



PATHOGENESIS OF BACTERIAL INFECTION

8.1 INTRODUCTION

In this chapter we would focus on how bacteria causes disease to human beings. This process of causing disease is termed as Pathogenesis. Pathogenesis is a multi-factorial process which depends on the immune status of the host, the nature of the species or strain (virulence factors) and the number of organisms in the initial exposure.

A limited number of bacterial species are responsible for the majority of infectious diseases in healthy individuals. Due to the success of vaccination, antibiotics, and effective public health measures, until recently, epidemics were felt to be a thing of the past. Due to the development of antibiotic resistant organisms, this situation is changing rapidly.

All humans are infected with bacteria (the normal flora) living on their external surfaces (including the skin, gut and lungs). We are constantly also exposed to bacteria (including air, water, soil and food). Normally due to our host defenses most of these bacteria are harmless. In compromised patients, whose defenses are weakened, these bacteria often cause opportunistic infectious diseases when entering the bloodstream (after surgery, catheterization or other treatment modalities). When initiated in the hospital, these infectious diseases are referred to as nosocomial. Some common bacteria found in the normal flora include *Staphylococcus aureus*, *S. epidermidis* and *Propionibacterium acnes* (found on the skin) and *Bacteroides* and *Enterobacteriaceae* found in the intestine (the latter in much smaller numbers).



OBJECTIVES

After reading this chapter, the student will be able to :

- describe the term pathogenesis.
- explain Koch's postulates.

Pathogenesis of Bacterial Infection

- differentiate colonization and pathogens
- explain steps involved in the bacterial pathogenesis
- describe toxins
- differentiate endotoxins and exotoxins
- discuss the various diseases caused by bacteria

8.2 PATHOGENICITY

Pathogenicity is the capacity to initiate disease. It requires the attributes of **transmissibility** or communicability from one host or reservoir to a fresh host, **survival** in the new host, **infectivity** or the ability to breach the new host's defenses, and **virulence**, a variable that is multifactorial and denotes the capacity of a pathogen to harm the host. Virulence in the clinical sense is a manifestation of a **complex bacterial–host relationship** in which the capacity of the organism to cause disease is considered in relation to the resistance of the host.

Types of bacterial pathogens

Bacterial pathogens can be classified into two broad groups, primary and opportunistic pathogens.

Primary pathogens are capable of establishing infection and causing disease in previously healthy individuals with intact immunological defenses. However, these bacteria may more readily cause disease in individuals with impaired defenses.

Opportunistic pathogens rarely cause disease in individuals' with intact immunological and anatomical defenses. Only when such defenses are impaired or compromised, as a result of congenital or acquired disease or by the use of immunosuppressive therapy or surgical techniques, are these bacteria able to cause disease. Many opportunistic pathogens, e.g. coagulase negative staphylococci and *Escherichia coli*, are part of the **normal human flora** and are carried on the skin or mucosal surfaces where they cause no harm and may actually have beneficial effects, by preventing colonization by other potential pathogens. However, introduction of these organisms into anatomical sites in which they are not normally found, or removal of competing bacteria by the use of broad-spectrum antibiotics, may allow their localized multiplication and subsequent development of disease.

The above classification is applicable to the vast majority of pathogens; however, there are exceptions and variations within both categories of bacterial pathogens. Different strains of any individual bacterial species can vary in their genetic makeup and virulence capacity. For example, the majority of *Neisseria meningitidis* strains are harmless commensals and considered opportunistic

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bacteria, however, some hypervirulent clones of the organism can cause disease in a previously healthy individual. Conversely, people vary in their genetic make-up and susceptibility to invading bacteria. For example, *Mycobacterium tuberculosis* is a primary pathogen but does not cause disease in every host it invades.

**INTEXT QUESTIONS 8.1**

1. The process of bacteria causing disease is termed as
2. Ability to affect the host's disease is
3. Capacity of a pathogen to harm the host is
4. Pathogens which causes disease in healthy individual is
5. Pathogens that causes disease in immune compromised individual is

8.3 KOCH'S POSTULATES (MODIFIED)

Koch forwarded four criteria designed to establish a causal relationship between a causative microbe and a disease. The postulates were formulated by Robert Koch and Friedrich Loeffler in 1884 and refined and published by Koch in 1890. Koch applied the postulates to establish the etiology of anthrax and tuberculosis, and now have been generalized to other diseases.

1. The organism must always be found in humans with the infectious disease but not found in healthy ones.
2. The organism must be isolated from humans with the infectious disease and grown in pure culture.
3. The organism isolated in pure culture must initiate disease when re-inoculated into susceptible animals.
4. The organism should be re-isolated from the experimentally infected animals.

Postulates 3. and 4. are extremely important in definite proof of the role of agent in human disease. However, this depends on the ability to develop animal models that resemble the human disease. In many cases such models do not exist.

Pathogenesis

The process of pathogenesis involves various steps beginning with the transmission of the infectious agent (bacterial) to the host, followed by colonization of the site. After the colonization of host, the bacteria remain adherent at the site of colonization then invades the host system. After surviving the host immune system it is ready to cause the disease.

Steps involved in the pathogenesis of the bacteria:

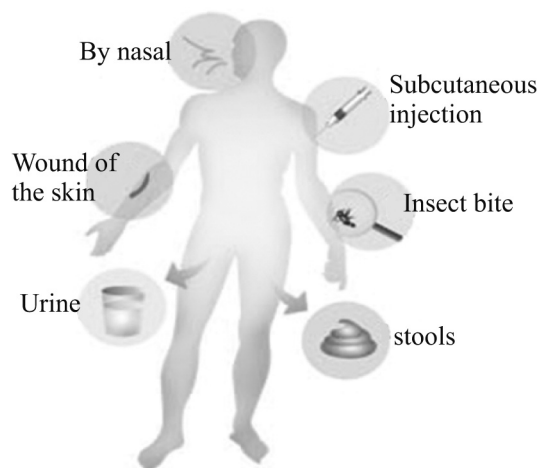
1. Transmission
2. Colonization
3. Adhesion
4. Invasion
5. Survival in the host
6. Tissue Injury



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Transmission

Potential pathogens may enter the body by various routes, including the respiratory, gastrointestinal, urinary or genital tracts. Alternatively, they may directly enter tissues through insect bites or by accidental or surgical trauma to the skin. Many opportunistic pathogens are carried as part of the normal human flora, and this acts as a ready source of infection in the compromised host (e.g. in cases of AIDS or when the skin barrier is breached). For many primary pathogens, however, transmission to a new host and establishment of infection are more complex processes.



Colonization

The establishment of a stable population of bacteria on the host's skin or mucous membranes is called colonization. For many pathogenic bacteria, the initial interaction with host tissues occurs at a mucosal surface and colonization normally requires adhesion to the mucosal cell surface. This allows the establishment of a focus of infection that may remain localized or may subsequently spread to other tissues.



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Adhesion

Adhesion is necessary to avoid innate host defense mechanisms such as peristalsis in the gut and the flushing action of mucus, saliva and urine, which remove non-adherent bacteria. For bacteria, **adhesion** is an essential preliminary to colonization and then penetration through tissues. Successful colonization also requires that bacteria are able to acquire essential nutrients—in particular iron—for growth. At the molecular level, **adhesion involves surface interactions** between specific **receptors** on the mammalian cell membrane (usually carbohydrates) and **ligands** (usually proteins) on the bacterial surface. The presence or absence of specific receptors on mammalian cells contributes significantly to tissue specificity of infection. Nonspecific surface properties of the bacterium, including surface charge and hydrophobicity, also contribute to the initial stages of the adhesion process. Several different mechanisms of bacterial adherence have evolved, all utilizing specialized cell surface organelles or macromolecules, that help to overcome the natural forces of repulsion that exist between the pathogen and its target cell. Many bacteria express pili (or fimbriae) which are involved in mediating attachment to mammalian cell surfaces. Different strains or species of bacteria produce different types of pili which can be identified on the basis of antigenic composition, morphology and receptor specificity.

Invasion

Invasion is penetration of host cells and tissues (beyond the skin and mucous surfaces), and is mediated by a complex array of molecules, often described as '**invasins**'. These can be in the form of bacterial surface or secreted proteins which target host cell molecules (receptors).

Once attached to a mucosal surface, some bacteria, e.g. *Corynebacterium diphtheriae* or *Clostridium tetani*, exert their pathogenic effects without penetrating the tissues of the host. These produce biologically active molecules such as toxins, which mediate tissue damage at local or distant sites. For a number of pathogenic bacteria, however, adherence to the mucosal surface represents only the first stage of the invasion of tissues. Examples of organisms that are able to invade and survive within host cells include *Mycobacteria*, *Salmonella*, *Shigella* and others. The initial phase of cellular invasion involves penetration of the mammalian cell membrane and many intracellular pathogens use normal phagocytic entry mechanisms to gain access. Inside the cell, they become surrounded by host cell-derived membrane vesicles. Many intracellular pathogens escape from these vesicles into the cell cytoplasm where they multiply rapidly before spreading to adjacent cells and repeating the process of invasion. The availability of specific receptors on host cells defines the type of host cells

that are involved. As a result, some pathogens can invade a wide range of cell types whilst others have a much more restricted invasive potential. The receptors for some of the invasive pathogens have been identified.

Virulence determinants

Both primary and opportunistic pathogens possess **virulence determinants or aggressins** that **facilitate pathogenesis**. Possession of a single virulence determinant is rarely sufficient to allow the initiation of infection and production of pathology. Many bacteria possess several virulence determinants, all of which play some part at various stages of the disease process. In addition, not all strains of a particular bacterial species are equally pathogenic. For example, although six separate serotypes of encapsulated *Haemophilus influenzae* are recognized, serious infection is almost exclusively associated with isolates of serotype b (hence Hib vaccine). Moreover, even within serotype b isolates, 80% of serious infections are caused by six out of > 100 clonal types.

Different strains of a pathogenic species may cause distinct types of infection, each associated with possession of a particular complement of virulence determinants. Different strains of *E. coli*, for example, cause several distinct gastrointestinal diseases, urinary tract infections, septicemia, meningitis and a range of other minor infections.

Many pathogens produce an impressive armoury of virulence determinants; however, their **expression is coordinated or regulated** by several nutritional and environmental factors. Among virulence regulators are the availability of nutrition (e.g. iron), oxygen, suitable temperature or other growth requirements. Importantly, differences in virulence between similar organisms may be due to additional cryptic phenotypic or genotypic variations. For example, some virulence factors are only expressed when indirect contact with host cells.

Virulence genes can move between bacteria via special genetic vehicles e.g. plasmids, bacteriophage and transposons. The horizontally transferred virulence factors (e.g. toxins) may or may not transform the recipient bacteria into better-adapted or more virulent pathogens.

8.4 SURVIVAL IN THE HOST

Many bacterial pathogens are able to resist the cytotoxic action of plasma and other body fluids involving antibody and complement (classical pathway) or complement alone (alternate pathway) or lysozyme. Killing of extracellular pathogens largely occurs within phagocytes after opsonization (by antibody and/or complement) and phagocytosis. Circumvention of phagocytosis by extracellular pathogens is thus a major survival mechanism. Capsules (many pathogens), protein A (*S. aureus*) and M protein (*S. pyogenes*) function in this regard.





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Protein A is a surface constituent of *S. aureus* as well as a secreted product and binds to the Fc portion of immunoglobulins. Bacteria, on binding antibody, activate the classical complement cascade which results in the attachment of fragments of C3. Phagocytosis occurs after binding of the opsonized bacteria to receptors for the Fc portion of IgG or C3 regions. Protein A is anti-complementary (since, on binding to IgG, the complement cascade is activated, depleting complement levels). Thus in the presence of protein A, interaction of bacteria (via bound complement) with C3 receptors will be inhibited. Free protein A binds to the Fc portion of IgG, thus phagocytosis via Fc receptors may not occur because of steric hindrance.

Peptidoglycan, like lipopolysaccharide, can activate the alternate complement cascade. In *S. pyogenes* peptidoglycan is sufficiently exposed that it is able to bind complement. The M protein of group A streptococci is the anti-phagocytic component of the fimbriae. M protein binds fibrinogen from plasma which blocks complement binding to the underlying peptidoglycan layer. Thus streptococci in non-immune serum are not phagocytosed.

Intracellular pathogens (both obligate and facultative) must be able to avoid being killed within phagolysosomes. This can occur from by-passing or lysing these vesicles and then residing free in the cytoplasm. Alternatively, they can survive in phagosomes (fusion of phagosomes with lysosomes may be inhibited or the organism may be resistant to degradative enzymes if fusion with lysosomes occurs).



INTEXT QUESTIONS 8.2

1. is used to establish the etiology of diseases
2. The establishment of a population of bacteria on host's skin is called
3. is necessary to avoid innate host defense mechanism
4. is penetration of host cells & tissues

8.5 TISSUE INJURY

Bacteria cause tissue injury primarily by several distinct mechanisms involving:

- Exotoxins
- Endotoxins and non-specific immunity
- Specific humoral and cell mediated immunity

Exotoxins

Many bacteria produce proteins (exotoxins) that modify, by enzymatic action, or otherwise destroy certain cellular structures. Effects of exotoxins are usually seen acutely, since they are sufficiently potent that serious effects (e.g. death) often result. Examples of this are botulism, anthrax, cholera and diphtheria. If the host survives the acute infection, neutralizing antibodies (anti-toxins) are often elicited that neutralize the affect of the exotoxin. Classes of exotoxins include:



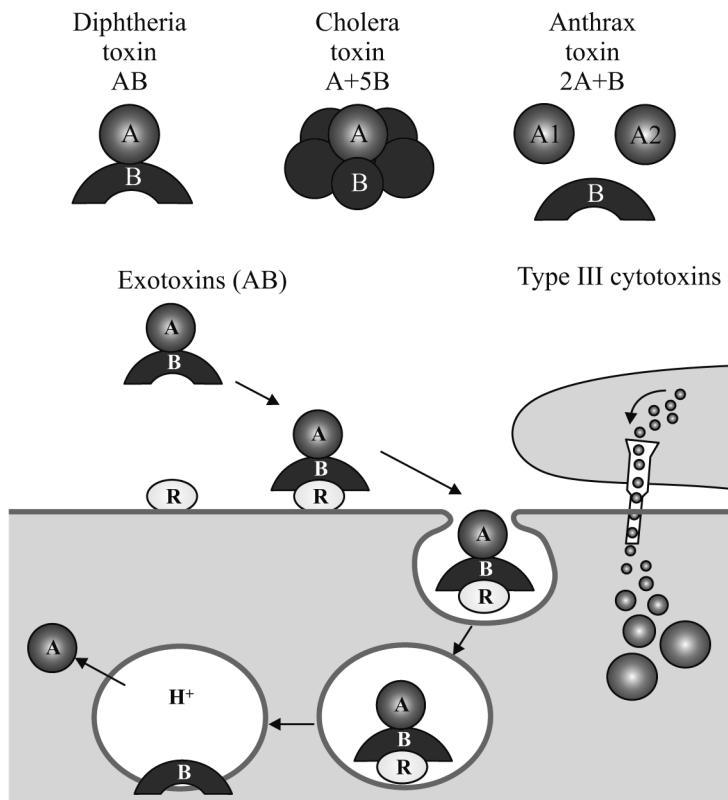
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Toxins that act on the extracellular matrix of connective tissuee.g. *Clostridium perfringens* collagenase, *Staphylococcus aureus* hyaluronidase.

Toxins that have a cell binding “B” component and an active “A” enzymatic component (A-B type toxins)

These include:

- a) Those with ADP-ribosylating activity e.g. cholera toxin, *E. coli* heat labile toxin, *Pseudomonas aeruginosa* and diphtheria toxins.
- b) Those with a lytic activity on 28S rRNA e.g. shiga and shiga-like (vero) toxins.
- c) Those with a partially characterized site of action e.g. botulinum toxin, tetanus toxin and anthrax lethal toxin.





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Membrane Damaging Toxins e.g. *Staphylococcus aureus* delta toxin

Toxins which act extracellularly. These include proteases, collagenases and hyaluronidases. For example, *Clostridium perfringens* produces a potent collagenase, whilst *Staphylococcus aureus* produces a hyaluronidase. Damage to the connective tissue matrix (by hyaluronidase and collagenase) can “loosen up” the tissue fibers allowing the organism to spread through the tissues more readily. Also included in this group is the exfoliatin of *Staphylococcus aureus* which causes separation of the layers within the epidermis and is the causative agent of scalded skin syndrome in the newborn.

A - B Toxins. Such toxins consist of two components. One binds to cell surfaces and the other passes into the cell membrane or cytoplasm where it acts. The classical toxins demonstrated to act in this fashion are those of cholera and diphtheria.

(i) ADP-ribosylating exotoxins

Diphtheria toxin (produced by *Corynebacterium diphtheriae*) is coded by the phage tox gene. The toxin is synthesized as one polypeptide chain and readily nicked into two chains held together by a disulfide bond. B binds to cells and A has the enzymatic activity. A is endocytosed and from the endosome passes into the cytosol. Diphtheria toxin ADP-ribosylates elongation factor (EF2) in ribosomes, thus inhibiting protein synthesis. *Pseudomonas* exotoxin A has a similar mode of action to diphtheria toxin.

Cholera toxin has several subunits which form a ring with one A subunit inserted in the center. B binds to gangliosides on the cell surface and appear to provide a channel through which A penetrates. A1 is formed by proteolytic cleavage and after internalization ADP-ribosylates a cell membrane regulator complex (using NADH as a substrate), in turn causing activation of adenylate cyclase. Activation of adenylate cyclase causes an increase in cyclic AMP production with resulting decrease in sodium chloride uptake from the lumen of the gut and active ion and water secretion with a watery diarrhea resulting. *E. coli* labile toxin has a similar mode of action.

(ii) Toxins that act on 28S rRNA

Shiga toxins (chromosomally encoded) are involved in the pathogenesis of shigellosis, whilst shiga-like toxins (phage encoded) are primarily produced by enterohemorrhagic *E. coli*. They share a common mode of action. A fragment of the A subunit passes to the ribosome where it has N-glycosidase activity on a single adenosine residue; i.e. the bond between the base and ribose is lysed. Diarrhea results not from active ion/water secretion, but poor water absorption due to death of epithelial cells from inhibition of protein synthesis.

(iii) Partially characterized site of action

Botulinum neurotoxins, tetanospasmin and the lethal toxin of *B. anthracis*, appear to be A-B type exotoxins. Botulinum toxin acts by causing inhibition of release of acetylcholine at the neuromuscular junction. Tetanus toxin is taken up at neuromuscular junctions and transported in axons to synapses. It then acts by inactivating inhibitory neurons. The exotoxins of tetanus and botulism appear to have B components, but the mode of action of their A subunits are not known. The B component of lethal toxin of *B. anthracis* is the protective antigen; interestingly, this also serves as the B subunit for edema toxin.

Membrane Damaging Toxins: These toxins enzymatically digest the phospholipid (or protein) components of membranes or behave as detergents. In each case holes are punched in the cell membrane and the cytoplasmic contents can leach out. The phospholipase (“toxin”) of *C. perfringens* is an example of a membrane damaging toxin. It destroys blood vessels stopping the influx of inflammatory cells. This also helps create an anaerobic environment which is important in the growth of this strict anaerobe. The delta toxin of *S. aureus* is an extremely hydrophobic protein that inserts into cell membranes and is believed to have a detergent-like action.

Endotoxins

Despite the advances of the antibiotic era, around 200,000 patients will develop Gram negative sepsis each year of whom around 25-40% will ultimately die of septic shock. Septic shock involves hypotension (due to tissue pooling of fluids), disseminated intravascular coagulation and fever and is often fatal from massive system failure. This includes lack of effective oxygenation of sensitive tissues such as the brain. There is no effective therapy to reverse the toxic activity of lipid A or peptidoglycan in patients.

Endotoxins are toxic components of the bacterial cell envelope. The classical and most potent endotoxin is lipopolysaccharide. However, peptidoglycan displays many endotoxin-like properties. Certain peptidoglycans are poorly biodegradable and can cause chronic as well as acute tissue injury. Endotoxins are “non-specific” inciters of inflammation. For example, cells of the immune system and elsewhere are stimulated to release cytokines (including interleukin 1 and tumor necrosis factor). Endotoxins also activate the alternate complement pathway. The production of these cytokines results in attraction of polymorphonuclear cells into affected tissues. PG and LPS and certain other cell wall components (e.g. pneumococcal teichoic acid) are also activators of the alternate complement cascade. Thus many bacteria will bind complement encouraging their uptake and killing by phagocytes in the absence of antibody. Certain complement by-products are also chemoattractants for neutrophils.



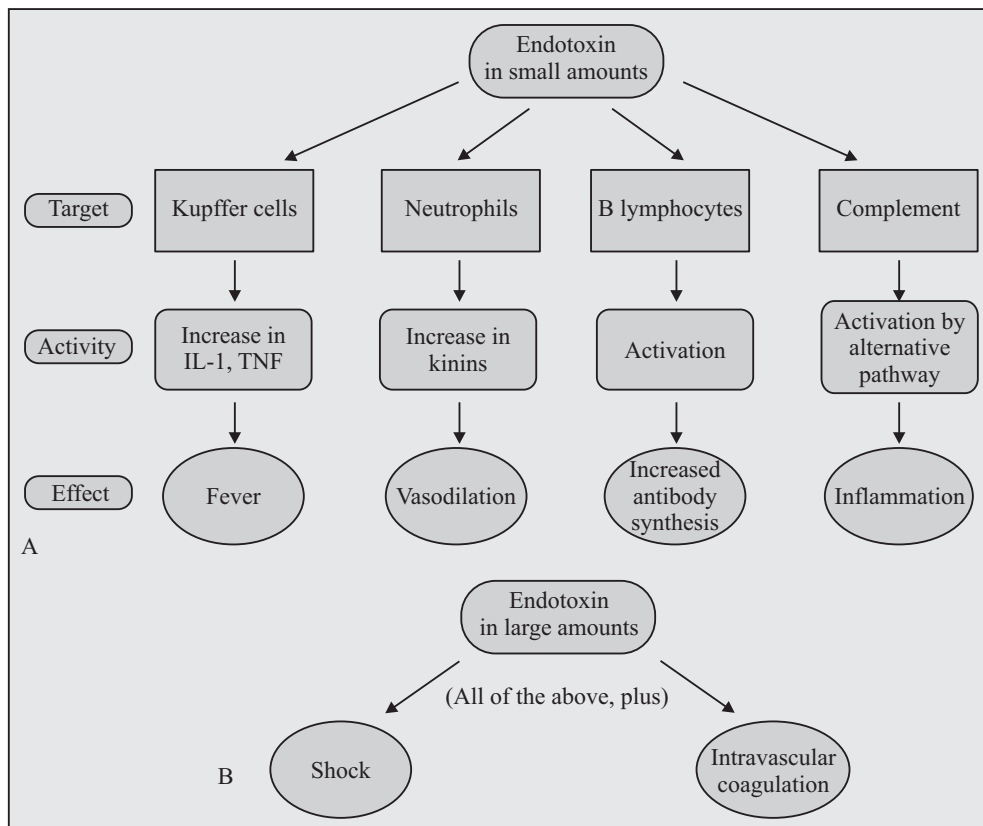
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Endotoxins are also potent B cell mitogens, polyclonal B cell activators and adjuvants (for both antibodies and cell mediated immunity); this plays a role in the development of a suitable chronic immune response in handling the microbes if they are not eliminated acutely.

In a “primary” infection during the acute phase “non-antigen specific” immunity will be of utmost importance in eradicating the infection. If the organism persists (or in a reinfection at a later date), specific immunity will be of greater significance in slowing growth of the organisms or in eliminating infection. This is important in chronic infections such as tuberculosis, leprosy, Lyme disease and syphilis.



INTEXT QUESTIONS 8.3

1. Bacteria produce that modify cellular structures
2. Toxins that act extracellularly are, &
3. are toxic components of bacterial cell envelope
4. Example of endotoxin is

8.6 IMMUNOPATHOLOGY

The infected tissue often serves as an innocent bystander and immunopathology results. This can occur in acute and chronic infections. Over stimulation of cytokine production and complement activation by endotoxins can cause tissue injury in the absence of an immune response. Continuously generated antigens released from persisting viable microbes will subsequently elicit humoral antibodies and cell mediated immunity resulting in chronic immunopathology. Certain poorly degradable antigens (e.g pneumococcal polysaccharide and group A streptococcal cell walls) can maintain immunopathology even in the absence of persistence of live agents. Other bacterial antigens cross-react with host tissue antigens causing the development of autoimmunity (e.g. the M protein of *S. pyogenes* cross-reacts with mammalian myosin). Thus immunopathology can persist even after the infection and microbial antigens are eliminated.



Notes

The immune system in resistance to infection - examples

1. Extracellular parasites. Antibodies cause lysis of the organism and/or their opsonization by phagocytes at which point they are rapidly killed.
2. Intracellular parasites are primarily killed by cell mediated immunity.
3. Exotoxins can be neutralized by antitoxins. These can be elicited using toxoid vaccines (toxoids are antigenic but not toxic). This occurs, for example, in vaccination against diphtheria.
4. Certain organisms produce IgA proteases (including *H. influenzae*, *S. pneumoniae*, *N. gonorrhoeae* and *N. meningitidis*) this helps survival on external surfaces.

Some Organisms of Medical Interest

Gram negative aerobic cocci Neisseria	Gram positive cocci (facultative anaerobes) Streptococcus Staphylococcus
Spirochetes Treponema Borrelia Leptospira	Gram negative bacilli <i>Pseudomonas</i> Bordetella Francisella
Spiral, Gram negative bacilli Campylobacter Helicobacter	Gram positive bacilli <i>Listeria</i> Erysipelothrix
Gram negative bacilli (a) <i>Enterobacteriaceae</i> Escherichia	<i>Actinomycetes</i> and related organisms Corynebacterium Mycobacterium

MODULE

Microbiology



Notes

Pathogenesis of Bacterial Infection

<ul style="list-style-type: none"> Salmonella Shigella Yersinia Enterobacter Proteus Serratia Edwardsiella 	<ul style="list-style-type: none"> Nocardia Actinomyces Corynebacterium-like in appearance Propionibacterium Fastidious Gram negative bacteria Brucella Rochalimeae/Bartonella Chlamydia Rickettsia Mycoplasma
<ul style="list-style-type: none"> (b) Others Vibrio Hemophilus Pasteurella 	
<ul style="list-style-type: none"> (c) <i>Legionellaceae</i> Legionella Tatlockia 	

Some major Exotoxins

Organism	Disease	Toxin
<i>Bacillus anthracis</i>	Anthrax	Edema toxin
		Lethal toxin
<i>Clostridium botulinum</i>	Botulism	Botulism toxin
<i>Clostridium difficile</i>	Pseudo membranous colitis	Enterotoxin
<i>Clostridium perfringens</i>	Gas gangrene	Alpha toxin Hyaluronidase
	Food poisoning	Enterotoxin
<i>Clostridium tetani</i>	Tetanus	Tetanospasmin
<i>Corynebacterium diphtheria</i>	Diphtheria	Diphtheria toxin
<i>Escherichia coli</i>	Diarrhea (ETEC)	Heat labile toxin
		Heat stable toxins
	Hemorrhagic colitis	Vero toxin
<i>Pseudomonas aeruginosa</i>	Diseases of compromised host	Exotoxin A
<i>Staphylococcus aureus</i>	Opportunistic infections	Alpha-gamma toxins, leucocidin
	Toxic shock	Toxic shock toxin
	Food poisoning	Enterotoxin
	Scalded skin syndrome	Exfoliatin
<i>Streptococcus pyogenes</i>	Scarlet fever Toxic shock	Erythrogenic/pyrogenic toxin
<i>Shigella dysenteriae</i>	Bacillary dysentery	Shiga toxin
<i>Vibrio cholera</i>	Cholera	Cholera toxin



INTEXT QUESTIONS 8.4

Match the following

Organism

1. Bacterial anthraxis
2. Clostridium botulinum
3. Staphylococcus aureus
4. Streptococcus pyogenes

Toxin

- (a) leucocidin
- (b) erythrogenic toxin
- (c) Edema toxin
- (d) Botulism toxin



WHAT HAVE YOU LEARNT

- The capacity to initiate disease is called pathogenesis
- Pathogenesis depends on the immune status of host, nature of species or strain (Virulence factor) & number of organisms in the initial exposure
- Bacterial pathogens are of two types namely primary and opportunistic pathogens
- Primary pathogens are capable of establishing infection and cause disease in previously healthy individuals with intact immune defense
- Opportunistic pathogens cause disease in individuals with impaired or compromised defenses
- Kochs postulate establishes a casual relationship between a microbe and disease
- The process of pathogenesis involves various steps beginning with the transmission of the infectious agent (bacterial) to the host, followed by colonization of the site.
- After the colonization host the bacteria remain adherent at the site of colonization then invades the host system.
- After being survived from host immune system it is ready to cause the disease.
- Pathogens possess virulence determinants or aggressins that facilitate pathogenesis
- Bacteria cause tissue injury by Exotoxins, Endotoxins & Non-specific immunity, specific humoral and cell mediated immunity.



TERMINAL QUESTIONS

1. What are pathogenic bacteria. Explain with suitable example?
2. What do you understand by the term opportunistic infections. Enlist some opportunistic infection seen in human being?



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Notes

Pathogenesis of Bacterial Infection

3. What are the reasons for opportunistic infections in human beings?
4. Enlist the steps involved in the pathogenesis of bacteria?
5. Explain every step involved in the pathogenesis of bacteria with suitable example?
6. Differentiate between endotoxin and exotoxins?



ANSWERS TO INTEXT QUESTIONS

8.1

1. Pathogenesis
2. Infectivity
3. Virulence
4. Primary pathogens
5. Opportunistic pathogen

8.2

1. Koch postulate
2. Colonization
3. Adhesion
4. Invasion

8.3

1. Exotoxins
2. Proteases, collagenases & hyaluroindes
3. Endotoxins
4. Lipopolysaccharide

8.4

1. (c)
2. (d)
3. (a)
4. (b)