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Streptococcus pneumoniae: Virulence Factors, Pathogenesis, and Vaccines

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INTRODUCTION

Streptococcus pneumoniae has been one of the most extensively studied microorganisms since its first isolation in 1881. It was the object of many investigations that led to important scientific discoveries (158). In 1928, Griffith observed that when heat-killed encapsulated pneumococci and live strains constitutively lacking any capsule were concomitantly injected into mice, the nonencapsulated could be converted into encapsulated pneumococci with the same capsular type as the heat-killed strain (158). Years later, the nature of this "transforming principle," or carrier of genetic information, was shown to be DNA (14). Other important discoveries resulting from investigations on pneumococci were the therapeutic efficacy of penicillin, the role of the bacterial capsule in resistance to phagocytosis, the ability of polysaccharides (PS) to induce antibodies (Ab), the first demonstration of antigen-specific tolerance or immunological unresponsiveness, the discovery of regulatory thymus-derived T lymphocytes, and the putative use of PS antigens as vaccines (15, 46).

In spite of the vast number of publications on *S. pneumoniae*, many questions about its virulence are still unanswered (78), and this pathogen remains a major causative agent of serious human disease, especially community-acquired pneumonia. In addition, in developing countries, the pneumococcus is responsible for the death of a large number of children under the age of 5 years from pneumococcal pneumonia. Although improved vaccines against *S. pneumoniae* are likely to be developed in the near future, a better understanding of the virulence factors determining its pathogenicity will be needed to cope with the devastating effects of pneumococcal disease in humans. This review focuses on the structural components and immunological properties of pneumococci thought to be responsible for their pathogenic capacity, and it highlights the potential use of some of these structures for the production of efficacious conjugate vaccines.

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EPIDEMIOLOGY AND SEROTYPE DISTRIBUTION

The incidence of pneumococcal disease is highest in infants under 2 years of age and in people over 60 years of age (51, 58). Pneumococci are the second most frequent cause (after *Haemophilus influenzae* type b) of bacterial meningitis (40) and otitis media (99) in children. With the recent introduction of conjugate vaccines for *H. influenzae* type b, pneumococcal meningitis is likely to become increasingly prominent. *S. pneumoniae* is the most important etiological agent of community-acquired pneumonia in adults and is the second most common cause of bacterial meningitis behind *Neisseria meningitidis*. In spite of the availability of antibiotics, the mortality of pneumococcal disease remains high. For instance, the mortality of pneumococcal bacteremia in the last four decades has remained stable between 25 and 29% (55).

On the basis of differences in capsular PS structure, pneu-

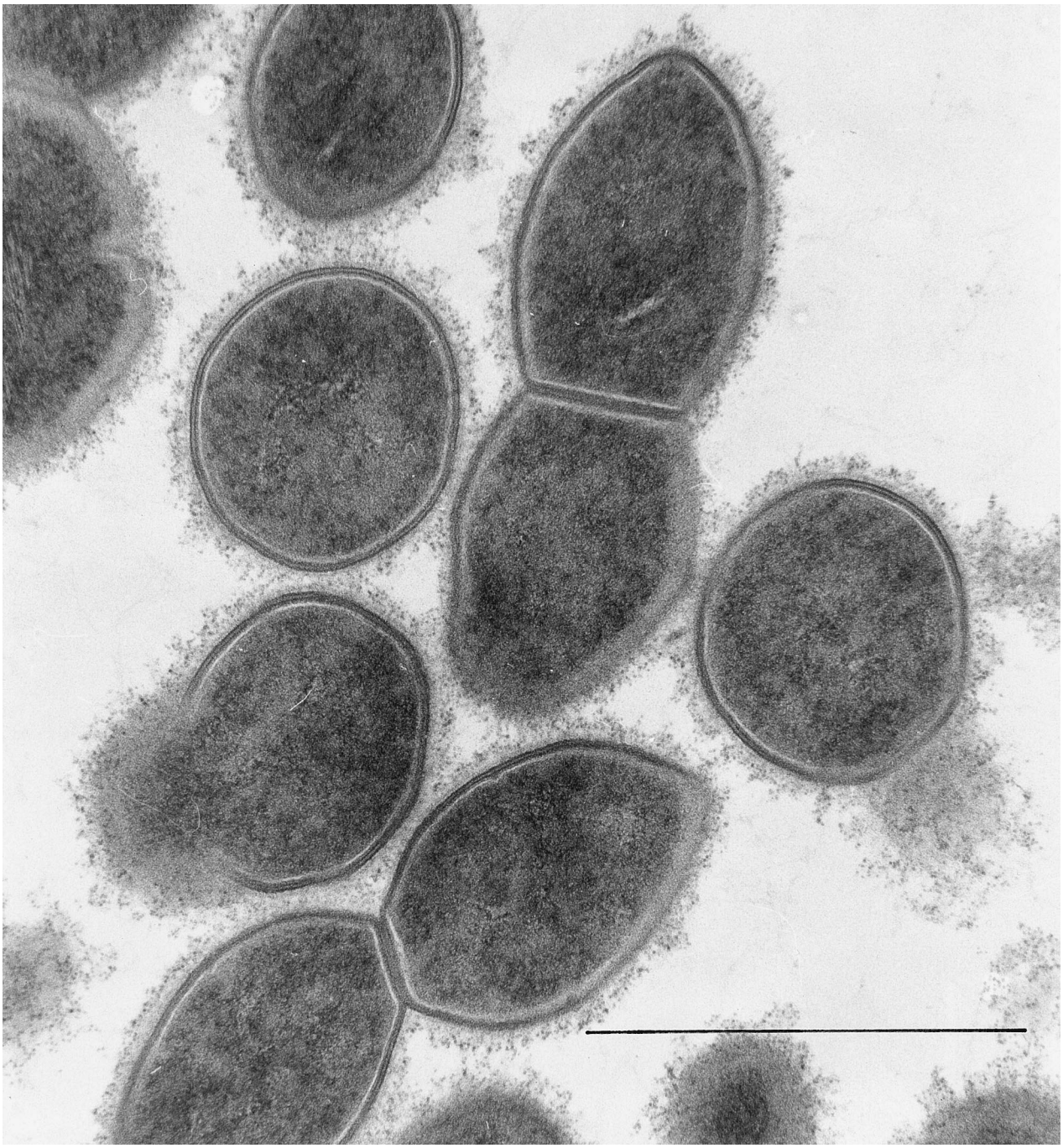

FIG. 1. Electron micrograph of nonencapsulated pneumococci stained with ferritin-labeled antibody against phosphorylcholine. Bar, 1 μm. The micrograph was kindly provided by U. B. S. Sørensen, Statens Serum Institute, Copenhagen, Denmark.

mococci can be divided into more than 80 serotypes. The Danish nomenclature classifies serotypes according to structural and antigenic characteristics; e.g., serotypes 6A and 6B differ only slightly from each other. The American nomenclature, however, assigns the numbers in sequence of first isolation. Thus, types 6A and 6B are types 6 and 26, respectively, in the American system. The Danish nomenclature, now widely adopted, is used throughout this review.

The distribution of types isolated from adults differs substantially from that of types isolated from children. Although considerable differences are observed among publications on this issue, some trends can be recognized. In children, a few types are responsible for a large proportion of pneumococcal disease. In one study, the most important pediatric serotypes (6A, 14, 19F, and 23F) were responsible for almost 60% of all infections. In adults, however, serotypes 3, 19F, and 6A accounted for only 31% of the isolations (58). The geographical distribution and prevalence of serotypes differ among the United States, Europe, and some Asian countries (86). Twenty serotypes are responsible for ~90% of all reported infections

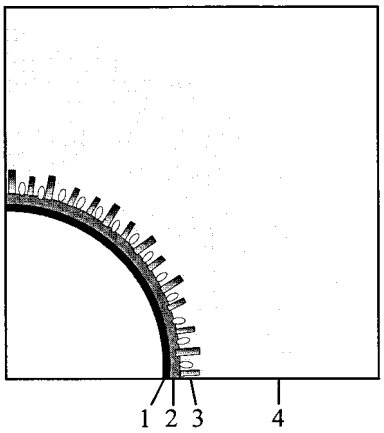

FIG. 2. Schematic structure of the surface of *S. pneumoniae*. 1, plasma membrane (9 nm); 2, peptidoglycan layer of the cell wall (20 nm); 3, CWPS and proteins (20 to 30 nm); 4, capsular PS (200 to 400 nm). CWPS is located on both the outer and inner surfaces of isolated cell walls.

in United States and Europe, whereas, for instance, the 23-valent whole-PS vaccine, which contains these serotypes, is effective against <70% of the pneumococcal infections in Asia (86, 153). Geographical differences also have been shown to occur in antibiotic resistance of pneumococci (9).

SURFACE COMPONENTS OF *S. PNEUMONIAE*

Pneumococci are lancet-shaped gram-positive bacteria, which grow in pairs or short chains. Three major surface layers can be distinguished in their surface: plasma membrane, cell wall, and capsule (Fig. 1 and 2). The cell wall consists of a triple-layered peptidoglycan backbone that anchors the capsular PS, the cell wall polysaccharide (CWPS) (141), and possibly also proteins. The capsule is the thickest layer, completely concealing the inner structures in exponentially growing pneumococci (140). Whereas CWPS is common to all pneumococcal serotypes, the chemical structure of the capsular PS is serotype specific.

The capsule consists of high-molecular-weight polymers made up of units of repeating oligosaccharides (OS), which can contain between two and eight monosaccharides. Many serotypes possess acidic components (like D-glucuronic acid or phosphate groups), ribitol, or arabinitol (153). In six serotypes, phosphorylcholine (PC) is part of the capsular PS (139).

The typical gram-positive cell wall is composed mainly of peptidoglycan: glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, cross-linked to each other through peptide side chains. The peptide chains have alanine as the first residue, linked to *N*-acetylmuramic acid. The CWPS, a complex teichoic acid containing PC residues (77), is also attached to peptidoglycan via *N*-acetylmuramic acid (150). The capsular PS is also linked to peptidoglycan. Although the bond between the two components has not yet been identified, it is probably covalent (141). The PC residues of CWPS are a recognition site for *N*-acetylmuramic acid-L-alanine amidase, an enzyme that cleaves peptidoglycan into separate glycan and peptide chains by hydrolyzing the bond between alanine and *N*-acetylmuramic acid. This enzyme is involved in the process of cell division by cleaving the peptidoglycan. This enzyme is also referred to as autolysin, because it induces lysis of pneumococci under certain conditions, i.e., during the stationary phase of growth (150).

Another important structure is the lipoteichoic acid or Forssman antigen, a teichoic acid similar to CWPS with additional covalently attached lipid material. This antigen, inserted into the plasma membrane via its lipid moiety, also contains PC (140, 150). The Forssman antigen is a powerful inhibitor of autolysin, and its PC residues are involved in its specific interaction with the enzyme. During the stationary phase of growth, pneumococcal cells release the Forssman antigen. It is thought that the loss of this enzyme-inhibitor, followed by unrestrained activity of autolysin, results in the destruction of the cell wall and finally in bacterial lysis (71). Immunization of mice with the Forssman antigen does not protect against pneumococcal infection (11).

VIRULENCE FACTORS

The pathogenicity of pneumococci has been attributed to various structures, most of which are situated on its surface. The high morbidity and mortality caused by this microorganism are still, however, poorly understood, and the list of virulence factors (Table 1) is probably far from complete. One group of factors, such as the capsule and a recently identified protein (104), provides resistance to phagocytosis and thus promotes the escape of pneumococci from the host immune defense. Other factors, including cell wall components and the intracellular toxin pneumolysin, are involved mainly in the inflammation caused by infection. The inflammation process probably fully develops only after lysis of bacteria by autolysin. Since inflammation is thought to induce most of the symptoms of pneumococcal disease (78, 99), this group of virulence factors may thus be more directly responsible for the morbidity and mortality caused by pneumococci once they have infected the host. However, since inflammation can facilitate the further spread of pneumococci to other tissues and organs, this distinction of virulence factors in two groups is obviously not absolute. Recently, the production of hydrogen peroxide by pneumococci and its toxic effects for rat alveolar epithelial cells were demonstrated in vitro (43). Since the amount of hydrogen peroxide produced by pneumococci is similar to that produced by activated neutrophils, this oxidant might be involved in the pathogenesis of pneumococci in vivo by causing lung injury.

Capsule

The capsule has long been recognized as the major virulence factor of *S. pneumoniae*. Experimental proof for this was provided by the difference in 50% lethal dose between encapsulated and unencapsulated strains. Encapsulated strains were found to be at least 10^5 times more virulent than strains lacking the capsule (12, 157). Recently, pneumococcal mutants that apparently differ only in the type of capsular PS expressed have been produced (81). The virulence of the mutants in relation to the parental strains was shown to be determined mainly by the capsular type. However, the genetic background of the recipient strain was also of importance, showing that other components besides the capsule are required for full virulence of pneumococci.

The chemical structure of the capsular PS and, to a lesser extent, the thickness of the capsule determine the differential ability of serotypes to survive in the bloodstream and possibly to cause invasive disease. This is probably due to differences between serotypes in terms of activation of the alternative pathway of complement (49, 54, 135), deposition and degradation of complement components on the capsule (8, 72), resistance to phagocytosis (35, 92, 135), ability to induce Ab (153), and clearance mediated by lectin-like structures on phagocytes (108).

TABLE 1. Characteristics of the major proposed virulence factors of *S. pneumoniae*

Virulence factor	Proposed mechanism of virulence[a]	Reference(s)
Capsule	Lack of activation of alternative complement pathway	49, 54, 135
	Resistance to phagocytosis	35, 92, 135
	Deposition of opsonically inactive complement components	8, 72
	No or low immunogenicity of some serotypes	153
Cell wall or CWPS	Inflammatory effects	
	Activation of the alternative complement pathway, resulting in anaphylatoxin production	160, 161
	Enhancement of vascular permeability, mast cell degranulation, PMN activation	78
	IL-1 production increased, cytopathic for endothelium	52, 123
	Mediator of attachment to endothelial cells	52
Pneumolysin	Cytolytic at high concentrations	24
	Cytotoxic at lower concentrations	24
	Inhibition of ciliary movement and disruption of epithelium	45, 126
	Inhibition of bactericidal activity of PMN	112
	Inhibition of lymphocyte proliferation	48
	Inhibition of Ab synthesis	48
	Complement activation	111
	IL-1β and TNF-α production by monocytes increased	73
	Binding of Fc fragment of Ab	111
PspA	Inhibition of complement activation?	104a
Complement factor H-binding component	Inhibition of complement activation	53, 104
	Inhibition of phagocytosis	53, 104
Autolysin	Release of pneumolysin and cell wall products	89
Neuraminidase[b]	Exposure of "receptors" for pneumococci?	74
Peptide permeases	Enhancement of adhesion	38
Hydrogen peroxide	Lung injury?	43
IgA1 protease	Counteracts mucosal defense mechanisms?	83

[a] The mechanisms marked "?" have been suggested but not demonstrated. Most of the mechanisms listed are observed only in vitro. Their significance for pneumococcal virulence remains unknown. IL, interleukin; TNF, tumor necrosis factor. For further explanation see text.
[b] This mechanism of virulence has been demonstrated only for viral neuraminidase, not for pneumococcal neuraminidase.

Hostetter (72) in 1986 proposed an interesting hypothesis that explained some of these differences: capsular serotypes which in some way allow the deposition of the complement component C3b but prevent its degradation to C3d are more easily taken up by phagocytes (via the receptor with high affinity for iC3b, complement receptor 3 [CR3]). As a result, however, they are poorly immunogenic, because they are rapidly cleared. On the other hand, serotypes on which capsule C3b is degraded to both iC3b and C3d are more resistant to phagocytosis but induce a stronger Ab response. Experimental support for the mechanism by which C3d might enhance the anti-PS Ab production, as suggested in 1986 by Hostetter, was provided some years later (63, 66). It should be noted that this explanation for the phagocytosis and immunogenicity of different serotypes is based on studies with a limited number of serotypes. It is therefore unlikely to apply to all of them.

The role of the capsule in the virulence of pneumococci is also well illustrated by the highly protective activity of anticapsular Ab (5, 56, 136, 138). Recently, survival of mice after challenge with a lethal dose of pneumococci was shown to be predictable from levels of anti-capsular PS Ab present in serum (2).

Cell Wall and Cell Wall Polysaccharide

In contrast to the capsular PS, purified peptidoglycan and especially CWPS have been found to induce inflammation similar to that seen after infection with whole pneumococci. Typical pneumococcal diseases such as otitis media, meningitis, and pneumonia can be mimicked in animals that have received injection of purified cell wall or its degradation products (33, 151, 152). CWPS activates the alternative pathway of complement (160, 161). During complement activation, the anaphylatoxins C3a and C5a, which enhance vascular permeability, induce mast cell degranulation, and recruit and activate polymorphonuclear leukocytes (PMN) at the inflammation site, are produced (78). Furthermore, purified cell wall is a powerful stimulus (even stronger than endotoxin) for the production of interleukin-1 by human monocytes (123). This cytokine, together with tumor necrosis factor, plays a pivotal role in the inflammation process. Cell wall also was shown to be involved in the attachment of unencapsulated pneumococci to human endothelial cells and to have (interleukin-1-mediated) cytopathic effects on these cells (52).

Anti-CWPS or anti-PC Ab have been demonstrated to protect animals against pneumococcal challenge (25–27, 82, 105),

although such a protective effect was not observed by others (106, 145). This discrepancy might be due to improper culture conditions of pneumococci, resulting in inocula containing partially unencapsulated bacteria (106). The protective activity of anti-PC Ab is substantially weaker than that of anti-capsular PS Ab (25, 27). Furthermore, protection induced by anti-CWPS Ab seems not to be mediated by the stimulation of phagocytosis (27, 156). In humans, no strong differences were observed in levels of anti-CWPS immunoglobulin G (IgG) found in healthy adults and patients at different stages of pneumococcal infection. This suggests that anti-CWPS IgG may not prevent or delay the progress of pneumococcal disease in humans (103). Nevertheless, since the control individuals in this study were not age matched, the lack of protective effect of anti-CWPS IgG might be due to qualitative differences between IgG from young individuals and IgG from elderly people. Such an age-dependent qualitative difference, in terms of affinity and protective efficacy, has been described for mouse Ab against PC (105). Whether similar differences play a role in the immune response to pneumococcal PS remains to be established (16).

Pneumococcal Proteins

Various proteins have been suggested to be involved in the pathogenicity of *S. pneumoniae* (Table 1). However, only a few of them have actually been confirmed as virulence factors (see below). Pneumococci produce an IgA1 protease that might interfere with host defense at mucosal surfaces (83). *S. pneumoniae* also produces neuraminidase, an enzyme that may facilitate attachment to epithelial cells by cleaving sialic acid from the host glycolipids and gangliosides (84). Partially purified neuraminidase was observed to induce meningitis-like symptoms in mice; however, the reliability of this finding has been questioned because the neuraminidase preparations used were probably contaminated with cell wall products (24). Nevertheless, immunization with pure neuraminidase slightly enhances the survival time of mice upon challenge with pneumococci, which confirms the contribution of this protein to the pathogenicity of pneumococci (90).

Other pneumococcal proteins besides neuraminidase are involved in the adhesion of pneumococci to epithelial and endothelial cells. These pneumococcal proteins have as yet not been identified but are able to recognize glycoconjugates on the surface of host cells. These glycoconjugates contain oligosaccharides with the following specificities: *N*-acetyl-D-glucosamine-β(1→3)galactose, *N*-acetyl-D-galactosamine-β(1→3) galactose, and glucosamine only (7, 38, 39). Recently, Cundell et al. reported that peptide permeases can modulate pneumococcal adherence to epithelial and endothelial cells. It was, however, unclear whether these permeases function directly as adhesions or whether they enhance adherence by modulating the expression of pneumococcal adhesions (38). Other proteins that may enhance the virulence of pneumococci, including hyaluronidase and neutrophil elastase inhibitor, have been reviewed by Gillespie (55) and Boulnois (24).

Recently, a protease-sensitive surface component of pneumococci that may be an important virulence factor has been described (53, 104). This component, likely to be a protein, is able to bind complement factor H, thereby inhibiting complement activation and phagocytosis. Protease treatment of pneumococci enhances their complement-activating ability and complement-dependent phagocytosis and diminishes their virulence in mice. Protease treatment has no effect on the presence of capsule. The production of a C3-degrading protease by exponentially growing pneumococci of different serotypes has been recently suggested by Angel et al. (8). The observed enzymatic activity is heat labile and independent of capsule production; it can be released readily from the pneumococcal surface by mutanolysin treatment.

Pneumolysin. Pneumolysin is an intracellular protein that belongs to the family of thiol-activated toxins (24). Conserved among pneumococcal isolates, it has a variety of toxic effects on different cell types. Although it is not secreted by pneumococci, it can be released upon lysis of pneumococci under the influence of autolysin. At high concentrations, pneumolysin oligomers are formed on mammalian cell membranes, giving rise to transmembrane pores that cause cell lysis (24). At lower concentrations, the toxin has several effects, most of which can be demonstrated in vitro. Pneumolysin stimulates the production of inflammatory cytokines like tumor necrosis factor alpha and interleukin-1β by human monocytes (73), inhibits the beating of cilia on human respiratory epithelial cells, disrupts the monolayers of cultured epithelial cells from the upper respiratory tract (45) and from the alveoli (126), decreases the bactericidal activity and migration of neutrophils (112), and inhibits lymphocyte proliferation and Ab synthesis (48). Furthermore, pneumolysin activates the classical complement pathway in the absence of anti-toxin Ab, an activity that seems to be mediated by binding of Ab via their Fc fragments (111).

In this regard, it is interesting that pneumolysin has some sequence homology with C-reactive protein (CRP), an acute-phase protein that partially protects mice against *S. pneumoniae* (27, 96, 162). Upon binding to the PC residues of pneumococcal CWPS, CRP activates the classical complement pathway through binding to C1q. Preliminary data indicate that pneumolysin also may bind C1q directly (24). Pneumolysin thus may enhance the inflammatory process through its capacity to activate complement, to disrupt the respiratory tract epithelium (thereby facilitating access of pneumococci to the blood), and to stimulate cytokine production. Furthermore, it also may contribute to the virulence of pneumococci by competing with CRP for binding to C1q, thereby abrogating the protective effects of CRP (24). Pneumolysin-negative mutants are less virulent than their parental strains (21), and immunization with pneumolysin prolongs the survival of mice after challenge with pneumococci of different serotypes (1, 90, 113). This toxin may be therefore considered an important virulence factor.

Pneumococcal surface protein A. Pneumococcal surface protein A (PspA) is a surface protein with structural and antigenic variability between different pneumococcal strains. It is found in most of the clinical isolates of pneumococci (37). Since it is probably a transmembrane protein (147), PspA has proven difficult to purify, and its functions are unknown. Nevertheless, it seems to be required for full virulence of pneumococci (29, 95). Preliminary experiments in our laboratory indicate that inhibition of complement activation (in a factor H-independent fashion) may be the mechanism by which PspA enhances pneumococcal virulence (104a). Passive immunization with polyclonal or monoclonal Ab against PspA and active immunization with recombinant PspA or its N-terminal fragment have both been shown to protect mice against challenge with pneumococcal strains of various capsular serotypes. These strains probably contain common protective PspA epitopes (27, 93, 94, 146).

Autolysin. Autolysin-negative mutants have been shown to be less virulent than wild-type pneumococci, and immunization with autolysin confers some protection against pneumococcal challenge in mice (20). The effects of autolysin, however, seem to be mediated by the release of pneumolysin (and perhaps also cell wall products) from the pneumococcal cytoplasm,

because immunization with autolysin does not protect against challenge with a pneumolysin-negative mutant (89). Furthermore, autolysin was recently shown to be triggered by human lysozyme, a defense factor released upon infection and inflammation (32), thereby inducing pneumococcal lysis and enhancing the inflammation. Autolysin thus seems to take advantage of the (protective) function of lysozyme.

Different Mechanisms of Virulence

When the mechanisms (summarized in Table 1) by which different virulence factors may contribute to pneumococcal pathogenesis are compared, paradoxes are observed. Although CWPS and pneumolysin activate the complement system, the factor H-binding component and PspA inhibit complement activation. This emphasizes our previous suggestion that virulence factors may be divided into two separate groups. One consists of factors present on the surface of intact pneumococci, which seem to act at the beginning of the infection, mainly by impeding phagocytosis via complement inhibition. The second group consists of factors that act at the stage of pneumococcal disintegration and lysis. At this stage, complement activation enhances the inflammation; this appears to be the "point of no return" of pneumococcal infection.

PATHOGENESIS OF PNEUMOCOCCAL DISEASE

Carriage and Infection

S. pneumoniae is carried in the upper respiratory tract by many healthy individuals. It has been suggested that attachment of pneumococci is mediated by a disaccharide receptor on fibronectin, present on human pharyngeal epithelial cells (7). Adherence of pneumococci to tracheal epithelial cells can be enhanced by prior influenza virus infection (120). This enhancement is thought to be mediated by viral neuraminidase. This enzyme cleaves sialic acid from glycosphingolipids, which is present in substantial amounts in human lung tissue (84). Thus, neuraminidase may expose other structures that function as receptors for adhering pneumococci.

The mechanisms by which pneumococci translocate from the nasopharynx to the lung, thereby causing pneumonia, or migrate directly to the blood, giving rise to bacteremia or septicemia, are poorly understood (24, 78). Most infections do not occur after prolonged carriage but follow the acquisition of recently acquired serotypes (59, 78). This suggests that the immune status of the host at the moment of colonization, as well as the virulence of the particular strain, determines whether pneumococci will remain confined to the nasopharynx or become invasive.

Failure of the specific (secretory IgA) and nonspecific (cough reflexes, mucosal secretion, and ciliary transport) defenses in the respiratory tract may facilitate access of pneumococci to the bronchi and the lungs (24, 99). The effects of pneumolysin on ciliary beating of epithelial cells and the effects of the IgA1 protease secreted by pneumococci (see the section on virulence factors, above) might impair these defense mechanisms. Simultaneously, damage of the epithelial monolayer by hydrogen peroxide (produced by pneumococci) and by pneumolysin may facilitate direct access of pneumococci to the blood. Epithelial damage, caused by previous (viral) upper respiratory infections, also increases the opportunity of pneumococci to reach the bloodstream. From the blood, they may migrate to the meninges and, after disrupting the endothelium (see the section of virulence factors, above), reach the subarachnoid space. Alternatively, they may reach the meninges via other routes, such as directly from the nasopharynx (24).

Unrestrained multiplication of pneumococci in the lungs, meninges, or middle ear will result in pneumococcal lysis, with the release of cell wall products and pneumolysin. The presence of lysozyme in secretion fluids at the sites of infection may contribute to pneumococcal lysis through the activation of autolysin (36). Pneumococcal lysis will in turn trigger the inflammatory process, directly by attracting and activating phagocytes and indirectly through complement activation and anaphylatoxin formation. There is increasing support for the hypothesis that such inflammation may be responsible for the morbidity and mortality of pneumococcal infection (99); this may account for the fact that elimination of pneumococci from the sites of infection by antibiotic therapy frequently does not improve the course or outcome of pneumococcal disease (24). Moreover, by inducing pneumococcal lysis, highly effective bactericidal antibiotics such as the β-lactams (especially when administered at late stages of infection) may even augment the harmful effects of pneumococcal disease (32).

Early Response of the Host

Unrestrained proliferation of pneumococci at the site of infection fortunately is not likely to occur in healthy individuals. Alveolar macrophages and infiltrated PMN will clear the bacteria providing specific Ab and complement are present (69). The importance of opsonization for the removal of pneumococci is strongly supported by the fact that impairment of either the phagocytic system or opsonin production predisposes to pneumococcal infection (78). Examples of such defects are the lack of Ab (hypogammaglobulinemia, inability to produce anti-PS Ab in children, IgA deficiency, etc. [32]), the absence of nonspecific opsonins (complement deficiencies), and defects of the phagocytic system (asplenia, neutropenia, Hodgkin's disease, and others [55]). Once ingested and entrapped in a phago(lyso)some, pneumococci are readily killed, even when PMN lack the ability to produce a normal oxidative burst, e.g., in patients with chronic granulomatous disease. This seems to be due to the inability of pneumococci to resist the toxicity of their own hydrogen peroxide (79).

Clearance of pneumococci in the absence of specific Ab may be facilitated by CRP-mediated complement activation. However, the anti-pneumococcal effects of CRP are presumably not mediated by the ability to induce phagocytosis, except for serotypes that possess PC in their capsular PS (35, 55, 70). Moreover, it has been suggested that pneumolysin may counteract the protective effects of CRP (see the section on virulence factors, above). Other non-Ab-mediated mechanisms may involve phagocytosis mediated by lectins, which are proteins that are abundant on macrophages from the liver and spleen and that specifically recognize carbohydrate structures (34, 108). However, lectinophagocytosis is not likely to be a general mechanism for all pneumococci; rather, it is dependent on the pneumococcal serotype, i.e., the structure of the capsular polysaccharide (4, 156a). Whether phagocytosis is lectin mediated or not, the use of nonimmune guinea pigs as a model has shown that the liver and spleen play a major role in Ab-independent pneumococcal sequestration and that an intact complement system is crucial for clearance. The rate of hepatic, as compared with splenic, sequestration of pneumococci was increased both by a functional complement system and by PS-specific Ab (30). Deposition of complement upon injection of pneumococci in nonimmune animals occurs preferentially on the pneumococcal cell wall because complement is activated by CWPS via the alternative pathway. The finding that, in the absence of anti-capsular Ab, pneumococci are relatively better cleared in the spleen has been explained by a diminished

ability of Kupffer cells in the liver to bind C3b deposited on the cell wall; this may be due to the barrier formed by the capsular PS (30). This explanation, however, has not been supported experimentally.

Antibody-Mediated Clearance

In the presence of anti-capsular Ab, pneumococci are rapidly cleared from the blood, mainly by the liver and to a lesser extent by the spleen; however, complement is necessary to achieve effective clearance. Whether activated by the classical or the alternative pathway, by CRP, Ab, or the PS itself, complement deposition on the capsule (but not on the cell wall) is essential for pneumococcal phagocytosis and clearance (30, 70). This supports the notion, described above, that the protective effects of anti-CWPS Ab are probably not mediated by phagocytosis of pneumococci. The most likely mechanism by which anti-CWPS Ab confer protection to animals (27) seems to be neutralization of the inflammatory effects of CWPS (55, 140). A properly functioning complement system and induction of anti-capsular PS Ab able to induce phagocytosis are thus necessary to achieve full protection against pneumococci.

IMMUNE RESPONSE TO PNEUMOCOCCAL ANTIGENS

Once an immunocompetent individual has been colonized or infected by *S. pneumoniae*, an immune response against different bacterial structures will be mounted. These structures include the capsular PS (60, 121), the CWPS (mainly its PC residues) (28, 62), pneumolysin (76), and other pneumococcal proteins (121) including PspA. Although Ab against many capsular serotypes have been found in serum from adults, the actual levels of these Ab may have been overestimated because of the codetection of anti-CWPS Ab in many immunoassays (100, 102). Ab against the capsular PS have the highest protective capacity, with an estimated protective level of 200 to 300 ng of antibody nitrogen (Ab N) per ml of serum in humans (128). The amount of human anti-capsular PS Ab needed to passively protect mice against 1,000 50% lethal doses of pneumococci was found to be 150 ng of antibody nitrogen (Ab N) per mouse (101). Ab specific for CWPS, pneumolysin, and PspA also have been shown to confer protection, mostly in animals; however, their protective ability was consistently shown to be less than that of anti-capsular PS Ab. It is also likely that Ab against neuraminidase and autolysin have at least some protective activity.

Detectable levels of IgM against PC are observed in infants after infection with *S. pneumoniae*, frequently even in the first year of life. Carriage also sometimes results in the development of anti-PC IgM in infants (62), although the induction of (cross-reactive) anti-PC antibodies by other organisms cannot be excluded. The amount of anti-PC Ab is age dependent: Ab are present in nearly all older children and adults, but their levels decrease after the age of 50 to 60 years (28, 103). It is likely that infants can elicit anti-capsular PS Ab of the IgG class following pneumococcal infection, although they rarely do so in the case of asymptomatic carriage with poorly immunogenic pediatric serotypes 23F and 19F (60). Anti-capsular IgG responses have been observed in infants under 2 years of age following *H. influenzae* type b meningitis (124). The ability to elicit an anti-PS IgG response against encapsulated bacteria contrasts with the typical responses of infants to purified capsular PS, which are weak and restricted mainly to IgM (see below). Such IgG responses, also observed after immunization of mice and rabbits with killed pneumococci (4; unpublished data), may be facilitated by association of the capsular PS with proteinaceous surface components of the bacterium (124). Indeed, pneumococcal capsular PS is covalently linked to peptidoglycan, which may also anchor pneumococcal proteins (140, 141). The finding that infants develop a clear anti-capsular PS Ab response upon reinfection or reacquisition of the same pneumococcal serotype, even when serotype-specific Ab was present at the time of reexposure (61), suggests the presence of a type of booster response similar to that seen upon reimmunization with a protein (see below).

TABLE 2. Major characteristics of the Ab response to TD, TI-2, and PS-protein antigens

Characteristic	Effect for:		
	TD antigens	TI-2 antigens	PS-protein conjugates
T cells required	Yes	No	Yes/no[a]
Memory induction	Yes	No	Yes
Response in infants	Yes	No	Yes
Main Ab (sub)isotype[b]			
Humans	IgG1, IgG4	IgM, IgA, IgG2	IgG2, IgG1
Mice	IgG1, IgG2a	IgM, IgG3, IgG1	IgG1, IgG3, IgM

[a] PS-protein conjugates induce TI responses in nude mice, but T cells are required to induce TD responses. For further explanations and references, see text.
[b] Listed in decreasing order of amount induced.

Thymus-Dependent Antigens

The immune response to protein antigens requires the cooperation of T and B lymphocytes, as shown by the lack of anti-protein Ab formation in nude mice. Therefore, protein antigens are referred to as thymus-dependent or T-cell-dependent (TD) antigens (Table 2). For the proper activation and differentiation of protein antigen-specific B cells to memory cells or to Ab-producing plasma cells, binding of the antigens to their antigen receptor (membrane Ig) is not sufficient; rather, the interaction between B cells and helper T (Th) cells and cytokine-mediated events are essential (107).

TD responses can be elicited in infants. When healthy infants are colonized or infected by *S. pneumoniae*, they consequently mount a normal Ab response to pneumococcal proteins, similar to that elicited by adults. TD responses are characterized by the induction of memory as evidenced by a booster effect upon subsequent immunizations, affinity maturation, and extensive Ab (sub)class switching. Primary Ab responses to proteins normally comprise IgM and IgG. The (sub-)classes in secondary Ab responses in humans are, in order of the amount induced, IgG1 > IgG4 >> IgM > IgG2 ≈ IgA ≈ IgG3 (133). In mice, the isotype distribution of secondary TD responses is IgG1 > IgG2a >> IgG2b ≥ IgM > IgG3 (119, 132) (Table 2). IgA levels in serum are almost undetectable (132).

Thymus-Independent Antigens

In contrast to proteins, most, if not all, pneumococcal capsular PS elicit Ab responses in nude mice and are therefore referred to as thymus-independent (TI) antigens (22, 97) (Table 2). They are further classified as TI type 1 and type 2 antigens based on their ability to induce Ab responses in CBA/N mice, a strain carrying X-linked immunodeficiency. TI type 2 antigens do not induce an immune response in this mouse strain and are therefore characterized as TI-2 antigens

(97). Typical TI-2 antigens, including various kinds of PS, polypeptides, and polynucleotides, are poorly metabolized polymers with high molecular weight (130). These antigens are weakly immunogenic in infants younger than 18 to 24 months, and their immunogenicity increases with age (23, 42).

Although T cells are not required to induce an Ab response against TI-2 antigens, different subsets of T cells are definitely involved in the stimulation or suppression of that response, as has been shown by different approaches (15, 64, 143). T cells may be activated in different ways to modulate anti-PS responses in an antigen-specific manner. Direct activation of T cells via stimulation of the T-cell receptor by PS, OS, or glycolipids complexed to major histocompatibility complex (MHC) class II molecules is unlikely (68, 75). Nevertheless, evidence is accumulating that the T-cell receptor can recognize glycopeptides complexed to MHC class II (67, 75). Recently, the saccharide moieties of glycopeptides were shown to influence the interaction of the peptide with MHC molecules (98). Another way in which regulatory T cells may be activated is by the recognition of idiotypic determinants from saccharide-specific B cells, as has been demonstrated for anti-dextran IgG responses and has been proposed to occur during the antibody response to pneumococcal PS (15, 142). T cells, as well as macrophages, mast cells, and natural killer cells, may also influence the response to TI-2 antigens by means of cytokines (137).

TI-2 responses, in contrast to TD responses, are oligoclonal (74), dependent on age, and characterized by the lack of affinity maturation, poor Ab (sub)class switching, and the inability to generate memory (97, 125, 143). Nevertheless, TI-2 antigens are capable of activating preexisting memory cells (97), as shown by the ability of PS to induce booster responses upon priming with TD PS-protein conjugates (74). PS induce mainly IgM and IgA, and the IgG subclasses elicited in humans are, in decreasing order, IgG2 >> IgG1 ≈ IgG3 > IgG4 (19). In some studies, IgA (mainly IgA2) was the most important isotype found in serum upon immunization with pneumococcal or other PS (148). In children, the IgG1/IgG2 ratio induced by PS is inversed: compared with adults, children produce relatively large amounts of IgG1 but small amounts of IgG2 (50). In mice, the anti-PS IgG response was mainly IgG3 > IgG2a (119). In another study, anti-dextran Ab had the following isotype distribution: IgM >> IgG1 = IgG3 > IgG2a; the isotypes IgG2b and IgA were undetectable (132).

The molecular mechanisms behind the activation and differentiation of B cells by TI-2 antigens are only partially understood (97, 137). The activation signals provided by Th cells in the case of TD antigens are supposed to be partially replaced by other factors. PS cross-links membrane Ig on B cells (65, 97), and C3d, deposited on the PS, is recognized by complement receptor 2 (CD21, CR2) on B cells in vitro. These two signals increase the numbers of B cells secreting anti-PS IgM (63, 66). Although the C3d-mediated activation mechanism is not definitely demonstrated to occur in vivo, its potential significance is supported by the finding that B cells of the marginal zone of the spleen in neonates have a low expression of CR2 (149). Marginal-zone B cells are involved in the initiation of TI-2 responses, which, in contrast to TD responses, do not frequently occur in infants younger than 2 years of age. The delay in the ontogeny of the immune response against these antigens may thus be explained by the inability of neonatal B cells to be triggered via CR2, a signal that is necessary for full activation. Interestingly, CBA/N mice, which do not respond to TI-2 antigens and therefore resemble neonates, also have a low CR2 expression on B cells (88).

PNEUMOCOCCAL POLYSACCHARIDE VACCINES

Attempts at the beginning of this century to induce protective immunity against *S. pneumoniae* in humans by vaccination with whole killed pneumococci were not very successful. This approach was abandoned because of the adverse side effects caused by the large amounts of inocula used. In the 1930s, when pneumococcal serotyping was being developed and the immunogenicity of purified capsular PS was demonstrated, studies were performed with PS from selected serotypes. Those trials yielded satisfactory results, which culminated in the licensing of a hexavalent pneumococcal PS vaccine. However, because of the enthusiasm in the medical world about the therapeutic efficacy of antibiotics, this vaccine was seldom used. Eventually, it was withdrawn from the market (31, 158).

Despite the use of antibiotics, the mortality rate of systemic pneumococcal disease remained high (55). Therefore, and because of the emergence of antibiotic-resistant pneumococci (9), renewed efforts were undertaken in the 1970s to develop better pneumococcal PS vaccines (136). In 1978, a 14-valent vaccine was licensed in the United States, and in 1983, a 23-valent vaccine became available; the latter included new serotypes based on the most current knowledge of serotype distribution and cross-reactivities between various serotypes (31, 86).

Pneumococcal PS elicit long-lasting Ab and protection in healthy adults. However, revaccination does not result in anamnestic responses, and the vaccine is poorly immunogenic in population groups that are at high risk for pneumococcal disease (31, 99). These groups include asplenic patients (153), patients with frequent respiratory tract infections (127), human immunodeficiency virus-infected individuals, elderly people, and young children (99, 153). Children produce smaller amounts of anti-PS Ab than do adults, and substantial responses against the most common pediatric serotypes (6A, 14, 19F, and 23F) are observed only after the age of 4 to 5 years (42). Therefore, the development of a new generation of pneumococcal vaccines is required to protect humans who are susceptible to pneumococcal infections.

PNEUMOCOCCAL SACCHARIDE-PROTEIN CONJUGATE VACCINES

Coupling of pneumococcal PS or OS to proteins has been shown to enhance the immune system response to the saccharide moiety in animals (13, 57, 115), thereby resulting in protective immunity against *S. pneumoniae* (5, 56, 138). The immunological basis for the increased immunogenicity of saccharide-protein conjugates, as compared with PS, is assumed to be related to the TD character of these conjugates (129) (Table 2). Upon repeated immunizations, increased numbers of activated protein-specific Th cells are thought to provide help to saccharide-specific B cells, resulting in their differentiation toward memory or plasma cells. For this cellular cooperation, internalization of the conjugates via the membrane-Ig on PS-specific B cells has to take place, followed by processing of the protein and presentation of peptides to Th cells (85).

The role of protein-specific Th cells in the immune response to saccharide-protein conjugates is suggested not only by the lack of TD responses in nude mice (22) but also by the carrier-priming effect (115, 129). Moreover, the ability to induce TD responses with constructs consisting of PS coupled to synthetic peptides representing T-cell epitopes (3, 87, 110) but not B-cell epitopes (87) also supports the fact that Th cells play a key role in the Ab response to conjugates. The anti-saccharide Ab re-

sponse induced by conjugates is in many aspects comparable to the TD responses to proteins (129) (Table 2). Conjugates are immunogenic in infants (74, 144), they induce memory (129), and the isotype restriction characteristic of TI-2 antigens is bypassed (132, 134), especially when the conjugates are administered in combination with adjuvants (154, 163).

Nevertheless, the Ab isotypes induced by conjugates are not the same as those induced by proteins (Table 2). The primary anti-PS Ab responses induced by PS-protein conjugates resemble TI-2 responses (132). Secondary responses consist of moderate amounts of IgM and IgA and relatively high levels of IgG, in which IgG2 and—to a lesser extent—IgG1 predominate. Children, however, produce relatively more IgG1 than IgG2, as they also do in response to unconjugated PS (see the section on thymus-independent antigens, above) (132). In mice, the anti-PS (sub)isotypes elicited by PS-protein conjugates are, in decreasing order, IgG1 > IgG3 > IgM > IgG2a > IgG2b ≈ IgA (118, 132). The most likely explanation for the difference in Ab isotype patterns between proteins and conjugates is that conjugates still possess some TI properties, e.g., the ability to induce anti-PS IgM in nude mice (22) and the dominance of IgG2 over IgG1 (134). However, one cannot exclude the possibility that saccharide-specific B cells belong to a lineage with different activation requirements from those of protein-specific B cells. Furthermore, clonal restriction, as shown for TI-2 responses, is still observed in response to conjugates. This phenomenon seems to be caused by the limitations of the B-cell repertoire; although conjugation to a protein indeed results in stronger activation of B cells and in extensive Ig (sub)class switching, the numbers of B-cell clones reactive with saccharides remain the same (74, 163).

Conjugate Properties That Influence Immunogenicity

The immunogenicity of saccharide-protein conjugates is dependent on their structural characteristics (reviewed in references 41 and 116). The saccharide length and terminal structures, the nature of the carrier protein, the saccharide/protein ratio, and the coupling chemistry used all have been shown to influence the amount and quality of the Ab induced (6, 109, 117, 131, 154, 155).

The length of the saccharide component of conjugates has a considerable effect on their immunogenicity and the biological efficacy of the Ab, in terms of stimulation of phagocytosis and protection. OS shorter than one repeating unit seem to elicit protective responses only if they are capable of inducing Ab recognizing the full-length PS. This ability depends on the presence of immunodominant epitopes in the OS and also on its molecular conformation (4, 5, 122). The TD characteristics of a conjugate seem to be enhanced by using short OS (134) because, in contrast to the original PS from which they are derived, short OS are probably not able to activate B cells in a TI fashion. On the other hand, OS that are too short may not fully express conformational epitopes, whose existence in a number of pneumococcal and other PS has been proven (80, 109, 159). Consequently, for PS containing conformational epitopes, conjugates with saccharides of intermediate length will induce optimal responses, combining TD properties with the expression of the desired conformation (109).

Choice of the Carrier

Until now, proteins to be used as carriers in conjugate vaccines were selected on basis of their Th-cell-activating capacity. Following this approach, effective pneumococcal and other conjugate vaccines were constructed with well-known heterologous proteins like diphtheria or tetanus toxoids (109, 117, 134, 144). However, an important disadvantage in using these carrier proteins might be the excessive production of anti-carrier Ab as a result of frequent immunizations in childhood. Anti-carrier Ab have been shown to suppress subsequent responses to conjugates in mice (118) as well as in humans (17, 18), an effect that has been attributed to FcγRII-mediated inhibition of Ab production (17). The risk of inducing suppressive amounts of anti-carrier Ab should not be underestimated, especially when dealing with polyvalent pneumococcal conjugate vaccines that contain multiple doses of carrier protein (one for each serotype included) in a single vaccine vial. Deletion of B-cell epitopes from these carrier proteins might be a method to overcome suppression while retaining Th-cell induction.

The possible application of peptides as carriers for conjugate vaccines has been explored successfully over the last few years (3, 87, 110). Peptides offer the advantage of being able to be selected on the basis of both their B- and Th-cell immunogenicities (44), thereby minimizing the risk of anti-carrier Ab-mediated suppression while still providing sufficient Th-cell activation. Major drawbacks for the application of peptides as carriers are their low immunogenicity and their restriction in MHC binding. The immunogenicity of peptides can be improved by the generation of linear polypeptides or of branched multiple antigenic peptides or by the addition of adjuvants (10, 47, 91). The problem of MHC restriction might be addressed via two (not mutually exclusive) approaches: the selection of promiscuous peptides lacking a strong MHC restriction and the mixture or colinear synthesis of different peptides with a broad MHC-binding pattern (110).

Other investigators have begun examining the possibility of using pneumococcal proteins as carriers. Since these proteins are not used in childhood vaccination programs, the risk of inducing anti-protein Ab capable of suppressing subsequent responses is probably low. Moreover, moderate amounts of Ab against these proteins may be useful to enhance the opsonin-mediated phagocytosis of pneumococci or to diminish the harmful effects of inflammation caused by their degradation products.

As described in the section on virulence factors (above), Ab against PspA and pneumolysin are at least partially protective against pneumococcal infection in mice (1, 94). The functions of PspA are, however, still largely unknown; its structure varies among different pneumococcal strains, and no data on the Th-cell-activating capacity of PspA are available. Furthermore, the mechanism of protection of anti-PspA Ab is probably similar to that of anti-capsular PS Ab, namely, improving opsonization and phagocytosis of pneumococci. It is therefore questionable whether anti-PspA Ab would confer additional protection in the presence of highly protective anti-capsular PS Ab.

Pneumolysin is to date the best-studied pneumococcal protein (24, 111). Virtually all clinical isolates of *S. pneumoniae* produce pneumolysin; its primary structure is remarkably stable as well as being independent of capsular serotype, geographic area, and time of isolation. Although native pneumolysin has strong toxic effects, several derivatives which are nontoxic but retain the immunogenicity and protective activity of the native protein have been engineered (114). These constructs therefore seem to meet many criteria for inclusion in a pneumococcal conjugate vaccine (1, 111). One such pneumolysin mutant already has successfully been conjugated to pneumococcal capsular PS of serotype 19F. The conjugate induced substantially higher Ab responses than did the PS alone; this response could be boosted by repeated immunization. This strongly suggests that Th cells are activated by the carrier

protein (114). Indeed, at least one Th-cell epitope has been found in pneumolysin (3). Moreover, Ab induced by this engineered pneumolysin are likely to exert their protective effect at a different stage in pneumococcal infection from that of anti-capsular PS Ab. In this way, protection can be achieved on two fronts. First, anti-capsular PS Ab will enhance opsonization and phagocytosis of invading pneumococci. Second, if pneumococci succeed in replicating at foci of infection, the inflammation—and hopefully the symptoms of the disease—will be reduced by anti-pneumolysin Ab. If that is the case, immunization with a PS-pneumolysin conjugate should provide partial protection against serotypes besides those included in the vaccine. This seems likely, since a recent study demonstrated a significant degree of protection against nine different serotypes upon immunization with pneumolysin toxoid (1).

Future pneumococcal conjugate vaccines may be the product of a combination of the different approaches described above. Recombinant polypeptides containing Th-cell epitopes (from pneumococcal or heterologous proteins) and protective B-cell epitopes from pneumococci may be optimal carriers, inducing strong Ab responses to the capsular PS and to protective peptides from pneumolysin and other pneumococcal proteins.

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