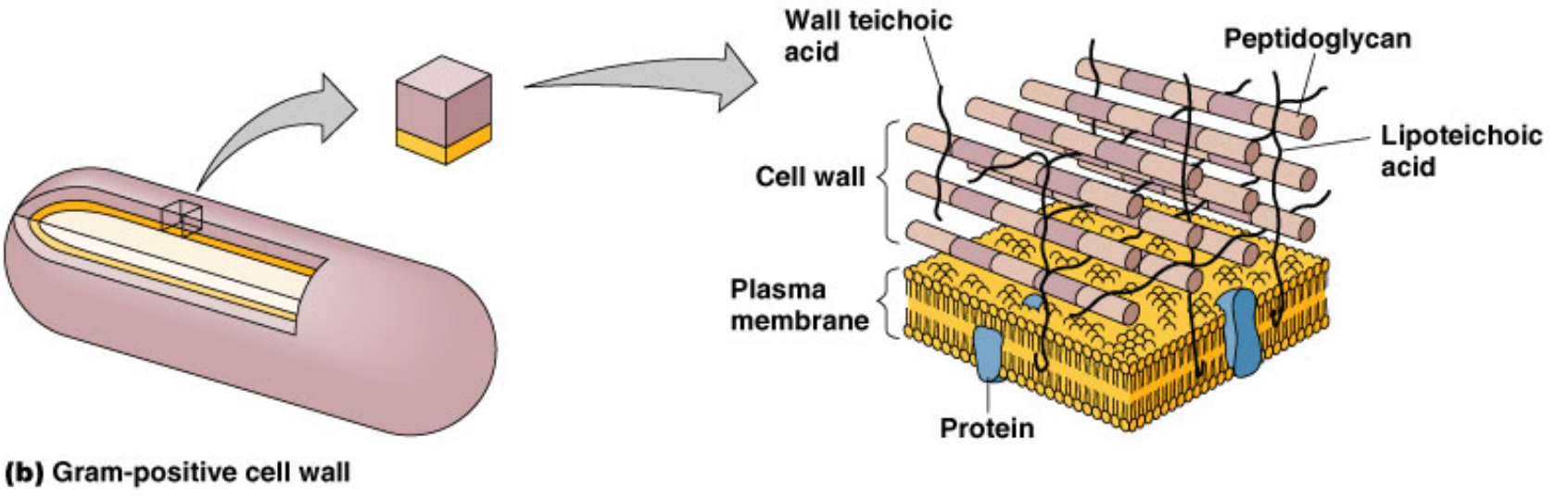


Gram-positive bacteria



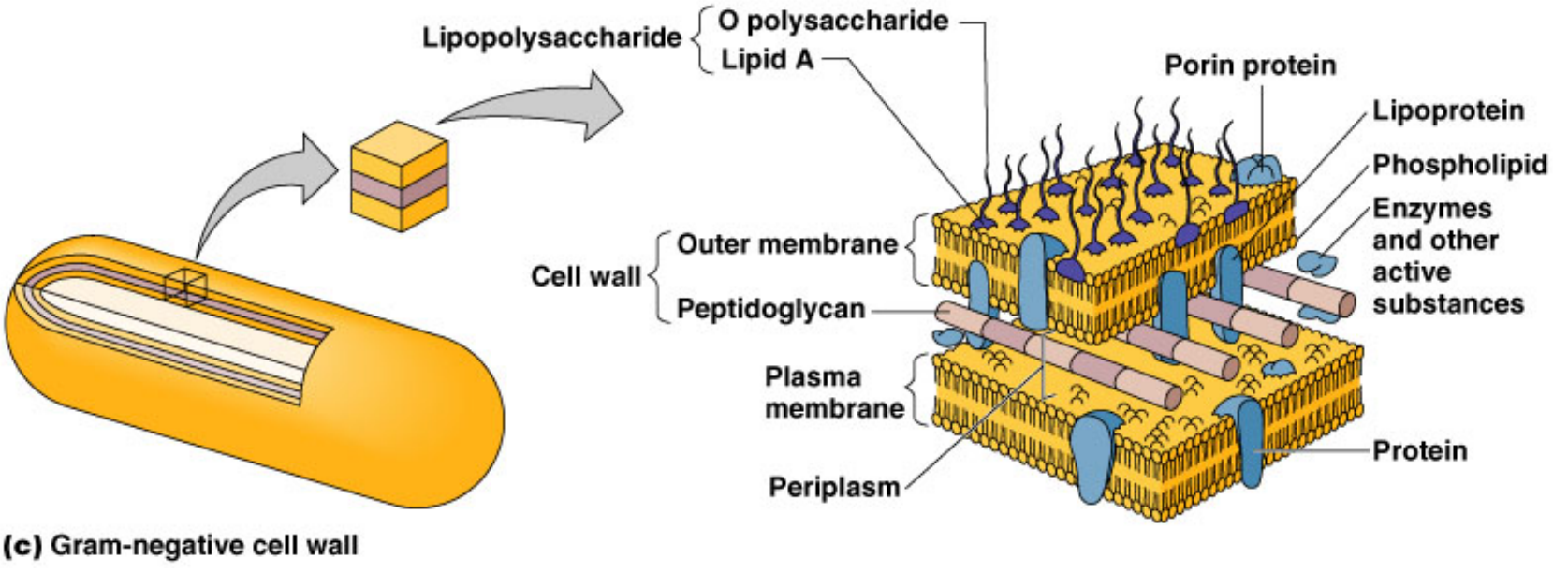
Gram-negative bacteria

Endotoxin - Lipopolysaccharide

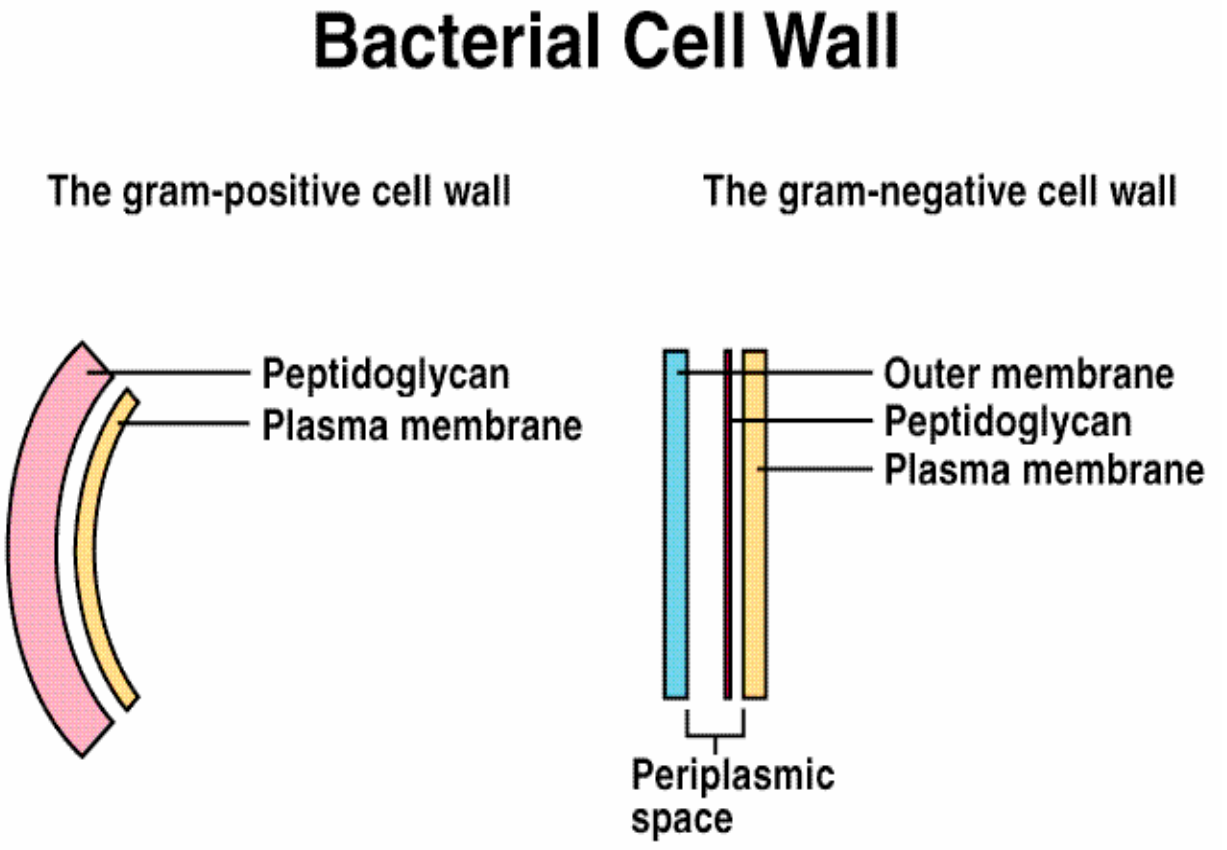


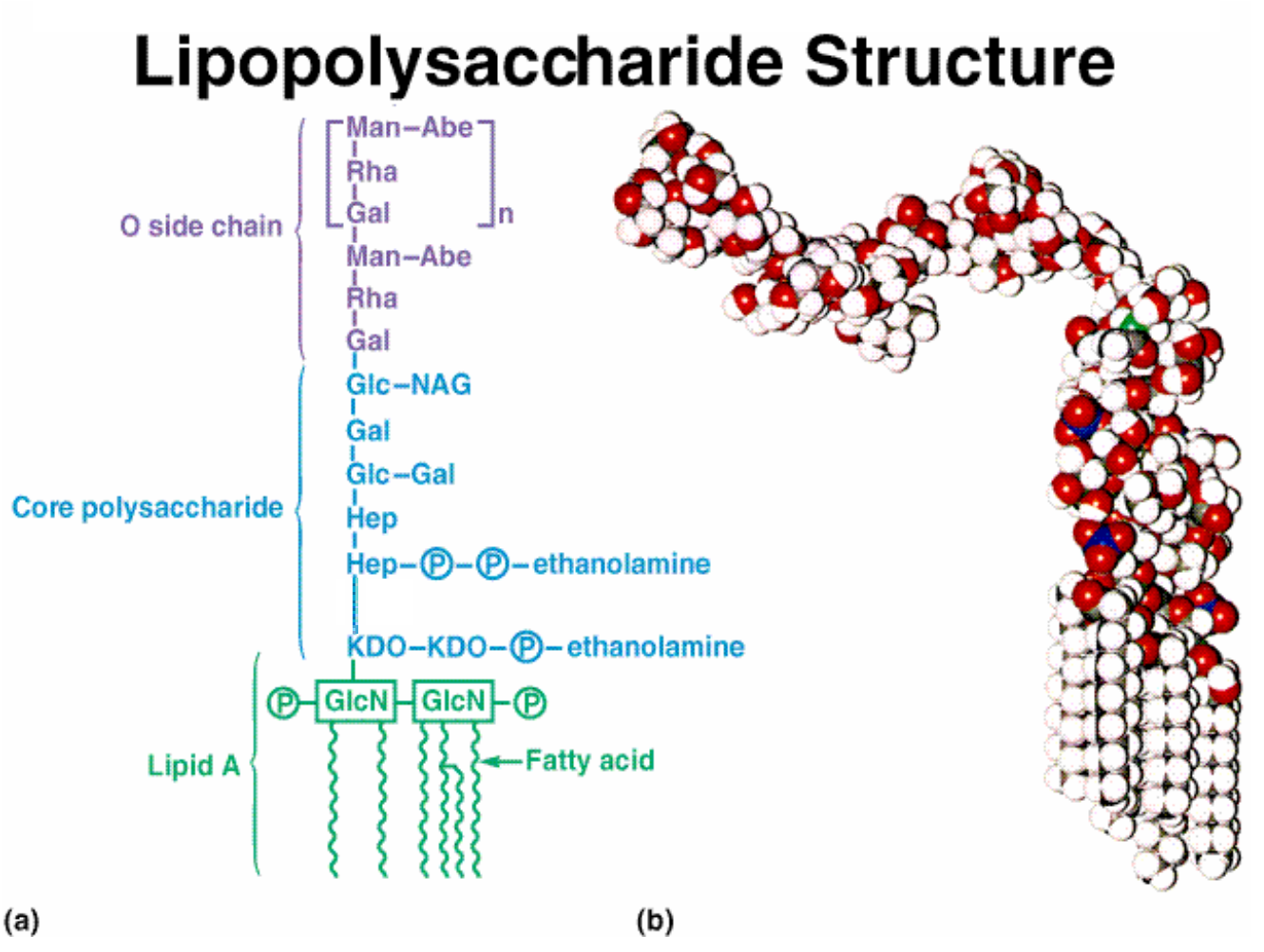
(b) Gram-positive cell wall

Endotoxin - Lipopolysaccharide

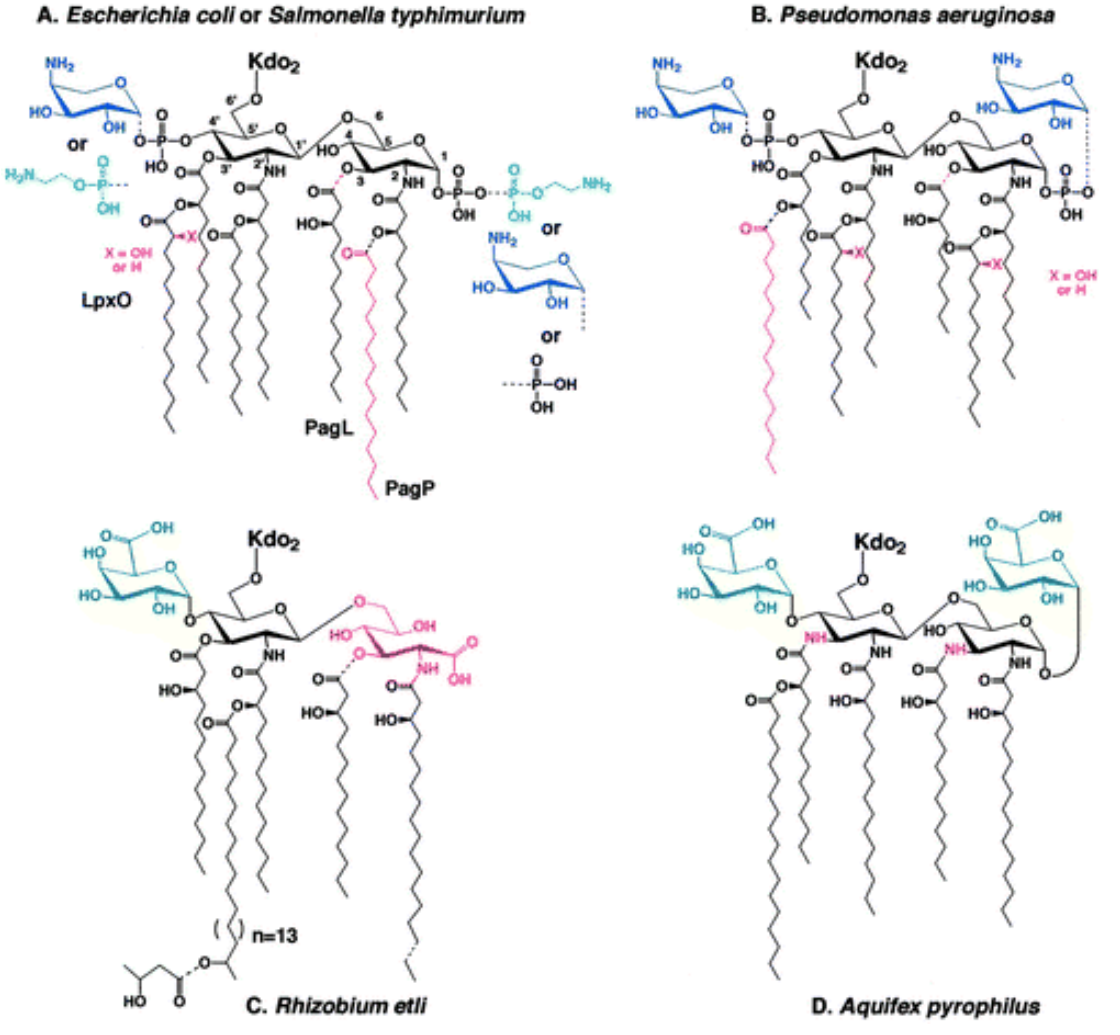


(c) Gram-negative cell wall

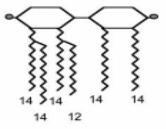
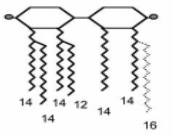
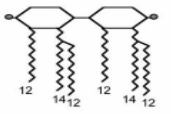
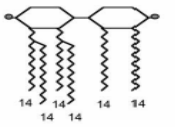
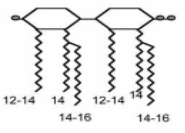
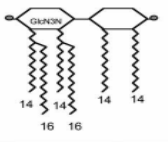
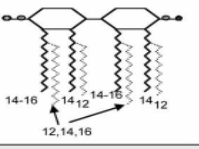
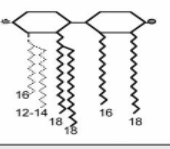
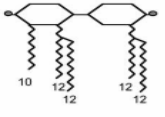
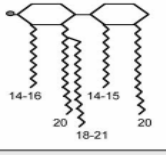
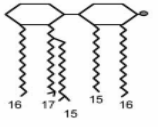
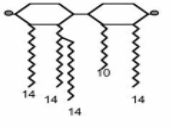
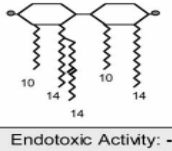
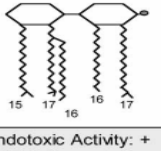
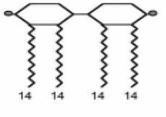
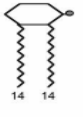

Bacterial Cell Wall
The gram-positive cell wall
The gram-negative cell wall
Peptidoglycan
Plasma membrane
Outer membrane
Peptidoglycan
Plasma membrane
Periplasmic space


Lipopolysaccharide Structure
Man–Abe
Rha
Gal
n
O side chain
Man–Abe
Rha
Gal
Glc–NAG
Gal
Glc–Gal
Hep
Core polysaccharide
Hep–Ⓟ–Ⓟ–ethanolamine
KDO–KDO–Ⓟ–ethanolamine
Ⓟ
GlcN
GlcN
Ⓟ
Lipid A
Fatty acid
(a)
(b)

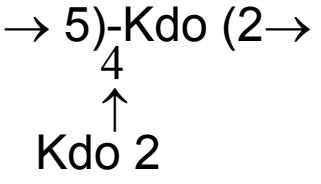
Endotoxin - Lipopolysaccharide



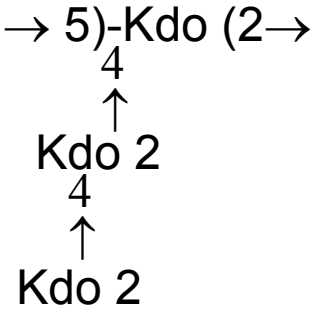
Endotoxin - Lipopolysaccharide

E.coli lipid A 	*S.minnesota* lipid A 	*N.meningitidis* lipid A 	*H.influenzae* lipid A 
Endotoxic Activity: **+++**	Endotoxic Activity: **+++**	Endotoxic Activity: **+++**	Endotoxic Activity: **+++**
K.pneumoniae lipid A 	*C.jejuni* lipid A 	*Y.pestis* lipid A 	*H.pylori* lipid A 
Endotoxic Activity: **+++**	Endotoxic Activity: **++**	Endotoxic Activity: **++(?)**	Endotoxic Activity: **++**
P.aeruginosa lipid A 	*C.trachomatis* lipid A 	*B.fragilis* lipid A 	*B.pertussis* lipid A 
Endotoxic Activity: **+**	Endotoxic Activity: **+**	Endotoxic Activity: **+**	Endotoxic Activity: **+ (?)**
R.sphaeroides lipid A 	*P. gingivalis* lipid A 	Compound 406 (Ia) 	Lipid X 
Endotoxic Activity: **-** **(LPS antagonist)**	Endotoxic Activity: **+** (TLR-2 agonist)	Endotoxic Activity: **-** **(LPS antagonist)**	Endotoxic Activity: **-** (Very weak antagonist)

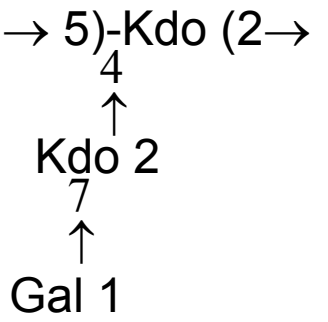
Carbohydrate backbone of the Kdo region of various *E. coli* strains lipopolysaccharides



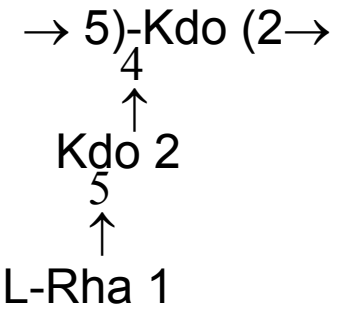
R1 core type



R2, R3, K12 core type



R2 core type



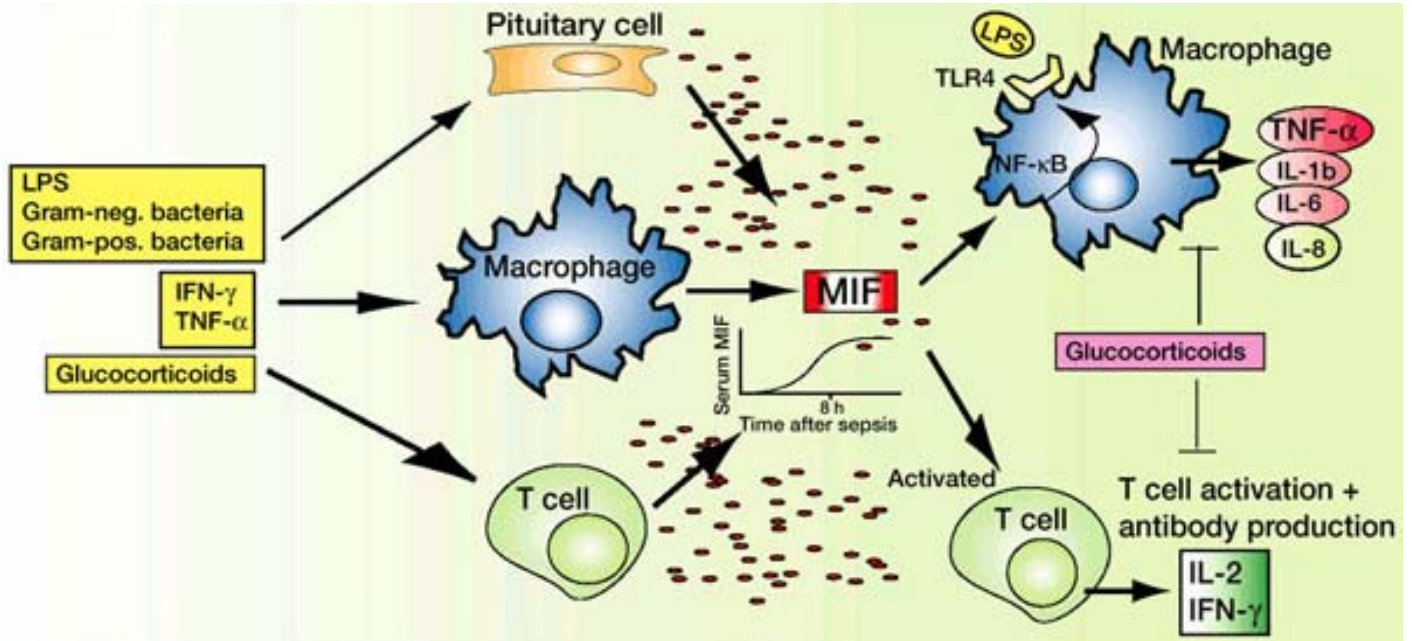
K12 core type

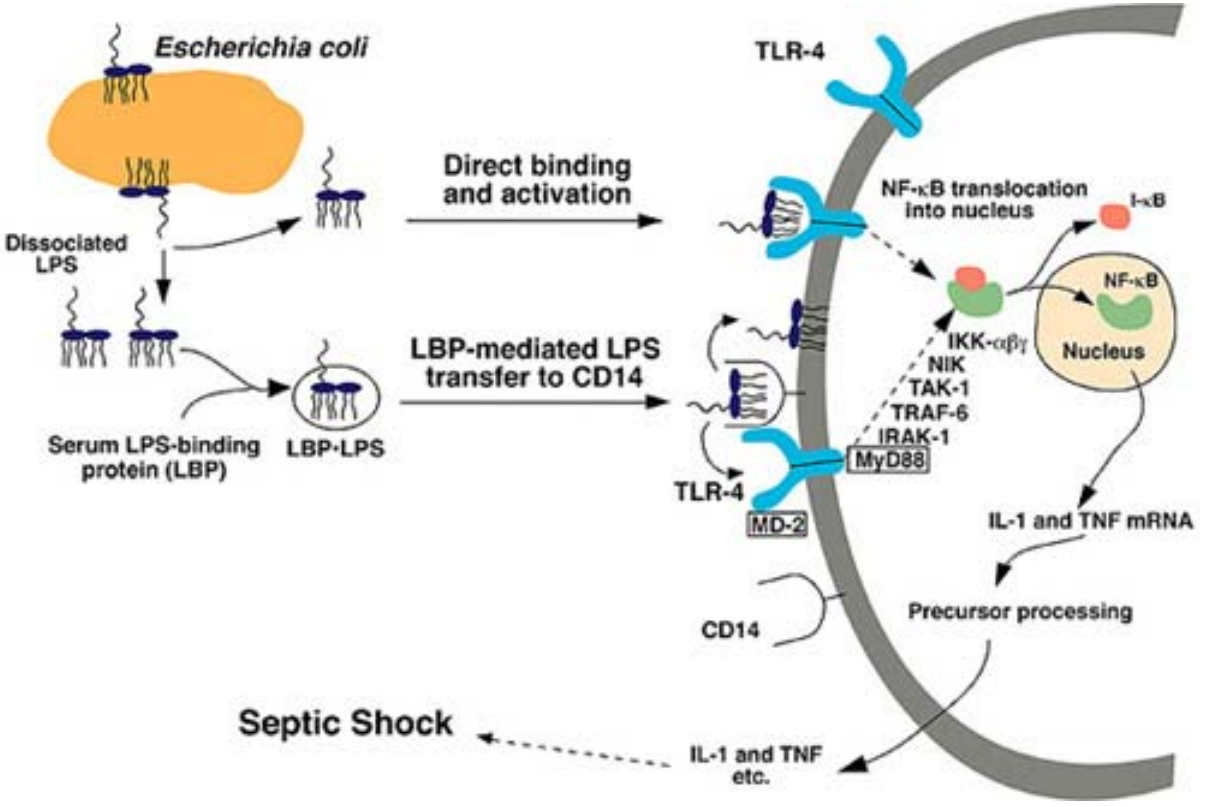
Rybka J, Zielinska-Kuzniarz K, Korzeniowska-Kowal A, Sondej A, Gamian A. Substitution pattern of 3-deoxy-D-manno-oct-2-ulosonic acid in bacterial lipopolysaccharides investigated by methylation analysis of whole LPS. *Carbohydr Res. 2003 Nov 14;338(23):2679-86*.

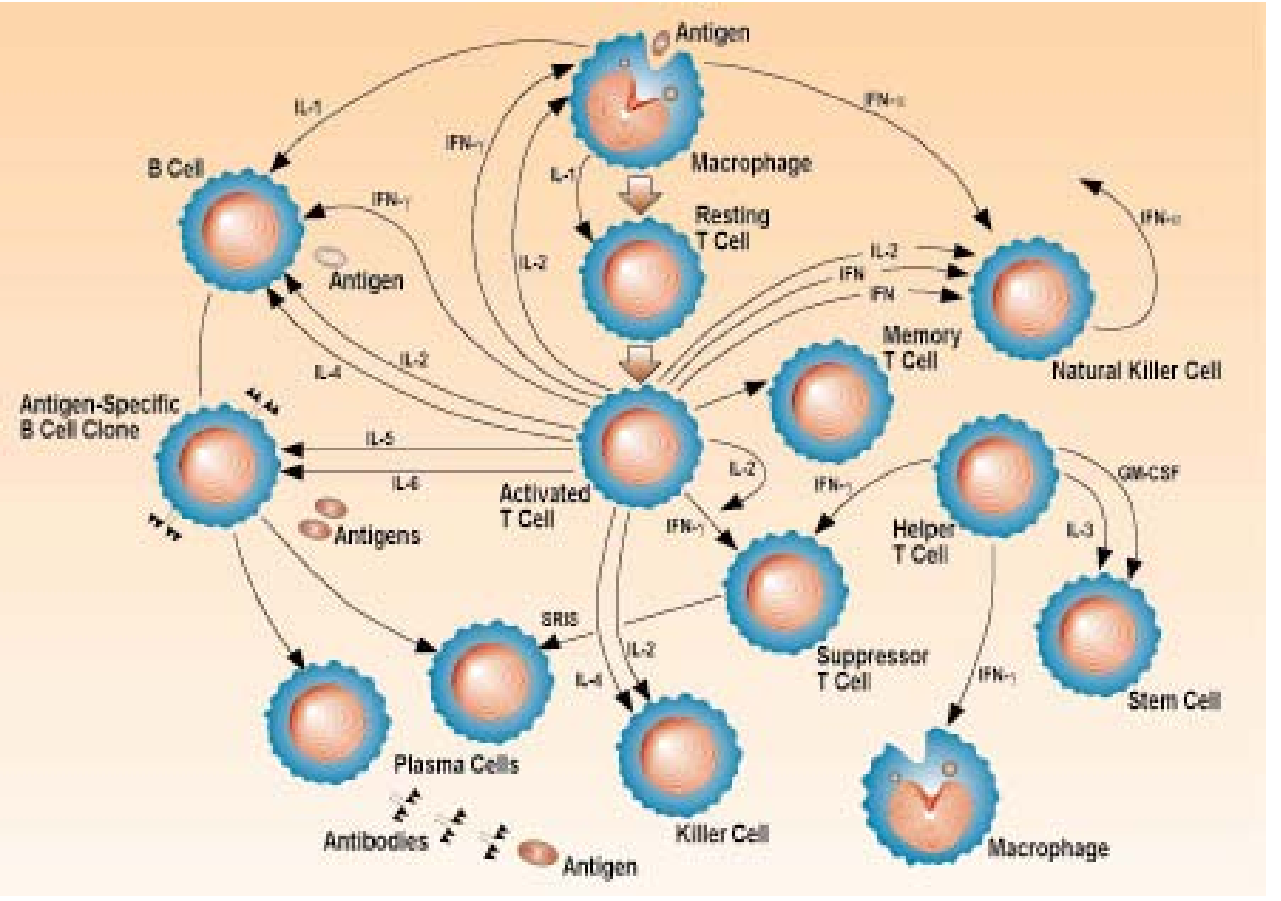
Katzenellenbogen E, Kocharova NA, Zatonsky GV, Bogulska M, Rybka J, Gamian A, Shashkov AS, Knirel YA. Structure of the O-specific polysaccharide from the lipopolysaccharide of Citrobacter gillenii O11, strain PCM 1540. *Carbohydr Res. 2003 Jun 23;338(13):1389-95.*

Lipinski T, Jones C, Lemercinier X, Korzeniowska-Kowal A, Strus M, Rybka J, Gamian A, Heczko PB. Structural analysis of the Lactobacillus rhamnosus strain KL37C exopolysaccharide. *Carbohydr Res. 2003 Mar 28;338(7):605-9.*

Kocharova NA, Mieszala M, Zatonsky GV, Staniszewska M, Shashkov AS, Gamian A, Knirel YA. Structure of the O-polysaccharide of Citrobacter youngae O1 containing an alpha-D-ribofuranosyl group. *Carbohydr Res. 2004 Jan 22;339(2):321-5.*


Pituitary cell
LPS
Gram-neg. bacteria
Gram-pos. bacteria
IFN-γ
TNF-α
Glucocorticoids
Macrophage
MIF
Serum MIF
8 h
Time after sepsis
T cell
Activated
T cell
LPS
TLR4
Macrophage
NF-κB
TNF-α
IL-1b
IL-6
IL-8
Glucocorticoids
T cell activation +
antibody production
IL-2
IFN-γ

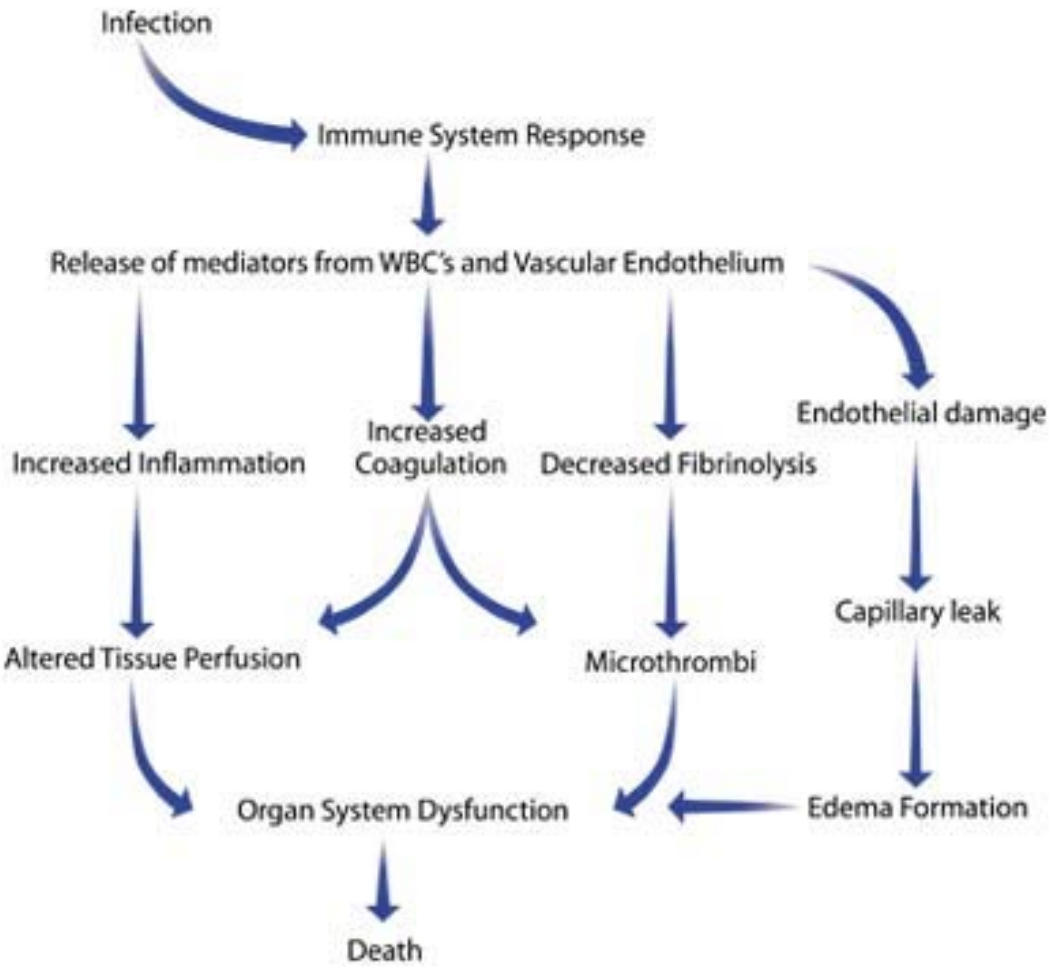

Escherichia coli
Dissociated LPS
Serum LPS-binding protein (LBP)
LBP·LPS
Direct binding and activation
LBP-mediated LPS transfer to CD14
TLR-4
NF-κB translocation into nucleus
I-κB
NF-κB
Nucleus
IKK-αβγ
NIK
TAK-1
TRAF-6
IRAK-1
MyD88
TLR-4
MD-2
IL-1 and TNF mRNA
Precursor processing
CD14
Septic Shock
IL-1 and TNF etc.


Antigen
IL-1
IFN-γ
B Cell
Macrophage
IFN-γ
IL-1
Resting
T Cell
IL-2
IFN-γ
Antigen
IL-2
IFN
IFN
Memory
T Cell
Natural Killer Cell
IL-4
IL-2
Antigen-Specific
B Cell Clone
IL-5
IL-6
Activated
T Cell
IL-2
IFN-γ
IFN-γ
Antigens
Helper
T Cell
GM-CSF
IL-3
SRIS
IL-2
IL-4
Suppressor
T Cell
IFN-γ
Stem Cell
Plasma Cells
Killer Cell
Macrophage
Antibodies
Antigen

Amounts of endotoxin which trigger immunological response are very low

They range from **pg/ml** in humans to **ng/ml** in rats

Sepsis


Infection
Immune System Response
Release of mediators from WBC's and Vascular Endothelium
Increased Inflammation
Increased Coagulation
Decreased Fibrinolysis
Endothelial damage
Altered Tissue Perfusion
Microthrombi
Capillary leak
Organ System Dysfunction
Edema Formation
Death

Facts About Severe Sepsis

- Affects more than 500,000 Europe inhabitants per year
- Mortality rates range from 28% to 50% or more
- Causes more than 150,000 deaths per year
- Costs associated with treating sepsis are estimated at almost 7 billion € a year in Europe

Detection of endotoxins - pyrogens

Pharmaceutical industry:
Intravenous and parenteral drugs, medical devices

Biomedical and pharmaceutical industry:
Tracking the bacterial content during technological process

Environmental monitoring:
Indoor and outdoor detection of air, water or dust contamination

Medicine:
Detection of Gram-negative bacterial infection, diagnosis of sepsis

Amounts of pyrogens allowed in various 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:

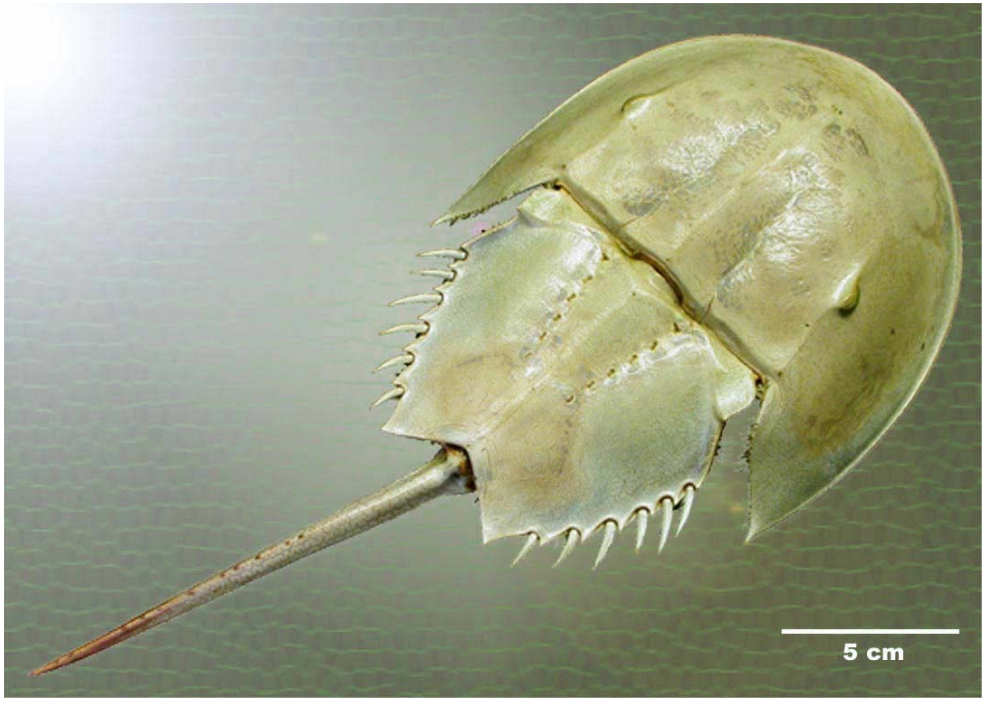
- Chemical markers (3-OH fatty acids, Kdo)
- Detection by molecules specifically recognizing LPS

Rabbit Pyrogen Test

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 four hours, is accomplished by injecting the drug being analyzed into a rabbit's ear. If the animal developed a fever, it confirmed the presence of pyrogens.

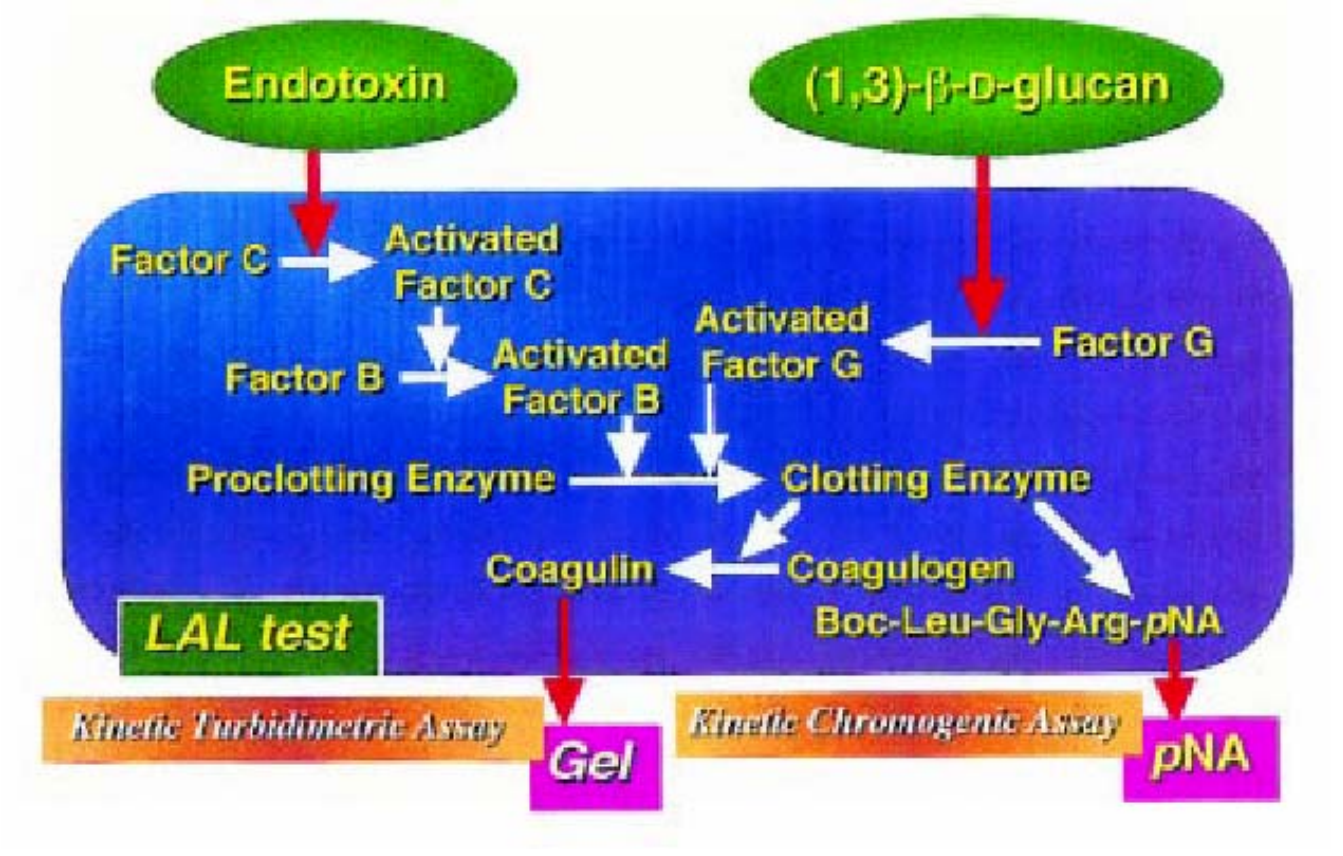
Limulus Amebocyte Lysate test (LAL)



The Atlantic horseshoe crab *Limulus polyphemus*

- 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.

Detection of endotoxin – biological tests



- Gel Clot LAL (PYROGENT®) provides a simple positive/negative result
- Chromogenic End-point LAL (QCL-1000®) 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 compatibility with many products.
- Kinetic Chromogenic LAL (Kinetic-QCL®) provides automation and greater sensitivity detecting as low as 0.005 EU/ml (1pg of LPS)

Classic methods (Rabbit pyrogen test and LAL) 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

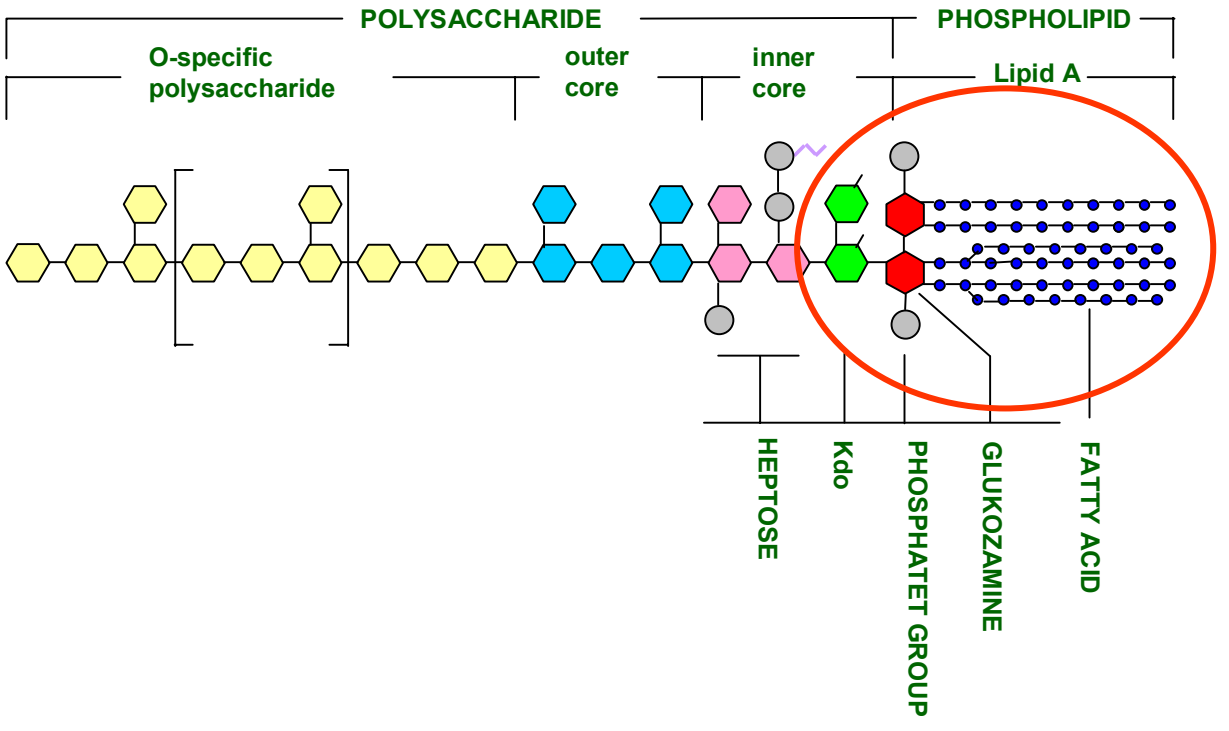
Neutrophil chemiluminescence assay

- A rapid, homogeneous assay for the detection of endotoxin activity (EA) in whole blood based on in vitro neutrophil activation.

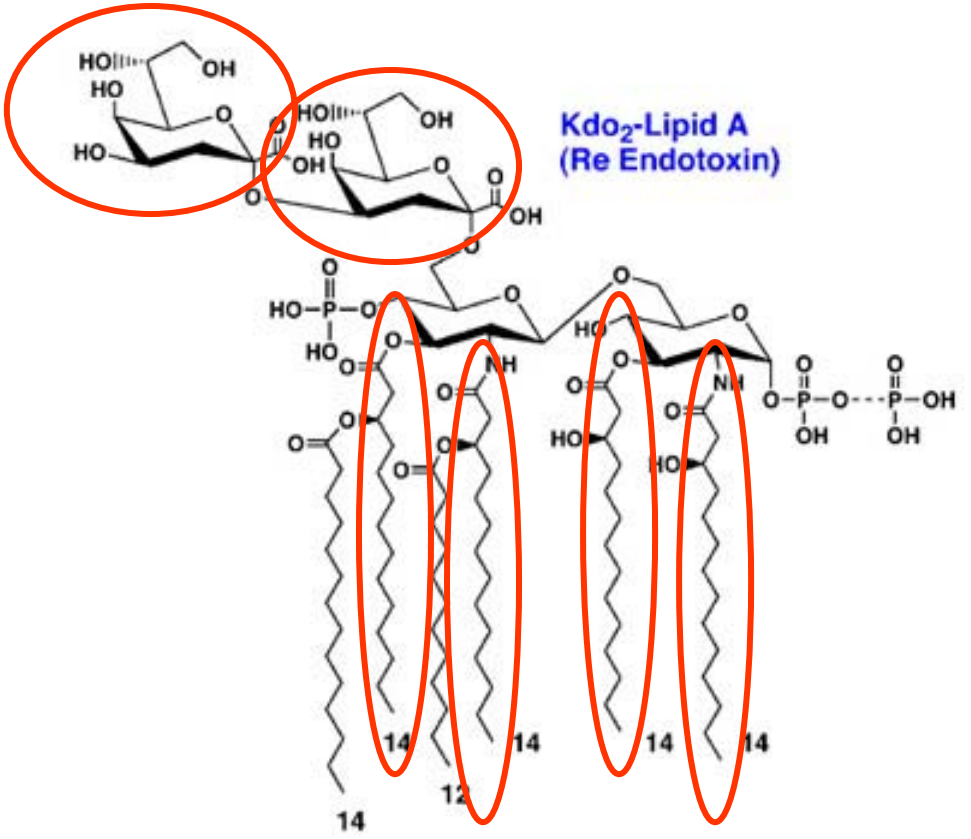
- This novel type of assay uses the priming effects of complement opsonized immune complexes on the respiratory burst activity of neutrophils as an analytical platform.

- Hypochlorous acid generated by the concerted activity of membrane-bound NADPH oxidase and azurophil granule myeloperoxidase of the neutrophil produces luminol chemiluminescence.

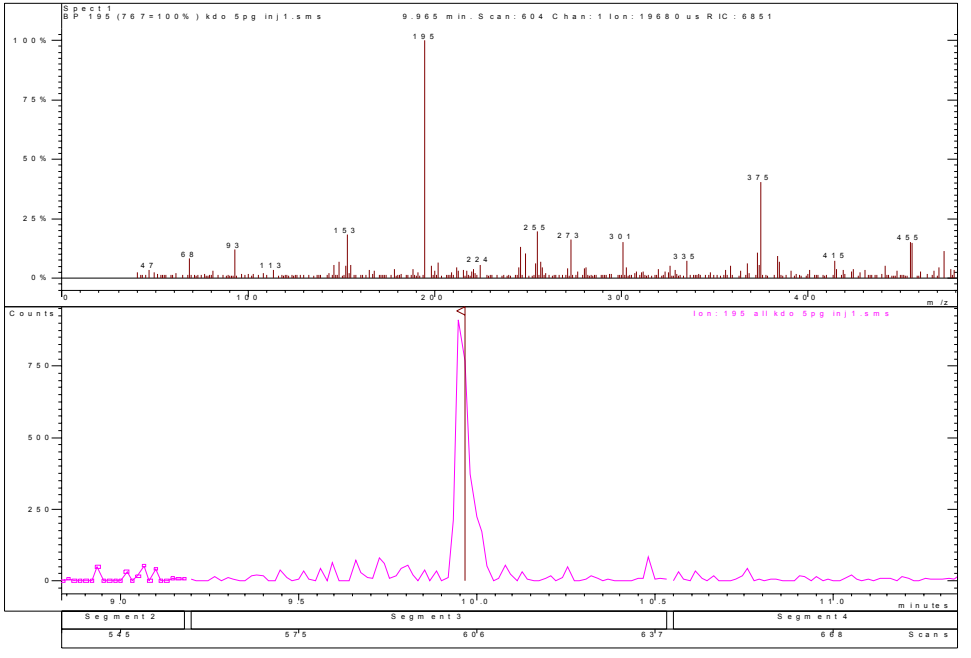
Detection of endotoxin – chemical markers



Detection of endotoxin – chemical markers



Detection of endotoxin – chemical markers



Szponar B, Krasnik L, Hryniewiecki T, Gamian A, Larsson L. Distribution of 3-hydroxy fatty acids in tissues after intraperitoneal injection of endotoxin.
Clin Chem. 2003 Jul;49(7):1149-53

Rybka J, Gamian A. Determination of endotoxin by the measurement of the acetylated methyl glycoside derivative of Kdo with gas-liquid chromatography-mass spectrometry.
J Microbiol Methods. 2005 May 30; [Epub ahead of print]

Detection by molecules with affinity to endotoxin

Proteins which specifically recognize the lipopolysaccharide molecule immobilized on Sol-Gel surface. LPS-protein binding is detected by the measurement of fluorescence anizotropy change.

Hreniak, A.; Maruszewski, K.; Rybka, J.; Gamian, A.; Czyzewski, J. A luminescence endotoxin biosensor prepared by the sol-gel method. Optical Materials, v. 26, iss. 2, p. 141-144.

Future methods for endotoxin detection ??