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The Pathogenesis of Disease Due to Type b *Haemophilus influenzae*

Ruth Aubrey and Christoph Tang

1. Introduction

1.1. Haemophilus influenzae

Haemophilus influenzae is a Gram-negative bacterium that was first described by Pfeiffer in 1892 ***(1)***. This ubiquitous, human-specific organism was originally thought to be the etiologic agent of "influenza." However, *H. influenzae* was not consistently isolated from autopsied lungs of individuals who had died during the influenza pandemic in 1918. The confusion that existed about the relationship between the prevalence of *H. influenzae* and human disease was relieved when Pittman discovered that strains of this bacterium could be divided into two groups, encapsulated (typeable) and nonencapsulated (nontypeable) strains ***(2)***. Pittman further distinguished six encapsulated types of *H. influenzae*, designated a–f, by the serological specificities of their capsular polysaccharide ***(2)***.

Mainly type b (Hib) strains cause serious invasive diseases including meningitis and septicemia, as well as epiglottitis, cellulitis, septic arthritis, pneumonia and empyema ***(3,4)***. Nonencapsulated strains cause otitis media, sinusitis, conjunctivitis, and acute lower respiratory tract infections, the latter causing many millions of deaths mainly in developing countries. Infants between the ages of 12 and 36 mo are most at risk of disease caused by *H. influenzae*. During this time, infants are no longer protected by transplacentally acquired maternal antibodies and have not developed their own serum antibodies ***(5)***.

Interest in the pathogenesis of *H. influenzae* infection waned with the advent of antimicrobial chemotherapy. Whereas the mortality of meningitis caused by *H. influenzae* in North America was almost 100% in 1930 ***(6)***, with the advent of antibiotics the mortality fell to 5–10% by the mid-1960s ***(4,7)***. However, at

From: *Methods in Molecular Medicine, vol. 71: Haemophilus influenzae Protocols*
Edited by: M. Herbert © Humana Press Inc., Totowa, NJ

least 30% of survivors were left with serious defects such as deafness, blindness, seizures, and mental retardation ***(8–11)***.

During the 1970s, antibiotic resistant strains of Hib arose and were propagated ***(12)***. By 1991 it was estimated that 11% of isolates of *H. influenzae* type b had acquired resistance to ampicillin, the most common antibiotic used at the time to treat infection by this organism ***(13)***. Jenner and colleagues reported the emergence of chloramphenicol-resistant strains of *H. influenzae* in 1990 ***(14)***.

H. influenzae has gained prominence not only as an important pathogen but also as the subject of fundamental genetic and molecular research. In the early 1950s, *H. influenzae* was found to be the second example (after the pneumococcus) of a naturally transformable organism. Then, in the late 1960s, Hamilton Smith's research on homologous recombination in *H. influenzae* led directly to the discovery of type II restriction endonucleases, which underlie much of recombinant DNA technology. Molecular and immunological research focusing on the polysaccharide capsule led to the development and commercial release of the first polysaccharide-conjugate vaccine in 1989. And finally, in 1995, *H. influenzae* became the first free-living bacterium to have its genome completely sequenced ***(15)***. This remarkable feat marked the beginning of widespread efforts to determine the whole genome sequences of many other bacteria, and initiated the application of genomics and bioinformatics in microbiological research.

1.2. Microbiology

Haemophilus belongs to the family Pasteurellaceae, which contains two other genera, *Actinobacillus* and *Pasteurella*. Bacteria belonging to this family are small (1×0.3 μm), non-spore-forming coccobacilli that have fastidious growth requirements, often needing supplemented media for isolation. The name of the genus, *Haemophilus* (meaning blood-loving) refers to the specific dependence of this organism on heme-related molecules for growth under aerobic conditions. The morphology of *H. influenzae* in clinical specimens is variable, ranging from coccobacilli to long filaments ***(16)***.

1.3. Classification of Typeable H. influenzae *Strains*

To understand the epidemiology of disease caused by typeable *H. influenzae*, investigators searched for marker systems to discriminate between strains. In the early 1980s, clinical Hib isolates were classified on the basis of variation in the electrophoretic mobility patterns of the major outer-membrane proteins (OMPs) ***(17–19)*** and lipopolysaccharide (LPS) ***(20,21)***. Hib strains were later assigned to one of four groups on the basis their reactivity with LPS-specific monoclonal antibodies (mAbs).

Musser and colleagues endeavored to measure genetic diversity and the evolutionary relationship between *H. influenzae* strains ***(22,23)***. Isolates were classified by multilocus enzyme electrophoresis (MLEE), in which the polymorphisms in essential metabolic enzymes were used to estimate genetic divergence from a presumed common ancestor. Over time, genes encoding essential metabolic enzymes accumulate neutral mutations that are not subject to selection. One hundred and seventy-seven type b strains, mostly isolated from the blood or cerebrospinal fluid of North American children with invasive disease, were grouped into several genetically distinct clusters or electrophoretic types (ETs), each of which showed strong nonrandom association of alleles ***(22)***. Strains having identical or very similar ETs were isolated from geographically distinct regions over a period of up to 40 yr. It was concluded that the population of type b strains was relatively clonal and that those causing most of the invasive disease episodes were a restricted subset of the genotypes of the species as a whole. Indeed, most of the North American isolates belonged to two closely related ETs and were distinct from a collection of isolates obtained from several European countries ***(24)***. These findings suggested an epidemiological pattern in which 'successful' type b clones swept through a host population and became hyperendemic over a period of yr, analogous to changes in *Neisseria meningitidis* populations ***(25,26)***.

More recently, the employment of techniques such as multilocus sequence typing (MLST) (Fiel and Spratt, personal communication), ribotyping, and RNA sequencing (Goldstein, personal comunication) has revealed substantial diversity within typeable and nontypeable strains of *H. influenzae*, which has raised doubts regarding the clonality of the species as a whole.

2. Colonization and Invasion

The pathogenesis of *H. influenzae* has been investigated through case studies and by using animal and in vitro models of infection. The investigation of *H. influenzae* has been a paradigm for understanding the pathogenesis of bacterial meningitis in general. Several distinct stages have been recognized during *Haemophilus* invasion.

2.1. Colonization of the Upper Respiratory Tract

H. influenzae typically colonizes human respiratory mucosal surfaces and occasionally the female genital tract ***(27)***. Children are more likely to be colonized than adults ***(28)***. Approximately 5% of healthy individuals are colonized with strains of serotypes a–f ***(28)***. From the nasopharynx, organisms are transmitted from one individual to another by airborne droplets or by direct contact with secretions ***(29)***. The primary interaction between *Haemophilus* and humans was shown in human organ cultures to be mediated by the binding of the bacte-

rium to mucin ***(30,31)***. In in vitro culture systems such as oropharyngeal epithelial cells, pili were shown to mediate adherence and were therefore proposed to be involved in colonization ***(32,33)***. It was later reported that nonpiliated *H. influenzae* also demonstrated significant levels of attachment to mammalian cells in vitro mediated by outer-membrane proteins (OMPs) ***(34,35)***. It has been shown by transmission electron microscopy (TEM) that, in the human nasopharyngeal organ culture system, both piliated and nonpiliated Hib attach themselves selectively to nonciliated epithelial cells ***(34)***. It was recently reported that specific gangliosides (sialylated glycosphingolipids) of human respiratory epithelial cells and of human macrophages serve as host receptors for *H. influenzae* ***(36)***.

It is speculated that during long-term carriage of *H. influenzae* the organism resides intracellularly. First, it is known that colonization is difficult to eradicate with antimicrobials ***(37)*** unless agents are used that are active within human cells, such as rifampicin and quinolones. Also, in vitro studies have shown that *H. influenzae* can be taken up by, and survive within, human epithelial cells ***(35)*** and macrophages ***(38,39)***.

2.2. Invasion of the Epithelium

In in vitro culture systems, strains of *H. influenzae* have been shown to exhibit a strong tropism for mucus, cause the breakdown of tight junctions of epithelial cells, and cause the sloughing off of ciliated cells and ciliostasis ***(29,40–42)***. Furthermore, this damage to epithelial cells led to exposure of the nonluminal cell surfaces underlying basal cells and the basement membrane ***(29)***. The strains demonstrated a much greater association with these surfaces than with intact epithelia ***(29)***. Despite the preferred intercellular route of entry into and through the mucosa, intracellular invasion has been observed ***(35,43)***.

2.3. Invasion of the Blood Stream

During the mid to late 1970s it was not known whether the transmission of *H. influenzae* to the central nervous system (CNS) occurred by spread from the nasopharynx along the olfactory nerve fibers or by the hematogenous route. To investigate this, isogenic strains of *H. influenzae* differing in antibiotic resistance were intranasally inoculated into rats. One strain was identified in the blood and cerebral spinal fluid (CSF) ***(44)*** demonstrating that the hematogenous route was taken by this organism. Furthermore, the occurrence of meningitis after an intranasal inoculation of *H. influenzae* in rats was shown to be directly related to the intensity of bacteremia ***(45)***.

Detailed investigations of the direct interactions between *H. influenzae* and endothelial cells were undertaken using the human umbilical vein endothelial cells (HUVECs) model ***(46)***. Using TEM, phagocytic ingestion of the organism by these cells could be visualized. It was shown that, upon internalization, the

bacteria remained viable within endothelial cell vacuoles and were then translocated within the vacuoles across the cell, emerging at the opposite surface.

As originally reported by Rubin and Moxon ***(47)***, it is now accepted that the passage of *H. influenzae* from the subepithelial tissue to the bloodstream occurs through direct invasion of capillaries supplying the epithelial tissue.

2.4. Survival in the Bloodstream

Innate and adaptive humoral immune responses elicited by the host renders the bloodstream a hostile environment in which *H. influenzae* must survive and replicate to cause prolonged infection. It has been shown that more than 90% of a population of *H. influenzae* cells inoculated intravascularly (iv) into the adult rat are cleared within a few minutes ***(48)***. Interestingly, it was shown that the remaining subpopulation that evaded clearance replicated sufficiently to invade the meninges of the rat and cause meningitis ***(48)***. Factors that aid the survival of subpopulations or variants of *H. influenzae* in the bloodstream are discussed later in this chapter. The clearance of encapsulated *H. influenzae* from blood involves deposition of C3 on the bacterium, and is independent of the later complement components, C5–C9. Bacteria are then removed from the circulation following phagocytosis by tissue macrophages. The type b capsule inhibits the initial binding of C3, thereby reducing uptake by phagocytic cells ***(49)***. The type b capsule appears to be more efficient than other capsular types at preventing bacterial clearance. This may largely account for the preponderance of type b strains among invasive disease isolates.

2.5. Invasion of the CNS

Specific interactions are believed to occur between *H. influenzae* and the blood-brain barrier (BBB). The BBB is a single layer of unique endothelial cells, which is largely responsible for the maintenance of biochemical homeostasis within the CNS ***(50)***. These endothelial cells exhibit continuous tight junctions and a marked paucity of pinocytosis ***(51)***. Interactions between *H. influenzae* and the BBB have been investigated in in vivo models such as the infant rat model of meningitis ***(52)***, and in in vitro BBB models such as the bovine microvascular endothelial cell model ***(51)***. The models show that *H. influenzae* cells adhere to the BBB and then translocate across or between the cellular tight junctions, to enter the CSF. In the rat, live or heat-killed Hib increase pinocytosis and disrupt interendothelial tight junctions ***(52,53)***. Damage to the BBB enhances *H. influenzae* entry into the CSF. Once in the CSF, the population of *H. influenzae* may continue to expand and infect the meninges of the brain, causing meningitis.

3. Virulence Determinants of *H. influenzae*

Each step in the pathogenesis of *H. influenzae* infection appears to depend on the expression of a combination of several specific virulence determinants.

These determinants include outer-membrane proteins (OMPs), pili, IgAl proteases, lipopolysaccharide (LPS), and capsule. It has been shown that many of these virulence determinants elicit a protective immune response to *H. influenzae* in rats and humans ***(17,54,55)*** and are relatively conserved between strains of this organism ***(56–58)***. They have therefore been investigated as vaccine candidates against diseases caused by *H. influenzae* ***(54,57)***.

3.1. OMPs

Strains of *H. influenzae* express between 10 and 20 OMPs ***(59)*** ranging from 16- to 98-kDa in size. The combination of expressed proteins varies between strains ***(60)***. The most abundant OMP of Hib is the porin protein, P2 ***(60,61)***. Cope and colleagues reported that P2 contributes to virulence of Hib as an isogenic mutant of a virulent Hib strain, incapable of synthesizing P2, was avirulent in the infant rat ***(62)***. This protein interacts with LPS ***(63)***. The P5 protein is thought to be involved in the invasion of the mucosal epithelium as inactivation of the P5 gene results in a decrease in bacteremia following intranasal inoculation of infant rats ***(64)***. The P6 and 98K OMPs have also been shown to be immunogenic in humans ***(54,65)*** and protective, as anti-P6 and anti-98K antibodies protect infant rats from *H. influenzae* disease ***(54,55)***.

3.2. Pili

H. influenzae pili are 4.7–18.0 nm in diameter, between 209 and 453 nm in length, and possess a hollow core ***(66,67)***. They appear as relatively thick flexible rods with a short, thinner fibrillum at the top, similar to *Eschericia coli* Pap pili. The pilus rods are composed of polymerized pilin proteins, which show slight interstrain variability in migration by polyacrylamide gel electrophoresis (PAGE) ***(67)***. At least 16 pili are expressed per bacterial cell ***(66)*** and are distributed in a peritrichous manner ***(68)***.

Pili appear to mediate bacterial adherence to mucosal surfaces and hence facilitate respiratory tract colonization. Anderson and co-workers observed that a piliated *H. influenzae* strain showed stronger adherence to buccal epithelial cells and was more effective in colonizing rats following intranasal inoculation than its nonpiliated variant ***(69)***. It was later shown that infant rats inoculated by the ip or iv route with piliated *H. influenzae* type b had decreased levels of bacteremia compared to rats inoculated with nonpiliated variants ***(70)***. Furthermore, piliated *H. influenzae* were shown to stimulate enhanced opsonization-dependent phagocytosis by neutrophils ***(71)***. It appears, then, that the expression of pili is important during the colonization stage of pathogenesis but detrimental at systemic stages.

The expression of pili in *H. influenzae*, like that in other organisms, is phase-variable ***(72)***. A single copy of the pilin locus, comprising *hifA* to *hifE* is present

in most *Haemophilus* strains studied, with the notable exception of Rd, the strain whose entire genome was sequenced and which is 1.8 Mb in size ***(15)***, 0.3 Mb smaller than the prototypic pathogenic strain, Eagan. The promoter regions of the two divergently transcribed pilus genes, *hifA* (encoding the major pilus subunit) and *hifB* (encoding the pilus chaperone) overlap. Tandem repeats of the dinucleotide 5'-TA-3' are located between the −10 and −35 regions of both promoters ***(73)***. The spacing between the –10 and –35 sequences is altered by changes in the number of repeats within this promoter region. This is thought to alter the efficiency of RNA polymerase binding and hence gene expression ***(74)***. A subset of piliated colonizing *H. influenzae* switch to the nonpiliated form by an alteration in TA repeat number between generations, and nonpiliated variants are presumed to have selective advantage for invasive disease ***(34)***.

3.3. Immunoglobulin Al Proteases

Immunoglobulin A1 (IgAl) protease is constitutively secreted by a number of mucosal pathogens, including *Neisseria meningitidis, N. gonorrhoeae*, and *Streptococcus pneumoniae* as well as *H. influenzae* ***(75,76)***. The *Haemophilus* IgAl proteases are serine type enzymes that are synthesized as 169-kDa proteins ***(75,77)***. The activity of IgAl proteases in cleaving and inactivating human IgAl, the predominant secretory antibody in the upper respiratory tract ***(78)*** is believed to facilitate colonization ***(79)***. IgAl proteases specifically cleave one out of four peptide bonds located within a limited amino acid sequence of the hinge region of the α chain of human IgAl, including the secretory form (S-IgAl). Thereby the antibody molecules are left as intact Fab (monomeric) fragments devoid of the Fc portion, which is particularly responsible for the protective properties of this immune factor ***(80)***. Upon cleavage, the 50-kDa C-terminal domain of the IgAl protease remains in the bacterial outer membrane, while the proteolytically active N terminus is secreted. For *H. influenzae*, at least two classes of IgAl proteases have been described based on cleavage at either a prolyl-seryl (designated type 1) or four amino acids away at a prolyl-threoryl bond (type 2) ***(81–83)***. In addition to differences in cleavage specificity, these proteins display considerable polymorphism and antigenic variation, so that more than 30 types have been described based on serological responses in humans ***(84,85)***.

3.4. Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. A hydrophobic lipid moiety, lipid A, constitutes about 60% (w/w) of the LPS of *H. influenzae* ,while the remainder of this molecule consists of hydrophilic polysaccharide ***(86)***. Lipid A is embedded in the outer membrane, while the polysaccharide portion extends outward from the

bacterial surface. As well as being an extremely important surface-exposed immunogen, LPS is essential for the integrity and functioning of the cell membrane. Unlike that of enteric bacteria, the LPS of *H. influenzae* lacks an O-antigen and thus consists of a simple set of monosaccharides ***(87)***.

3.4.1. Structure of LPS of H. influenzae

The lipid A of *H. influenzae* consists of a β-(1,6)-1inked D-glucosamine disaccharide substituted by phosphate groups at C-1 of the reducing, and C-4 of the non-reducing D-glucosamine. The diglucosamine is further substituted by four molecules of (R)-3-hydroxytetradecanoic acid linked by amide linkages at the 2 and 2' positions and ester linkages at the 3 and 3' positions. The two 3-hydroxytetradecanoic acid molecules on the nonreducing glucosamine carry a further two tetradecanoic acid molecules on their hydroxy groups, so that there are six fatty acid molecules in all ***(88)***. This pattern is very similar to that of *Escherichia coli*, which differs only in that the 2'-amide linked 3-hydroxytetradecanoic acid is substituted by dodecanoic acid ***(89,90)***. It appears that the structure of lipid A in different strains of *H. influenzae* is relatively conserved. Up to 50% of a large collection of Hib strains were shown to share at least one epitope in the lipid A region as determined by reactivity with a Hib lipid A-directed Mab ***(91)***. Mass spectrometric (MS) and nuclear magnetic resonance (NMR) analyses of the LPS of several strains of *H. influenzae* reveal that lipid A is linked via a single 2-keto-3-deoxyoctulosonic acid (KDO) to a conserved triheptose backbone ***(92–96)***. From this backbone there is interstrain variation in the presence, number, arrangement, and linkages of hexose sugars, phosphate groups, and sialic acid. The structure of LPS of two Hib strains, RM7004 (Richards, personal communication) and RM 153 ***(95)*** is shown in **Fig. 1.**

3.4.2. Involvement of LPS in Pathogenesis

Despite absence of the O-antigen, the LPS of *H. influenzae* plays an important role in pathogenesis. Isogenic strains with mutations in single LPS genes and therefore differing in LPS structure were constructed, and survival was compared in the infant rat ***(97–99)***. Likewise, natural LPS variants, isolated by LPS-specific Mabs ***(100,101)***, as well as chemically ***(62)*** or genetically mutagenized strains ***(102)*** with altered LPS, were compared with parental strains for survival. The findings confirmed that LPS indeed contributes to the virulence of *H. influenzae*.

The role of LPS in causing the symptoms of meningitis has also been shown by inoculating both rabbits and rats with Hib LPS alone rather than the whole organism ***(53,103)***. Following intracisternal inoculation of LPS, a dose- and time-dependent increase in BBB permeability was observed and a correlation established between CSF pleocytosis and BBB permeability. There was a close

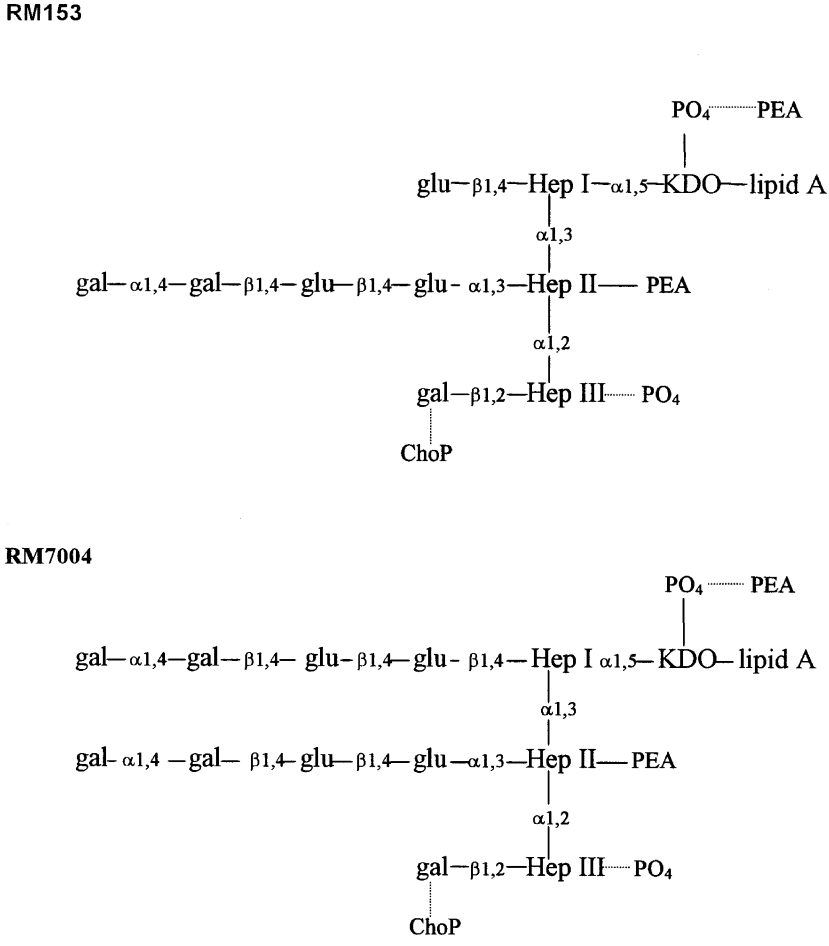

Fig. 1. Schematic representation of the structure of LPS of RM153 ***(95)***, RM7004. Heptose sugars are represented by "Hep," glucose and galactose sugars are denoted by "glu" and "gal," respectively. Phosphate groups are shown in red, and a hashed line indicates those that are variably present. Phosphorylcholine and phosphoethanolamine are abbreviated to ChoP and PEA, respectively (Richards, personal communication).

relationship between numbers of white blood cells in the CSF and the degree of BBB permeability. The toxicity of LPS was shown to be attributed mainly to the activity of the lipid A portion, since the deleterious effect of LPS was significantly inhibited by prior treatment with polymyxin B (which binds to the lipid A domain) or by deacylating LPS (to remove nonhydroxylated fatty acyl chains from the lipid A). Since lipid A is embedded in the outer membrane of the organism, its endotoxic activity is exerted mostly when the organism is

lysed. In order for LPS to strongly activate host cells, LPS must bind a plasma LPS-binding protein, LBP ***(104)***. The LPS–LBP complex binds membrane CD14 (mCD14), present mainly on myeloid cells ***(105)*** and soluble CD14 (sCD14), a secreted form that circulates in the plasma ***(106,107)***. CD14 then interacts with Toll-like receptor proteins (TLR) ***(108–110)*** culminating in the transduction of a cytoplasmic signal ***(110,111)***. Through the activation of a complex cascade of events, the production of cytokines is triggered. The activity of cytokines and complement components may lead to septic shock.

Specific components of the polysaccharide portion of LPS have been shown to be important at different stages of pathogenesis. For example, the expression of phosphorylcholine has been shown to be important for colonization of the nasopharynx ***(101)***, while expression of a specific digalactoside (α-D-galactose[1–4]-β-D-galactose) ***(101,112–114)*** and sialic acid ***(43)*** are important during systemic stages of infection, permitting resistance against immune-mediated clearance.

Several loci involved in the assembly of the polysaccharide domain of LPS were identified by classical genetics. Four of these loci were shown to contain tetranucleotide repeats near the 5' end. It is thought that during DNA replication homologous strands mis-pair in the repeat region, culminating in the loss or gain of one or more repeats. This places the downstream coding sequence in or out of frame with the upstream initiation codon. Thus a translational switch mechanism is generated. The availability of the whole genome sequence of *H. influenzae* (strain Rd) permitted the identification of another tetranucleotide repeat-containing LPS gene along with up to 30 more non-repeat-containing candidate LPS genes. Importantly, the high-frequency on–off switching of phase-variable loci permits the generation of a plethora of LIPS glycoforms. The most appropriate form may then be selected at each stage of pathogenesis.

3.5. Capsule

Polysaccharide capsules are considered important determinants of pathogenicity in several species of bacteria ***(115)***. *H. influenzae* may express one of six antigenically and chemically distinct capsular polysaccharides, designated a–f ***(2)*** **(Fig. 2)**. Type a and b polysaccharides differ from types c, d, e, and f in that they contain the five-carbon sugar, ribitol ***(116)***. The type a capsule consists of a polymer of glucose-ribitol phosphate, while type b consists of poly-ribose ribitol phosphate (PRP). Types c and f contain 2-acetamido-2-deoxyhexose and are O-deacylated ***(117,118)***. Type d and e polysaccharides contain 2-aceta mide-2-deoxy-D-mannose uronic acid ***(119,120)***.

3.5.1. Contribution of Capsule to Pathogenesis

A strong correlation was established between expression of capsule by *H. influenzae* and invasive disease in humans ***(4)***. Interestingly, it was reported

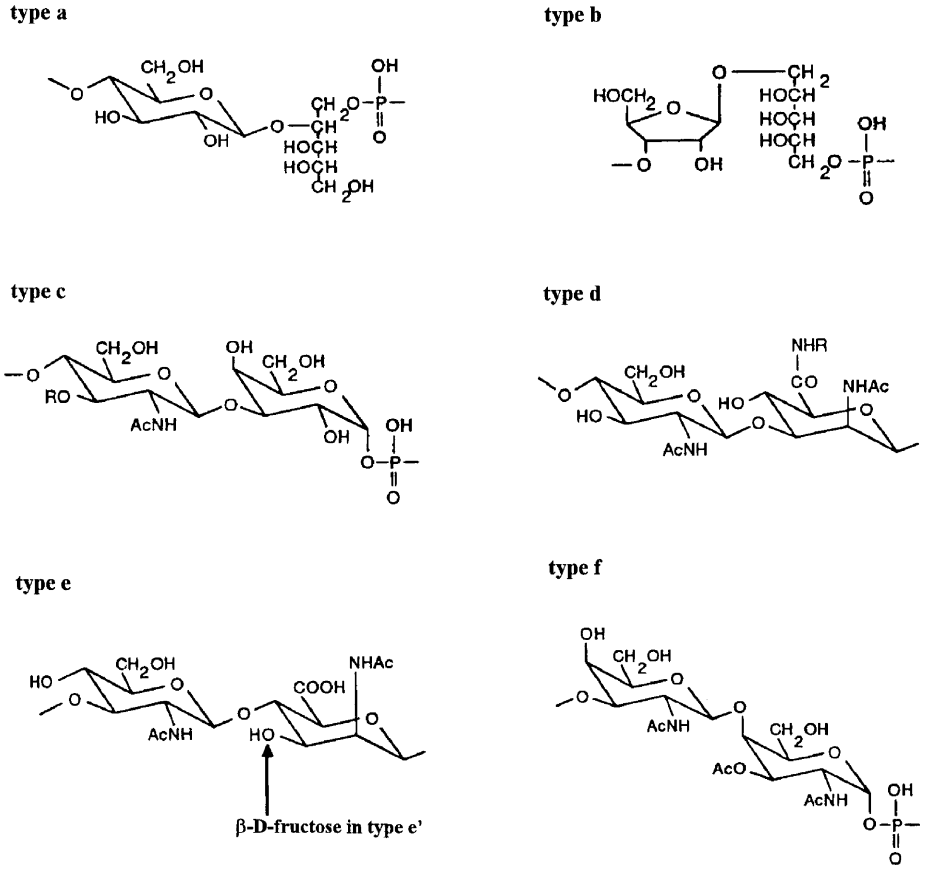

Fig. 2. Structure of the capsular polysaccharides of *H. influenzae* (serotypes a–f).

that whereas each serotype could successfully colonize the nasopharynx, type b strains accounted for more than 95% of systemic disease in humans ***(4,121)***. Similar findings were reported in the infant rat model of infection ***(48)***. After intraperitoneal (ip) inoculation, all encapsulated strains possessed the potential for systemic infection, but type b strains were found to be the most virulent. Noncapsulated strains were noninvasive. Furthermore, after intravenous (iv) inoculation, only type b strains caused persistent bacteremia. This investigation was extended by the construction of a series of capsular transformants representing all six serotypes of *H. influenzae* which, other than capsule, were identical with respect to OMP profiles and LPS ET ***(99)***. After intranasal inoculation, all strains colonized the nasopharynx, but bacteremia was generally detected only in serotype a and b strains. After ip administration, the type b strain was

found to be significantly more virulent than each of the other transformants as assessed by the magnitude of bacteremia 48 h after inoculation.

3.5.2. Genetics of Capsule Expression

A single clone of *H. influenzae* can synthesize only one capsular serotype, which does not show antigenic variation. However, the quantity of capsule expressed by an individual bacterium can vary. This has been shown in serotype b strains of phylogenetic lineage 1, which are the majority of strains that cause invasive infection ***(122,123)***. Capsule production depends on a cluster of genes in an 18-kb chromosomal locus termed *cap*. The *cap* locus may be divided into three regions. Region 1 contains the bex genes *(bexA-D),* of which *bexA*, at least, is essential for the capsule export to the cell surface. Region 2 contains four genes involved in polysaccharide biosynthesis. This region is capsule-type specific. Region 3 contains two open reading frames (ORF)s that are thought to be involved in polysaccharide export. It is reported that in about 98% of type b strains there is a deletion of part of one copy of *bexA* in an otherwise duplicated cap locus, that is flanked by direct repeats of the insertion sequence IS1016 ***(124,125)***. The type b *cap* locus in lineage I strains exists mostly in duplicate form, one copy of which has a deletion in *bexA*. As a result of the duplication, recombinatorial loss of one copy of *cap* occurs, leaving the copy with the *bexA* deletion. Irreversible loss of capsule expression occurs ***(126)***. These "class 1" mutants are generated at a frequency of approx 20% during late exponential liquid culture growth. Secondary mutations arise that alleviate potentially lethal consequences of the buildup of PRP within the cytoplasm ***(126)***. The presence of the insertion element also facilitates amplification of the copy number of *cap* and up to five copies have been detected in clinical isolates ***(123)***. The quantity of expressed capsule is increased in a gene-dose manner, which may be crucial to serotype b pathogenesis. Organisms producing more capsule may have a selective advantage in the respiratory tract, for example, where the hydrophilic capsule may provide a physical barrier that protects them from desiccation and promotes resistance against nonspecific attack by neutrophils and macrophages ***(127)***. Organisms that lose the ability to express capsule may have a selective advantage in invasion of host cells. Several research groups have provided evidence that capsule-deficient mutants show enhanced adherence to and invasion of human epithelial cells compared with wild-type strains ***(128–130)***. This has also been demonstrated for endothelial cells. Virji and co-workers investigated interactions of capsulate (b+) and capsule deficient (b−) Hib with HUVECs ***(46)***. The presence of type b capsule resulted in decreased bacterial association with endothelial cells. More b− bacteria were internalized by the HUVIECs compared with b+.

3.5.3. Development of Capsule-Based Vaccines

Antibodies to the capsular polysaccharide of Hib mediate protective immunity. Adults make highly effective thymic (T)-independent responses to these polysaccharide antigens, including IgM and IgG2 responses, but T-cell-independent responses are weak in the immature immune system of infants until after 118–24 mo old ***(131–134)***. Young infants are therefore more susceptible to infection. To achieve immunoprophylactic control of Hib meningitis caused by Hib, attempts to generate a more effective vaccine were developed that stimulated the production of T-dependent antibodies. As T-cell help is recruited in response to protein antigens, chemically linking the capsular polysaccharide to a protein carrier induces T-cell responses such that B cells proliferate and produce antibodies to the polysaccharide. During the 1980s, several conjugate vaccines were developed for use against disease caused by Hib. These consisted of PRP conjugated to T-dependent immunogens such as diphtheria toxin (PRP-D) ***(135,136)***, an OMP of *N. meningitidis* (PRP-OMP) ***(137)*** and tetanus toxoid (PRP-T) ***(138)***. The immunogenicity of these vaccines was modified by adjusting the structure and length of the polysaccharide, the ratio of protein to carbohydrate, as well as the method of coupling the protein with the polysaccharide. Each of these vaccines was found to induce an enhanced antibody response to PRP and a vigorous booster response when administered to young children ***(139,140)***.

Since 1989 in North America and 1992 in the United Kingdom, the Hib vaccine has been administered to infants, typically PRP-T at 2, 3, and 4 mo of age in the UK. Booy and colleagues studied the efficacy of this vaccine over a 3-yr period (1992–1995) in the UK and reported it to be overall greater than 98% effective in preventing *H. influenzae* type b disease ***(141)***.

Despite the success in preventing Hib diseases, there is still a need to develop vaccines that are effective against nontypeable *H. influenzae*. LPS and OMPs have been investigated in this capacity ***(142,143)***.

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