Diphtheria

*Corynebacterium diphtheriae*

Corynebacteria are Gram-positive, aerobic, nonmotile, rod-shaped bacteria classified as *Actinobacteria*. Corynebacteria are related phylogenetically to mycobacteria and actinomycetes. They do not form spores or branch as do the actinomycetes, but they have the characteristic of forming irregular, club-shaped or V-shaped arrangements in normal growth. They undergo snapping movements just after cell division, which brings them into characteristic forms resembling Chinese letters or palisades.

The genus *Corynebacterium* consists of a diverse group of bacteria including animal and plant pathogens, as well as saprophytes. Some corynebacteria are part of the normal flora of humans, finding a suitable niche in virtually every anatomic site, especially the skin and nares. The best known and most widely studied species is *Corynebacterium diphtheriae*, the causal agent of the disease diphtheria.

Figure 1. Stained *Corynebacterium* cells. The "barred" appearance is due to the presence of polyphosphate inclusions called metachromatic granules. Note also the characteristic "Chinese-letter" arrangement of cells.

Diphtheria is an upper respiratory tract illness characterized by sore throat, low fever, and an adherent membrane (called a pseudomembrane on the tonsils, pharynx, and/or nasal cavity. Diphtheria toxin produced by *C. diphtheriae*, can cause myocarditis, polyneuritis, and other systemic toxic effects. A milder form of diphtheria can be restricted to the skin.

Diphtheria is a contagious disease spread by direct physical contact or breathing aerosolized secretions of infected individuals. Once quite common, diphtheria has largely been eradicated in developed nations through wide-spread use of the DPT vaccine. For example, in the U.S., between 1980 and 2004 there were 57 reported cases of diphtheria. However, it remains somewhat of a problem worldwide (3,978 reported cases to WHO in 2006) in the face of efforts to achieve global vaccination coverage.

Diphtheria is a serious disease, with fatality rates between 5% and 10%. In children under 5 years and adults over 40 years, the fatality rate may be as much as 20%. Outbreaks, although very rare, still occur worldwide, even in developed nations. Following the breakup of the former Soviet Union in the late 1980s,
vaccination rates in the constituent countries fell so low that there was a surge in diphtheria cases. In 1991 there were 2,000 cases of diphtheria in the USSR. By 1998, according to Red Cross estimates, there were as many as 200,000 cases in the Commonwealth of Independent States, with 5,000 deaths.

Figure 2. This figure shows the reported global incidence of diphtheria between 1980 and 2006. Generally, as vaccine coverage with DPT has increased, the incidence of diphtheria has decreased. However, note the spike between 1993 and 1997, attributable to a drop in vaccine coverage in new Independent States of the former Soviet Union, as explained in the text above. WHO.

History and Background

No bacterial disease of humans has been as successfully studied as diphtheria. The etiology, mode of transmission, pathogenic mechanism and molecular basis of exotoxin structure, function, and action have been clearly established. Consequently, highly effective methods of treatment and prevention of diphtheria have been developed.

The study of Corynebacterium diphtheriae traces closely the development of medical microbiology, immunology and molecular biology. Many contributions to these fields, as well as to our understanding of host-bacterial interactions, have been made studying diphtheria and the diphtheria toxin. Some of the milestones along this path are given below.

Hippocrates provided the first clinical description of diphtheria in the 4th century B.C. There are also references to the disease in ancient Syria and Egypt.

In the 17th century, murderous epidemics of diphtheria swept Europe; in Spain the disease became known as "El garatillo" (the strangler"), in Italy and Sicily as "the gullet disease".

In the 18th century, the disease reached the American colonies where it reached epidemic proportions about 1735. Often, whole families died of the disease in a few weeks.
The bacterium that causes diphtheria was first described by Klebs in 1883, and was cultivated by Loeffler in 1884, who applied Koch’s postulates and properly identified *Corynebacterium diphtheriae* as the agent of the disease.

In 1884, Loeffler concluded that *C. diphtheriae* produced a soluble toxin, and thereby provided the first description of a bacterial exotoxin.

In 1888, Roux and Yersin demonstrated the presence of the toxin in the cell-free culture fluid of *C. diphtheriae* which, when injected into suitable lab animals, caused the systemic manifestation of diphtheria.

Two years later, von Behring and Kitasato succeeded in immunizing guinea pigs with a heat-attenuated form of the toxin and demonstrated that the sera of immunized animals contained an antitoxin capable of protecting other susceptible animals against the disease. This modified toxin was suitable for immunizing animals to obtain antitoxin, but it was found to cause severe local reactions in humans and could not be used as a vaccine.

In 1909, Theobald Smith, in the U.S., demonstrated that diphtheria toxin that had been neutralized by antitoxin (forming a **Toxin-Anti-Toxin complex, TAT**) remained immunogenic and eliminated local reactions seen in the modified toxin. For some years, beginning about 1910, TAT was used for active immunization against diphtheria. TAT had two undesirable characteristics as a vaccine. First, the toxin used was highly toxic, and the quantity injected could result in a fatal toxemia unless the toxin was fully neutralized by antitoxin. Second, the antitoxin mixture was horse serum, the components of which tended to be allergenic and to sensitize individuals to the serum.

In 1913, Schick designed a skin test as a means of determining susceptibility or immunity to diphtheria in humans. Diphtheria toxin will cause an inflammatory reaction when very small amounts are injected intracutaneously. The Schick Test involves injecting a very small dose of the toxin under the skin of the forearm and evaluating the injection site after 48 hours. A positive test (inflammatory reaction) indicates susceptibility (nonimmunity). A negative test (no reaction) indicates immunity (antibody neutralizes toxin).

In 1924, Ramon demonstrated the conversion of diphtheria toxin to its nontoxic, but antigenic, equivalent (**toxoid**) by treating with formaldehyde. He provided humanity with one of the safest and surest vaccines of all time, the diphtheria toxoid.

In 1951, Freeman made the remarkable discovery that pathogenic (toxigenic) strains of *C. diphtheriae* are lysogenic, (i.e., are infected by a temperate Beta phage), while non lysogenized strains are avirulent. Subsequently, it was shown that the gene for toxin production is located on the DNA of the Beta phage.

In the early 1960s, Pappenheimer and his group at Harvard conducted experiments on the mechanism of a action of the diphtheria toxin. They studied the effects of the toxin in HeLa cell cultures and in cell-free systems, and concluded that the toxin inhibited protein synthesis by blocking the transfer of amino acids from tRNA to the growing polypeptide chain on the ribosome. They found that this action of the toxin could be neutralized by prior treatment with diphtheria antitoxin.

Subsequently, the exact mechanism of action of the toxin was shown, and the toxin has become a classic model of an ADP-ribosylating bacterial exotoxin.

**Diphtheria in the United States**

At the turn of the century, in the United States, diphtheria was common, occurring primarily in children and was one of the leading causes of death in infants and children. In the 1920's, when data were first gathered, there were approximately 150,000 cases and 13,000 deaths reported annually. After diphtheria immunization was introduced, the number of cases gradually fell to about 19,000 in 1945. When diphtheria immunization became widespread in the late 1940's, a more rapid decrease in the number of cases and deaths occurred.
From 1970 to 1979, an average of 196 cases per year were reported. Seventeen outbreaks of 15 or more cases occurred in the United States between 1959 and 1980, but there have been none since 1980. During 1980-1995, a total of 41 respiratory diphtheria cases were reported; of these, four (10%) were fatal, and all occurred in unvaccinated children.

Since 1988, five of the six culture-positive diphtheria cases reported in the United States have been associated with importation of *Corynebacterium diphtheriae*, an organism believed to have become rare or to have disappeared from the United States. However, a case of infection with toxigenic *C. diphtheriae* discovered in 1996 showed that the bacterium remains present in areas where the disease was once endemic, such as the Northern Plains Indian Community of South Dakota. On June 1, 1996, the discovery of a 62-year-old American Indian woman infected with diphtheria led to increased surveillance of the disease among the community. *C. diphtheriae* was isolated in 5% of 133 patients surveyed during August-October 1996. The findings underline the need for timely vaccination by people of all ages throughout the US.

**Human Disease**

CDC describes diphtheria as an upper respiratory tract illness characterized by sore throat, low-grade fever, and an adherent membrane of the tonsil(s), pharynx, and/or nose. Diphtheria is a rapidly developing, acute, febrile infection which involves both local and systemic pathology. A local lesion develops in the upper respiratory tract and involves necrotic injury to epithelial cells. As a result of this injury, blood plasma leaks into the area and a fibrin network forms which is interlaced with rapidly-growing *C. diphtheriae* cells. This membranous network, called a pseudomembrane, covers over the site of the local lesion leading to respiratory distress, even suffocation.

![Figure 3. Diphtheria pseudomembrane. CDC.](image)

The diphtheria bacilli do not tend to invade tissues below or away from the surface epithelial cells at the site of the local lesion. However, at this site they produce the toxin that is absorbed and disseminated through lymph channels and blood to the susceptible tissues of the body. Degenerative changes in these tissues, which include heart, muscle, peripheral nerves, adrenals, kidneys, liver and spleen, result in the systemic pathology of the disease.
Pathogenicity

The pathogenicity of *Corynebacterium diphtheriae* includes two distinct phenomena:

1. **Invasion**: the local tissues of the throat, which requires colonization and subsequent bacterial proliferation. Little is known about the adherence mechanisms of *C. diphtheriae*, but the bacteria produce several types of pili. The diphtheria toxin, as well, may be involved in colonization of the throat.

2. **Toxigenesis**: bacterial production of the toxin. The diphtheria toxin causes the death of eucaryotic cells and tissues by inhibition of protein synthesis in the cells. Although the toxin is responsible for the lethal symptoms of the disease, the virulence of *C. diphtheriae* cannot be attributed to toxigenicity alone, since a distinct invasive phase apparently precedes toxigenesis. However, it has not been ruled out that the diphtheria toxin plays an essential role in the colonization process due to short-range effects at the colonization site.

Three strains of *Corynebacterium diphtheriae* are recognized, **gravis**, **intermedius** and **mitis**. They are listed here by falling order of the severity of the disease that they produce in humans. All strains produce the identical toxin and are capable of colonizing the throat. The differences in virulence between the three strains can be explained by their differing abilities to produce the toxin in rate and quantity, and by their differing growth rates.

The gravis strain has a generation time (in vitro) of 60 minutes; the intermedius strain has a generation time of about 100 minutes; and the mitis stain has a generation time of about 180 minutes. The faster growing strains typically produce a larger colony on most growth media. In the throat (in vivo), a faster growth rate may allow the organism to deplete the local iron supply more rapidly in the invaded tissues, thereby allowing earlier or greater production of the diphtheria toxin. Also, if the kinetics of toxin production follow the kinetics of bacterial growth, the faster growing variety would achieve an effective level of toxin before the slow growing varieties.

![Figure 4. Corynebacterium diphtheriae colonies on blood agar. CDC.](image)
Toxigenicity

Two factors have great influence on the ability of Corynebacterium diphtheriae to produce the diphtheria toxin: (1) **low extracellular concentrations of iron** and (2) the **presence of a lysogenic prophage** in the bacterial chromosome. The gene for toxin production occurs on the chromosome of the prophage, but a bacterial repressor protein controls the expression of this gene. The repressor is activated by iron, and it is in this way that iron influences toxin production. High yields of toxin are synthesized only by lysogenic bacteria under conditions of iron deficiency.

**The role of iron.** In artificial culture the most important factor controlling yield of the toxin is the concentration of inorganic iron (Fe\(^{++}\) or Fe\(^{+++}\)) present in the culture medium. Toxin is synthesized in high yield only after the exogenous supply of iron has become exhausted (This has practical importance for the industrial production of toxin to make toxoid. Under the appropriate conditions of iron starvation, *C. diphtheriae* will synthesize diphtheria toxin as 5% of its total protein). Presumably, this phenomenon takes place in vivo as well. The bacterium may not produce maximal amounts of toxin until the iron supply in tissues of the upper respiratory tract has become depleted. It is the regulation of toxin production in the bacterium that is partially controlled by iron. The tox gene is regulated by a mechanism of negative control wherein a repressor molecule, product of the DtxR gene, is activated by iron. The active repressor binds to the tox gene operator and prevents transcription. When iron is removed from the repressor (under growth conditions of iron limitation), derepression occurs, the repressor is inactivated and transcription of the tox genes can occur. Iron is referred to as a **corepressor** since it is **required for repression of the toxin gene**.

**The role of B-phage.** Only those strains of *Corynebacterium diphtheriae* that are lysogenized by a specific Beta phage produce diphtheria toxin. A phage lytic cycle is not necessary for toxin production or release. The **phage contains the structural gene for the toxin molecule**. The original proof rested in the demonstration that lysogeny of *C. diphtheriae* by various mutated Beta phages leads to production of nontoxic but antigenically-related material (called CRM for “cross-reacting material”). CRMs have shorter chain length than the diphtheria toxin molecule but cross react with diphtheria antitoxins due to their antigenic similarities to the toxin. The properties of CRMs established beyond a doubt that the tox genes resided on the phage chromosome rather than the bacterial chromosome.

Even though the tox gene is not part of the bacterial chromosome, the regulation of toxin production is under bacterial control since the DtxR (regulatory) gene is on the bacterial chromosome and toxin production depends upon bacterial iron metabolism.

![Figure 5. The Beta phage that encodes the tox gene for the diphtheria toxin.](image)
It is of some interest to speculate on the role of the diphtheria toxin in the natural history of the bacterium. Of what value should it be to an organism to synthesize up to 5% of its total protein as a toxin that specifically inhibits protein synthesis in eucaryotes and archaea? Possibly the toxin assists colonization of the throat (or skin) by killing epithelial cells or neutrophils. There is no evidence to suggest a key role of the toxin in the life cycle of the organism. Since mass immunization against diphtheria has been practiced, the disease has virtually disappeared, and *C. diphtheriae* is no longer a component of the normal flora of the human throat and pharynx. It may be that the toxin played a key role in the colonization of the throat in nonimmune individuals and, as a consequence of exhaustive immunization, toxigenic strains have become virtually extinct.

**Figure 6. The Diphtheria Toxin (DTx) Monomer.** A (red) is the catalytic domain; B (yellow) is the binding domain which displays the receptor for cell attachment; T (blue) is the hydrophobic domain responsible for insertion into the endosome membrane to secure the release of A. The protein is illustrated in its "closed" configuration.

The diphtheria toxin (DTx) is a two-component bacterial exotoxin synthesized as a single polypeptide chain containing an A (active) domain and a B (binding) domain. Proteolytic nicking of the secreted form of the toxin separates the A chain from the B chain. The B chain contains a hydrophobic T (translocation) region, responsible for insertion into the endosome membrane in order to secure the release of A. The toxin binds to a specific receptor (now known as the HB-EGF receptor) on susceptible cells and enters by receptor-mediated endocytosis. Acidification of the endosome vesicle results in unfolding of the protein...
and insertion of the T segment into the endosomal membrane. Apparently, as a result of activity on the endosome membrane, the A subunit is cleaved and released from the B subunit as it inserts and passes through the membrane. Once in the cytoplasm, the A fragment regains its conformation and its enzymatic activity. Fragment A catalyzes the transfer of ADP-ribose from NAD to the eucaryotic Elongation Factor 2 which inhibits the function of the latter in protein synthesis. Ultimately, inactivation of all of the host cell EF-2 molecules causes death of the cell. Attachment of the ADP ribosyl group occurs at an unusual derivative of histadine called diphthamide.

![Figure 7. The Mechanism of action of Diphtheria toxin DTxA.](image)

Figure 7. The Mechanism of action of Diphtheria toxin DTxA.

![Figure 8. Uptake and activity of the diphtheria toxin in eucaryotic cells. The figure is redrawn from the Diphtheria Toxin Homepage at UCLA. A represents the A/B toxin's A (catalytic) domain; B is the B (receptor) domain; T is the hydrophobic domain that inserts into the cell membrane.](image)

Figure 8. Uptake and activity of the diphtheria toxin in eucaryotic cells. The figure is redrawn from the Diphtheria Toxin Homepage at UCLA. A represents the A/B toxin’s A (catalytic) domain; B is the B (receptor) domain; T is the hydrophobic domain that inserts into the cell membrane.

In vitro, the native diphtheria toxin is inactive and can be activated by trypsin in the presence of thiol. The enzymatic activity of fragment A is masked in the intact toxin. Fragment B is required to bind the native toxin to its cognate receptor and to permit the escape of fragment A from the endosome. The C terminal
end of Fragment B contains the peptide region that attaches to the HB-EGF receptor on the sensitive cell membrane, and the N-terminal end is a strongly hydrophobic region which will insert into a membrane lipid bilayer.

The specific membrane receptor, heparin-binding epidermal growth factor (HB-EGF) precursor is a protein on the surface of many types of cells. The occurrence and distribution of the HB-EGF receptor on cells determines the susceptibility of an animal species, and certain cells of an animal species, to the diphtheria toxin. Normally, the HB-EGF precursor releases a peptide hormone that influences normal cell growth and differentiation. One hypothesis is that the HB-EGF receptor itself is the protease that nicks the A fragment and reduces the disulfide bridge between it and the B fragment when the A fragment makes its way through the endosomal membrane into the cytoplasm.

**Immunity to Diphtheria**

Acquired immunity to diphtheria is due primarily to toxin-neutralizing antibody (antitoxin). Passive immunity in utero is acquired transplacentally and can last at most 1 or 2 years after birth. In areas where diphtheria is endemic and mass immunization is not practiced, most young children are highly susceptible to infection. Probably, active immunity can be produced by a mild or inapparent infection in infants who retain some maternal immunity, and in adults infected with strains of low virulence (inapparent infections).

Individuals that have fully recovered from diphtheria may continue to harbor the organisms in the throat or nose for weeks or even months. In the past, it was mainly through such healthy carriers that the disease was spread, and toxigenic bacteria were maintained in the population. Before mass immunization of children, carrier rates of *C. diphtheriae* of 5% or higher were observed.

Because of the high degree of susceptibility of children, artificial immunization at an early age is universally advocated. Toxoid is given in 2 or 3 doses (1 month apart) for primary immunization at an age of 3 - 4 months. A booster injection should be given about a year later, and it is advisable to administer several booster injections during childhood. Usually, infants in the United States are immunized with a trivalent vaccine containing diphtheria toxoid, pertussis vaccine, and tetanus toxoid (DPT or DTaP vaccine).

The relative absence of diphtheria in the United States is due primarily to the high level of appropriate immunization in children, and to an apparent reduction in toxin-producing strains of the bacterium. However, the increasing percentage of diphtheria cases in adults suggests that many adults may not be protected against diphtheria, because they have not received booster immunizations within the past ten years. A similar situation exists with tetanus.