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Pneumococcal Diversity: Considerations for New Vaccine Strategies with Emphasis on Pneumococcal Surface Protein A (PspA)

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INTRODUCTION

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide. In the United States, there are over 100,000 cases of bacteremic pneumococcal pneumonia each year, primarily in the elderly, with at least 40,000 resultant deaths (34a). In the developed world, fatal pneumococcal respiratory infections are most frequently seen in the elderly and are rare in the very young. In contrast, in the developing world, pneumococcal respiratory infections are a major cause of death in children younger than 5 years. In children, the pneumococcus is a major cause of otitis media, while pneumococcal meningitis occurs in both children and the elderly. Pneumococcal sepsis also occurs with high frequency in those infected with the human immunodeficiency virus (47).

Since *S. pneumoniae* was found to be sensitive to penicillin, pneumococcal disease was not considered to be a problem after penicillin came into routine use for the treatment of pneumonia patients. Deaths in elderly individuals with pneumonia who were treated with penicillin were assumed to be due to viral infections. Through the efforts of Robert Austrian and others, it was demonstrated that despite the availability of penicillin therapy, pneumococcal pneumonia caused more deaths in this country than almost any other infectious disease (6, 7, 35, 52). From a comparison of mortality data from the pre- and postantibiotic eras, Austrian and Gold demonstrated that patients who die during the first few days after being diagnosed with bacteremic pneumonia are generally not protected by treatment with antibiotics. However, patients who would have died at later times after the diagnosis can frequently be saved by antibiotic treatment. The early deaths apparently occurred because of the very rapid progress of the disease in some of the infected individuals. Austrian and Gold reasoned that the best way to protect these individuals was to develop a vaccine that would prevent disease progression and the associated fatal infections (6). Their studies and those of others led to the development of the 14-valent and subsequently the 23-valent capsular polysaccharide vaccines for the prevention of infection in adults (99). The need for pneumococcal vaccines has been further emphasized by more recent studies demonstrating a recent rapid increase in both the prevalence and level of resistance of multiple-antibiotic-resistant pneumococci (4, 33).

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PNEUMOCOCCAL VACCINES

Ideally, a vaccine that could protect against pneumococcal pneumonia would also protect against pneumococcal meningitis, otitis media, and bacteremia in young children. Since pneumococci are generally acquired from carriers and since nasopharyngeal carriage generally precedes infection, much of the recent thinking regarding the development of a pneumococcal vaccine is that the optimal vaccines would be those that protected against carriage as well as invasive disease.

The original decision to develop a capsular polysaccharide vaccine was based on a large body of research originating in the laboratories of Avery and others, which indicated that the outer surface of the pneumococcus was covered with a polysaccharide capsule. Antibodies generated against the capsular polysaccharide are typically highly protective against lethal infection (9, 110). The vaccine design was complicated, however, by the fact that protection elicited by the capsule is type specific and there are at least 90 different capsular types of *S. pneumoniae* (64). Taking advantage of the fact that certain capsular types are more commonly associated with human disease than are others, a vaccine composed of polysaccharides of the 14 (and later the 23) most common types was constructed (99). This vaccine was highly effective in young adults working in gold mines in South Africa, whose high rate of pneumococcal infection was caused largely by their working and living environments (5). However, the vaccine is only about 60% effective in preventing fatal pneumococcal bacteremia in the elderly (110). Unfortunately, the vaccine is unable to elicit adequate antibody responses to most of the capsular polysaccharides in children younger than 2 years (40). A recent survey of urgently needed vaccines in the developing and developed world places an improved pneumococcal vaccine among the top three vaccine priorities of industrialized countries (39).

Polysaccharide-Protein Conjugate Vaccines

Polysaccharide-protein conjugates are being developed as a potential solution to the less than adequate immunogenicity of the multivalent capsular vaccine in children and the elderly. This approach is based on early work with animals, which showed that polysaccharides could be rendered more immunogenic by conjugating them to proteins (8, 10, 56). This process endows the conjugated polysaccharide with certain properties of a T-cell-dependent antigen, allowing it to be antigenic in infants (68, 114). Pneumococcal conjugates have been prepared by coupling capsular polysaccharides to several carriers including tetanus toxoid, diphtheria toxoid, CRM_{197} (a nontoxic mutant of diphtheria toxin), pneumolysin, and meningococcal outer membrane proteins (112). Of these strategies, conjugates to tetanus toxoid and diphtheria toxoid have progressed the farthest in human trials. In general, conjugates of pneumococcal polysaccharides and proteins have been found to be more immunogenic in children than have the soluble polysaccharides (70, 131). Whether they will be more efficacious in adults than the soluble polysaccharides and whether they will elicit protection against carriage is still under study (112).

The basic approach of using polysaccharide-protein conjugates has proven highly successful with the *Haemophilus influenzae* type b polysaccharide vaccine in children, where only a single conjugate is necessary (118). However, for *S. pneumoniae*, it will be important that the vaccine contain conjugates of as many of the polysaccharide types as possible. Since each conjugate requires unique conjugation substrates and reaction conditions, individual conjugates must be separately constructed. Due to the amount of conjugated protein required to elicit immunity to a single polysaccharide, the number of different conjugates included in a single vaccine will of necessity be limited. In addition, one must be mindful that there is some variation in the most common pneumococcal serotypes in different parts of the world. This could be addressed by further increasing the number of polysaccharides in the vaccine or by making different mixtures of polysaccharides and conjugates for different regions of the world.

Immunogenic Considerations for Conjugate Vaccines

The finding that very young children failed to respond to the pneumococcal polysaccharide vaccine led to the discovery that human infants and infant young of other vertebrate species exhibit late maturation of their antipolysaccharide immune responsiveness (in comparison to their responsiveness to protein antigens). Children do not become adequately responsive to polysaccharides until after 2 years of age. In mice, antipolysaccharide responsiveness does not occur until after 2 weeks of age (40, 57, 71, 93).

Since the ability of infants to make antibodies to polysaccharides would undoubtedly aid in their resistance to encapsulated bacteria, the absence of antipolysaccharide responsiveness in infants raises the possibility that there is some selective immunologic or developmental disadvantage in making such responses (46, 63). Hayrinen and colleagues suggest that tolerance induced by host tissue polysialic acid and bacterial polysaccharide cross-reactivities may play a role in the poor immunogenicity of meningococcal capsular polysaccharides (63). Polysialyated glycoproteins have been found in human embryonal brain (63) and newborn rat kidney, heart, and muscle but not in the corresponding adult tissues (49). This suggests that the absence of responsiveness to this and other polysaccharide antigens in children may prevent the production of antibodies reactive with developing tissues. Based on these data, Finne and coworkers have raised caution regarding meningococcal group B vaccine development for children (49, 50). Whether similar concerns should be raised about other conjugate vaccines in infants is a matter of speculation.

Another possible problem with early vaccination of mice with polysaccharides may come from deleterious modulation of the murine antibody response (46, 129, 130). This effect is well demonstrated by the response to phosphocholine (PC). In the mouse, the antibody response to PC has served as a model for the effects of antibody specificity on neonatal immunization after mice reach maturity. PC is present in both F-antigen and C-polysaccharide (29, 32) and is an immunodominant determinant of these molecules in the mouse. C-polysaccharide is the name given to pneumococcal cell wall teichoic acid, which is released by the activity of the pneumococcal autolysin. F-antigen is a lipoteichoic acid of the pneumococcus which possesses a teichoic acid which is essentially identical to the cell wall teichoic acid (128). When mice are immunized with killed pneumococci, their immune response is dominated by antibodies to PC (84, 134); these antibodies are quite protective against subsequent *S. pneumoniae* infection (17, 24, 72, 90, 117). The majority of BALB/c antibodies to PC are of the T15 idiotype and are encoded by germ line light- and heavy-chain genes (98). Although BALB/c mice make antibodies to PC of other idiotypes, only the T15 idiotype antibodies are highly protective against pneumococcal infection (19, 20). In this context, "idiotype" refers to the tertiary structure of the complementary determining regions of antibodies. Although other tests can be used, the expression of different idiotypes is generally determined by the use of antisera or monoclonal anti-

TABLE 1. Modulation of the expressed adult repertoire of BALB/c mouse antibodies to PC by injection of C-polysaccharide or F-antigen 2 days after birth

Antigen injected 2 days after birth[a]	Immune response of mice to heat-killed pneumococcal vaccine at 7 weeks of age[b,c]			Median time (h) to death afforded by passive protection with 3 μg of PC antibody[b,d]
	Heat-killed pneumococcal immunogen	Geometric mean concn of anti-PC antibody (μg/ml) (SE factor)[e]	Geometric mean % T15 antibody response (SE factor)[e]	
Saline	Yes	63 (1.1)	72 (1.2)	166 ($P < 0.05$)[g]
F-antigen	Yes	135 (1.3)	20 (1.5)	161 ($P = 0.05$)
C-polysaccharide	Yes	224 (1.1)	<2	53
None	No	<5		36[f]

[a] Doses of C-polysaccharide and F-antigen were adjusted to contain the same amounts of antibody-detectable PC.
[b] Twelve mice per group were used in immunization and passive-protection studies.
[c] Adult mouse immunizations were as described previously (129).
[d] CBA/N mice were challenged with 350 CFU of capsular type 3 *S. pneumoniae* WU2. Passive-protection studies were conducted with the sera described in the first four columns..
[e] The SE factor is the number by which the geometric mean may be multiplied and divided to calculate the upper and lower bounds of standard error.
[f] For passive protection, a volume of normal mouse serum was injected comparable to that containing 3 μg of anti-PC in immune mice pretreated with saline.
[g] *P* values in comparisons with no antigen.

bodies (MAb) specific for the antigen binding site of the antibody in question (22).

In mice, the adult repertoire of antibodies is strongly affected by idiotype interactions during fetal and early neonatal development (46). To test the effect of neonatal exposure to C-polysaccharide, mice were injected with heat-killed, protease-treated pneumococci 2 days after birth and then immunized with the same killed vaccine as 7-week-old adolescents (129). It was observed that pretreatment with killed pneumococci resulted in an anti-PC response of 7-week-old mice that was largely deficient in antibodies of the T15 idiotype. In contrast, nonimmunized control mice were able to mount a primarily T15 response when immunized with killed pneumococci at 7 weeks of age. When sera from these mice were tested by passive injection into naive CBA/N mice, the sera from immunized, normal mice were protective against pneumococcal infection whereas the sera from mice also injected neonatally with killed pneumococci were not (129).

In subsequent studies, 2-day-old mice were injected with F-antigen, C-polysaccharide, or saline only. Strong suppression of the adult T15 response was observed when the mice were immunized with isolated C-polysaccharide but not F-antigen (Table 1). The protective capacity of these sera was evaluated by passive immunization of CBA/N mice with serum containing 3 μg of antibody to PC followed by challenge with capsular type 3 strain WU2. As expected from its low T15 content, the antibody to PC from mice that had received C-polysaccharide at 2 days of age was relatively nonprotective, whereas antibodies to PC elicited in mice treated with F-antigen or saline at 2 days caused significant extension of life (Table 1).

When adult mice were immunized with the same two preparations, F-antigen elicited the strongest anti-PC responses and C-polysaccharide appeared to be tolerogenic at high doses (Table 2). The greater immunogenicity of F-antigen than of C-polysaccharide has been known since the 1940s (55) and is presumed to be due to the lipid content of F-antigen. Taken together, these results demonstrate that neonatal exposure of mice to polysaccharide antigens can lead to idiotypic modulation and nonprotective immune responses. Interestingly, the strongest effect on the adult antibody repertoire was mediated by the form of the antigen that was least immunogenic. If this is found to be true for human infants, rigorous removal of nonconjugated tolerogenic polysaccharide fragments might minimize any immunomodulatory effects of polysaccharide-protein vaccines.

TABLE 2. Antigenicity of F-antigen and C-polysaccharide

Dose of antigen (μg)	Amt of anti-PC antibody (μg/ml)[a] elicited by:	
	F-antigen	C-polysaccharide
125	26	2
6.3	20	11
0.3	41	16

[a] Data for pools of serum from groups of five mice.

In spite of these concerns, it must be pointed out that millions of infants have been immunized at 3 months of age with the *H. influenzae* group B polysaccharide-protein conjugate vaccines and there have been no reports of deleterious immunologic or developmental effects. For a pneumococcal vaccine, however, five or more different conjugates would be required, possibly increasing the risk of deleterious consequences, especially if they are dependent on idiotype network interactions that may interfere with B-cell differentiation (46, 130).

Pneumococcal Protein Vaccines

In addition to the capsule, a number of protein antigens are either exposed on the surface or released from the pneumococcus (Fig. 1). Several laboratories have examined the possibility that antigens other than capsular polysaccharides elicit protection against pneumococcal infection (113). One approach has been to immunize animals with known toxins and surface proteins to see if they elicit immunity against infection. Using this approach, it has been shown that antibodies to pneumolysin, autolysin (pneumococcal amidase), and neuraminidase are protective (77, 78). Of these, pneumolysin and autolysin are the most effective at eliciting protection against sepsis. Antibodies to autolysin appear to exert their effect in part because they prevent the autolysin-dependent release of pneumolysin. Recent studies, however, indicate that strains with a mutation in autolysin may be less virulent than strains with a mutation in pneumolysin (34). If this is the case, autolysin may play other roles in virulence, such as the release of virulence factors in addition to pneumolysin.

Based on in vitro studies, a number of biologic functions have been ascribed to pneumolysin, including complement fix-

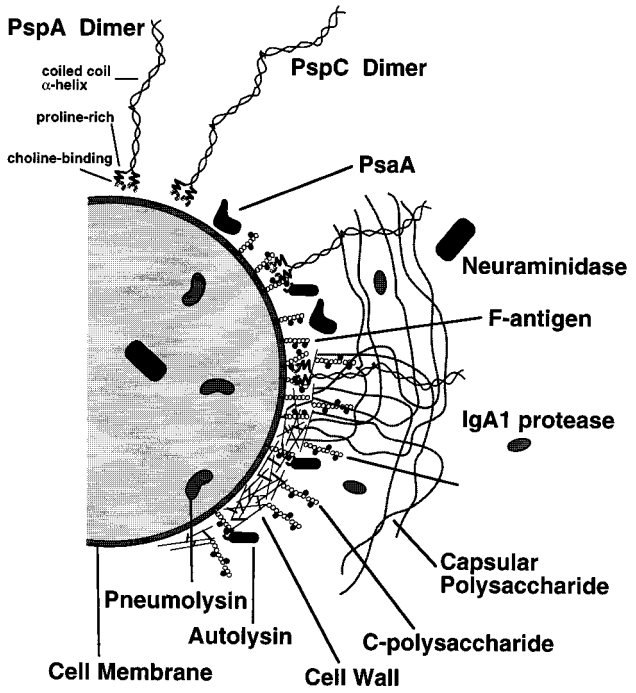

FIG. 1. Hypothetical representation of the pneumococcal surface depicting several noncapsular antigens shown to elicit protective immune responses in mice. C-polysaccharide (teichoic acid) attached to the cell wall is thought to be similar in structure to F-antigen (lipoteichoic acid), except that the latter contains lipids allowing it to insert in the cell membrane. Neuraminidase has been depicted both in the cytoplasm and beyond the capsule, since it is thought to be secreted by pneumococci. Although its role in virulence is mediated at a distance from pneumococci, pneumolysin is depicted in the cytoplasm of the cell shown here, since its release is dependent on the autolytic activity of autolysin. For PspA, an effort has been made to draw its extension from the surface to scale with respect to the thickness of the cell wall and capsule. It has been hypothesized that the lysines of the PspA α-helix interact with the capsular polysaccharides to stabilize the coverage of the surface by the capsule. This hypothetical function is depicted by showing individual capsular polysaccharide strands interacting with more than one PspA molecule. Since the location of PsaA with respect to other cell surface structures is unknown, its depiction here is completely hypothetical. Reprinted from reference 21 with permission of the publisher.

ation (97), Fc binding (91), and damage to epithelial cells (48, 105). It has also been shown that pneumolysin plays a role in ocular infections (65, 69) and pneumococcal infections initiated in mice by the intranasal, intratracheal, and parenteral routes (1, 14, 34, 77, 96, 104). It is clear that in the presence of pneumolysin, capsular type 2 strains are able to grow exponentially in mice and cause fatal disease, whereas in the absence of pneumolysin, pneumococci increase in number only until they reach 10^6 CFU/ml of blood. From that point on, the mouse is largely able to control their numbers to about 10^6 CFU/ml, with death being delayed for several days and at times prevented altogether. Although the mechanism by which pneumolysin acts is unclear, it appears to regulate the host inflammatory response. In the absence of pneumolysin, the inflammation that develops is highly effective and can even protect against subsequent infections with pneumolysin-producing strains. In the presence of pneumolysin, the elicited inflammatory response is not effective in curtailing the *S. pneumoniae* infection (12).

Another approach used for identifying protection-eliciting pneumococcal proteins has been to produce MAb to proteins by immunizing with whole nonencapsulated pneumococci or with pneumococcal extracts. Protective MAb were then used to identify the eliciting proteins and to aid in the cloning of their structural genes. This approach was used originally to identify pneumococcal surface protein A (PspA) (84) and, more recently, a 37-kDa protein, PsaA (106, 126). PspA immunization can protect against pneumococcal sepsis following either intravenous (i.v.) or intraperitoneal (i.p.) pneumococcal challenge doses of $>10^5$ times the 50% lethal dose (LD_{50}) of pneumococci (18, 23). As in the case of pneumolysin, immunization with PspA has been found to elicit protection against strains of more than one capsular type (1, 125). PsaA was named "pneumococcal surface adhesin A" based on the similarity of its predicted amino acid sequence to those of adhesins of certain oral streptococci (107). PsaA has been shown to elicit protection against fatal infection in mice with a single capsular type 3 strain (120). PspC and several other choline binding proteins have been identified through the homology of their genes to the structural genes for autolysin (21, 103, 111).

In the development of protein-based vaccines, it seems likely that the optimal vaccine might contain more than one protein antigen. In this regard, it is important to note that many of the proteins, such as PsaA, pneumolysin, and PspA, might be found to play even more important roles in protection against carriage, lung infection, or invasion than they do against sepsis. Until the studies of their roles in virulence are completed, the full value of these proteins as immunogens will not be known.

PNEUMOCOCCAL SURFACE PROTEIN A (PspA)

Identification of PspA by Using MAb

PspA was first identified by using MAb produced from lymph node cells of CBA/N mice immunized with heat-killed nonencapsulated R36A pneumococci (84). Nonencapsulated pneumococci were used as the immunogen to maximize the chance of eliciting antibodies to noncapsular antigens. CBA/N mice express an X-linked immunodeficiency (XID) trait which prevents them from making immune responses to most polysaccharide antigens, including the PC determinants of C-polysaccharide and F-antigen (2, 24, 93). The failure of these mice to make antibody responses to PC was especially relevant to this study, since PC is a major immunologic determinant when mice are immunized with killed nonencapsulated pneumococci (36).

Growing hybrids were screened for antibodies against heat-killed R36A *S. pneumoniae*. In later fusions, mice were immunized with whole encapsulated pneumococci or cell wall extracts of pneumococci. Although XID mice were used, about 60% of the MAb reactive with R36A were specific for the PC epitope (84). The observation that antibody responses to polysaccharides are seen more readily in MAb than in serum antibody of XID mice has also been reported by others (109). In almost every case, MAb elicited by immunization with whole pneumococci or pneumococcal cell wall extracts that were nonreactive with PC were found to react with proteins (81, 84). The only exceptions observed in MAb from numerous independent fusions were a single MAb reactive with the type 5 capsule that was generated by immunization with a heat-killed capsular type 5 pneumococcus (27) and a MAb reactive with a non-PC component of pneumococcal teichoic acids (88, 135). All but one of the anti-protein MAb reacted with the same protein. This protein was designated PspA (27, 85). The only other protein we identified by this approach was PspB, which was serologically variable like PspA. Unlike PspA MAb, the MAb to PspB was not protective against pneumococcal infection (82). Once it became available, recombinant PspA was used to elicit additional MAb (83). PspA is now identified by

over 12 MAb. Each of these has been shown to react with immunologically distinct epitopes and recognizes different but overlapping subsets of pneumococcal strains. This finding was the first evidence for the complex serological diversity and cross-reactivity of PspA (43, 83, 84).

Because R36A is a nonencapsulated pneumococcus, the protective capacity of the MAb was evaluated by challenge with capsular type 3 pneumococcal strain WU2. It was observed that five of nine MAb tested were protective against fatal WU2 infection (83) and that the four MAb which did not protect against this strain reacted poorly with WU2 PspA (83).

Role of PspA in Virulence

To determine if PspA is required for full virulence of pneumococci, insertion duplication mutagenesis (80, 123) was used to disrupt a gene required for PspA expression. Mutants of nonencapsulated strain Rx1 were derived by transformation with a library of restriction-digested pneumococcal DNA fragments ligated to pVA891. This plasmid carries an erythromycin resistance gene and lacks a functional origin of replication in *S. pneumoniae* (80). Thus, erythromycin-resistant colonies contain plasmid DNA integrated into the chromosome by homologous recombination. If the DNA fragment is internal to a gene, that gene will be interrupted by the integrated plasmid and a truncated gene product will be produced. By screening colony blots of erythromycin-resistant pneumococci with a PspA-specific MAb, transformants which had lost the ability to produce detectable PspA were identified (89).

From one of these mutants, WG44.1, we isolated pKSD300, a derivative of pVA891 that contained a 583-bp fragment of pneumococcal DNA. By transforming pKSD300 back into Rx1, it was possible to eliminate the expression of surface PspA in 100% of transformants (89). By adapting the transformation procedure for use with encapsulated pneumococci (145), it was possible to use pKSD300 to produce similar mutations in the virulent strain D39 (89). Later studies demonstrated that the D39 and Rx1 mutants prepared by transformation with pKSD300 actually produced a PspA fragment comprising the N-terminal 245 amino acids of the protein. However, these fragments were not surface attached and were secreted into the medium (144). Similar mutations were prepared in encapsulated type 3 *S. pneumoniae* WU2. The reduced virulence of the D39 PspA$^-$ and WU2 PspA$^-$ mutants was manifest as increased LD_{50}s and more rapid clearance of PspA$^-$ pneumococci from the blood of infected mice (27, 89). Originally it was also reported that PspA$^-$ mutants were also prepared from a type 5 strain (27). Later it was discovered that, for reasons that are still unclear, these particular mutants had also lost their ability to make capsule. Thus, the lack of virulence in the PspA$^-$ type 5 strain cannot be attributed solely to a loss of PspA. In the D39 and WU2 mutants, however, capsule was present in quantities essentially identical to those in the parent strains (27).

Analysis of Nucleotide and Amino Acid Sequences

The first amino acid sequence determined for PspA was that of the N-terminal 45 amino acids of an isolated N-terminal fragment of Rx1 PspA (121). The fragment was produced by an Rx1 strain that had been transformed with pKSD300. The fragment migrated with an apparent molecular mass of 43 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In later cloning experiments, the deduced amino acid sequence indicated that the actual size of this PspA protein fragment was 27 kDa and that PspA fragments could have some tertiary structure even in SDS-containing gels (143, 144). The N-terminal sequence of PspA was unblocked. It contained a proline in amino acid position 4, and the remainder of the 45-amino-acid sequence was consistent with that of an α-helical coiled-coil protein (121).

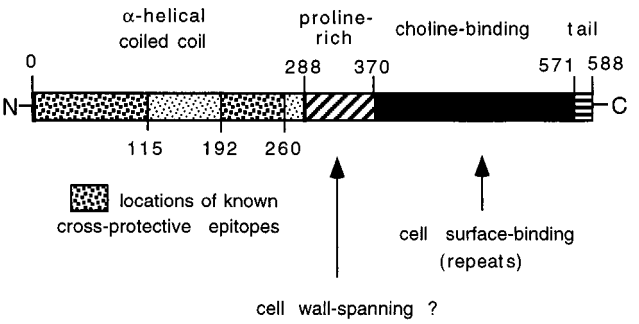

FIG. 2. Schematic presentation of the domains of PspA delineated from the deduced amino acid sequence of Rx1 *pspA*. The locations of cross-protective MAb epitopes, determined by mapping studies, are shown. Numbers indicate amino acid residues. The two arrows point to the proline-rich (amino acids 289 to 370) and choline-binding (amino acids 371 to 571) regions, respectively. The functions of these regions are largely unknown but may include mechanistic roles, in addition to those described herein.

Numerous attempts to clone the *pspA* gene, using MAb to detect expression in *Escherichia coli*, were unsuccessful. In retrospect, our failures to clone *pspA* by this method are probably explained by the fact that expression of full-length PspA greatly retards the growth of *E. coli*. The complete *pspA* gene was finally cloned, however, by a circuitous approach. It was known that insertional inactivation, directed by the 583-bp pneumococcal DNA fragment present in pKSD300, resulted in an absence of PspA surface expression (89). It was assumed that the insertion directed by this fragment either interrupted a necessary regulatory gene, interfered with the promotion of the *pspA* structural gene, or interrupted the *pspA* structural gene itself. To examine these possibilities, the WG44.1 chromosome containing the insert was cloned, mapped, and sequenced (144). DNA sequencing and subsequent cloning revealed a previously undescribed 2-kb open reading frame which included the 583-bp fragment (143). Guided by the sequence of the open reading frame and knowledge of the restriction enzyme sites that had been identified during the mapping and cloning, the two halves of the *pspA* gene were reassembled and cloned into *E. coli*. Even though recombinant PspA was somewhat toxic, full-length PspA was readily detected in the transformed *E. coli* (143, 144). While these studies were in progress, *pspA*-containing DNA from strain D39 was successfully cloned into lambda (86). However, again, the slow growth of *E. coli* producing the intact gene product thwarted attempts to move the functional *pspA* gene from the lambda clone to a plasmid in *E. coli*.

Although the sequence of the Rx1 *pspA* gene predicted an amino acid sequence for a 65-kDa protein, the PspA of strain Rx1 migrated in SDS-PAGE with an apparent molecular mass of 84 kDa (121, 143, 144). It seems likely that the larger than expected apparent size of the PspA in SDS-containing gels is due to tertiary structure rather than to polysaccharide substitutions, since substituted proteins are thought to be rare in bacteria. Moreover, the size of PspA is similar whether it is produced in *E. coli* or pneumococci (86, 144). Most cloned truncated PspA fragments were also smaller than their apparent size in SDS-PAGE (144).

Based on its deduced amino acid sequence, it is possible to divide PspA into four domains (Fig. 1 and Fig. 2). The N-

terminal 50% of the molecule has a sequence consistent with the α-helical coiled-coil structure frequently found in surface fibrillar proteins of gram-positive bacteria (143). The surface exposure of the α-helical region is supported by mapping studies in which individual MAb have been localized to epitopes in this region (43, 83). PspA MAb have been shown to bind intact pneumococci in enzyme-linked immunosorbent assay studies with immobilized whole bacteria (84), immunofluorescence microscopy (142), and electron microscopy (58). The surface exposure of PspA was also confirmed by surface labeling of pneumococci with radioactive iodine before immunoprecipitation with PspA MAb (85).

There is a proline-rich region, C-terminal to the α-helical sequence, in which the predominant sequence motif contains alternating proline and alanine residues. It is anticipated that this proline-rich region of PspA spans the cell wall of *S. pneumoniae*, as is the case for proline-rich regions of other surface proteins of gram-positive bacteria (45). C-terminal to the proline-rich region are 10 20-amino-acid repeats followed by a 17-amino-acid nonrepeating tail at the C terminus. The repeat region is composed of hydrophobic and hydrophilic amino acids that form a binding site for choline. The repeat region forms a binding site that attaches PspA to choline residues of F-antigen (also referred to as lipoteichoic acid) in the pneumococcal cell membrane (146). The polysaccharide chains of the F-antigen extend into the cell wall (128), and therefore the attachment of PspA to the F-antigen may occur within the cell wall. Such a scenario would require at least the start of the proline-rich region to reside within the pneumococcal cell wall. If this were the case, the α-helical region might extend at least 43 nm from the pneumococcal cell wall surface (143). Although the bulk of PspA is known to be associated with F-antigen, the possibility cannot be excluded that some small fraction of PspA binds to choline residues in the C-polysaccharide (also known as teichoic acids). Should that be the case, PspA might extend even further from the bacterial surface.

Putative Function of PspA

Although the role of PspA in virulence remains unresolved, it is known that it retards the clearance of pneumococci from the blood (89). It seems likely, therefore, that the major effect of PspA on virulence involves interference with the antiphagocytic properties of pneumococci. In the absence of PspA, pneumococci fix complement more effectively as measured by the bystander complement assay (21). However, this assay does not measure complement deposited on the pneumococcal surface, and thus the significance to pathogenesis requires additional study. With regard to antibody-mediated protection, it is clear that antibody to PspA enhances the clearance of pneumococci from the blood and that prior treatment with cobra venom factor largely eliminates the protective effects of passively administered PspA antibody (26).

A possible virulence mechanism for PspA is suggested by the amino acid composition of the PspA sequence. The α-helical region of Rx1 PspA contains a higher concentration of lysines than that generally found in coiled-coil proteins. The positions of the heptad repeat of coiled-coil proteins exposed to the fluid phase are b, c, e, f, and g. In PspA, 26% of these positions are occupied by lysines, while in other fibrous proteins, only about 12% of the exposed positions contain lysines (38). Even M protein of group A streptococci contains only about 18% lysines in positions b, c, e, f, and g (51). Capsular polysaccharides of almost all pneumococci that are virulent in humans are negatively charged (75). Since the flexible side chains of lysines carry a positive charge, they are ideally suited for interactions with the negatively charged capsular polysaccharide. If such interactions occur, they could serve to stabilize the capsular structure and maximize the ability of capsule to mask the pneumococcal cell wall. Alternatively, the proposed ionic interactions might simply permit PspA to extend into and protrude through the capsule layer.

It should be noted, however, that the overall net charge of the α-helical half of Rx1 PspA is negative at neutral pH. In the α-helical region, there are 61 amino acids (glutamic acid and aspartic acid) with negatively charged side chains and 56 (including 50 lysines) with positively charged side chains. The predicted isoelectric point is approximately 4.9 (143). Thus, even if there are charge interactions between individual lysines and the capsular polysaccharide, the net charge of the surface would remain negative. Any negative charges of the polysaccharide that are neutralized by lysines would be more than compensated for by the negatively charged amino acids of PspA.

Protective Effect of Immunity to PspA

As previously indicated, the first evidence that immunity to PspA might be protective was that PspA-specific MAb protected mice from otherwise fatal sepsis. It was observed that passive protection by the injection of immunoglobulin G (IgG) or IgM MAb to Rx1 PspA protected mice from death following i.v. or i.p. injection with 10^5 times the LD_{50} of *S. pneumoniae* WU2 (18). The passive antibody resulted in clearance of pneumococci from the blood of challenged mice (18, 84) in a complement-dependent manner (26).

The observations that MAb to PspA passively protected mice against experimental pneumococcal sepsis suggested that active immunization with PspA might elicit a protective response. Unfortunately, PspA proved difficult to isolate in pure form because the native molecule aggregates readily (122). Thus, the original analyses of the ability of immunization with PspA to elicit protection were indirect. One approach was to immunize XID mice with heat-killed Rx1 or with heat-killed WG44.1, a $PspA^-$ mutant of Rx1 containing an inactivated *pspA* gene (89). Immunization with rough pneumococci elicits strong antibody responses to PC in immunologically normal mice. Anti-PC antibody of mice, especially that of the T15 idiotype, can be quite protective against pneumococcal infection (17, 24, 134). Thus, immunodeficient XID mice were chosen for this study, since they lack the ability to produce serum antibody to PC (24) but still make antibody responses to proteins. At the time, it was known that *S. pneumoniae* WG44.1 lacked PspA on its surface but it was not known if the mutation was in the *pspA* structural gene or a regulatory gene. It was later found that WG44.1 unexpectedly lacks the 5′ half of the *pspA* gene, including the sequence encoding the *pspA* promoter region (144). Thus, WG44.1 is completely unable to make PspA or fragments of PspA.

Mice were immunized with heat-killed pneumococci suspended in saline and then challenged i.v. with 25 to 50 LD_{50} of type 3 strain WU2. The nonencapsulated $PspA^+$ strain Rx1 elicited protection against fatal infection, whereas the $PspA^-$ strain WG44.1 did not (89). This finding made it clear that immunization with heat-killed nonencapsulated pneumococci elicited protection in mice only if PspA was present. This study did not rule out the involvement of or the requirement for other pneumococcal molecules as adjuvants or coimmunogens.

Immunity to PspA in the Absence of Other Pneumococcal Antigens

Before the completion of the cloning studies of Yother et al. (143, 144), the *pspA* structural gene was cloned by using a lambda *gt*11 library (86). Efforts to resolve the full-length gene from lambda into an expression plasmid in *E. coli* were unsuccessful due to the toxicity of full-length PspA in *E. coli*. The lambda clone did, however, provide a means of evaluating the ability of full-length PspA to elicit protection. The *pspA* gene was expressed during lysogeny, and the PspA produced was recovered as the "lambda lysate" by washing the agar plate with buffer. As a control, lysates were prepared from clones of the lambda library that contained random unidentified (non-PspA encoding) pneumococcal DNA fragments. The lysates were emulsified in complete Freund's adjuvant and used as immunogens. Mice immunized with the PspA-containing lysates were protected from fatal infection following i.v. pneumococcal infection of CBA/N mice with 3,000 CFU (300 LD_{50}) of capsular type 3 strain WU2. Similar immunization with preparations from non-PspA-producing lambda lysates did not protect mice from pneumococcal infection (86).

Immunization with the 27-kDa N-terminal fragment of the α-helical region of Rx1 PspA first demonstrated that an isolated PspA protein could elicit protection. This fragment, composed of amino acids 1 to 245 of PspA, was originally produced in pneumococci by insertion duplication mutagenesis with pKSD300 (143, 144). The 27-kDa PspA protein fragment was isolated by preparative SDS-PAGE from a periplasmic extract of *E. coli* expressing the cloned product (121). When the 27-kDa PspA fragment was used for immunization, its complete structure had not been determined; due to its electrophoretic mobility, it was referred to as a 43-kDa fragment of Rx1 PspA (121). Immunization of CBA/N (XID) mice with 5 μg of the 245-amino-acid fragment was carried out with a primary subcutaneous injection of 5 μg of material in complete Freund's adjuvant followed by an i.p. booster dose in phosphate-buffered saline, while control mice were immunized likewise with adjuvant and phosphate-buffered saline but no antigen. Mice immunized with the fragment were protected from subsequent i.v. challenge with 300 CFU (30 LD_{50}) of *S. pneumoniae* WU2 (121). We subsequently demonstrated that immunization with a fragment containing the N-terminal 243 or 260 amino acids of Rx1 PspA was unsuccessful without adjuvant (23, 139). The antigenicity of the 243-amino-acid fragment was greatly enhanced however, by expressing it as a fusion protein with interleukin-2 (139). More recent studies involving immunization with 1 or 5 μg of a fragment comprising the N-terminal 303 amino acids of Rx1 PspA in the absence of adjuvant have shown it to be immunogenic in mice and able to elicit protection against fatal infection following i.v. challenge with 480 CFU of A66.1, a strain that is significantly more virulent than WU2. The greater immunogenicity of the 303-amino-acid fragment in the absence of adjuvant may be due to its larger size (67).

It seems likely that the major mediator of protection following PspA immunization is antibody rather than some form of cell-mediated immunity. It is clear that immunization with PspA elicits IgG antibody in serum and that passive anti-PspA IgG and IgM MAb can protect against fatal infection (84). Moreover, immune sera from immunized mice (86) or rabbits (124) can passively protect mice from otherwise fatal sepsis with injection doses of 10^2 to 10^4 times the LD_{50} of the challenge pneumococci.

Isolation and Immunogenicity of PspA

Initial attempts to isolate full-length PspA from cell wall extracts of pneumococci were greatly hampered by the fact that native PspA aggregates (122). Isolation of PspA was greatly enhanced after the discovery that the repeat region of PspA was homologous to the choline-binding domain of autolysin (143). For autolysin, binding to the PC epitope of teichoic acid is necessary for activation of its enzymatic activity (108) and is sufficient for attachment of purified autolysin to the cell wall (53). However, elution with high choline concentrations or growth in medium containing ethanolamine rather than choline did not release autolysin for cell surfaces (15, 146), suggesting that autolysin may also have a non-choline-dependent method of attachment to the pneumococcal surface.

For PspA, however, the repeat region was found to be both required and sufficient for binding PspA to the pneumococcal surface by attachment to choline residues of F-antigen. This finding led to several successful approaches to the release of PspA from pneumococci and its subsequent isolation (23, 146). The most direct isolation protocol was to grow pneumococci under normal culture conditions, wash them with buffered saline, and then elute PspA from the bacterial surface with 2% choline chloride. An alternative approach was to grow the organisms in 1.2% choline chloride, a previously known means of blocking the action of autolysin by preventing its binding to the choline of cell wall teichoic acids (29, 54, 127). As for PspA, growth in 1.2% choline chloride results in a significant proportion of PspA being released into the medium (146). Additionally, PspA is released from the surface of pneumococci when they are grown in a defined medium containing ≤0.00001% choline chloride and 0.03% ethanolamine (146). This latter technique also leads to the loss of activity of autolysin because of the absence of choline in teichoic acids (128). Growth in either high concentrations of choline chloride or 0.03% ethanolamine media also inhibits the action of the enzyme(s) responsible for separation of cells during growth (28). As a result, pneumococci grown in these media remain in long chains and do not autolyse.

Each of these techniques releases PspA from the pneumococcal surface, but the PspA is in the presence of other bacterial proteins. Even so, the contribution of PspA to the protection elicited by the fractions could be clearly demonstrated by comparing the resistance of mice immunized with material prepared from $PspA^+$ Rx1 and $PspA^-$ WG44.1 strains (23). In every case, the PspA-containing preparations were able to elicit protection in the absence of Freund's complete adjuvant whereas the preparations lacking PspA were not. It was observed, however, that in the presence of adjuvant, non-PspA components present in the medium of $PspA^-$ pneumococci grown in 1.2% choline chloride or 0.03% ethanolamine (in place of choline) medium were able to elicit protection if the spent medium was injected at high concentrations. These results indicated that these preparations contained additional non-PspA protection-eliciting molecules (23). The purer PspA preparations were obtained when pneumococci were washed before elution with 2% choline chloride (146). If similar elutions were made from $PspA^+$ Rx1 and $PspA^-$ WG44.1, protection was elicited only by preparations made from the $PspA^+$ pneumococci, even in the presence of adjuvant (23).

The most successful strategy for purification of PspA has been to run PspA-containing extracts over a choline (or choline analog) column and subsequently elute the bound PspA with 2% choline chloride. The spent medium from cultures grown in ethanolamine can be chromatographed directly on the choline-Sepharose column. If PspA is to be isolated on a

TABLE 3. Ability of three PspAs to elicit protection against 14 diverse *S. pneumoniae* challenge strains[a]

Strain	Pneumococcal challenge		% of mice alive 21 days after challenge of mice immunized with[b]:			
	Capsule type	PspA type	D39	WU2	BG9739	None[c]
D39	2	25	38	60		3
WU2	3	1	100	100	100	2
A66	3	13	75	100	80	5
EF10197	3	18	100		80	0
ATCC 6303	3	7	100			0
BG9739	4	26	11	60	13	0
EF3296	4	20	25	20	10	0
EF5668	4	12	22	25	60	9
L81905	4	23	10	0	31	0
DBL5	5	33	10		14	0
EF6796	6A	1	100			0
DBL6A	6A	19	67	25	33	4
BG9163	6B	21	89			20
BG7322	6B	24	100	60	25	6

[a] Immunized and control CBA/N mice (groups of 10 to 50 animals) were challenged with ~100 LD_{50} of each strain.

[b] Where no data are shown, no experiment was conducted. The results of the same studies have been previously presented as median days alive after challenge (26). Underlined numbers are statistically different from those in the right-hand column ($P < 0.05$).

[c] "None" includes unimmunized mice and mice immunized with mock preparations from *S. pneumoniae* lacking the *pspA* gene.

choline-Sepharose column from the spent medium of pneumococci grown in 1.2% choline chloride or the 2% choline chloride eluate of live pneumococci, prior dialysis to remove excess choline is required. PspA isolated from a choline-Sepharose column is more than 95% pure based on SDS-PAGE with silver staining (23, 146). The isolated material is immunogenic in mice at doses as low as 0.1 μg in the absence of adjuvant. When a mock isolation is conducted with spent medium from a $PspA^-$ strain, even a 100-fold-greater volume of the choline-eluted material does not elicit protection. Even in the presence of adjuvant, the mock-isolated material fails to elicit protection. These results verify that the only protection-eliciting molecule isolated by this procedure is PspA (23).

Cross-Reactivity and Cross-Protection

Even though PspA can be divided into numerous serologically distinguishable groups, it is a very cross-reactive protein. Sera from a single rabbit immunized with PspA can recognize PspA in Western blots of all pneumococci (43). Moreover, seven MAb to individual epitopes were each observed to react with 20 to 50% of PspAs and collectively with 95% of PspAs examined (43). The best evidence for cross-reactions between PspAs is the observation that immunization with a single PspA can protect mice against strains with serologically distinguishable PspAs (86, 125). In fact, cross-protection appears to be the rule rather than the exception. This was seen when mice were challenged with a panel of 14 *S. pneumoniae* strains following immunization with individual PspAs (Table 3) (25). Although certain PspAs elicited better protection against some strains than others, each PspA exhibited cross-protection against most challenge strains. Another major finding is that it is easier to protect against some pneumococcal strains than others. For example, it was difficult to obtain complete protection against capsular type 2 and 4 strains, even when mice were immunized with the homologous PspA. This finding may be partially due to the use of hypersusceptible, immunodeficient CBA/N mice. Preliminary results, however, indicate that protection against death due to capsular type 2 strain D39 and capsular type 4 strain EF3296 can be readily elicited by PspA in immunologically normal BALB/cByJ mice (25).

Location of Protection-Eliciting Epitopes of PspA

From the first cloning studies, it was apparent that MAb that were able to protect against pneumococcal infection reacted with fragments of PspA containing the majority of the α-helical region of PspA (43, 121, 144). The first evidence that this region could elicit protective immunity was not obtained, as was described above, until an insertionally inactivated mutant PspA, comprising the N-terminal 245 amino acids, was used for immunization (121). The same general region of PspA was shown to elicit protection against diverse strains of *S. pneumoniae* when mice were immunized with a recombinant *Mycobacterium bovis* BCG vaccine expressing a fragment containing amino acids 4 to 299 of Rx1 PspA (74). Subsequently, a fragment of PspA comprising only the first 115 amino acids was also shown to elicit protection against pneumococcal infection (23).

By using recombinant fragments of PspA, five MAb protective against the mouse-virulent encapsulated strain WU2 have been localized in two regions of Rx1 PspA (83). One of the five cross-protective MAb reacted with the fragment containing the N-terminal 115 amino acids of PspA. The other four MAb reacted with epitopes located between amino acids 192 and 260. No significant reactivity was observed with any of the cross-protective MAb in the region between 115 and 192 or in the portion of the α-helical region from amino acids 260 to 288 (Fig. 2).

It has also been shown that the PspA fragment of *S. pneumoniae* Rx1 extending from amino acid 192 to the C terminus (amino acid 588) is highly immunogenic and capable of eliciting statistically significant cross-protection against a diversity of pneumococci with very different PspAs (125). By using Rx1-specific primers for the respective ends of the sequence from amino acids 192 to 588, a number of homologous fragments of diverse PspAs were amplified and cloned (125). Three of these clones were expressed, and the recombinant PspAs were used to immunize mice. In all three cases, cross-protection was elicited against unrelated strains of pneumococci (125). These results indicate that cross-protection can be efficiently elicited by epitopes C terminal to amino acid 192 of PspA. Additionally, an Rx1 *pspA* fragment, BAR416, which expressed amino acids 192 to 299 was cloned (83). This PspA peptide proved capable of eliciting protection against capsule type 3 strain WU2 (83) and five additional strains expressing diverse PspAs. Moreover, PspA fragments homologous to BAR416 from other pneumococcal strains also elicit protection against infections with *S. pneumoniae* WU2 (26).

Immunity to Pneumococcal Carriage Elicited by PspA

The human reservoir of pneumococci is maintained by asymptomatic nasopharyngeal carriage. Strains acquired by adults and children are thought to be generally obtained from carriers, and carriage is known to precede infection (59). Therefore, a vaccine that could block carriage would be able to protect not only the immunized individual but also individuals who have not been immunized or who are unable to respond effectively because of immunodeficiency or age. This principle has been well established for the case of type b *H. influenzae*, where immunization with protein conjugates of the type b polysaccharide have virtually eliminated both carriage and disease (118).

We have developed a mouse model of pneumococcal carriage whereby carriage can be achieved with strains of at least five capsule types, including type 6B and 4 strains, which are highly virulent in mice when injected parenterally (141). By using this model, protection against nasopharyngeal carriage can be obtained by immunization with full-length PspA or tetanus toxoid conjugates of capsular type 6B polysaccharide in the presence of cholera toxin B subunit as an adjuvant (140). Studies with other pneumococcal surface molecules such as PsaA (120) and PspC (21), a molecule very similar in structure to PspA, are needed to ensure that an optimal vaccine is developed to protect against carriage.

Existence of a PspA-Related Protein, PspC

In virtually all pneumococci, there are two chromosomal loci homologous to the Rx1 *pspA* gene (87). Both of these loci, *pspA* and *pspC* (originally called the *pspA*-like locus), have homology to regions of the *pspA*/Rx*1* gene encoding the α-helical, proline-rich, and repeat regions of Rx1 PspA (21, 87, 115). Evidence now exists that most strains produce products from both genes (31, 42). The *pspC* gene has also been discovered by others and has been designated *spsA* and *cbpA* (62, 103).

Potential Use of PspA as a Human Vaccine

It has been known since 1992, from the unpublished work of Marilyn Crain, that the sera of virtually all adult humans and many children contain antibody to PspA as detected by Western blotting (41). The results of quantitative studies of human response to natural exposure and active immunization with PspA are not yet available. If vaccination with PspA is found to elicit an even higher antibody response in humans than does natural exposure and if the elicited antibodies are found to be efficacious against pneumococcal infections, PspA could be used as a vaccine in one of several ways. If it is found to be highly efficacious against all pneumococcal infections, it might be used by itself as a 3- to 7-valent PspA vaccine. It could also be used as a carrier for pneumococcal polysaccharides, provided that the conjugation did not significantly impair the protection elicited by PspA itself. Alternatively, it could be used as a nonconjugated protein in combination with a limited number of protein-polysaccharide conjugates or with other pneumococcal proteins such as pneumolysin or PsaA, which might elicit protection that is complementary to that elicited by PspA.

DIVERSITY IN PspA AND PNEUMOCOCCI

With regard to vaccine development, antigenic variation may be an important consideration. While it would be much easier to prepare a vaccine by using an invariant molecule, the existence of variability in a protein suggests that there is immunity to the molecule in the normal host that exerts selective pressure for variation. Thus, the protein molecules that elicit the best immunity may be those which, like capsular polysaccharides of pneumococci and M-proteins of group A streptococci, exhibit considerable serologic variation within the population. If variable proteins are used as vaccines, however, it becomes essential to understand the extent of this variation and the degree of cross-protection elicited by different variants of each protein.

Understanding the variability of pneumococci themselves is important because it is becoming clear that not all pneumococci cause equivalent infections or use identical infection strategies. This is quite apparent for animal infections (13, 16, 141) but is also seen with regard to capsular type differences for human infections and carriage in adults and children.

Variability of PspAs and *pspA* Genes

PspA is one of the more variable gene products of pneumococci. By using MAb to PspA, it has been possible to define more than 40 different serotypes (43, 95). By examining the apparent molecular weight of the PspAs, it is evident that differences can frequently be observed and that most individual isolates have unique combinations of PspA serotype and PspA molecular weight (43, 138). As expected, the *pspA* gene is also highly variable at the level of DNA sequence (66). *pspA* genes from more than 50% of independent isolates analyzed have been amplified by PCR. Examination of more than 70 amplified *pspA* genes revealed 17 different restriction fragment length polymorphism (RFLP) patterns (30, 116). Closely related but unique amino acid sequences have been identified in the 5′ half of each of over 25 different *pspA* genes (66).

The variability in chromosomal DNA containing *pspA* and *pspC* sequences can also be detected by Southern blot analysis of restriction fragments with full-length *pspA* or *pspA* oligonucleotide probes (87, 111, 115). The full-length *pspA* probe detects both the *pspA* and the *pspC* loci in virtually all strains, even at high stringency. An oligonucleotide probe from within the α-helical region of *pspA* hybridizes with DNA from a minority of strains at high stringency. These findings demonstrated that for both genes, the 5′ half is more variable than the 3′ half. As the stringency is decreased, the probe for the α-helical region recognizes one or both of the *pspA*-homologous sequences in most strains (115).

Other genes exhibiting variability among pneumococci are IgA1 protease and genes for certain penicillin binding proteins of penicillin-resistant isolates (60, 61, 79). However, the genes for pneumococcal neuraminidase, pneumolysin, and PsaA of all pneumococci, as well as those for penicillin binding proteins of penicillin-sensitive strains, appear to be relatively invariant. In Southern blots, probes for the neuraminidase gene (116) and the pneumolysin gene (11) generally hybridize with single bands of similar size in the pneumococcal strains analyzed. Moreover, pneumolysin genes have been sequenced from several pneumococci and the deduced amino acid sequences are >99% identical (92, 133).

Variability of Pneumococci

The first demonstration that pneumococci are genetically quite diverse, even within capsular types, came from studies of the serologic and molecular weight diversity of PspA (43, 136–138). The fact that individual isolates of *S. pneumoniae* of the same capsular type generally differed either in PspA serotype or in the apparent molecular weight of their PspA suggested that a large number of different pneumococcal clones of each capsular type exists (43, 137). This conclusion was confirmed at the molecular level by RFLP analysis of the Rx1 *pspA* gene (87), as well as RFLP analysis of PCR-amplified *pspA* genes from many pneumococcal strains (115). Since PspA (and, by analogy, probably PspC) is a virulence factor, it could even be argued that differences in these genes may be greater than those at other loci.

Although this is probably true, it turns out that pneumococci are quite diverse even within capsular type and even when the analysis is not based on the diversity of PspA or PspC. This diversity has been shown in part by the use of multilocus enzyme electrophoresis (95, 119). More recently, restriction enzyme-digested DNAs of independent pneumococcal isolates have been shown to generally exhibit different restriction pat-

terns by Southern analysis with the IS*1167* insertion sequence (116). The IS*1167* element, first identified in Morrison's laboratory (147), has been found by us to be present in copy numbers ranging from 3 to 12 in virtually all pneumococci (100, 101). This considerable diversity of pneumococci is also apparent from other techniques including BOX- and repetitive extragenic palindromic (REP)-PCR (73, 102) and pulsed-field gel RFLP patterns of genomic DNA (76, 132).

The great diversity of pneumococci appears to have accrued over an extended period, since changes have not been observed with repeated isolates from patients or from outbreaks (43, 87, 94, 95, 138). One particularly interesting group of strains is that containing D39 and its descendants, including R36A and Rx1. These strains have been separated by more than 130 years of laboratory and animal passage, yet they all have identical *pspA* genes and gene products by all analyses described above, including identical IS*1167* patterns (43, 87, 100, 138, 147). PspA was monitored in sets of pneumococcal strains of the same capsular types recovered from individual patients over a period of 1 year or more. In each case, all had identical PspAs by serologic and molecular weight determinations (136, 138). Examination of strains from two outbreaks showed no variation in IS*1167* pattern or PspA type or *pspA* chromosomal RFLP pattern within either outbreak (101).

Panmictic Nature of Pneumococci

It would be anticipated that mutational mechanisms undoubtedly account for much of the variability seen in pneumococci. However, the observation that the same PspA epitopes occur in different combinations on PspAs from different strains suggests that there have been numerous crossovers within the *pspA* locus (43, 137). Moreover, the observation that different PspA types and different PspA epitopes appear to be found in strains of many, if not all, different capsular types suggests that genetic exchange within pneumococci has been important in the development of present-day pneumococcal genotypes (43, 137). Additional evidence for this exchange comes from studies showing that genes for penicillin resistance and genes for capsular type have been exchanged to different genetic backgrounds in certain highly penicillin-resistant strains (37, 44). More recent studies using variability at the IgA protease locus and multienzyme typing have concluded that pneumococci are panmictic; that is, the diversity in their genotypes created by genetic exchange is substantial compared to the diversity created by mutation alone (79).

CONCLUSIONS

Human disease resulting from *S. pneumoniae* infection has been and continues to be associated with a significant degree of morbidity and mortality and with a significant economic burden. Improved pneumococcal vaccines are badly needed to help combat this threat. Although major efforts are being directed at the development of polysaccharide-protein conjugate vaccines, the immunogenic nature of pneumococcal proteins also makes them prime targets for new vaccine strategies. Although several pneumococcal proteins are capable of eliciting protective immunity in mice, PspA is unique in that it can elicit antibodies that protect mice against inocula at least 100-fold greater than the LD_{50}. PspA enhances the virulence of pneumococci and is able to elicit protective immunity against both pneumococcal sepsis and nasopharyngeal carriage. Although PspA is serologically variable, it is sufficiently cross-reactive that immunization with a single PspA elicits protection against diverse strains of *S. pneumoniae*. The design of the pneumococcal vaccine must take into consideration the extreme variability of pneumococci. Due to the cross-protective nature of PspA, it is anticipated that an appropriately chosen mixture of different PspAs or PspA fragments (possibly in combination with other pneumococcal proteins) might be able to elicit broad protection against pneumococcal infection.

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