

Nontypeable *Haemophilus influenzae*: Pathogenesis and Prevention

A. RUTH FOXWELL, JENNELLE M. KYD, AND ALLAN W. CRIPPS*

*School of Human and Biomedical Sciences, Faculty of Applied Science,
University of Canberra, Belconnen, Australia*

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INTRODUCTION

Respiratory tract infections associated with nontypeable *Haemophilus influenzae* (NTHi) are a major cause of morbidity and mortality in both developed and nonindustrialized nations. The success of this organism as a colonizer and pathogen is due to its lack of reliance on any single mechanism of attachment and its ability to respond rapidly to host defense mechanisms by antigenic variation of proteins and enzymes. First we review the interaction between NTHi and the human host, with particular emphasis on mechanisms of adhesion, increased mucin production, and evasion of host defenses via immunoglobulin A (IgA) proteases, epithelial cell entry, and antigenic variation. Then we review vaccine strategies with emphasis on the potential of outer membrane components of NTHi to stimulate appropriate humoral and cellular immune mechanisms for prevention of infection or immunomodulation of chronically infected individuals.

NONTYPEABLE *HAEMOPHILUS INFLUENZAE* AS A HUMAN PATHOGEN

Efforts expended on the development of a vaccine for *H. influenzae* type b came to fruition in 1985. Studies have shown the efficacy of this preventative strategy by a subsequent drop in morbidity and mortality due to infections with *H. influenzae* type b (174, 178).

The focus in this area of human health has now turned to NTHi. Since the vaccine for *H. influenzae* type b is directed against its type-specific polysaccharide capsule, it has no ability to prevent infections caused by the nonencapsulated NTHi. Good reviews of infections caused by NTHi are given by Murphy and Apicella (138) and St. Geme III (186); however, a brief highlight of the more important infections is presented below.

Lower Respiratory Tract Infections

Lower respiratory tract infections, associated with NTHi, are a major cause of mortality in both infants and children in developing countries (13, 59, 60, 179), with morbidity and the ability to predispose the individual to more serious disease being highly significant in both developed and developing na-

* Corresponding author. Mailing address: Faculty of Applied Science, P.O. Box 1, Belconnen, ACT 2616, Australia. Phone: 61 6 201 5047. Fax: 61 6 201 5402. E-mail: cripps@science.canberra.edu.au.

tions (23, 88, 146, 183, 193, 212, 213). Particularly prevalent are infections in hosts with an underlying disease which affects the innate mucosal immune system, such as chronic obstructive pulmonary disease and cystic fibrosis (146, 186).

Otitis Media

The prevalence of otitis media during the first 3 years of life has enormous effects on intellectual ability, school achievement, speech, and language (193). Up to 100% of children in developing communities and 62% of children in more developed communities will have their first episode of otitis media by the age of 1 year (184, 194). NTHi is responsible for between 27 and 37% of these episodes (88, 138, 188).

Other Infections

The parasitic nature of NTHi on the upper respiratory mucous membranes (103) results in the majority of cases of infection with this organism being located in the middle ear, conjunctiva, sinus, and lower respiratory tract (138). NTHi and *Streptococcus pneumoniae* account for the vast majority of acute and chronic infections in these areas (138, 186).

NTHi colonization of the genitourinary tract also predisposes prepubescent females to infection and is a source of maternally derived neonatal infection (211).

BALANCED PATHOGENESIS

The questions must surely arise how and why this organism changes from a relatively harmless commensal into an agent of infection. Survival of the bacterial species depends on the ability of the NTHi to parasitize the mucous membranes of the human host (103). Infection and disease represent an imbalance of colonization. From this perspective, NTHi should coexist in a balanced relationship with its human host. Of course, the spread of different subtypes to allow exchange of genetic information and subsequent evolution of adaptive diversity for NTHi is essential, and it may be due to this requirement that the balanced state is disturbed.

One can speculate that the imbalance in this state is due to the host, the microenvironment, and the organism. This paper will review and comment on aspects of the organism's virulence factors known to affect colonization by and subsequent disease due to NTHi. It will also look at outer membrane components of NTHi and discuss the potential of these antigens as suitable vaccine components.

CLASSIFICATION AND SUBTYPING

NTHi strains are small gram-negative coccobacilli, which neither produce nor have the genetic material to code for a polysaccharide capsule. They grow aerobically or as facultative anaerobes; require protoporphyrin IX or iron-containing protoheme, NAD, or NAD phosphate; and grow optimally between 35 and 37°C. They are chemorganotrophic and occur as obligate parasites on the mucous membranes of humans (103).

Eight major systems for classifying *H. influenzae* are currently available. Capsulated strains are divided into six serologic types, a through f, based on the differences in their polysaccharide capsular material (159). The seven remaining classification systems deal with noncapsulated *H. influenzae* (i.e., NTHi) and are elegantly summarized by Murphy and Sethi (146). They include biotyping (102); outer membrane protein (OMP) differentiation by molecular weight (143) or antigenic heterogeneity (137); classification by lipooligosaccharide (LOS) antigenic heterogeneity (19); and determination of

genetic differences by electrophoretic typing (147, 161), restriction fragment length polymorphism (74, 117, 125), and PCR amplification (202).

Subtyping relates not only to predicting the virulence factors and the pathogenesis of the disease process in an individual but also to the origin and prevention of spread of the microorganism. The methods of subtyping used so far are helping to develop an epidemiologic picture; however, the results of surveys need to be interpreted with caution due to diversity in NTHi genotypes, the ability to change OMPs under immunological pressure, and, as yet, the small number of reports linking subtype to disease group or geographical location.

OMP typing has been successfully used to show clonality in a nursing home nosocomial infection (64), yet OMP P2 has demonstrated variation in molecular size and antigenicity in serial isolates of the same strain from chronic obstructive pulmonary disease patients (75), and from NTHi in long-term tissue infections set up in rabbits (40).

Musser et al. (147) noted that the majority of infections from *H. influenzae* type b strains were clonal in origin, while several groups have demonstrated that infections caused by NTHi strains show a marked genetic and phenotypic diversity (74, 117, 160, 199). The diversity of NTHi is particularly pronounced when organisms have been isolated from infections rather than as part of the normal flora (199). Subdivision of *Haemophilus* species into biotypes on the basis of biochemical reactions to indole, urease, and ornithine decarboxylase (102) mirror the genetic findings of diversity by showing that the majority of *H. influenzae* type b isolates fall into Kilian's biotype I group whereas the majority of clinical NTHi isolates are in biotype I, II, III, or IV of the eight biotypes (11, 18). Musser et al. (147) suggested that Kilian's biotype IV be placed in a separate group due to its marked genetic diversity; however, a higher mean genetic diversity appears to be common in disease-producing NTHi isolates, as discussed by van Alphen et al. (199).

The relative importance of NTHi within the *Haemophilus* group may have been underestimated. Wallace et al. (212) cast doubt on the reliability of the statistics, noting that serotyping performed by slide agglutination, rather than counterimmunoelectrophoresis or immunofluorescence, falsely classified many NTHi strains as *H. influenzae* type b.

ANIMAL MODELS

A number of infection models in the rat, mouse, and chinchilla have been developed to help understand the pathogenesis of NTHi infection and to assess potential vaccine candidates for their ability to prevent both colonization and infection by NTHi. Since NTHi is not a natural colonizer or pathogen in any of the animal models used, there are significant limitations to the usefulness of these models when attempting to make comparisons with human infections (153). Useful data has been provided, however, by in-depth histological and antibody analysis of the pathogenic events related to infection and of the development of vaccine strategies.

Of particular interest to this review are the otitis media models in chinchillas (4–6) and rats (120), and mouse and rat models of pulmonary infection (84, 85, 112, 197, 208, 210).

The middle ear of rats is structurally similar to that of humans, and direct inoculation of both NTHi and *S. pneumoniae* into the tympanic bulla has produced pathologic changes similar to those seen in humans (120). Chinchillas have been used extensively in studies of otitis media due to their susceptibility to NTHi, the ability to induce otitis media after colonisation of the nasopharynx, and the large size of their bullae (4, 6).

Infection has been achieved through either intranasal inoculation after viral infection or direct inoculation of NTHi into the bulla (4–6). Prevention of the development of otitis media in the chinchilla model has successfully helped in the assessment of potential vaccine candidates, as stated in the section on outer membrane proteins (below).

Pulmonary NTHi infections in mice and rats have been hampered by the rapid clearance of bacteria from the lungs (197, 208). The only chronic pulmonary model established so far required chemically induced damage before long-term NTHi infection could be established (182). However, pulmonary models in these rodent species have been useful in establishing baselines for differences in the pathogenesis of disease in immune and nonimmune animals (84, 85, 208, 209), looking at immunity induced by mucosal versus systemic delivery of immunogens, and assessing the potential of individual proteins as vaccine candidates (109–111). All rodent pulmonary models discussed in this review involve direct bolus inoculation of NTHi into the lungs via the trachea (197, 208). A reliable model which parallels acute exacerbations in animals with chronic lung conditions would be most helpful in studying pulmonary infections with NTHi.

MECHANISMS OF COLONIZATION

The ability of NTHi to attach to the mucous membranes or extracellular matrix is essential to allow the organism to multiply in a particular host (51, 121, 219). This host-organism interaction is very tissue specific and is dependent on the receptors produced by the mammalian tissue type, the particular ligands produced by the bacterium, the microenvironment of the tissue, and other organisms colonizing that particular area (93).

Interaction with Mucin

NTHi colonizes the nasopharyngeal region in up to 80% of humans (198). Its ability to colonize other tissue types, even in close proximity to areas of normal colonization, appears to be dependent largely on the presence of an underlying disease state in that particular patient (138).

In vitro studies suggest that NTHi preferentially adheres to respiratory epithelial cells that either lack cilia or are structurally damaged (52, 165). Cellular damage in the host may occur as a result of numerous factors unrelated to the presence of bacteria. However, the presence of NTHi can cause stasis and loss of cilia, as well as damage and sloughing of epithelial cells due to the secretion of heat-stable substances such as LOS (38, 98). LOS occurs in both cell-bound and secreted forms (76). The LOS released from NTHi appears to be a far more potent activator of inflammatory cytokine production by monocytes, more toxic to mice, and more active in the *Limulus* ameocyte lysate assay than is the cell-bound LOS (76). The production of heat-labile substances, via a nontoxic process, also culminates in a cycle that increases mucin production from the host cells lining the respiratory tract (1). It may well be that this heat-labile substance, with a molecular mass between 50 and 200 kDa (1), is responsible for the vigorous activity seen in goblet cells in the middle ear mucosa of rats after infection with NTHi (120).

Certain OMPs of NTHi such as P2, P5, and as yet an unidentified 21- to 31-kDa OMP have the ability to bind to the sialic acid-containing oligosaccharides of mucin in the nasopharynx, thus initializing the binding process of colonization (166). The process of binding is specific and enhances the ability of some NTHi strains to adhere to mucin-coated naso-

pharyngeal epithelial cells (32, 167). Examination of the eustachian tube in chinchillas after NTHi infection demonstrated that adherence and growth of NTHi in the mucus could account for the onset of otitis media in the chinchillas (128).

Interestingly, this ability to bind mucin appears to be down-regulated by NTHi once the microbe enters the bloodstream (108). Isolates from the bloodstream show marked binding to lactoferrin, in contrast to variable binding by respiratory isolates. Kubiet and Ramphal (108) speculated that the down-regulation in mucin binding and the subsequent upregulation in lactoferrin binding was an active process initiated by the bacteria to escape the respiratory tract and invade the bloodstream. This may represent a naive attempt by the NTHi to escape from a hostile environment to an environment that has increased levels of nutrients. Alternatively, the organism may be upregulating this receptor as a mechanism for obtaining iron in an iron-restricted environment or as a decoy mechanism to compete for lactoferrin. Lactoferrin has the ability to bind the lipid A portion of the LOS, resulting in a complex that disrupts the integrity of the outer membrane and kills the bacterium (see below) (44, 45).

Interaction with Mammalian Surface Structures

NTHi can interact with mammalian surface structures either through secreted substances such as the Hap protein (see the section on IgA proteases, below) or through direct adhesion of NTHi to mammalian epithelial cells.

Barenkamp and Bodor (8) noted bactericidal antiadhesion antibodies in sera from otitis media patients and subsequently defined two surface-exposed high-molecular-weight proteins (HMW1 and HMW2) related to the filamentous haemagglutinin of *Bordetella pertussis*. Antiserum raised against a recombinant protein binding with both HMW1 and HMW2 recognized high-molecular-weight proteins in 75% of the 125 heterologous NTHi strains tested (9). These proteins were shown to mediate attachment to human conjunctival cells in vitro. HMW1- and HMW2-negative mutant NTHi strains showed a marked reduction in their ability to adhere to the conjunctival cells; however, some adhesion was noted, reaffirming that adhesion of NTHi to mammalian epithelial cells is multifactorial.

HMW1 and HMW2 proteins on NTHi strains bind mammalian surface structures containing glycoproteins related to heparan sulfate (152, 187). This allows binding to a wide variety of mammalian cells including mononuclear phagocytes. It also helps the bacteria to colonize damaged tissue sites and invade subepithelial tissues due to the predominance of collagen proteoglycans containing protein-bound glycosaminoglycan (gag) chains in the extracellular matrix (219). HMW1 has an enhanced cellular binding specificity for oropharyngeal cells, whereas HMW2 binds with greater affinity to genital tract epithelial cells, allowing NTHi strains expressing these proteins in different concentrations to potentially bind to either respiratory or genital tract epithelium (93). A study indicating HMW1 or HMW2 dominance in wild-type isolates from the respiratory and genital tracts would enhance the understanding of the pathogenesis of NTHi with respect to these proteins. In NTHi isolates not expressing HMW1 or HMW2, an alternative high-molecular-weight protein (molecular mass, 114 kDa), coded for by the *hia* gene, has been identified. The Hia immunodominant protein appears to mediate adhesion to Chang epithelial cells, and the *hia* gene was present in 13 of the 15 strains which were deficient in HMW1 and HMW2 (12).

Some NTHi strains also possess the gene encoding the pilin protein (57). In strains that express the protein, the adherence

and antigenic properties are similar to those of *H. influenzae* type b. Assembly of the pilin protein into a pilus may be different in NTHi. This may account for the relatively weak correlation between the presence of a pilus and binding to epithelial cells (61) and indicates that the pili of NTHi have additional mechanisms of buccal cell adherence. The genes encoding the major structural subunit of the pili, HifA, show homology across NTHi and *H. influenzae* type b strains; however, the conserved immunodominant epitopes present on *H. influenzae* type b strains so far identified are unavailable to bind antibody in their native form (62). Pilin-mediated binding occurs only to sialic acid-containing lactosylceramide structures (GM₃) on oropharyngeal epithelial cells and erythrocytes (201). The abundance of GM₃ receptors in the upper respiratory tract leads to speculation that once the normal microbiota of the respiratory tract is disrupted, lactosylceramide binding NTHi may colonize the respiratory tract in excessive numbers, causing a greater imbalance in the normal microbiota. *H. influenzae* also binds to asialoglycosphingolipids that are found extensively in the respiratory tract (107). This possible strategy allowing NTHi dominance by increasing the number of host cell sites to which they bind, at the expense of a normal mixed microbiota, can be likened to the overgrowth of *Clostridium difficile* in the gut after antibiotic treatment. Therapy with *Lactobacillus* spp. addresses the microbial imbalance due to *C. difficile* and successfully treats the colitis (66, 100). The use of recolonization by another member of the normal respiratory tract microbiota may help address overgrowth by NTHi before degeneration into a disease state occurs.

A less well characterized adhesin for NTHi is the heat-modifiable fimbriin protein with a molecular mass of 25 kDa. This protein has a high degree of homology to OmpA of several other gram-negative bacteria and P5 from *H. influenzae* type b and has been observed on all clinical isolates to date. The disruption of the fimbrial gene reduces but does not eliminate the in vitro ability of the bacteria to adhere to oropharyngeal cells (181). This reduction in adhesion corresponds with a reduction in virulence in the chinchilla otitis media model.

Tissue tropism, orchestrated by the surface-exposed adhesins of NTHi, also can modulate the immune response mounted by the host. Limited experiments with genetically engineered NTHi have shown that adhesins specify both the inflammatory cell to which the organism will bind and the consequences for both microbe and mammalian cell. In these experiments, clinically isolated NTHi strains remained extracellular and viable when they bound to macrophages via their high-molecular-weight proteins. This was in contrast to the laboratory-manipulated nonencapsulated *H. influenzae* strains, derived from encapsulated organisms, which readily bound to macrophages but were readily phagocytosed independent of serum opsonins (151).

Lipooligosaccharide

The ability of LOS to contribute to the interaction between NTHi and the mammalian host is multifactorial. The LOS of *Haemophilus* species is a major surface antigen and makes up approximately 4% of the organism's dry weight. Despite the lipid portion being similar to that of other gram-negative bacteria, the LOS contains short oligosaccharide side chains compared with the longer polysaccharide side chains of the members of the family *Enterobacteriaceae* (56). Functionally, the LOS from *Haemophilus* spp. appear very similar to the lipopolysaccharide (LPS) purified from the *Enterobacteriaceae*, with rabbits exhibiting both the typical endotoxic Shwartzman

reaction and a biphasic febrile response on exposure (56). The between-strain heterogeneity of the LOS structure aids in the evasion of host immune defense mechanisms (19, 106, 226).

Despite the heterogeneity seen, certain oligosaccharide epitopes are conserved among different species of *Haemophilus*, *Neisseria*, and *Branhamella* (20, 122, 157, 214). A broadly cross-reactive epitope, identified by the monoclonal antibody 3F11, has been observed in 10 of 16 NTHi strains studied (122) and also recognizes the disaccharide Gal β 1-4GlcNAc, which is present on human cell surfaces. Further studies have found NTHi strains with an LOS epitope which binds to Gal β 1-4Glc β 1, a disaccharide present on lactosylceramide, a precursor of human blood group antigens (157, 205). The presence of these digalactose moieties on both NTHi and mammalian cells suggests host mimicry as a mechanism of evading the mammalian defense system (130).

Phase variation to a higher-molecular-weight LOS enhances the adhesion of *Haemophilus* strains to rat nasopharyngeal cells (215). Similar LPS- and LOS-based adhesion characteristics have also become apparent for many other gram-negative bacteria (for a review, see reference 95). The ability to express the higher-molecular-weight phase of LOS may be an advantage to NTHi by initially increasing its ability to adhere; however, it has been suggested that this higher-molecular-weight phase also increases bacterial clearance once dissemination occurs. An inefficient ability to alter the LOS to a lower-molecular-weight phase would thus increase the likelihood of mucosal colonization rather than systemic infection (215, 217).

LOS contributes directly and indirectly to lung damage. The lipid A portion, found as a heat-stable soluble product, inhibits the movement of cilia in rats (38, 98). LOS also induces an inflammatory response from the host (226). Rapid recruitment of polymorphonuclear leukocytes (PMNs) to the pulmonary spaces can occur by LOS-mediated enhancement of mammalian heat shock protein 70 production (224) or by the production of tumor necrosis factor alpha (TNF- α) from activated alveolar macrophages (149).

Once the PMNs arrive in the lungs, LOS priming of the PMNs enhances the ability of the bacteria to cause damage that results in respiratory distress or endotoxic shock (78). The increase in the number of PMNs in the lungs is accompanied by a general inflammation of the site that increases the level of proteins such as transferrin, acute-phase proteins, and lactoferrin in plasma. Binding of LOS to these proteins downregulates the PMN response to LOS, providing a mechanism for regulating the PMN response during inflammation (207). Lactoferrin, found in PMN granules and mucosal secretions, binds to the lipid A moiety of LOS, resulting in both bacterial stasis and death (3, 44, 45). The binding of LOS to the lactoferrin decreases the ability of the bacteria to prime PMNs without affecting the ability of lactoferrin to act as a free radical (26).

LOS and peptidoglycan from NTHi have also been implicated in chronic low-grade inflammation at mucosal surfaces. LOS and peptidoglycan from the NTHi cell wall remaining as cellular debris, after clearance of the bulk of infecting organisms, may be involved in long-term disruption of clearance of transient bacteria (113). Increased levels of LOS in chinchillas with NTHi-induced otitis media also were associated with increased amounts of middle ear fluid (96). Limited production of antibody to LOS may demonstrate induction of tolerance to that organism (37).

MECHANISMS OF EVASION OF HOST DEFENSES

The human host has developed a barrage of innate and acquired forms of defense to retain the pathophysiological bal-

ance that allows the tissues to function in their optimal form. Bacteria such as NTHi have developed a number of systems to either evade these host immune defenses, use them to their own advantage to enable them to exist as commensals, or take advantage of altered surroundings and cause disease. As was seen with adhesion, NTHi has utilized a multifactorial approach to achieve this goal.

IgA Protease

IgA is an extremely important antibody at all mucosal surfaces, with IgA1 being the dominant antibody in the upper respiratory tract (101). Children with atopic conditions have increased levels of cleaved IgA1 in nasopharyngeal secretions compared to healthy children of the same age range (104). This coincides with a larger number of IgA1 protease-producing bacteria colonizing the upper respiratory tract. One of the major colonizers in this situation is NTHi (104). Strains of *H. influenzae* produce three types of IgA1 protease, all of which cleave the heavy chain of IgA1 in the hinge region at one of several postproline sites (105, 133). The cleavage of the heavy chain results in a monomeric Fab fragment, which retains its ability to bind the antigen, and either a mono- or dimeric Fc fragment, which loses its biologic functions (162). Despite the loss of function of the Fc portion, the Fab portion of the antibody may still perform a partial neutralizing function, after binding of the NTHi, through altering the ability of the NTHi to bind to its host cell by steric hindrance of the binding site. The majority of capsulated *H. influenzae* strains produce only one of the three types of IgA1 protease mentioned above. This correlates with its serotype but not its biotype. In contrast to the encapsulated strains, NTHi strains produce proteases from any of the three types (133).

The IgA1 proteases derived from *H. influenzae* are antigenically quite diverse, with more than 30 antigenic types having been recognized. The polymorphism is more pronounced in NTHi than in encapsulated *Haemophilus* spp. (116). In a limited clinical study it was found that healthy children show frequent clonal exchanges by replacing one NTHi clone expressing IgA1 protease with an antigenic type of IgA1 protease not previously encountered by that host (116). Patients with chronic obstructive pulmonary disease, however, retain the same clone of NTHi in their lower respiratory tract over a longer period. The NTHi strains remaining in the lower respiratory tract have demonstrated the ability to change their IgA1 protease under immunological pressure, so that it not only cleaves IgA at a different site but also changes its antigenic properties. The restriction fragment length polymorphism analysis of strain variation used by Lomholt et al. (116) and the reversion seen to the original strain would indicate that the change is a point mutation; however, production of a new gene product could not be totally ruled out. This ability to change antigenic presentation to the host can render the previously derived neutralizing antibodies inactive against the newly formed IgA protease (116). The same evasion mechanism is used by some outer membrane proteins of NTHi, such as P2, in response to pressure from neutralizing antibodies (75).

A specific function or advantage of possession of IgA1 protease has not yet been demonstrated in the pathogenesis of NTHi (52). It has been suggested that the IgA protease may belong to a larger family of proteins which have a dual function of protease activity and adhesion (189). The products of the *hap* gene of NTHi demonstrated significant homology to the IgA proteases. Not only did they share catalytic domains and processing and secretion pathways with the proteases, but also both endowed the bacteria with a 45-kDa protein inserted into

the outer membrane after cleavage of a 100- to 110-kDa secreted protein (189). Possession of the *hap* gene allowed NTHi to attach and invade epithelial cells in vitro. *Escherichia coli* was also shown to produce an homologous protein which was important in its adherence to the avian respiratory tract (163). Schmitt and Haas (175) have also described a protein from *Helicobacter pylori* that demonstrates marked structural homology to the IgA proteases of NTHi. This *H. pylori* protein induces the formation of intracellular vacuoles in epithelial cells. It has been demonstrated, however, that IgA1 protease-deficient mutants behave in a similar manner to the wild-type strains in terms of mechanisms of attachment, invasion and ability to remain within phagocytic vacuoles (52). As mentioned above, the ability of NTHi to be associated with the respiratory epithelium is multifactorial, and it is therefore conceivable that secreted products, such as the Hap protein and IgA1 protease, could alter either the mammalian or the bacterial surface to facilitate bacterium-host interaction.

Entry into Epithelial Cells

NTHi strains have not traditionally been thought of as invasive. More recent work, however, has demonstrated that the bacterium may enter the host cell, presumably to evade the local immune response. This response has been demonstrated with several *Haemophilus* species.

Capsule-deficient *H. influenzae* strains invade endothelial cells and remain within membrane-bound vacuoles over an extended period with no apparent effect on the host cell (205). Wild-type NTHi strains have been found, in vitro, to adhere to, invade, and persist in Chang epithelial cells (190). The relationship between the bacterium and the mammalian cell appeared to be a dynamic interaction, since both adherence and invasion of the NTHi increased over time. Intracellular entry was also accompanied by penetration of the mucosal surface at points of necrosis and cell junction. Following on from this, Van Schilfgaarde et al. (204) coined the phrase "paracytosis" when describing NTHi adherence to and subsequent passage through lung epithelial cell lines. In these experiments, highly adherent strains demonstrated greater paracytosis. The passage time was independent of inoculum size, fimbriae, or capsule but, was dependent on the rate of bacterial multiplication, with rapidly growing strains taking 10 to 18 h and slower growing strains taking 30 h to pass between the apical and basolateral chambers. Chloramphenicol addition prevented paracytosis, indicating that de novo protein synthesis was required for the process to occur.

Paracytosis is both an in vitro and in vivo phenomenon. Adenoids removed from children with persistent otitis media or adenoidal hypertrophy were shown to have up to 10^5 viable intracellular *H. influenzae* cells in the reticular crypt epithelium and macrophage-like cells in the subepithelial layer of tissue (58).

The mechanism for uptake into the cell is unknown; however, under strategies outlined by Falkow (51) and Isberg and Tran Van Nhieu (94), it may be associated with the Hap protein or indeed the gag-dependent binding of the high-molecular-weight proteins on the bacterial surface (187, 189).

Antigenic Variation

Within the host, the successful organism must be able to avoid or adapt to evolving host defenses. As a commensal and pathogen, NTHi has demonstrated its ability to adapt by varying both the proteins and the LOS that constitute the major components of the outer membrane.

Several groups have demonstrated the heterogeneity that

exists in the OMPs of NTHi (11, 74, 75, 117, 143, 185). At any one time, the human oropharyngeal area appears to be colonized by one predominant strain of NTHi; however, up to seven different strains may be present (49, 116, 185). The dominant strain remains in the nasopharynx during episodes of otitis media (117) and increases in number (50), providing a selective advantage (16).

The importance of antigenic differences in proteins among strains of NTHi has been demonstrated in protection of chin-chillas against otitis media. It was found that resolution of infection after intrabullar inoculation of one ear produced a protective response to rechallenge in the other ear with a homologous but not a heterologous strain of NTHi (99). Likewise, recurrent otitis media in humans has been caused by NTHi strains with only minor changes in their OMPs (142). Some studies further indicate that NTHi strains cause infection in patients despite the apparent presence of specific antibodies in serum and sputum (73, 86). However, the crudity of the outer membrane preparations used in these studies would probably not allow differences to be determined at the epitope level (146).

Certain major OMPs, such as P2, are dominant in inducing an effective antibody response during disease states such as exacerbations of chronic obstructive pulmonary disease (139). The development of antigenic drift in the immunodominant epitopes of P2 has allowed NTHi to avoid complement-dependent killing. The antigenic drift is caused by small changes in amino acid composition within the variable region of the protein, allowing the organism to persist for a longer time despite an apparent appropriate immune response from the host (75, 129). In vivo experiments have further demonstrated antigenic drift in P2 in a model of subcutaneous chronic infection with NTHi in immunized and nonimmunized rabbits over a period of 948 days (206). The postinfection drift in the P2 protein occurred more rapidly with animals immunized with a homologous rather than a heterologous NTHi strain.

Proteins P1 and P5 are also targets of bactericidal antibody in humans (10, 47, 63, 86) and have demonstrated the ability to change to avoid the host immune system during times of persistent infection (10, 74, 75, 129, 134). The model of chronic infection in tissue cages in rabbits, described by Vogel et al. (206), did not demonstrate alterations in the P5 protein, despite the drift in P2 mentioned above. The authors suggested that antigenic drift in P5 may be dependent on a particular host environment in addition to persistence. Development of an animal model with persistent NTHi lung infection would allow investigation of P5 variation in NTHi in a more natural environment.

As with the protein antigens in the outer membrane, NTHi exhibits a more heterogeneous population with respect to LOS than do the encapsulated forms. *H. influenzae* type b isolates can be placed in three separate groups, depending on their ability to bind two monoclonal antibodies directed against LOS epitopes on two distinct molecules (156). NTHi strains, on the other hand, could not be split into fewer than 14 groups based on their LOS composition (155). As with *Neisseria* species and *B. pertussis*, a single strain of NTHi usually produces a heterogeneous range of LOS (157). In both NTHi and *H. influenzae* type b, the LOS variation is partially due to phase variation encoded for by the *lic-1* and *lic-2* loci (216, 217). The invasive capacities of *Haemophilus* strains are enhanced by this method of phase variation (217). This may be a positive selection process due to the relatively elevated levels of LOS expressed during the log phase of growth (225).

COMPONENTS OF ANTIGENIC INTEREST

As mentioned above, secreted substances and adhesins appear to be important for establishing a niche for NTHi in the ciliated regions of the respiratory tract (1, 38, 165). Molecules making up the outer membrane of NTHi may also be useful in preventing colonization and subsequent infection. The outer membrane of *H. influenzae* is very similar to that of the majority of gram-negative bacilli, containing phospholipid, LOS, peptidoglycan, and protein. However, it has a more fragile cytoplasmic membrane surrounded by a more wrinkled outer membrane (29, 115).

Outer Membrane Proteins

Interest in the OMPs has centered mainly on their antigenic qualities as potential vaccine candidates and in their ability to split NTHi into subtypes that correspond to the location of infection for epidemiological purposes (11, 115, 138).

Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of both encapsulated and nonencapsulated *H. influenzae* strains show the protein component to contain up to 36 proteins of which 6 represent the major protein content (115). The major proteins seen on the SDS-PAGE protein migration have molecular weights between 50,000 and 15,000 and are labelled P1 to P6 (136) or a to f (115) in order of decreasing molecular mass. The proteins of the NTHi strains show greater variability in their migration patterns than do those of the *H. influenzae* type b strains (11, 115, 138); however, the general terminology of P1 to P6 has been transposed to the NTHi strains (112, 186).

Major outer membrane proteins. P1, or protein a, is a heat-modifiable surface exposed protein found in *H. influenzae* type b and NTHi with a molecular mass of 35 kDa at room temperature and 46 to 50 kDa when heated to 100°C (10, 115, 143). Significant variability in the primary protein sequence in the variable region of P1 and its ability to be modified by heat allowed it to be used as a form of subtyping for *H. influenzae* type b strains (10, 134). However, monoclonal antibodies raised against 8 epitopes of P1 protein from *H. influenzae* type b demonstrated significant areas of conservation among typeable and nontypeable strains (22).

The potential for P1 as a vaccine candidate against NTHi shows mixed results at this stage. Antisera raised in rabbits to any of the eight conserved epitopes showed no bactericidal activity against NTHi (22). However, P1 extracted from NTHi enhanced homologous bacterial clearance from the lungs of rats after mucosal immunization (31). Animal studies investigating the ability of P1 to protect against heterologous NTHi strains and investigations on antibody titer after NTHi infections in humans must be pursued to determine the potential for P1 as a vaccine candidate.

P2, or protein b/c, present in *H. influenzae* type b and NTHi (115, 136, 143), is the most dominant band on SDS-PAGE OMP preparations. It has been used to form the basis of a subtyping system because its molecular mass varies between 36 and 42 kDa (143). This protein exists as a trimer and functions as a porin, allowing molecules of up to 1,400 Da to pass through the membrane (30). P2 has also been reported to be a target for immunoprotection (8, 75). Bactericidal antibodies to P2 are present in normal human sera (63, 139). Immunization with a P2-LOS complex in mice (84) and purified P2 preparation in a rat model (31, 110) showed enhanced pulmonary clearance of a homologous strain of NTHi. Immunization with P2, however, did not enhance clearance as much as did immunisation with P1 or P6 (31, 112). Since P2 is the predominant protein on the surface of NTHi, the poor clearance ob-

served after immunization suggests that an immune response to P2 may be inappropriately targeted. Modulation of the B- and T-cell response in rats has been shown to influence the ability of P2 to protect them from challenge with a homologous NTHi strain (110). The interstrain heterogeneity (143) and antigenic drift (74, 75) of the organism and its ability to survive in the absence of P2 (17, 27, 171) may preclude the use of P2 as a vaccine candidate except as a carrier of other proteins inserted into its hypervariable regions (136a).

P4, or protein e, is a 28- to 30-kDa lipoprotein that is thought to be present in all encapsulated and nonencapsulated *H. influenzae* strains (11, 115). One surface-exposed epitope was conserved across the 28 clinical isolates tested. In vitro experiments have suggested that this protein has little importance for NTHi colonization by showing that the expression of this protein is downregulated during adhesion and invasion of Chang epithelial cells (190); however, it has been indicated that P4 is essential for the utilization of hemin, protoporphyrin IX, or hemaglobin as exclusive sources of porphyrin (169).

The ability of P4 to induce antibodies protective against infection with NTHi is still controversial. Antiserum raised against this protein has been shown to be both bactericidal, when antibodies were raised against the purified P4 in mice or rabbits (70), and nonbactericidal, when antibodies were raised against a mixture of P4 and recombinant PCP and *H. influenzae* peptidoglycan-associated lipoprotein (Hi-PAL) in chinchillas (72). This contradiction could be due to different host reactions to the antigen or denaturation of the antigen during its preparation as a mixture. The lack of production of bactericidal antibodies coincided with a lack of protection against otitis media in the chinchillas (72). Further studies with P4 extracted from NTHi also failed to enhance the clearance of homologous bacteria from the lungs of rats after mucosal immunization (109).

P5, or protein d, is a heat-modifiable 27-kDa OMP (115, 135). This protein is the lower-molecular-weight OMP that forms the basis of the NTHi subtyping system proposed by Murphy et al. (143). As mentioned above, this protein appears to be a fimbrial adhesion in NTHi and has demonstrated the ability to afford partial protection against a homologous strain after passive or active immunization in the chinchilla otitis media model (181). However, Munson and Granoff (135) found that rabbit antisera raised to P5 purified from *H. influenzae* type b did not elicit protective immunity in the infant-rat bacteremia model. The discrepancy in protection seen could indicate a difference in the antigenic nature of the two proteins, the response of the host, or the type of protection required in the two models, i.e., mucosal protection in the chinchilla otitis media model versus systemic protection in the rat bacteremia model. More recent studies have demonstrated that parenteral immunization with either P5 or a synthetic peptide of P5 linked to a T-cell epitope (labelled LB1) successfully enhanced elimination of NTHi from the nasopharynx of colonized chinchillas. Parenteral immunization with the P5-fimbrin also enhanced the clearance of a heterologous NTHi strain in the chinchilla otitis media model (5).

An appropriate mucosal response to a given antigen is extremely important when looking for protection against infection at the mucosal surface. It is of interest that despite both *E. coli* and NTHi being mucosal colonizers and having a related potentially protective protein (OmpA and P5, respectively) (200), they both have the ability to cause infection at mucosal sites adjacent to their areas of colonization (138, 218). The lack of immunity to these colonizers may well be related to the inability of the immune system to sample antigenic sites such as P5 or OmpA in a way in which it can mount a response to

prevent infection when innate mechanisms temporarily break down at the mucosal surface. The interstrain variation of P5 seen in NTHi (136) may be expressed as surface epitopes, and thus similar limitations of this protein as a vaccine candidate may exist to those for P2. Further studies are under way in this laboratory.

P6 (PAL), or protein g, is a 16-kDa lipoprotein found in all *H. influenzae* type b and NTHi strains (10, 115, 143, 145). It elicits bactericidal antibody in both its native (135, 141) and recombinant (71) forms. Since this protein is highly conserved among encapsulated and nonencapsulated *H. influenzae* strains (140, 148), surface exposed, and immunogenic (31, 112, 135), there has been much interest in it as a potential vaccine candidate. Experiments demonstrating enhanced pulmonary clearance of homologous and heterologous strains of NTHi, after gut immunization with the purified protein are encouraging (112). The enhanced clearance postimmunization appears to be multifactorial. Our studies (112) suggest that the enhanced clearance is partially attributable to antigen-specific B- and T-cell responses following mucosal immunization, while several authors also correlate phagocytic recruitment to the lungs (84, 112, 208) and the presence of the C5 complement protein molecule (196) with enhanced pulmonary clearance. While these results appear promising for P6 as a vaccine candidate in inducing appropriate responses to immunization, variations in the host response to P6 may reduce its usefulness. Mice and rabbits have been shown to produce bactericidal antibodies after systemic immunization with P6 and recombinant P6 (34, 222). Chinchillas, however, have demonstrated a mixed reaction to P6 immunization. In studies conducted by Green et al. (72), P6 immunization neither induced bactericidal antibodies nor protected chinchillas from challenge with a homologous NTHi strain postimmunization. The lack of induction of protection could be due to the host and/or the presentation of antigen. In these studies, combined antigen immunization was used, compared with the use of single antigens in the rabbits and mice. Parenteral immunization with P6 lipoprotein alone has recently been shown to induce bactericidal antibody against heterologous NTHi strains and also to partially protect against otitis media in chinchillas (36). A similar experience has been noted for humans, with studies indicating that children prone to otitis media may not recognize the P6 protein as an immunogen during acute bouts of infection (220). This lack of P6 recognition is further demonstrated by otitis media-prone children showing a highly variable IgG subclass response to P6 (15) and a correlation between the length of colonization and the ability to produce protective titers of secretory IgA to P6 (49). Studies in both defining the optimal presentation of P6 and assessing immunological maturity in otitis media-prone children warrant continued investigation.

The high-molecular-weight proteins (HMW1 and HMW2) identified by Barenkamp and Bodor (8) appear to be major adhesins for NTHi to epithelial cells, mononuclear cells, and the extracellular matrix (152, 219). Immunization with purified HMW1 and HMW2 proteins afforded partial protection against infection in the chinchilla otitis media model (7). Interestingly, the immunized animals all showed similar levels of antibody in serum postimmunization; however, 50% of the immunized group were infected with the homologous strain of bacteria postchallenge. Bacteria isolated from immunized animals which were infected showed downregulation of the HMW1 and HMW2 proteins, suggesting a bacterial response to immunologic pressure.

Following the identification of a second family of high-molecular-weight adhesins encoded by the *hia* gene, Barenkamp

and St. Geme (12) have proposed a vaccine combining HMW1- and HMW2-like proteins and Hia-like proteins to prevent the initial steps in NTHi colonization. St. Geme (188) suggests that construction of such a vaccine would provide protection encompassing the majority of NTHi strains.

Minor outer membrane proteins. Minor OMPs have also demonstrated potential to be recognised as immunogens by the mammalian host. The *pcp* gene product (PCP) comigrates on SDS-PAGE with P6 (PAL). It makes up only 0.5% of the OMP in *H. influenzae* and is antigenically conserved in encapsulated and nonencapsulated strains of *H. influenzae* (34). In vitro studies have demonstrated the ability of the recombinant protein to elicit in mice and rabbits a polyclonal antiserum that was bactericidal to several heterologous strains of NTHi (34). Antiserum to recombinant PCP and P6 (PAL) demonstrated synergistic bactericidal levels. Later studies, however, have indicated a possible problem with induction of bactericidal antibodies in all species, when it was demonstrated that antiserum recovered from chinchillas immunized with the purified recombinant protein had no bactericidal activity against NTHi despite the organism fixing complement on its surface. The lack of bactericidal antibodies corresponded to a lack of protection against bacterial challenge in the chinchilla otitis media model (72).

A protein with a molecular mass of 26 kDa, termed OMP 26, has been identified in our laboratory and elicits homologous and heterologous clearance of NTHi after mucosal immunization in a rat pulmonary model (111). Significant anti-OMP 26-specific IgA is induced. The gene encoding this protein is present in the *H. influenzae* Rd genome in all 20 strains tested so far (43). This protein demonstrates significant potential as a vaccine candidate, and further studies on strain conservation, immunogenicity, and identification of human antibodies postinfection are being undertaken.

A high-molecular-weight, surface-exposed protein (103 kDa), termed D15, was found to be conserved across the 32 *H. influenzae* type b and NTHi strains tested (195). Cloning and sequencing of the *d15* gene followed by Southern blot analysis further demonstrated the presence of *d15* gene across multiple serotypes and NTHi (55, 119). Antibodies to the native protein were found in eight of nine convalescent-phase sera from young children (195). Antibodies raised to recombinant D15 confer protection against bacteremia in infant rats when the animals are challenged with homologous or heterologous bacterial strains (119). Since the majority of NTHi infections are at mucosal surfaces, it would be useful to assess the ability of D15 to protect against otitis media or infection in the lungs.

The transferrin binding proteins (Tbp) have also attracted much interest in studies of *Haemophilus* spp. These proteins act as bacterial surface receptors to directly interact with the mammalian host iron binding glycoprotein transferrin (132, 176). At present, two Tbps have been identified, the iron-repressible Tbp1 with a molecular mass of approximately 100 kDa and the more variable Tbp2 with an approximate molecular mass of 85 kDa (176). Cloning and sequencing of several isolates showed Tbp1 to be highly conserved among *H. influenzae* type b and NTHi strains (118). Initially, it was thought that these proteins were essential for *H. influenzae* type b to survive as an invasive pathogen because soluble-iron chelators (siderophores) were not found in invasive *H. influenzae* type b strains (177). More recently, Hardie et al. (89) found seven invasive and two commensal isolates of *H. influenzae* type b that appeared to be able to utilize iron from a transferrin source without the use of Tbps. The mechanism of utilization was undetermined since the absence of siderophores in these strains has been established. Of the 34 commensal NTHi

strains tested in this study, only 14 (41%) demonstrated transferrin binding ability, 18 (53%) produced siderophores, and the remainder were found to be unable to utilize transferrin as the sole iron source. The Tbps of *H. influenzae* type b have been suggested as vaccine candidates because they are essential in the majority of cases of invasive disease (69, 89, 177) and because of the presence of antibodies to them in convalescent-phase sera (90). Six of eight sera from healthy adults also contained antibodies to Tbp1 and Tbp2 (91). Antiserum raised to purified recombinant Tbp2 has shown protection against bacteremia in a infant-rat model after challenge with an *H. influenzae* type b strain expressing a homologous Tbp2 protein in its outer membrane. In a similar experiment, antiserum raised to recombinant Tbp1 was not protective (118).

The value of a vaccine against the Tbps of NTHi has yet to be determined. The majority of commensal NTHi strains use several mechanisms to obtain iron (89). The notion of the pathogenic NTHi strains using Tbps as the sole source of binding iron is unlikely because this would reduce the flexibility of the bacteria to adapt to different microenvironments and thus would not be a selective advantage. It would also be interesting to further investigate the effect of Tbp antibody on the levels of NTHi nasopharyngeal colonization in healthy children. The presence of high levels of NTHi and early colonization in the nasopharynx are thought to predispose to otitis media (48, 183, 184); thus, control of early colonization is essential for early protection against otitis media.

NTHi and *H. influenzae* type b secrete a conserved 100-kDa heme:hemopexin binding protein (HxuA) in a soluble form that provides heme as a nutrient for the growing bacteria by an as yet unidentified mechanism (28). This protein interacts with a heme carrier from children with meningitis, thus demonstrating its presence in vivo (77). Examination of convalescent-phase sera also confirms its immunogenicity (77).

Lipooligosaccharides

Antibodies to LOS appear to be limited in their ability to protect against infection. As mentioned above, NTHi strains show marked heterogeneity in their LOS structures (155). This variation occurs both between different strains and during the growth of the individual bacterium (215, 216) and would thus make a cross-strain-protective epitope difficult to find.

Systemic immunisation with a protein-LOS complex resulted in enhanced pulmonary clearance after challenge with an homologous strain (84). Antibodies to both protein and LOS were detectable in the serum and the alveolar spaces. The exact mechanisms of enhanced clearance may not be attributable to antibodies formed to LOS, since LOS antibodies present in acute-phase sera of children with *H. influenzae* type b meningitis were not protective (180). Similarly, 4 of 11 chinchillas with specific acute-phase LOS antibody showed the same susceptibility to bacterial challenge as did the chinchillas that developed antibody only after challenge (99). Recent studies with a detoxified form of LOS conjugated to a high-molecular-weight protein of NTHi also proved to be highly effective in inducing an antibody response to the LOS and protein from mice and rabbits (76). It will be essential to extend these studies in protective animal models of mucosal infections such as pneumonia or otitis media models. The use of the detoxified LOS alone and the LOS-protein conjugate will help answer questions about the mechanisms by which LOS enhances bacterial clearance.

If the antibodies to LOS do not directly help clear infecting bacteria, it is conceivable that a limited amount of LOS could still be used as a carrier for an antigen or a combination of

antigens to be used as a vaccine. The nonspecific host defense mechanisms for clearance of gram-negative bacteria from mucosal surfaces rely, to a degree, on interaction of the lipid A portion of the bacterium with the host (42), as is seen by *Lps*-deficient mice being hyporesponsive to the toxic inflammatory and immunomodulatory effects of lipid A, resulting in lack of clearance of the gram-negative bacteria. The ability to induce a significant mucosal immune response (39, 42) and to attain adult antibody titers to LOS in children (180) without affecting the future ability of an organism to cause infection would mean that it could be used many times as a carrier.

VACCINE STRATEGIES

Strategies to prevent host tissue damage due to infection by NTHi must take into account the differences between prevention of acute respiratory infection and prevention of chronic respiratory infection. The occurrence of an acute exacerbation, indicates a breakdown in the innate immune response, while chronic infection indicates that the immune response mounted to infection is either acting inappropriately or being countered by NTHi. Therefore, while prevention of acute infections may focus on prevention of attachment, invasion, and multiplication of the bacteria, dealing with an established chronic infection requires a strategy of modulating the immune response for successful treatment.

Prevention of infection by NTHi is based on innate humoral and cellular mechanisms. Many of the innate factors have already been addressed in the general text above. There is obviously extensive interaction between the mechanisms of innate and acquired immunity which cannot be dealt with in this review; however, models of protection against NTHi infections may give us a window of opportunity to further investigate this area. This was demonstrated by the cross-protection shown for NTHi as a result of a resolved *S. pneumoniae* infection in the ears of rats, despite no cross-protective antibodies being demonstrated (124).

Mucosal Solution for a Mucosal Problem

NTHi is a mucosal pathogen, and, as such, any effective preventive strategy must target the mucosal surface. The development of technologies for the oral delivery of vaccine antigens that does not result in tolerance but will induce a response that provides protection from colonization and invasion has provided a significant scientific challenge.

Proteins that preferentially bind to glycolipids and glycoproteins on the intestinal mucosa have proved to be successful as immunogens rather than causing tolerance to the antigen (33). This specific binding facilitates the uptake of the antigen by the M cells of the follicle-associated epithelium in the intestinal tract. Vesicles within the M cells transport materials from the gut lumen to the underlying mucosal lymphoid tissue (150, 168). The specific secretory IgA subsequently produced can provide protection against colonization and invasion (150).

Orally delivered antigen can induce antigen-specific B and T cells at mucosal effector sites such as in the lungs (reviewed in reference 123). In some cases, successful induction of antigen-specific protection has been relatively short-lived, leading to speculation that mucosal immunization does not induce memory B cells (23). Further evidence, however, now demonstrates that the nature of the antigen is extremely important for B-cell memory. Combination vaccines such as polysaccharide-protein conjugates or specific carrier systems can solve the dilemma of lack of B-cell memory for carbohydrate-based oral vaccines (123). However, carriers that induce an immunogenic response

to themselves are not ideal due to the relatively long time required between doses to allow for a decrease in the immune response to the carrier (79, 158).

Protein versus Carbohydrate Antigens

The use of protein or carbohydrate antigens for the induction of immunity also affects the predominance of the IgA and IgG subclass produced (80, 127). Plasma cells in the upper respiratory tract produce significantly larger amounts of IgA1 than of IgA2 (96 and 4% respectively), compared with cells in the lower bronchus (69 and 31%, respectively) (35, 101, 126, 127). It can therefore be argued that the ability of NTHi to produce IgA1 protease helps restrict its colonization to the upper airways. Enhancing the production of IgA2 in the upper respiratory tract with the use of carbohydrate immunogens (81) may help prevent NTHi colonization and subsequent disease.

Humoral versus Cellular Response

The issue of humoral versus cellular mechanisms being dominant in protection against infection is far from being resolved. Bactericidal antibody production appears to correlate with protection (87, 221), yet naive animals are protected from homologous challenge in the rat pulmonary model after transplantation of primed CD4⁺ T cells (41, 209). Enhanced bacterial clearance from the lungs is seen with a corresponding decrease in antibody levels after immunization with a P2-SDS combination compared to the clearance after immunization with P2 alone (110). The mechanisms involved appear to be linked to a switching of antibody isotype in serum. This resulted in a decrease in the IgG2a and IgA levels and an increase in the IgG1 level. The enhanced bacterial clearance may be indicative of a switch in T-helper subtype as reflected by the decrease in IgA production (44).

The relative amounts of IgG2a and IgG1 are indicators of a predominantly Th1- or Th2-type response, respectively (54). Th2-type responses appear to be optimal in prevention of colonization as a result of the interleukin-5 (IL-5) and IL-6 requirement for IgA production (123). Th1-type responses are also required, later, for the presence of gamma interferon and IL-10 as modulators of the Th2 response (54, 191) and are also needed to induce CD8⁺ cytotoxic lymphocytes to help clear infected host cells (123).

Effect of Age

Responses to vaccine antigens are significantly different in neonates than in adults. A predominance of the Th2-type response, with IgG1 being dominant, is induced in neonates, compared with a predominant Th1-type response and subsequent dominance of IgG2 in adults when the same vaccine antigens are used (14, 83). This correlates well with the observation that young children demonstrate poor responses to polysaccharide antigens (82), possibly due to the delay in development of adult levels of IgG2 until early adolescence (83). The delay in B-cell maturity and the subsequent low antibody levels do not result from an induction of tolerance to polysaccharide in the neonate (92); however, memory imprinting occurs and the polarization to IgG1 can remain if a booster vaccination is administered in adult life (14). Eskola et al. (46) demonstrated that linking of a polysaccharide to a protein carrier improves the overall antibody response to the polysaccharide, as seen with *H. influenzae* type b capsular polysaccharide-diphtheria toxoid conjugate vaccine; however, the IgG subtypes were not specified.

Role of Immunodeficiency and Allotype

Genetic susceptibility, along with age-related immune system immaturity, may help explain Murphy's observations that children who preferentially develop antibody to conserved proteins, such as P6 of NTHi, are less likely to experience repeated infections, whereas, those who preferentially make antibody to variable proteins, such as P2 of NTHi, are not protected (144). A study by Samuelson et al. (172) has also shown that patients with common variable immunodeficiency are subject to recurrent infections and persistent colonization with NTHi. Despite patients with multiple immunodeficiencies being more prone to NTHi infection than those with single deficiencies, individual variability in haplotype and allotype should be considered in the development of vaccine strategies to prevent infections by NTHi in children.

Individuals with IgG2 deficiencies have a predisposition to chronic infections (as reviewed by Papadea and Check [154]), and this may indicate the significance of an IgG2 response for vaccines to prevent infection. This can be further narrowed to possession of the G2m(n) allotype. G2m(n) allotype antigen carriers produce a better IgG antibody response to polysaccharide than do G2m(n)-negative individuals (2, 68, 131). Children possessing the G2m(n) phenotype have a lower relative risk of *H. influenzae* type b vaccine failure (67). Furthermore, very young G2m(n)-negative Caucasian children are genetically predisposed to nonpiglotitis *H. influenzae* type b infections (2). This phenomenon dissipates with age (131).

The age at which the presence or absence of G2m(n) becomes critical for response to bacterial antigens is a matter of debate. Allotype-dependent differences in IgG3 concentrations are measurable in some children as early as 6 months of age (164), whereas allotype differences in IgG2 concentrations can be difficult to quantitate before 5 years of age (173). In children, the combination of being Gm-f negative (and subsequently having suboptimal IgG3 levels [21, 164, 170, 223]) and G2m(n) negative may have more significance on predisposition to infection by noncapsulated bacteria than being solely G2m(n) negative. This is demonstrated in studies involving *Branhamella catarrhalis*, a noncapsulated gram-negative organism, which preferentially causes otitis media in young children with Gm phenotype-linked IgG3 deficiencies (21, 65). This may well be linked to bacterial proteins predominantly inducing IgG1, IgG3, and IgG4 (21, 53, 97), whereas antibody responses to polysaccharides tend to be mainly IgG2 (2, 68).

The phenomenon of maturation of antibody subtype with increasing age is not restricted to IgG. The IgA response to polysaccharide antigen is also highly age dependent, especially when an animal is primed via the gastrointestinal system (170). Unlike the Gm haplotypes mentioned above, the frequencies of A2m allotypes are race dependent (203). A genetic linkage between Gm and A2m results in A2m allotypes being inherited in Gm-Am haplotypes (203). A2m(1) is highly dominant in Caucasian populations. On the rare occasions when the A2m(2) allotype is seen, it is often linked with the Gm(n)-negative haplotypes (2, 81, 203). This leads to the hypothesis that A2m(2) infants have an increased susceptibility to infection, as was found with the G2m(n)-negative infants (2).

Vaccine Trials in Humans

Randomized controlled human trials in adults with histories of susceptibility to chronic upper respiratory tract infections have had different degrees of success in decreasing the severity and frequency of acute bronchitis following orally delivered, killed whole-cell NTHi vaccine (23–25, 114, 192). Most of the trials demonstrated that partial protection against bronchitis

corresponded to a reduction in the number of NTHi organisms carried. Protection against acute infection was observed in the 3-month period immediately following immunization (23, 192). However, with the exception of one study (114), patient surveillance was not extended beyond 3 months. In this study, which was conducted in an environment of continued exposure to NTHi, protection was observed for the 9 months of surveillance postimmunisation. Clearly, further trials must be conducted to determine the actual efficacy and long-term respiratory protection which can be induced by oral immunization.

Lack of a detailed analysis of antibody responses in these trials leaves the mechanisms of temporary protection to speculation alone; however, Clancy et al. (23) found no correlation between salivary or serum antibodies, using a crude antigen preparation, and clinical protection from NTHi. Studies such as these, in which crude antigen was used to analyze the immune response, may mask the underlying picture of useful antibody to specific epitopes or proteins (146).

Future Directions

Development of the ideal vaccine to prevent infection from this member of the normal microbiota is a great challenge. Vaccine strategies can be targeted toward prevention of primary infection or immunomodulation of the host response that has resulted in either repeated acute exacerbations or chronic infection. Mucosal presentation should be included in the development of these vaccine strategies. The combination of a carbohydrate moiety with a glycolipid or glycoprotein binding protein may maximize antigen uptake, humoral and cellular responses, and the development of immunological memory in both the young and old.

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