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Characterization of the protective antibody responses to PSPA in Streptococcus pneumoniae.

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CHARACTERIZATION OF THE PROTECTIVE ANTIBODY RESPONSES TO PSPA IN *STREPTOCOCCUS PNEUMONIAE*

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Characterization of the Protective Antibody Responses to PspA in *Streptococcus pneumoniae*

Streptococcus pneumoniae is a common cause of respiratory tract infections, meningitis, and septicemia worldwide. It is a major cause of morbidity and mortality, especially in children and the elderly. The emergence and progressive increase in incidence of antibiotic resistance coupled with the less-than-adequate polysaccharide vaccine warrants further investigations of protective pneumococcal protein antigens.

Pneumococcal surface protein A (PspA) is a promising candidate, alone or with other immunogens, in a future vaccine. Based on sequence homology, *pspA* sequences have been divided into two major families and further into six different clades. The major cross-protective region of a family 1 PspA was shown to be located at the distal part of the N-terminal domain, a region that has been called the clade-defining region (CDR).

The first study described in this dissertation examines the protection and cross-protection eliciting regions of a family 2 PspA. Recombinant fragments spanning the α-helical domain were produced from the family 2 PspA of *S. pneumoniae* EF3296. Certain recombinant fragments provided better protection and cross-protection than others. Fragments that included the CDR were better able to protect and cross-protect than fragments that lacked the CDR. This study indicated that, although dissimilar, the sequences

in PspAs of families 1 and 2 still possess protection-eliciting regions with enough conformational similarity to elicit cross-protection..

The second study discusses the contribution of PspA to eliciting protection from death against a type 4 encapsulated strain. Types 2, 4, and 5 encapsulated strains have historically been harder to protect against with PspA immunization. PspA immunization is able to prevent death in type 3 (WU2) strains but not in type 4 strains, here TIGR4. The *pspA* genes were exchanged between these two strains. Immunization with PspA fragments from either the type 3 or the type 4 strain showed that PspA immunization was generally very successful in protecting against infection but that strains of the type 4 background were somewhat harder to protect against than the type 3 strains, regardless of which PspA was expressed.

The dissertation thus shows that PspA immunization can be very successful in conferring protection against pneumococcal infection and identifies the CDR of the protein as the major cross-protective region.

DEDICATION

I dedicate this dissertation to my family and my husband, Anders Håkansson.

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AP	Alkaline phosphatase
BCIP	5-Bromo-4-chloro-3-indolylphosphate
BSA	Bovine serum albumin
CbpA	Choline-binding protein A
CDR	Clade-defining region
CFU	Colony-forming unit
DLDH	dihydrolipoamide dehydrogenase
DHFR	Dihydrofolate reductase
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluoroscein isothiocyanate
Hib	*Haemophilus influenzae* type B
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
LD_{50}	Lethal dose 50
NBT	Nitroblue tetrazolium
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline containing 0.05%Tween 20
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpsA	Immunoglobulin-A binding protein
THY	Todd-Hewitt yeast medium containing 0.5% yeast extract
TIGR	The Institute for Genomic Research
XID	X-Linked immunodeficiency

INTRODUCTION

History and Background

The pneumococcus has been an integral and important part of early discoveries in the fields of microbiology, immunology, and microbial genetics. Although probably first observed and reported by Klebs in 1875 (188), the pneumococcus was not reportedly isolated until 1881, when Pasteur and Sternberg independently described it. Pasteur isolated the pneumococcus from rabbits inoculated with saliva from an infant who died of rabies, and Sternberg did the same from rabbits that were injected with his own saliva, which was used as a control in one of his experiments (139, 168). In the late 1880s the pneumococcus was implicated as the causative agent of pneumonia, meningitis, and otitis media (188); this report initiated the quest for a better understanding of the workings of this organism. In the next decade a few seminal discoveries followed. In 1891 Klemperer and Klemperer (102) were the first to show passive protection against pneumococcus with serum therapy by demonstrating that protection could be obtained in offspring of immunized animals against challenge with a homologous strain of pneumococcus. These researchers then injected immune serum into patients to demonstrate protection against infection (102). Metchnikoff was the first to demonstrate agglutination of pneumococci by immune serum and thus the first to establish a method of differentiating pneumococci by serotype (188). This led to an early differentiation of three types of pneumococci. Other ways of better differentiating pneumococci for diagnostic purposes came with the

discovery by Neufeld that pneumococci were bile soluble (188). This finding was later followed by differentiation of capsular material by the Quellung reaction, which increased the sensitivity of strain distinction. Outside the realm of pneumococcal infection, studies of the pneumococcus have led to a number of other significant discoveries in biology. In 1884, Christian Gram used the pneumococcus to demonstrate the vital but now routine Gram stain (77). In 1925, Avery and colleagues discovered that the soluble substance that surrounds the pneumococcus and was used for strain differentiation, was made of polysaccharides, providing the first description of the capsular composition in any bacterial organism (188). In 1928, Griffith found that when he injected into mice a mixture of virulent, encapsulated, heat-killed pneumococci with live unencapsulated, avirulent bacteria, the unencapsulated strain was capable of being converted to the same serotype as the heat-killed strain. Avery and Dubos initiated the quest for the molecule responsible for this transformational process in the 1930s (11); in 1944, Avery et al. elucidated this phenomenon by demonstrating that the genetic carrier of information, or the "transforming principle" was DNA (12).

Epidemiology

Streptococcus pneumoniae is one of the most common bacterial species causing respiratory tract infections. The pneumococcus is also a major cause of septic infections and meningitis and is occasionally an etiological agent of endocarditis.

Worldwide, there are an estimated 4 million deaths in children because of pneumonia; about 1 million of these deaths are caused by *S. pneumoniae,* mostly in children who live in developing countries and are below the age of 1 year (166).

In the US for the year 1999, the pneumococcus was ranked 6th among the top 10 leading causes of disease (4). In the general population, *S. pneumoniae* infections cause 100,000–135,000 hospitalizations for pneumonia; 6 million cases of otitis media; and over 60,000 cases of invasive disease, including 3,300 cases of meningitis (5). In children below the age of 5 years, *S. pneumoniae* remains the leading cause of infections, including bacterial pneumonia, bacteremia, and otitis media, (101, 109); where about 17,000 cases of invasive disease in these children are reported every year including 700 cases of meningitis and 200 deaths (4).

S. pneumoniae has become the most common invasive pathogen in infants and children because of the emergence of antibiotic-resistant strains during the 1990s (see below) and because of the fact that the vaccine against *Haemophilus influenzae* type b (Hib) has proven highly effective in eliminating Hib as the primary pathogen in immunized children (24).

Risk factors

Pneumococcal infections increase with age and previous hospitalizations. Workers and residents at nursing homes, workers and children at day-care centers, and persons with underlying disorders like liver disease, heart disease, or chronic obstructive pulmonary disorder are all predisposed to pneumococcal infections (49, 110). Previous infection with influenza virus or other respiratory tract viruses is thought to promote pneumococcal infection (13, 49, 80, 87, 143, 178). Malnutrition, especially zinc deficiency, increases susceptibility to pneumococcal infection. Also, anatomical factors such as narrow eustachian tubes in children are responsible for increased incidence of otitis media

(98, 156). Finally, splenectomized patients who are unable to clear bacteria because of their inability to filter blood, and persons with sickle cell disease are also more susceptible to pneumococcal infections (46, 136, 149, 192, 193).

Disease process

Carriage. Pneumococci are part of the normal microflora of the upper respiratory tract in humans. Five to 10% of healthy adults carry pneumococci in their nasopharynx, but carriage in children is much higher (78). Carriage rates have been estimated to be as high as 44% among children aged 6 years or younger (199), 60%-80% for children at day-care centers (27, 28, 68), and greater than 70% for children with otitis media (59).

Both colonization and adherence to the respiratory tract are thought to be important at the beginning of the pathogenic process. Nasopharyngeal carriage is reported to be the single most important factor in predisposing children to pneumococcal disease. Still, only a few individuals carrying pneumococci develop symptomatic otitis media, pneumonia, bacteremia, or meningitis. It is not uncommon for individuals to carry more than one strain, and it has been reported that as many as four serotypes can be carried simultaneously by a child ((9) and references therein). Carriage with a specific strain can last anywhere from a few weeks to 6 months; although prolonged carriage has not been shown to increase the risk of infection, acquisition of new serotypes has been implicated (78, 79, 97). Infants are thought to acquire a new serotype every 4 months on an average, thereby increasing their chances of developing disease.

Adherence and colonization. Pneumococci bind to host structures through a variety of mechanisms. In the 1980s, Glc-NAc (β1-3) Gal and Gal-NAc (β1-4) Gal were identified as receptors for pneumococci on buccal epithelial cells and the epithelial cells from the nasopharynx (2, 105). Later, the oligosaccharide structure containing the disaccharide unit NeuAc α2-3 (or 6) Galβ was shown to block adherence of pneumococci to cultured epithelial cells (16), and a study by Riise et al. showed adherence inhibition by N-acetylcysteine (148), which is known to reduce the number of infectious exacerbations in patients with chronic bronchitis. The adhesins for these binding specificities have not yet been identified. The in vivo efficacy of anti-adherence carbohydrates based on Glc-NAc (β1-3) Gal-containing structures indicated that these carbohydrates could indeed prevent experimental pneumonia in a rabbit infection model (94, 201). A later, randomized, placebo-controlled study in children did not show any differences between placebo- and receptor-analog- treated groups with acute otitis media being the end point (180).

Pneumococci also bind to activated pneumocytes and endothelial cells expressing the platelet-activating factor receptor (56). Adherence of pneumococci to platelet-activating factor receptors and activated endothelial cells is somehow thought to be mediated by phosphoryl choline because pneumococci grown in media where choline was replaced with ethanolamine or pneumococci pretreated with phosphoryl choline antibody exhibited greater than 60% decrease in adherence (56). Cleavage of terminal sialic acid residues by neuraminidase is implicated in exposing otherwise cryptic receptors and hence promoting adherence. *S. pneumoniae* may be assisted by influenza and parainfluenza viruses that may employ their neuraminidases to alter receptors synergistically with pneumococcus and thereby increase adherence. Peptide permeases are implicated in in-

creasing adherence of *S. pneumoniae* to epithelial cells and endothelial cells during initial colonization of the lung or the vascular endothelium (57).

Transition from carriage to invasion. Exactly how the pneumococcus succeeds in translocating from the nasopharynx, from being a carrier to becoming an invader in the lung, in the blood or to the middle ear is not completely clear (97). Although adherence seems to be important for establishing and maintaining colonization, the relationship between adherence and infectivity of individual strains is not easily discerned. It has been observed that carrier strains and acute otitis media strains generally show high adherence to epithelial cells whereas meningitis-and sepsis-causing strains generally exhibit low adherence (3). This finding may indicate that a high level of adherence will restrict the bacteria to the mucosal surface, whereas a lower level of adherence to mucosal structures may make the strain capable of slipping into the blood stream; however, the association cannot be taken that far. Other factors most likely determine whether a colonizing strain becomes a disease-causing strain. Phase variation (186, 187) or previous viral infection (80, 143) is believed to aid the entry of pneumococci to the alveolar space and result in inflammation and pneumonia. Spontaneous phase variation is widespread among strains of pneumococci (186). Transparent variants are less virulent than opaque colonies of pneumococci. Opaque variants are incapable of colonizing the nasopharynx and do not adhere to lung cells because they are unable to bind to the receptors on these cells (58). Invasion from the nasopharynx directly to the bloodstream and the brain (114) is also known to occur. Recently, it has been suggested that pneumococci may use choline-binding protein A (CbpA), also known as pneumococcal surface protein C (PspC)

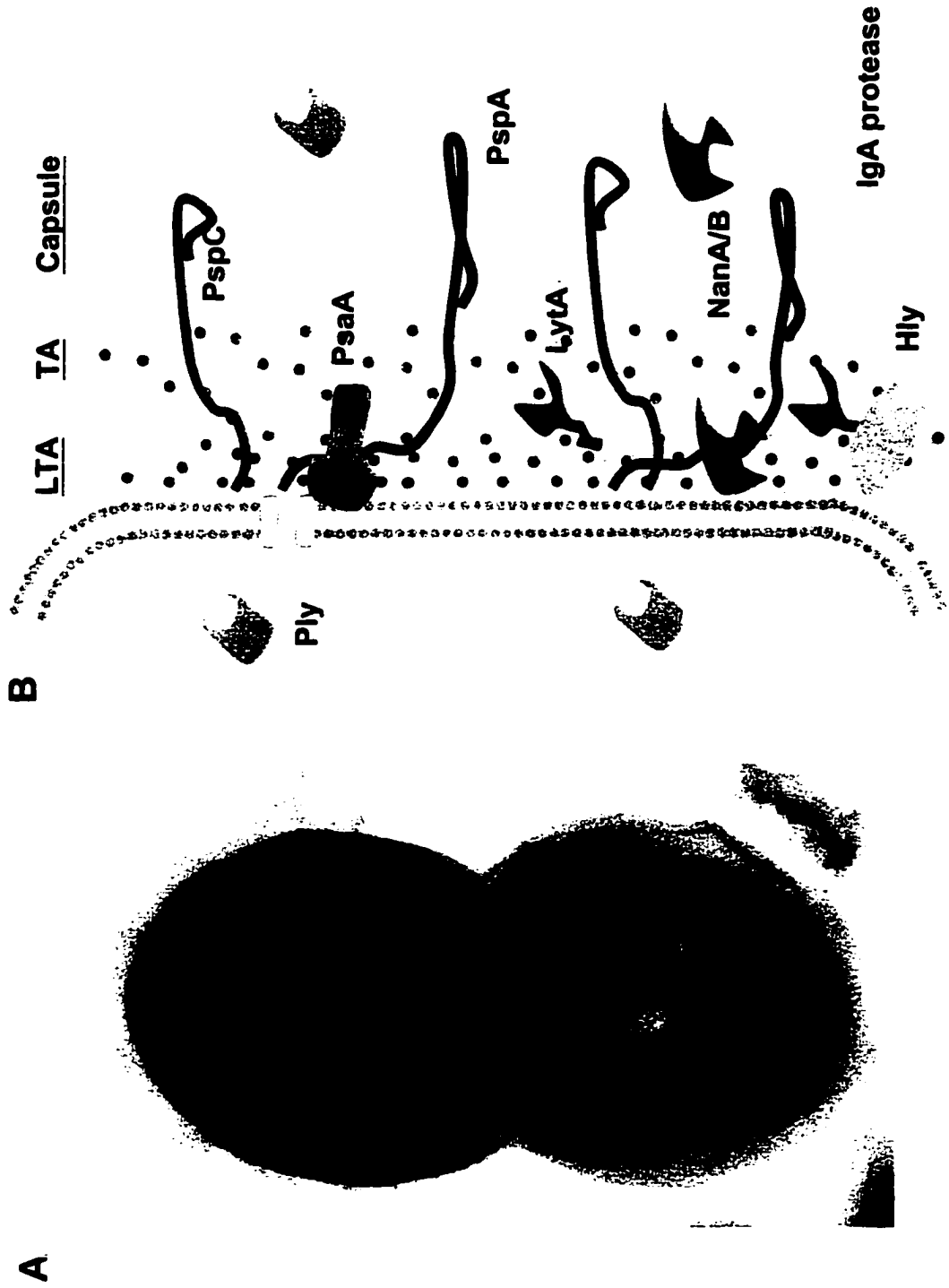
and immunoglobulin A-binding protein (SpsA), to bind to human pIgR to cross the epithelial barrier (200).

Pneumococcal structure and virulence factors

S. pneumoniae is a gram-positive bacterium that expresses several structural proteins as well as proteins, with other functions. This section is intended to present the overall structure of the pneumococcus and its virulence factors. A depiction of the bacterial surface can be seen in Fig. 1.

Capsule. The capsule in *S. pneumoniae* is different from those in other streptococci. So far, there are 90 different serotypes with varying capsular polysaccharide structures (89, 181). The capsule is important for the survival of pneumococci, both in the nasopharynx and in the bloodstream (11, 43, 86, 113, 190). It is well known that non-encapsulated pneumococci are readily cleared when used to infect mice (11, 86). Additionally, the pneumococcal capsule has recently been shown to be required for colonization of the nasopharynx (113). Several mechanisms have been proposed for the role of the capsule as a virulence factor where all the mechanisms relate to the capsule's ability to confer resistance to opsonization and phagocytosis (43, 44, 52, 92, 115, 163, 190). The capsule may function as a physical barrier to prevent the interaction between complement and its receptors on phagocytes (44) and may also protect against attack of circulating antibodies on the surface proteins expressed by the pneumococcus in a manner similar to the capsular protection of C5a peptidase in Group B streptococcus (26, 183).

FIG. 1. Morphology and virulence factors of *S. pneumoniae*. (A). Electron micrograph of *S. pneumoniae* EF3296. The micrograph shows a diplococcus with almost full septation and condensed chromatin. (B). A schematic drawing of the pneumococcal surface displaying the main virulence factors of the organism. LTA, lipoteichoic acid; TA, teichoic acid; Ply, pneumolysin; Hly, hyaluronidase; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C; PsaA, pneumococcal surface adhesin A; LytA, autolysin; NanA/B, neuraminidase A and B.



Although 90 different serotypes are present with modifications in structure and chemical composition, only a few are reported to be involved in causing disease. It is estimated that 10 serotypes are responsible for causing up to 62% of pneumococcal inva- sive disease worldwide; serotype prevalence however, varies with the age group examined (88). In the US, children are usually infected with one of seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) that account for 80% of infections in children and 50% of infections among persons > 6 years old (47, 48). These serotypes are common also in other parts of the world, although some variation has been shown. For example, in children from Africa and South America, types 1 and 5 are the common serotypes associated with infection, which is not the case in the US. In adults, types 1, 3, 4, 6, 8, 9, 12, 14, and 23 are prevalent for infection or colonization (73).

Although type-specific antibodies are known to clear pneumococcal pneumonia, the two currently available 7-valent and 23-valent vaccines cover only 7 or 23 different capsule types (see discussion below).

Cell wall. The pneumococcal cell wall consists of two major components, teichoic acid and peptidoglycan, in almost equal proportions. Attached to the cell membrane by a lipid moiety is the lipoteichoic acid. Teichoic acid and lipoteichoic acids of pneumococci have identical repeat carbohydrate chain structures in which phosphocholine is incorporated and the presence of phosphocholine and identity of repeats distinguish them from other gram-positive bacteria (74, 75). These phosphocholine residues are essential for binding of a family of choline-binding proteins and are also required for the activity of autolysins (175). The cell wall composition changes when the bacteria be-

come resistant to penicillin or other antibiotics and in the phase variants (76, 158, 159, 185). In penicillin-resistant strains, the normally linear stem peptides were exchanged for a more complex branched stem peptide structure (76). In phase variants the ratios of teichoic to lipoteichoic acid were affected (185). Transparent colonies have increased quantities of teichoic acids and choline in the cell wall, and opaque colonies have more lipoteichoic acids.

Pneumolysin. Pneumolysin (*ply*) is a 53 kDa thiol-activated pore-forming cytolysin present in all pneumococci and is a key virulence factor. Mutants inactivated for pneumolysin were avirulent in mice (23). The main function of pneumolysin is to interact with cholesterol in the cell membrane and insert itself into the lipid bilayer. This cytolysin then oligomerizes to form transmembrane pores, resulting in cell lysis (140). Pneumolysin is cytotoxic to varying cell types, including monocytes, neutrophils, endothelial cells (153), and alveolar epithelial cells (152). Other functions have been associated with different domains of the protein (128). In addition to being cytotoxic to respiratory tract cells, pneumolysin disrupts surface integrity and slows the ciliary beating of human respiratory tract epithelium (72). Additionally, the toxin is known to activate the classical complement pathway (141). This activation reduces serum opsonic activity and is believed to be partially caused by the ability of pneumolysin to bind to the immunoglobulinG's (IgG) Fc region nonspecifically (129). The ability of pneumolysin to modulate these different functions makes it a crucial factor in pathogenesis.

Autolysin. Autolysin (*lytA*) is the major cell wall hydrolase mediating the lysis and death of pneumococci induced by, for example, bile salts and β-lactam antibiotics. Activation requires the presence of choline in the cell wall teichoic acids, as has been elegantly presented by Diaz et al. (61, 62), who showed that changing the C-terminal choline-binding domain of LytA to the non-choline-binding domain of the CPL1 phage lysozyme made LytA choline independent. Autolysin has a functional role in cell separation at the end of cell division and in the autolytic event seen at the end of the pneumococcal growth phase (150). LytA-negative mutants have been shown to be greatly reduced in virulence in intranasal and intraperitoneal mice models of infection (17, 20, 22). When the *lytA* gene alone was mutagenized, there was significant decrease in virulence but in a pneumolysin (*ply*) deletion background, it failed to increase attenuation in virulence; however, in an intraperitoneal mouse infection model, suggesting that LytA's major role would be to cause release of pneumolysin (20). The long-held view that autolysin was required for release of pneumolysin needs to be revised in light of a new report that suggested that this was not the case at least for one strain of pneumococcus (14). It has been suggested that antibiotic tolerance results from the modulation of autolysin. In multiply drug-resistant strains of pneumococci, autolysin is downregulated and pneumococci are able to survive but not grow in the presence of penicillin (111, 176).

Pneumococcal surface adhesin A (PsaA). PsaA has been suggested to be an adhesin through its homology with adhesins of certain oral streptococci (155). Some adherence data are available, although a definitive role as an adhesin has not yet been established (21). PsaA is a 37-kDa surface protein that is proposed to be part of an ATP-

binding cassette transport complex and is likely involved in Mn^{2+} and probably Zn^{2+} uptake (21, 107). A *psaA*-negative mutant has been shown to be avirulent in mice in an intraperitoneal model and resulted in reduced carriage in an intranasal challenge model and in a 10-fold reduction in the ability to bind to type II pneumocytes when compared with wild type strain (21, 31, 132). Additionally, mutations in *psaA* were reported to decrease autolysis and transformation efficiency (53, 132). PsaA is known to be immunogenic, and antibodies against PsaA are protective against pneumococcal infection in experimental animal models (31, 33, 134, 171). Increased immunogenicity has been observed in mice when PsaA has been used in conjunction with other pneumococcal proteins such as pneumolysin and PspA (31, 33, 134). Antibodies to PsaA are found in human sera, and children previously exposed to pneumococcal infection have higher titers of antibodies to PsaA (33, 134). It has also been reported that pneumococcal carriage and acute otitis media induce local production of anti-PsaA antibodies early in life (145, 146, 164).

Pneumococcal surface protein A (PspA). PspA is one of 12 known choline-binding proteins and the gene is present in all pneumococci (55). PspA has been shown to be required for complete virulence. Strains where PspA has been inactivated show reduced virulence in murine infection models (125). Immunization with PspA has been shown to protect against pneumococcal infection (32, 34, 36, 37, 119, 121, 125 , 172). Being the topic of this dissertation most known aspects of PspA will be discussed in greater detail later in this section.

PspC. The presence of a surface protein with structural features similar to PspA was identified during studies with PspA and named pneumococcal surface protein C (PspC) (124, 169). PspC has also been referred to in the literature as CbpA (151), Hic (95), or SpsA (83). PspC is known to cross-react with PspA, which may in part be the reason antibodies elicited by PspC are protective against infection with pneumococci (35, 42). CbpA/PspC mediates adherence to activated lung cells and is shown to play an important role in nasopharyngeal colonization in an infant rat model (151). It was recently reported that CbpA/PspC alone or together with a genetic toxoid of pneumolysin, PdB, protected mice against infection with *S. pneumoniae* (135). CbpA/PspC has been shown to bind the secretory component of human IgA (83), human factor H (60) and the complement component C3 (91, 95). CbpA/PspC is suggested to be expressed in higher quantities during carriage rather than during bacteremia (185). Also, PspC interacts with the polymeric Ig receptor, which may enable invasion of the mucosa (200).

Neuraminidase. The presence of one or more neuraminidases has been reported for most clinical isolates of pneumococcus. Neuraminidases have the capacity to modify host structures by cleaving terminal sialic acid residues on glycolipids, glycoproteins on cell surfaces, or in body fluids. They could also reveal otherwise cryptic cell surface receptors and thus make the receptors accessible to adhesins (105). At least two neuraminidase genes, *nanA* and *nanB* have been cloned and sequenced (18, 51). The exact role of these enzymes in pathogenesis has not been elucidated, although NanA is believed to play a role in the colonization of the nasopharynx and the development of otitis media (177).

Hyaluronidase. Hyaluronidase is present in all strains of pneumococcus. This enzyme is likely to assist in pathogenesis by degrading the connective tissues and allowing invasion (19, 20, 104). A recent report has shown that, although mutation in the hyaluronidase (*hly*) gene alone did not affect virulence in a mouse infection model, *hly* contributed to virulence by decreasing the virulence of a pneumolysin mutant even further (20).

Other known virulence factors. The availability of the genomic sequence of *S. pneumoniae* has led to the identification of several new proteins that may function as virulence factors (174). The putative proteinase maturation protein A (PpmA), which shows homology to members of the family of peptidyl-prolyl cis/trans isomerases, is a surface protein that elicits species-specific opsonophagocytic antibodies that are cross-reactive with various pneumococcal strains. In a mouse pneumonia model, *ppmA*-deficient mutants showed reduced virulence (137, 138).

Enzymes involved in virulence.

(i) Pyruvate oxidase and hydrogen peroxide. *S. pneumoniae* produces hydrogen peroxide, primarily through the action of pyruvate oxidase (SpxB). Hydrogen peroxide is a free-radical intermediate that is produced as it grows, in quantities comparable with those produced in activated neutrophils. It has been reported to cause middle-ear mucosa damage in acute otitis media (170), and studies also show that the damage hydrogen peroxide causes to alveolar epithelium contributes to the cellular injury in pneumococcal pneumonia (67). Another function for hydrogen peroxide may be the suppres-

sion of growth of *Haemophilus influenzae* and other respiratory tract pathogens like *Moraxella catarrhalis* and *Neisseria meningitides* (142).

spxB of *S. pneumoniae* encodes a pyruvate oxidase that decarboxylates pyruvate to acetyl phosphate, hydrogen peroxide, and carbon dioxide (167). A mutation of *spxB* led to a reduction of virulence in both pneumonia and sepsis models in mice. The decreased virulence was attributed to a decrease in acetyl phosphate levels, resulting in downregulation of adhesive proteins (167), and could also be associated with a decreased ability of the bacteria to produce hydrogen peroxide.

(ii) Nicotinamide adenine dinucleotide (NADH) oxidase. NADH oxidase is an enzyme encoded by the *nox* gene in *S. pneumoniae*, and is reported to be an oxygen sensor that modulates competence and virulence in pneumococci (10, 69, 70). The role of NADH oxidase in virulence was demonstrated when *nox* mutants used in a respiratory tract infection mouse model showed markedly decreased virulence (198).

(iii) Dihydrolipoamide dehydrogenase (DLDH). DLDH is usually involved in the three-step conversion of 2-oxo acids to their respective acyl-CoA derivatives. In addition to being the E3 component of these enzyme complexes, DLDH has also been shown to have other functions. DLDH-negative bacteria that were shown to grow normally in vitro were avirulent in both sepsis and lung infection models in mice, indicating that DLDH activity was necessary for survival of pneumococci within the host (165).

Vaccines

Despite the advent of antibiotics, the incidence of disease caused by pneumococci has not been eradicated. Vaccines for *S. pneumoniae* have been available for a long time, but the emergence of drug-resistant strains has been cause for serious concern in recent years. It is estimated that about one-third to one-half of all the pneumococcal strains recovered from humans have become at least partly resistant to penicillin (6, 63). New control measures need to be implemented to combat the rise of antibiotic-resistant strains and the subsequent increase in morbidity and mortality caused by *S. pneumoniae*. Widespread use of the vaccine coupled with judicious administration of drugs will go a long way in stemming disease caused by pneumococci.

Antibiotic resistance. The discovery of penicillin in the 1940s, followed by the discovery of streptomycin, dramatically decreased death and illness from infectious diseases. Today, in stark contrast, almost all pathogenic bacteria are becoming resistant. It is well known that antibiotic use promotes the development of antibiotic resistance (127) and that widespread or indiscriminate use of antibiotics promotes the spread of antibiotic resistance. Initial reports of multiple drug resistance in *S. pneumoniae* came from South Africa in 1977 and were quickly followed by worldwide reports (6, 84, 85, 93, 100). In the US, penicillin-resistant pneumococci emerged in the early 1990s (15, 30, 64, 189). Currently, more than 35% of pneumococci isolated from clinical specimens in the US are penicillin resistant (6). Although rare in the US, reports of fluoroquinolone resistance have been made (7, 126, 184). Judicious use of antimicrobials, combined with optimal use of pneumococcal vaccines, may slow the development of resistance in pneumococci.

Pneumococcal capsular polysaccharide vaccines. The earliest vaccine for pneumococcus was administered in 1911 in the form of heat-killed whole cells (195). Soon after World War II, two different 6-valent vaccines became available in the US; however, interest in them waned with the discovery of penicillin as the panacea for bacterial infections. The vaccines were then withdrawn from the market because of lack of demand. A 14-valent vaccine was licensed for use in 1977 in the US, and not until 1983 did the two currently available 23-valent pneumococcal polysaccharide vaccines came into the market. Pneumovax 23 consists of a purified capsular mixture (25 μg of each capsular polysaccharide from the 23 most prevalent invasive serotypes. The capsular types included in the vaccine are 1, 2 ,3 4, 5, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 17F, 19A, 20, 22F, 23F, and 33F. Although the 23-valent vaccine is expected to cover 85%-90% of the serotypes that cause invasive disease in adults and children in the US (50), the polysaccharide vaccine is ineffective in the very young and only about 60% effective in the elderly (160). One plausible reason for this diminished effectiveness is the poor immunogenicity of the polysaccharide vaccine (because of its T-cell-independent nature) in the elderly and the failure in young children below 2 years of age to make antibodies to polysaccharides. It is also known that certain serotypes such as 6B, 9V, 19F and 23F elicit relatively weak antibody responses and that the levels fall to pre-vaccination titers rather quickly (157). Additionally, qualitative differences in the antibodies made have been reported in some vaccinated adults (154).

Polysaccharide conjugate vaccines. The existing polysaccharide vaccine is less than ideal because of its low efficacy in the target population, especially children, and

because the protection is afforded only against serotypes present in the vaccine (50). The success of *H. influenzae* type B oligosaccharide conjugate vaccine prompted the development of a more effective pneumococcal conjugate vaccine (8, 147). Invasive pneumococcal disease rates are the highest in children under the age of 2 years. Because pneumococcal polysaccharides are T-cell-independent antigens, antibody responses induced against them are limited. However, because conjugating a carrier protein to the polysaccharide vaccine induces a T-cell-dependent response and can induce higher levels of antibodies in infants, this approach has been tried; however, the problem of serotype switching limits its efficacy (65, 103, 108). Carrier proteins that have been used include diphtheria toxoid, tetanus toxoid, a non-toxic mutant derivative of diphtheria toxin (CRM_{197}), pneumolysin, and meningococcal outer membrane proteins (161).

Prevnar, the 7-valent conjugate vaccine licensed for use in the US, is composed of polysaccharides from serotypes 4, 6B, 9V, 18C, 19F, and 23F, each conjugated to the diphtheria CRM_{197} protein. One study in northern California with 37,868 children estimated the efficacy of the vaccine at 94% against invasive disease for serotypes included in the vaccine (162).

The heptavalent vaccine had a less dramatic effect on otitis media among the vaccinated children, decreasing episodes by about 7% (25). A Finnish study demonstrated 57% reduction in episodes of acute otitis media caused by vaccine serotypes and 6% reduction in overall otitis media, and infection with absent serotypes increased by 33% (71). Although the vaccine has been shown to reduce the carriage of vaccine serotypes, the cleared niche is quickly occupied by non-vaccine serotypes (133). This, coupled with the limited number of serotypes that can be included in the vaccine and with the high cost

will limit the use of conjugate vaccines in the developing countries where the need for them is acute.

Protein vaccines. Protein vaccines are of particular interest because they are immunogenic in young children, who do not respond to polysaccharide vaccines, and they are potentially less variable. Some of the well-studied protein vaccine candidates are discussed below. Sixty-nine potential surface proteins have been found by the pneumococcal-genomic-sequencing project. Based on sequence homology, it was estimated that 25 of these were likely contributors to the pathogen's virulence, capsule synthesis, and colonization of the host (131). Additionally, these studies have also led to the identification of several potential protein vaccine candidates (191).

Pneumococcal protein vaccine candidates. Vaccines directed against pneumococcal non-capsular antigens common to all serotypes hold great promise for preventing pneumococcal infections. A number of pneumococcal proteins have been identified as likely candidates, including autolysin, neuraminidase, pneumolysin, PspA, PsaA, and CbpA (34, 112, 121, 125, 134, 135, 173). In addition to providing protection against all pneumococcal serotypes, these proteins will induce a T-cell-dependent response with immunologic memory. PspA and pneumolysin have been the most extensively studied candidates for eliciting protection against pneumococcal infection. PsaA is another candidate that has naturally immunogenic properties and alone or in concert with PspA or pneumolysin has been shown to be protection eliciting (31, 33, 145, 164).

PspA

Identification of PspA. In an attempt to elicit antibody responses to non-capsular antigens, protective monoclonal antibodies to proteins were developed as a way to probe the identity of protection-eliciting proteins. CBA/N mice were hyperimmunized with heat-killed non-encapsulated R36A pneumococci (121). Since the capsule is known to elicit a protective antibody response, a non-encapsulated strain was used to obtain higher levels of antibodies to non-capsular protein antigens. To overcome the predominant immune response seen in normal mice to polysaccharides, including the phosphocholine in teichoic acids and lipoteichoic acids (1, 38, 130), CBA/N mice were used because they have an X-linked immunodeficiency (XID) that precludes this phosphorylcholine (PC)-induced response. Antibody-secreting hybridoma cell lines made from lymph node cells of immunized mice were screened for production of monoclonal antibodies (MAb) against R36A. Even with the use of XID mice, a large proportion of the monoclonl antibodies were made to the PC component (121). The MAb generated that were not reactive with the PC epitope reacted with proteins (117, 121). All the MAb except one reacted with the same protein, and this protein was called PspA (41, 122). It was also shown that passive administration of these antibodies caused protection against pneumococcal infection, making this molecule a potential vaccine candidate.

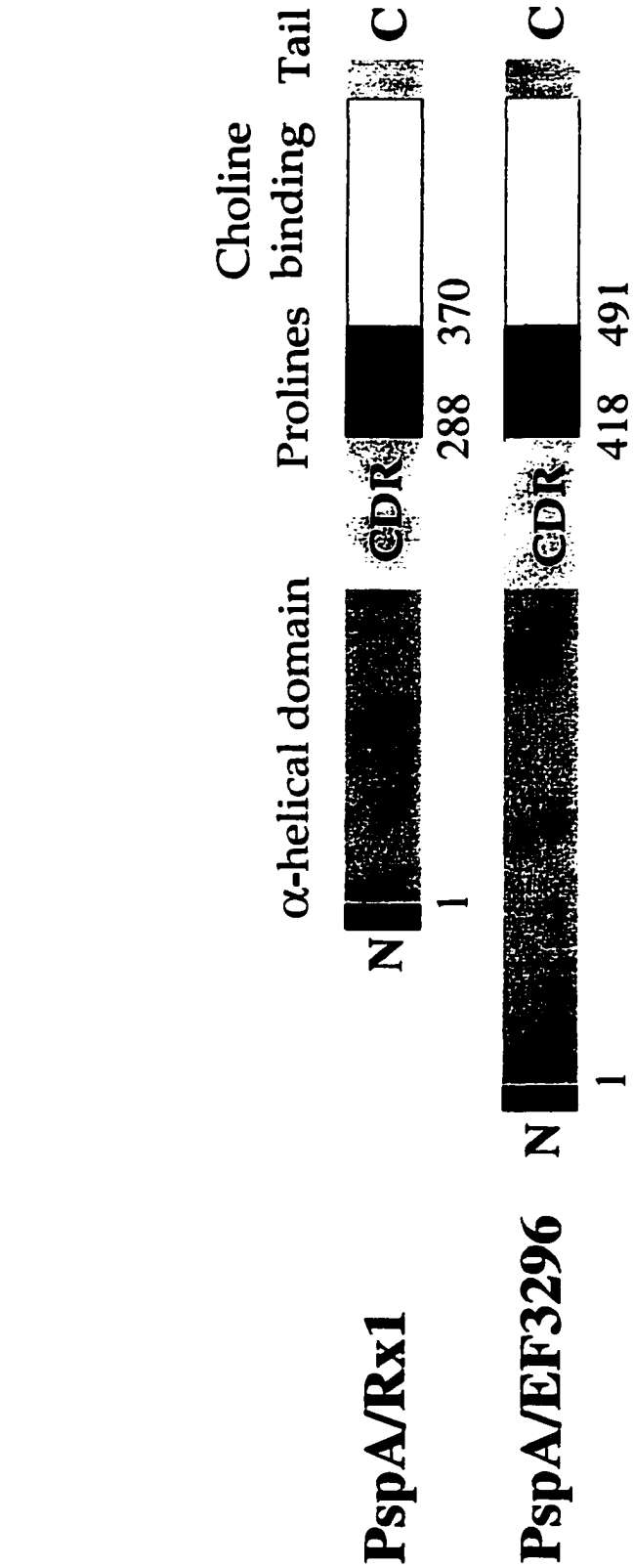
PspA's role in virulence. To see whether PspA had any effect on the virulence of pneumococci, the *pspA* sequence was mutated by insertion duplication mutagenesis and the resulting strains were tested for virulence in a murine sepsis model. PspA-negative D39 and WU2 cells had increased lethal dose 50s (LD_{50}) and higher clearance

rates (41, 125). The absence of PspA resulted in more rapid clearance of the pneumococci from the blood of the infected mice (125, 179).

Biological function of PspA. PspA research was first approached from the protection-eliciting or immunogenic aspect of the molecule. More recently, some functional aspects of the protein have been studied. Many properties have been assigned to PspA, but its actual role in virulence has not been elucidated. Recent findings have suggested that the role of PspA in virulence is to interfere with deposition of complement factor C3b on the pneumococcal surface, inhibiting the formation of a fully functional alternative pathway C3 convertase (179). Data from the same study showed that the PspA-negative strain caused more complement activation in mice, implicating an obstructive interaction between PspA and complement. PspA has also been shown to bind to human lactoferrin, which is produced abundantly during inflammation (81, 82). Since lactoferrin is known to downregulate the immune system and to block complement activation (45, 99, 116, 182), the elucidation of this interaction may offer more clues about the actual function of PspA during pneumococcal infection.

Structure. PspA is expressed on the surface of all pneumococci (55) and varies in size from 65 kDa to about 95 kDa (90). Knowledge of the structural features of PspA has come from the sequences of PspA/Rx1 and PspA/EF5668 (96, 119, 196, 197). PspA has four structural domains (Fig. 2) (120). The α-helical region at the N-terminus is thought to take on an anti-parallel coiled-coil conformation. This region contains the surface-exposed, protection-eliciting (albeit variable) region. Major cross-protective epi

FIG. 2. A schematic drawing of the major structural domains of PspA. The cartoon displays the structural domains of PspA/Rx1 (family 1) and PspA/EF3296 (family 2). Both proteins have a conserved N-terminal signal sequence (in black), followed by the α-helical domain (darker gray) that is exposed on the bacterial surface. The α-helical domain of PspA/EF3296 is considerably longer than the same region of PspA/Rx1. Within the α-helical domain is marked the B region, which contains cross-protective epitopes and is also the clade-defining region based on sequence analysis. The α-helical domain is followed by a proline-rich domain (black) that is thought to span the cell wall. This region is followed by the choline-binding domain (light grey) which contains 10 choline-binding repeats that attaches the protein to the lipoteichoic acid of the cell wall. Finally, each protein has a tail sequence (grey) at its C-terminal end.



topes have been mapped to the most C-terminal 100 amino acids of the α-helical (coiled-coil) domain (120). The N-terminal domain comprises the N-terminal half of PspA (90, 119, 196), and is followed by the proline-rich region, which in some strains is interrupted with a non-proline block of residues. This region may help to span the cell wall as has been demonstrated in other gram-positive bacteria (66). After this region is the C-terminal end of the molecule, which is composed of 9 to 10 conserved choline-binding repeats of 20 amino acids each; and this region is required for the attachment of PspA to the choline residues of lipoteichoic acids. The C-terminal tail of the molecule consists of a highly conserved 17 amino-acid residue.

Diversity. Sequencing of the α-helical region of PspA from 24 unrelated clinical isolates representing 13 capsular types led to the emergence of two major families, which were subdivided into five clades (90). The differentiation of the molecule into clades and families is based on the sequence within the clade-defining region (CDR) of the α-helical domain. An examination of 2,000 strains from around the world revealed that at least 98% are in families 1 and 2, which are made up of clades 1and 2 and clades 3-5, respectively (54). Because of the variability of PspA, the ideal formulation for a PspA vaccine has been hypothesized to be one that contains PspAs from the major sub-divisions of PspA families and/or clades. However, most PspAs appear to share conformational epitopes and are adequately cross-reactive to be able to elicit protection against strains of pneumococci that express different PspA serotypes (40, 123, 173).

Protection-eliciting domains. As mentioned above, mice can be protected from pneumococcal sepsis and death by immunizing passively with monoclonal antibodies and polyclonal antibodies against PspA (32, 119, 121, 125). Immunizing actively with native and recombinant PspA also shows robust protection against pneumococcal infection (34, 36, 37, 172). DNA immunizations have been shown to confer protection as well (29, 118).

The very first demonstration of PspA being a protection-eliciting molecule came from studies of mice immunized with the 27 kDa fragment of the α-helical region of PspA/Rx1 (172). This fragment was composed of amino acids 1-245 of PspA; 5 μg of the purified protein was administered to mice subcutaneously in complete Freund's adjuvant (CFA). Mice were then given a booster dose without adjuvant intraperitoneally; upon infection with 300 colony-forming units (CFUs) of *S. pneumoniae* WU2, themice were protected against death (172). Follow-up experiments demonstrated that the N-terminal 245 or 260 amino acid fragments, when used as immunogens without adjuvant were not protective (36). However, when interleukin-2 was used as an adjuvant, the immunogenicity was greatly improved (194).

Monoclonal antibodies were made to assess and map the protection-eliciting epitopes of PspA/Rx1. Nine MAb reacted with epitopes in the α-helical region of PspA. Five of these reacted with PspA/WU2 and protected against infection with the strain WU2. Four of these cross-reactive monoclonal antibodies identified epitopes between amino acids 192 and 260 of PspA/Rx1 and one reacted with an epitope at the N-terminal end within the first 115 amino acids. These results suggested that the region spanning amino acids 192-260 was good at eliciting cross-protection (Fig. 2) (120). Further stud-

ies with different fragments of PspA/Rx1 supported this conclusion. A fragment composed of amino acids 192-299 elicited protection against a panel of challenge strains expressing diverse PspAs. Immunization of mice with fragments that corresponded to amino acids 192-299 from six different PspAs all elicited cross-protection against the strain WU2 (120, 144). Immunization of mice with fragments of different PspAs comprising amino acids 192-588 were highly immunogenic and elicited protection against different strains of *S. pneumoniae* (120, 173). Pneumococcal lysates that only had the N-terminal 115 and 245 amino acids of Rx1 have also been shown to elicit protection against WU2 but not cross-protection (36). Recombinant PspA/Rx1 was able to elicit cross-protection when phage lysates were used for immunization (123), when expressed by live BCG (106), and when injected with CFA (40). Recombinant PspA from EF5668 elicited cross-protection when used for immunization (119). These studies showed that single PspAs were able to elicit protection in CBA/N mice against challenge strains irrespective of clade. However, protection was more readily seen against serotypes 3 and 6 strains than against types 2, 4, and 5 strains (39, 40).

Statement of purpose

Until the start of this dissertation work only one PspA molecule (PspA/Rx1) had been investigated in detail for its protection-eliciting regions. Since disease causing strains are almost equally distributed between the two PspA-families, we wanted to characterize in detail a strain belonging to the other major family. For a future vaccine to be efficacious, it is important to understand the way different PspA molecules protect against infection. The strain Rx1 belongs to family 1 PspA, whereas the strain studied in

this dissertation, EF3296, belongs to family 2. The PspAs of strains Rx1and EF3296 differ in the α-helical region. The α-helical region of PspA/EF3296 is about 68% longer than that of PspA/Rx1 and consists of 418 amino acid residues. Also, unlike PspA/Rx1, this region lacks the non-proline segment in the center of the proline-rich region (90). These discrepancies may cause differences in protective abilities between the families.

The EF3296 strain (serotype 4) was also chosen based on the historical difficulties seen when trying to protect PspA-immunized mice against infection with serotypes 2, 4, and 5 strains (173, 144).

The first part of this dissertation deals with elucidating the protection-eliciting regions of EF3296 against homologous infection and the ability of these regions to cause cross-protection against strains with different clade specificity of their PspA. The second section addresses the differences observed when the family 2 PspA from the serotype 4 strain was swapped with the family 1 PspA from a serotype 3 strain. Specifically, we investigated the effect PspA immunization has on protection against the resultant strains.

REGIONS OF PSPA/EF3296 BEST ABLE TO ELICIT PROTECTION AGAINST *STREPTOCOCCUS PNEUMONIAE* IN A MURINE INFECTION MODEL

by

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ABSTRACT

Pneumococcal surface protein A (PspA) can elicit protection against *Streptococcus pneumoniae* in mouse infection models. PspA is serologically variable and has been classified by serology and sequence into two major families, which are divided by sequence into 5 clades. The most variable portion of the molecule is the α-helical region, which comprises the N-terminal half of the molecule and differs by about 60% in sequence between family 1 and family 2 PspA. Prior studies of a family 1 PspA demonstrated that protective antibodies are almost always reactive with the epitopes in the α-helical domain. Moreover, cross-protection was best elicited by fragments that contained the 108-most C-terminal amino acids of the α-helical region. In the present studies we have used 6 recombinant fragments of a family 2, clade 3 PspA (EF3296) to map the protection-eliciting regions within its α-helical domain. The three overlapping fragments that included amino acids 314 to 418 (the 104-most C-terminal amino acids of this α-helical region) were able to elicit protection against EF3296. A fragment extending from amino acids 75 to 305 failed to elicit protection. A fragment containing the N-terminal 115 amino acids did elicit protection against EF3296 but only in BALB/c mice and not CBA/N mice. All three fragments containing amino acids 314 to 418 were able to elicit cross-protection against pneumococci expressing PspAs of clades other than clade 3. Of the 5 PspA clades, we were able to elicit cross-protection against all but clade 1, which may not share sufficient epitopes cross-reactive with those of EF3296. Cross-protection was easier to demonstrate in CBA/N mice than in BALB/c mice. The 1-115 fragment elicited some cross-protection in BALB/c mice against clades 2 and 4 but not in CBA/N

mice. These studies provide strong support for the importance of C-terminal 104 to 108 amino acids of the α-helical region of PspA in the elicitation of cross-protection.

INTRODUCTION

Streptococcus pneumoniae is a common cause of respiratory tract infections, otitis media, sepsis, and meningitis in young children and in the elderly. It is a major cause of mortality in the developing world and the major cause for hospital visits among children in the US (18, 31, 38).

The incomplete protection offered by the current polysaccharide vaccine and the recently developed protein-conjugate vaccine against otitis media, carriage, and bacteremic disease caused by non-vaccine types (4, 5, 7, 17, 21, 28, 33) has increased the importance of studies of other vaccine candidates. Pneumococcal surface protein A (PspA) and pneumolysin have been the most extensively examined pneumococcal proteins used for protective immunization in animal models (10, 34), although a number of other pneumococcal proteins have been reported (6, 8, 16, 23, 26, 27, 36, 39).

PspA is present on all pneumococci (20) and is serologically variable, cross-reactive (24, 29, 32), and cross-protective (12). The mature protein contains three main domains based on its sequence. Upstream of the choline-binding domain, which attaches the protein to the cell surface, is the proline-rich domain, which is thought to span the cell wall (12, 41). N-terminal to the proline-rich domain and exposed on the surface is the α-helical domain, which is thought to form an anti-parallel coiled-coil structure (25, 41) reminiscent of many other fibrillar surface proteins on gram-positive bacteria. Most of the epitopes detected by a panel of protective monoclonal antibodies to PspA reacted

with epitopes that mapped to the C-terminal 119 amino acids of the alpha-helical region of PspA/Rx1. Overlapping fragments that contained this region of PspA/Rx1 (referred to as the clade-defining region or CDR) were found to elicit protection (29) against strains of different capsular types.

The most variable portion of PspA is the α-helical domain, and this variability extends into the CDR. Based on the variability in the CDRs of the different PspAs it was possible to classify PspA sequences into two major families comprising 5 different clades (24). Despite the fact that the members of the two major PspA families can differ by as much as 60% of their amino acids in the CDR, immunity to family 1 PspA/Rx1 fragments containing this region was cross-protective against strains of both PspA families (9, 12). The studies with PspA/Rx1 demonstrated that cross-protection could be elicited by an 108 amino acid fragment (amino acids 192 to 299) (15). It was also observed that the N-terminal 115 amino acids of PspA/Rx1 could elicit protection, but no cross-protection was measured (11). The ability of fragments in the middle of the α-helical domain to elicit protection was not examined.

The classification of PspAs into families and clades based on B-region structure could assist in the formulation of a PspA vaccine containing different PspAs, if it were known that the B-region of PspAs in PspA family 2 was also important in the elicitation of cross-protection. In this study we have characterized the protection elicited by overlapping fragments in a family 2 molecule, PspA/EF3296. In EF3296, the α-helical domain extends from amino acids 1 to amino acid 418. The B window region has been considered to extend from amino acids 314 to 418 (24). Recombinant fragments composed of overlapping segments of the α-helical domain were produced and used to im-

munize BALBc/ByJ and CBA/N mice. The results show that the protective regions were located at both the N-terminal end of the α-helical domain, as well as the CDR. However, the CDR elicited the best cross-protection, as had been observed for PspA/Rx1.

MATERIALS AND METHODS

Reagents. NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) were from Fisher Scientific (Atlanta, Ga.). Streptavidin-alkaline phosphatase (AP), AP-conjugated goat anti-mouse, and AP-conjugated goat anti-rabbit antibodies were from Southern Biotechnology Associates (Birmingham, Ala.). Bacto-Todd Hewitt media and yeast extract were from Difco Laboratories (Detroit, Mich.). Protein markers and ready-gels were from Bio-Rad Laboratories(Hercules, Calif.).

Monoclonal anti-PspA antibodies were produced as described (29). An anti-PspA family 2 antiserum was produced as described (22). Monoclonal antibody PC3.1 reactive with EF3296 was a gift from Aventis Inc. (Toronto, Canada) (19).

Bacteria. The pneumococcal strains were stored at –80°C in 12% glycerol (1), transferred to blood agar plates, and incubated at 37°C in 5% CO_2 atmosphere overnight. Colonies grown on blood agar were used to inoculate liquid growth medium (Todd-Hewitt medium containing 0.5% yeast extract [THY]). Upon reaching late log phase, the bacteria were harvested by centrifugation at 1,500 x *g* for 15 min. and suspended in sterile 60 mM phosphate buffered saline (PBS, pH 7.2). The bacterial concentration was adjusted by measuring absorbance at 600 nm and confirmed by viable counts.

PspA and PspA fragments. Full-length PspA was purified from *S. pneumoniae* EF3296 as described (11, 42). Fragment SW102 (amino acids 1 to 478) of PspA/EF3296 constituting amino acids 1-478 was kindly provided by Aventis Inc. (Toronto, Canada). PspA fragments HR101 (primer pair ABW23/LSM12), HR102 (primer pair HR10/HR11), HR104 (primer pair HR12/HR14), HR107 (primer pair HR10/HR14), and HR108 (primer pair HR19/HR20) were amplified from *S. pneumoniae* EF3296. The primers used for PCR have been described earlier (22) except for HR19 (5'-agctgcatgcTTAGCAAAAAAACAAACAGA-3') and HR20 (5'-agctctgcag AGTTTCTTCTTCATCTCCAT-3'). Amplicons of *pspA* were cloned either into *Bgl*II-*Hind*III or *Sph*I-*Sal*I-digested pQE40 vector (Qiagen Inc, Valencia, Calif. USA) and transformed into M15 (pREP4), a K-12 derived *Escherichia coli* strain containing a plasmid that encodes a *lac* repressor allowing control over expression. Clones containing the different *pspA* inserts were identified by Southern blot analyses with digoxigenin-labeled PspA probes (29). Expression of positive clones was induced with 1 mM isopropylthio-galactopyranoside (IPTG) during growth at room temperature. The over-expressed protein fragments were purified by affinity chromatography with a nickel resin according to the manufacturer's instructions. The different constructs encoded PspA fragments with predicted masses of 38.6 kDa (HR101), 52 kDa (HR102), 59.3 kDa (HR104), 71.8 kDa (HR107), and 11.4 kDa (HR108), which were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoreis (SDS-PAGE) and quantified using the Bio-Rad *DC* protein assay (Bio-Rad Laboratories).

Mouse immunization and challenge. Five-8 week old CBA/CAHN/XID or BALBc/ByJ mice (Jackson Laboratories, Bar Harbor, Me.) were used for protection studies. The mice were immunized subcutaneously with 1-5 μg of purified recombinant proteins PMC, HR101, HR102, HR104, HR107, or HR108 in alum for primary immunization. A booster dose in alum was administered subcutaneously 2 weeks later. Fourteen days after the boosts, the animals were challenged intravenously through the tail vein with the *S. pneumoniae* strains A66.1, BG7322, EF3296, 3JYP2670, and ATCC6303 at a dose that was 2 logs higher than the lethal dose 50 (LD_{50}). The challenge doses of these strains in CBA/N mice were 200, 400, 2000, 700, and 800 colony-forming units (CFUs) for *S. pneumoniae* A66.1, BG7322, EF3296, 3JYP2670, and ATCC6303, respectively. The challenge doses in BALBc/ByJ mice were 4.8×10^5, 3.3×10^6, 2.0×10^6, 1.0×10^5, and 2.8×10^6 CFUs for A66.1, BG7322, EF3296, 3JYP2670, and ATCC6303, respectively. The infected animals were then monitored for 21 days, and mice that survived this period were considered protected against death.

Western blot. PspA and fragments of PspA (0.5 μg) were run on polyacrylamide gels (Bio-Rad Ready gels, Bio-Rad Laboratories, Hercules, Calif.), and the gels were electroblotted to a 0.45-μm nitrocellulose membrane (Bio-Rad) in Tris-glycine buffer (20% methanol, 25 mM Tris, and 192 mM glycine, pH 8.1-8.4) at 100 V for 1 h at 4°C. The blotted membrane was incubated with 1% bovine serum albumin (BSA) in PBS-T (PBS containing 0.05% Tween-20) for 1 h at room temperature and washed three times (5 min each) with PBS-T. The membranes were overlaid with anti-PspA antibodies (PC3.1 monoclonal or polyclonal anti-PspA family 2 antisera) for 30 min at 37°C and

washed three times in PBS-T. The anti-PspA exposed membrane was further incubated with a mix of biotinylated goat anti-mouse or anti-rabbit antibodies (1:1,000 in PBS-T) and alkaline phosphatase conjugated streptavidin (1:500 dilution in PBS-T) for 30 min at 37°C. After washing, the membrane was developed by using 0.1 mg/ml NBT and 0.5 mg/ml of BCIP in 0.15 M Tris-HCl (pH 8.8).

Determination of anti-PspA serum levels. Mice were bled retro-orbitally 24 h before challenge. Titers were evaluated by enzyme-linked immunosorbent assay (ELISA) using the homologous recombinant fragment and recombinant full-length α-helical fragment (SW102) to coat plates and the coated plates were incubated overnight at 4°C. The plates were blocked by incubation for 2 h with 1% BSA in PBS-T. After 3-h incubation at 37°C with different dilutions of the mouse sera, the plates were washed with PBS-T and biotinylated goat anti-mouse antibodies (1:1,000 dilution in PBS-T) were used to detect the mouse serum antibodies. After further incubation with AP-conjugated streptavidin (1:1,000 dilution in PBS-T), *p*-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Co. St. Louis, Mo.) was used for color development. Absorbance was read at 405 nm after 30 min. Antibody reactivity to PspA was determined in micrograms per milliliter by using a serum standard of pooled serum from mice immunized with HR101 and HR107 with known PspA-antibody concentration.

Binding anti-PspA serum antibodies to the bacterial surface. Bacteria were grown on blood agar plates or in THY medium and were suspended in PBS at a concentration of approximately 1 x 10^8 bacteria/ml. The bacterial suspension (80 μl) was mixed

with 20 µl of mouse pre-immune or post-immune sera (final dilution 1:40) or with monoclonal anti-PspA PC3.1 antibodies (20 µg/ml) for 30 min at room temperature and washed by centrifugation at 1,500 x *g* for 5 min in PBS. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (1:100 dilution in PBS) were added for an additional 30 min at room temperature; after a final wash in PBS, the cells were inspected by epi-fluorescence microscopy with a Leitz upright microscope (Leitz, Wetzlar, Germany). The binding was quantitated by flow cytometry with a FACSCalibur Flow Cytometer (Becton-Dickinson Biosciences, Rutherford, N. J.).

Statistical Analyses. Antibody levels and days to death were compared with the two-tailed Mann-Whitney U test (non-parametric, two sample rank) and 2X2 contingency tables evaluated by Fisher's Exact test were used to compare alive versus. dead in survival experiments.

RESULTS

Production of recombinant fragments of PspA/EF3296. The α-helical domain of PspA is known to be exposed on the bacterial cell surface (22, 29). Studies of PspA/Rx1 (family 1 protein) have shown that the most cross-protection-eliciting region is located in the CDR of the α-helix located in the C-terminal part of this domain (29). To investigate the protection-eliciting regions of PspA/EF3296 (family 2/clade 3), overlapping fragments of PspA/EF3296 were produced spanning the α-helical domain (Fig. 1). PCR products were cloned in-frame into pQE40, an expression vector in which the cloned fragments are located C-terminus to the mouse dihydrofolate reductase (DHFR),

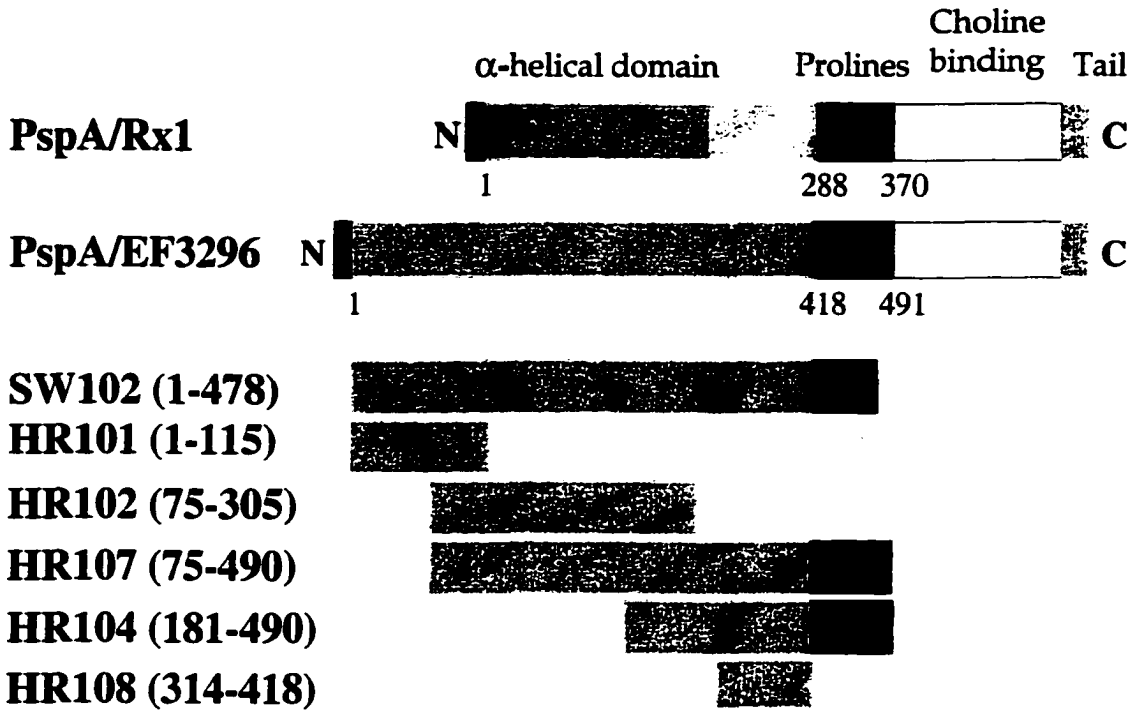

FIG. 1. Cartoon of PspA/EF3296 fragments used in the study.

which has a poly-histidine tag at the N-terminal end. The DHFR gene in the vector is expected to be non-immunogenic. For HR108, pQE30, an expression vector similar to pQE40 but without the DHFR, was used. Recombinant plasmids with inserts were selected initially based on antibiotic resistance, the insert was verified by sequencing, and the recombinant protein fragments were expressed and purified.

All fragments were of the expected sizes in a Western blot developed with anti-His antibodies (Fig. 2A). Western blots visualized with polyclonal anti-family 2 PspA antibodies or PC3.1 anti-PspA/EF3296 monoclonal antibodies showed the same recognition pattern except for HR101 (amino acids 1-115), which was not recognized by the polyclonal serum (Fig. 2B). The non-reactivity of polyclonal serum against the N-terminal domain of PspA in Western analyses has been observed before; for both PspA/Rx1 and PspA/EF3296 (22). This result is not totally surprising based on our current model of the protein's structure, which indicates that this domain is most probably hidden in the structure (12, 25).

Immunogenicity and functional antibody responses against the PspA/EF3296 fragments. Immunization with fragments of PspA/EF3296 provides an opportunity to identify regions of PspA that elicit protective antibody responses. To assess the protection-eliciting capacity of the recombinant fragments, groups of BALBc/ByJ and CBA/N mice were immunized and boosted 2 weeks apart with 1 μg of specific fragments adjuvanted with alum (SW102, HR101, HR102, HR104, HR107, or HR108) (Table 1). Two weeks after the boost, the mice were bled and serum was collected for analysis with ELISA. The immune sera to the fragments showed no reactivity to microtiter plates

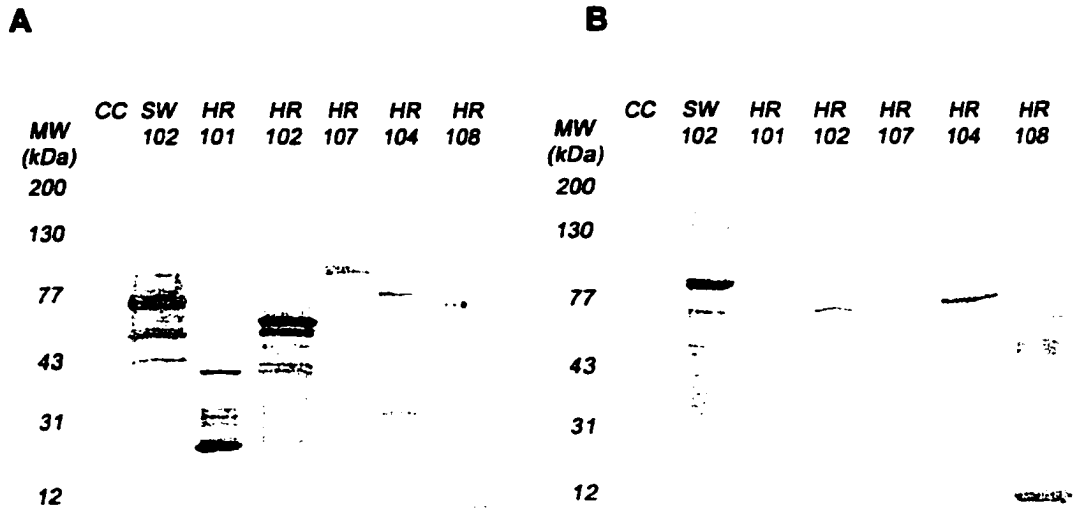

FIG. 2. Western blot of fragments of PspA/EF3296. Fragments of PspA/ EF3296 were run on 12% gels and subjected to Western blot by using anti-His antibodies (A) or anti-PspA antibodies (B). Both blots display a choline eluate from strain EF3296 (CC), as well as the fragments SW102 (lane 2), HR101 (lane 3), HR102 (lane 4), HR104 (lane 5), HR107 (lane 6), and HR108 (lane 6).

coated with recombinant DHFR, suggesting that most or all elicited antibody was against the PspA-sequences (Table 1).

TABLE 1. Immune-sera reactivity in ELISA and flow cytometry.

Mouse strain	Immunogen	ELISA titer (μg/ml ± SD)*		Cell surface binding
		SW102	Immunizing fragment	Times the control (± SD)**
BALBc/ByJ	None	0.00 (± 0.00)	-***	1.00
	DHFR	0.00 (± 0.00)	-	-
	SW102	507 (± 565)	508 (± 566)	9.25 (± 2)
	HR101	0.003 (± 0.001)	0.22 (± 0.13)	1.51 (± 0.09)
	HR102	37 (± 19)	88 (± 90)	6.36 (± 2)
	HR104	0.09 (± 0.05)	50 (± 25)	0.94 (± 0.03)
	HR107	48 (± 17)	74 (± 76)	6.64 (± 1)
	HR108	23 (± 13)	225 (± 139)	2.24 (± 0.3)
CBA/N	None	0.00 (± 0.00)	-	1.00
	DHFR	0.00 (± 0.00)	-	-
	SW102	43 (± 14)	43 (± 14)	5.41 (± 1.92)
	HR101	0.01 (± 0.003)	3.73 (± 5)	1.06 (± 0.06)
	HR107	172 (± 118)	187 (± 119)	6.61 (± 1.15)

* Immune sera from BALBc/ByJ or CBA/N mice were tested for their anti-PspA antibody titer either against the full-length α-helical PspA/EF3296 (SW102) or against the fragment used for immunization. The antibody concentration and standard deviation (SD) were determined as described in Materials and Methods.

** Binding to the EF3296 bacterial surface was determined for each serum as fluorescent signal of sample divided by the binding of a pre-immune serum control. Sixteen sera from each immunization were used, and an average binding was determined.

*** - = not done.

The immune reactivity varied between the fragments. In BALBc/ByJ mice, the highest reactivity was seen after immunization with SW102, with an average concentra-

tion of 507 μg/ml of PspA-antibodies. Fragments HR102, HR107, and HR108 also consistently resulted in significantly high concentrations (20-50 μg/ml), whereas HR101 and HR104 only elicited antibody responses in nanogram levels (Table 1). For all fragments, ELISA on plates coated with full-length α-helical fragment SW102 as detecting material consistently showed lower levels than when the sera were tested against the fragment used for immunization. Especially high discrepancies were seen for fragments HR101 and HR104, which elicited few if any antibodies capable of binding SW102. HR101 had a 70-fold higher reactivity when probed against the homologous fragment, and HR104 had a 500-fold higher reactivity against the homologous fragment (50 μg/ml). The same trend was seen in sera from immunized CBA/N mice. The levels of antibody-binding full-length SW102 were high from immunizing with SW102, HR107, and HR108; however, the levels of these antibodies capable of binding SW102 were low from immunizing with HR101. These results indicate that the immune response elicited by the different fragments differed, with HR101, constituting the N-terminal 115 amino acids, showing low antigenicity. The results also suggest that the conformation of some of the fragments is different from the conformation of the whole α-helical domain.

To examine the functional antibody activity, we also determined whether the antibodies produced against the different fragments could bind the antigen on the surface of the bacteria. *S. pneumoniae* EF3296 were incubated with sera from the immunized mice, and antibodies that bound were detected by fluorescent reagents. The amount of binding was quantitated by flow cytometry analysis (Table 1), and the pre-immune sera were used as a non-binding control.

All sera from mice immunized with fragments SW102, HR107, and HR108 showed from 2 to 10 times higher binding to the bacterial cells when compared with the pre-immune serum control, showing clearly that the antibodies produced could recognize their epitopes on PspA because PspA is presented on the bacterial surface (Table 1). Sera from mice immunized with HR102 showed a different pattern characterized by highly variable binding capacity in individual sera. Eight of 16 serum samples tested had a binding less than 1.5 times the control, six showed some binding, and two showed very high binding to the bacterial surface. Finally, none of the sera from 28 mice immunized with fragment HR101, and none of the sera from mice immunized with HR104 recognized the protein on the bacterial surface. This result was not surprising because these two immunogens had very little reactivity in the ELISA against the full α-helical domain.

Protection of BALBc/ByJ and CBA/N mice immunized with PspA/EF3296 fragments and challenged with *S. pneumoniae* EF3296. To evaluate the protection induced by immunization with family 2 PspA, BALBc/ByJ mice were first immunized with recombinant SW102 and challenged with *S. pneumoniae* EF3296 at a dose two logs higher than the LD_{50} (2×10^6 CFUs). Immunization with this fragment resulted in a prolonged median time to death of the mice from 5 days in the control group to 14.5 days in the immunized mice ($P < 0.05$) (Table 2). Five of 10 immunized mice survived the challenge compared with none in the non-immunized group ($P < 0.05$). This partial protection is different from what is seen in protection experiments with strains of most other capsular types and supports earlier data suggesting that type 4 encapsulated *S. pneumoniae* are harder to protect against by immunization with full-length PspA (35, 37).

TABLE 2. Protection of BALB/c mice with recombinant PspA/EF3296 fragments challenged with *S. pneumoniae* EF3296

Mice strain immunized	Immunogen* (amino acids)	Median days to death**			Alive:Dead***		
		Non-immune	Immune	*P***	Non-immune	Immune	*P*
BALBc/ByJ	SW102 (1-478)	5	14.5	**0.036**	0:10	5:5	**0.032**
	HR101 (1-115)	3	21	**<0.001**	0:15	14:7	**<0.001**
	HR102 (75-305)	5	5	0.570	0:23	3:20	0.230
	HR104 (181-490)	5	21	**0.011**	3:13	11:5	**0.011**
	HR107 (75-490)	5	9	**0.008**	1:22	9:13	**0.004**
	HR108 (314-418)	2.5	21	**0.008**	3:7	9:1	**0.020**
CBA/N	SW102 (1-478)	2	21	**0.001**	0:7	5:2	**0.020**
	HR101 (1-115)	3	4	1.000	0:7	0:7	1.000
	HR107 (75-490)	4	21	**<0.001**	1:9	10:0	**<0.001**

*Mice were immunized and boosted 2 weeks apart with 1 μg of protein and challenged 2 weeks after the boost with $2x10^6$ EF3296.

**Median days to death were compared with the two-tailed Mann-Whitney U test (non-parametric, two sample rank).

***Fisher's Exact test was used to compare alive versus dead ratios in survival experiments. Boldface *P* values indicate values that were significant.

To investigate the location of protective epitopes of PspA/EF3296, BALBc/ByJ mice were immunized with 1 μg of overlapping PspA/EF3296 fragments HR101, HR102, HR104, HR107, and HR108, where HR108 is the CDR of PspA/EF3296 (24). Two weeks after the second immunization, the mice were challenged intravenously with *S. pneumoniae* EF3296. Immunization with HR101 showed the highest degree of protection, with 14 of 21 mice surviving the challenge (Table 2). Immunized mice had a median survival time significantly longer than control mice (21 days for the immunized mice compared with 3 days for control mice; $P < 0.001$). The protection elicited with this fragment was somewhat surprising. The protection-eliciting epitopes recognized appear to be hidden in the native structure since the antibodies produced did not recognize PspA on the bacterial surface and showed low reactivity in ELISA, suggesting that the action of the antibodies in vivo may be more complicated than just direct binding to or recognition of a surface-exposed portion of PspA.

Immunization with HR104, HR107, and HR108 resulted in significant protection of the mice in terms of both median time to death and survival (Table 2). Again, there was no direct association between the recognition of the antigen in ELISA or on the bacterial surface and the ability to protect. For example, HR104 elicited low antibody concentrations when probed against SW102 and the antibodies did not bind to the bacterial surface (Table 1). On the other hand, HR102 did not protect the mice significantly from death but elicited high titers of surface-binding antibodies. The failure of HR102 to elicit protection was surprising because this fragment contained amino acids spanning those present in the HR107 and HR108 fragments. An attempt to immunize with 5 μg of

HR102 showed better protection although the small number of mice did not permit a useful test of statistical significance (data not shown).

Fragments SW102, HR101, HR107, and H108 were further studied for protection against EF3296 infection in CBA/N mice (Table 2). The results for fragments HR107 and HR108 showed that the protection of immunization in CBA/N mice was better than that seen in BALBc/ByJ mice ($P < 0.01$ and $P < 0.01$ for HR107 when median days to death and alive:dead ratios were compared, respectively). The same trend was seen for SW102, although the data did not reach significance. For HR101, immunization did not protect against challenge with EF3296; thus, protection was significantly less in CBA/N mice ($P < 0.05$ and $P < 0.01$ when comparing median days to death and alive:dead ratios, respectively).

These results make it clear that PspA can elicit protection against this type 4 encapsulated strain, although a lower degree of protection is seen when compared with that against capsular types 3 and 6 immunized with homologous fragments (35). The results also indicate that most fragments of PspA/EF3296 confer better protection in the immunodeficient mice strain CBA/N than in the immunocompetent BALBc/ByJ strain.

Ability of immunity to PspA/EF329 fragments to elicit protection against *S. pneumoniae* expressing different clades of PspA. To evaluate the protective responses of PspA/EF3296 further, BALBc/ByJ or CBA/N mice were immunized and challenged with strains of capsular types 3 and 6 expressing different clades of PspA (Table 3).

When median days to death and alive:dead ratios were compared, BALBc/ByJ mice immunized with SW102 were protected against BG7322 (clade 1/family 1),

TABLE 3. Cross-protective immunity of PspA/EF3296 immunization in BALBc/ByJ mice.

Immunogen*	Strains (capsule type)	PspA clade	Median days to death**			Alive:Dead***		
			Non-imm	Imm	*P*	Non-imm	Imm	*P*
SW102	A66.1 (3)	1	2	2	0.456	0:7	0:7	1.000
	BG7322 (6B)	2	7	21	**0.026**	0:7	5:2	**0.020**
	3JYP2670 (3)	4	4	21	**0.026**	0:7	5:2	**0.020**
	ATCC6303 (3)	5	3	21	**0.018**	0:7	6:1	**0.005**
HR101	A66.1 (3)	1	5	21	0.360	3:5	6:4	0.630
	BG7322 (6B)	2	4	21	**0.043**	0:7	5:5	**0.044**
	3JYP2670 (3)	4	6	21	**0.036**	0:3	5:0	**0.017**
	ATCC6303 (3)	5	5	7.5	0.515	2:6	4:6	0.640
HR107	A66.1 (3)	1	4	4	0.561	1:10	4:8	0.310
	BG7322 (6B)	2	8	13.5	0.621	3:8	5:7	0.660
	3JYP2670 (3)	4	2	2	0.920	0:4	0:5	1.000
	ATCC6303 (3)	5	3	12	0.136	2:9	6:6	0.190
HR108	A66.1 (3)	1	4.5	3	0.111	2:18	2:17	1.000
	BG7322 (6B)	2	9	21	**<0.001**	2:13	14:3	**<0.001**
	3JYP2670 (3)	4	2	3	0.110	2:15	6:12	0.230
	ATCC6303 (3)	5	3	21	**<0.001**	0:17	11:6	**<0.001**

*Mice were immunized and boosted 2 weeks apart with 1 μg of protein and challenged 2 weeks after the boost.
**Median days to death were compared with the two-tailed Mann-Whitney U test (non-parametric, two sample rank).
***Fisher's Exact test was used to compare alive versus dead ratios in survival experiments. Boldface *P* values indicate values that were significant.

3JYP2670 (clade 4/family 2), and ATCC6303 (clade 5/family 2). No protection was seen against A66.1 (clade 2/family1).

When HR101 was used, mice were protected against death induced by BG7322 and 3JYP2670. For the remaining two strains, the immunized mice showed an increased survival time when compared with non-immunized mice; however, the difference was not significant (Table 3). HR108 showed the same pattern, with protection seen against BG73322 and ATCC6303 and with a trend toward protection against 3JYP2670. HR107 immunization did not result in protection against any of the strains, although an increased survival was seen for ATCC6303.

When protective effects of the different fragments were compared, we found that immunization with HR101protected significantly better against challenge with A66.1 than immunization with either HR108 or SW102 ($P < 0.01$ and $P < 0.01$ when comparing median days to death and alive:dead ratios, respectively, for both fragments), and SW102 immunization protected better than HR108 immunization ($P < 0.05$ when comparing median days to death). Furthermore, immunization with SW102 protected better against BG7322 than immunization with either of HR101 or HR107 ($P < 0.05$ and $P < 0.05$, respectively, when comparing alive:dead ratios) and HR108 immunization protected better than HR107 immunization ($P < 0.05$ and $P < 0.05$ when comparing median days to death and alive:dead ratios, respectively). Both HR101 and SW102 immunization protected better against 3JYP2670 than HR107 immunization, and HR108 immunization protected better than HR101 immunization ($P < 0.01$ and $P < 0.05$ when comparing median days to death and alive:dead ratios, respectively).

The cross-protective ability of immunization with PspA/EF3296 fragments was also investigated in CBA/N mice, which lack a functional response to polysaccharides and are thus devoid of natural anti-phosphocholine antibodies (3, 13, 30). Immunization of CBA/N mice resulted in the same level of protection against the four strains after immunization with SW102. SW102 totally protected against challenge with ATCC6303 and also protected against BG7322 and 3JYP2670 (6 of 7 mice and 5 of 7 survived the challenge) (Table 4). There was no significant protection seen against A66.1, although 3 of 7 mice survived the challenge when compared with none in the non-immunized group. The same protection was seen when HR107 was used as immunogen. For HR107 an increased survival time was seen when BG7322, 3JYP2670, and ATCC6303 were used as challenge strains (Table 4). When alive:dead ratios were compared, significant protection was seen only for 3JYP2670. An increased survival was seen also for BG7322 and ATCC6303, but the low number of mice used did not permit a useful test of statistical significance.

In contrast to the results in BALBc/ByJ mice, immunization of CBA/N mice with HR101 failed to elicit meaningful protection against any of the challenge strains (Table 4). When comparing the protective effects between the immunogens, we found that SW102, HR107, and HR108 immunizations were significantly more protective against challenge with all strains when compared with HR101 immunization.

To conclude, the data indicate that the whole α-helical domain (SW102) confers the greatest cross-protective antibody response, with a high level of protection in both mouse strains, followed by the fragment HR108. HR107 confers the best protection

TABLE 4. Cross-protective immunity of PspA/EF3296 immunization in CBA/N mice.

Immunogen*	Strains (capsule type)	PspA clade	Median days to death**			Alive:Dead***		
			Non-imm	Imm	*P*	Non-imm	Imm	*P**
SW102	A66.1 (3)	1	2	4	0.62	0:7	3:4	0.191
	BG7322 (6B)	2	6	21	**0.018**	0:7	6:1	**0.005**
	3JYP2670 (3)	4	1	21	**0.002**	0:7	5:2	**0.020**
	ATCC6303 (3)	5	2	21	**<0.001**	0:7	7:0	**<0.001**
HR101	A66.1 (3)	1	2	2	1.000	0:7	0:7	1.000
	BG7322 (6B)	2	5	6	0.379	0:7	0:7	1.000
	3JYP2670 (3)	4	1	1	0.945	0:7	0:7	1.000
	ATCC6303 (3)	5	2	3	**0.002**	0:7	0:7	1.000
HR107	A66.1 (3)	1	2	3	0.692	0:5	0:5	1.000
	BG7322 (6B)	2	6	21	**0.016**	0:5	3:2	0.167
	3JYP2670 (3)	4	2	21	**<0.001**	0:10	9:1	**<0.001**
	ATCC6303 (3)	5	2	21	**0.016**	0:5	3:2	0.167

*Mice were immunized and boosted 2 weeks apart with 1 μg of protein and challenged 2 weeks after the boost.
**Median days to death were compared with the two-tailed Mann-Whitney U test (non-parametric, two sample rank).
***Fisher's Exact test was used to compare alive versus dead ratios in survival experiments. Boldface *P* values indicate values that were significant.

against infection in CBA/N mice, with only slight, (albeit not significant) protection in the BALBc/ByJ mice. HR101 showed better protection in BALBc/ByJ mice, with no detectable protection seen in CBA/N mice. These protection results emphasize the need for using more than one model when examining protection-eliciting immunization.

DISCUSSION

PspA's potential as a pneumococcal vaccine makes it important to determine which portions of the molecule are of maximum importance for the elicitation of protective antibodies. This determination will be an important aspect of studies designed to understand exactly how antibody to PspA leads to protection. PspAs are serologically variable yet capable of eliciting cross-protection, and it appears that the cross-protective antibody is actually reactive with one of the more variable portions of PspA. It is thus necessary to clearly define which portions of PspA elicit the most cross-protective antibodies. This knowledge should in time help us determine how such a serologically variable molecule can elicit antibodies to highly variable sequence structures that are able to cross-react and cross-protect. Finally, knowledge of the cross-protective regions of PspC could also be very helpful in the construction of PspA vaccines by permitting a vaccine composition that elicits the highest proportions of protective versus non-protective antibodies.

The potential to develop superior PspA immunogens is made readily apparent by comparing the levels of antibody elicited by the different fragments that are reactive with the full α-helical region (SW102), with the levels of protection obtained. By this measure, SW102 was 100,000 times as immunogenic as HR101 in BALB/cJ mice; but the an-

tibodies elicited by HR101 protected a higher percentage of the immunized mice from EF3296 infection. HR102 was 10,000 times as immunogenic as HR101, but the response was not protective, suggesting that, per microgram, the antibody elicited by HR101 was much more effective than that elicited by the other two fragments.

In terms of cross-protection, HR108 elicited half as much antibody in BALB/cJ mice as did HR107 and resulted in good cross-protection against the clade 2 and clade 5 strains. In contrast, the response to HR107 was not as cross-protective in these mice with the same challenge strains. Many other similar examples could be cited, all of which raise the possibility that careful selection and construction of PspA fragments could even further improve the efficacy of a PspA-containing vaccine.

As had been observed for the clade 2 immunogen Rx1 (29), a fragment composed of the N-terminal 115 amino acids and fragments containing the CDR of EF3296 were able to elicit good protection against the challenge strains of the same PspA clade. In fact, both the N-terminal 115 amino acids and the isolated CDRs of EF3296 were protection-eliciting against EF3296, as had been seen for cognate protection in the case of PspA/Rx1. HR102 (75-305AA), the fragment between the two ends of the α-helical region, was not protection eliciting. When the original studies were conducted with Rx1 PspA (29), a similar central fragment was not examined. Thus, the present study is the first to report that the amino acids in the center of the molecule are not protection-eliciting, at least when immunized in the absence of adjoining PspA amino acids that may be important for proper conformation.

When cross-protection was examined, the N-terminal fragment of EF3296 and the CDR were similarly cross-protective in BALB/c mice. They were somewhat comple-

mentary in the strains against which they protected, a finding that suggests that the inclusion of both fragments or possibly a fusion fragment in a PspA vaccine might be important.

One of the most interesting findings of the study was the fact that very different results were obtained with BALB/c than with CBA/N mice. In BALB/cJ mice the first 115 amino acids of PspA/EF3296 was very protection eliciting. In CBA/N mice, this fragment elicited no less antibody but failed to elicit protection. In contrast, fragment HR107, which contained all but the N-terminal-most 74 amino acids of the mature α-helical region, was very cross-protective in CBA/N mice but was not cross-protective at all in BALB/cJ mice.

This result suggests either that the antibodies produced by the two strains to the same molecule have different specificities or that other factors are involved. For example, BALB/cJ mice are known to have levels of antibody to phosphocholine in their serum that can protect against low-challenge inocula. These antibodies are not in the serum of CBA/N mice that express the Xid trait (14). It is possible that these antibodies can synergize with antibodies to PspA elicited by some of the epitopes of the N-terminal 115 amino acids but not with the antibody elicited by epitopes of the other fragments.

On the other hand, the broad protection seen in CBA/N mice by immunization with fragment HR107 may be because the antibody elicited can protect against the low levels of pneumococci needed to kill CBA/N mice but not the higher levels of antibody needed to kill BALB/cJ mice. It is known that high levels of systemic infection with pneumococci can decrease complement levels and that PspA acts by interfering with complement deposition on pneumococci (2, 40). Thus, these particular antibodies to

PspA may be able to prevent the development of an infection but might be much less effective at protecting against an existing infection once the mice become septic.

A final, very intriguing observation is the fact that expression of immunogenic protection-eliciting epitopes of PspA EF3296 can be highly effected by the context in which the sequence is presented. For example, fragment 75-490 (HR107) contains fragment 314-418 (HR108); however, HR107 failed to elicit cross-protection against pneumococci expressing clades 2 and 5 in BALB/cByJ mice; whereas the smaller fragment HR108 elicited good protection. This difference may be due to one of two factors: 1) The cross-protective epitopes of HR108 may not be expressed in the larger fragment because of conformational problems in HR107. 2) HR107 may contain some highly immunogenic but non-protection-eliciting epitopes that are absent in HR108. Thus, antigenic competition within the HR107 epitopes might prevent responses to the protection-eliciting epitopes of HR108.

For a future vaccine to be successful, it is important to elicit protection against all types of *S. pneumoniae*. Unfortunately, type 2-, 4-, and 5-encapsulated *S. pneumoniae* have so far been notoriously hard to protect against with PspA immunization (35, 37). Our results with EF3296 suggest that, by using smaller fragments encoding the protective portions of the molecule, it may be possible to enhance protection. Both fragments HR101 and HR108 confer higher protection against EF3296 than the full-length SW102 fragment confers. Still, the protection seen in the present study is not as complete as is usually the case with homologous PspA immunization by using challenge strains of other capsular types. Further studies will be required to understand this phenomenon fully.

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RELATIVE ROLES OF GENETIC BACKGROUND AND VARIATION IN PSPA ON THE ABILITY OF ANTIBODIES TO PSPA TO PROTECT AGAINST TYPE 3 AND TYPE 4 PNEUMOCOCCI

by

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ABSTRACT

Pneumococcal surface protein A (PspA) is immunogenic in mice and humans and is able to elicit antibodies that are protective against fatal infection of mice with *Streptococcus pneumoniae*. It has been observed that immunization with a single PspA molecule can protect against infections of mice with diverse PspAs. However, several studies have shown that immunity to PspA is a little less effective at protecting against several capsular type 4 strains than against strains of capsular types 3, 6A, and 6B. To address the relative role of differences in PspA versus differences in genetic background we used four strains of *S. pneumoniae*: a PspA family 1 capsular type 3 strain, a PspA family 2 capsular type 4 strain, and genetically engineered variants of these two strains that expressed the PspA of the opposite strain. Before infection with one of the four challenge strains, the mice were immunized with recombinant family 1 PspA, recombinant family 2 PspA, or alum only. The results revealed that part of the difficulty in protecting against capsular type 4 strains was eliminated when mice were immunized with a homologous PspA but that, regardless of which PspA the strains expressed, the capsular type 4 strains were still a little harder to protect against than the capsular type 3 strains. These results point out the importance of including more than one rPspA in any PspA vaccines developed for human use.

INTRODUCTION

Streptococcus pneumoniae is a human pathogen that is still among the leading causes of morbidity and mortality in the US (10). The current vaccine strategy for protection of adults relies on the 23-valent capsular polysaccharide vaccine. Unfortunately,

this vaccine is not highly efficacious in the target groups most affected by this pathogen, small children and the elderly (12, 19, 29). Thus, other approaches have been taken to identify several vaccine candidates that will protect equally well in both of the main target populations. Besides a protein-conjugated polysaccharide vaccine, certain protein vaccine candidates currently being investigated include PspA, pneumolysin, pneumococcal surface adhesin A (PsaA) and pneumococcal surface protein C (PspC) (3, 6, 9, 21, 25, 26, 28, 30, 33).

PspA is a surface protein present on all pneumococci (13). It has been shown to be highly immunogenic in mice and to elicit protection against pneumococcal challenge (6, 21, 27, 30); (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation). PspA has also been shown to elicit antibodies in humans that will passively protect mice against pneumococcal sepsis (6). PspA's role in virulence probably includes its ability to decrease complement activation in vivo (2, 24, 32). PspC also interferes with complement activation but by a different mechanism (17).

The PspA sequence is variable among pneumococcal strains, especially in the α-helical N-terminal domain that is exposed on the surface (16, 20). Based on sequence similarities, PspA sequences have been broadly classified into three main families, with over 95% of strains belonging to either family 1 or family 2 (11, 16). Despite the variability in their sequences, individual PspAs elicit antibodies that protect against strains with serologically diverse PspAs on their surface, suggesting that the major cross-protective epitopes may be conformationally conserved.

During studies of PspA protection, it has been noticed that pneumococcal strains of capsular types 2, 4, and 5 are harder to protect against with PspA immunization than

strains of other capsular types (7, 21, 27, 30). We recently characterized the protective effects of PspA fragments from the capsular type 4 strain EF3296 and confirmed the difficulty in obtaining full protection against type 4 pneumococci; however, we also found that certain recombinant fragments may elicit even better protection than full-length protein (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation).

In the present study, we examined the protection against a PspA family 1, clade 2, type 3 strain (WU2) and a PspA family 2, clade 3, type 4 strain (TIGR4) after immunization with fragments including the α-helical domain of their respective PspA clades. To assess the role of PspA in the protection against the type 3 and type 4 strains, a WU2 mutant carrying PspA/TIGR4 (BR93.1) and a TIGR4 mutant carrying the family 1, clade 2 PspA/Rx1 (BR6.1) were produced. Both the parental wild-type strains and the mutant strains were used to challenge mice immunized with PspA/TIGR4 or PspA/Rx1.

MATERIALS AND METHODS

Reagents. Alexa-Fluor-conjugated streptavidin was from Molecular Probes Inc, (Eugene, Or.) alkaline-phosphatase (AP) conjugated streptavidin, biotin-conjugated goat anti-mouse, and biotin-conjugated goat anti-rabbit antibodies were from Southern Biotechnology Associates (Birmingham, Ala..). Protein markers and Ready gels were from Bio-Rad Laboratories (Hercules, Calif.)

Monoclonal anti-PspA antibody (Xi126) was produced as described (20). Monoclonal anti-PspA/EF3296 and screening antibody (PC3.1) was a gift from Aventis Inc. (Toronto, Canada) and was elicited by immunization with PspA/EF3296. (11).

Bacteria. The bacterial strains and plasmids used for this study are described in Table 1. The pneumococcal strains were stored at –80°C in 12% glycerol, streaked on blood agar plates, and incubated at 37°C in 5% CO_2 atmosphere overnight. Colonies grown on blood agar were used to inoculate liquid growth medium (Todd-Hewitt medium containing 0.5% yeast extract, THY). Upon reaching early stationary phase, the bacteria were harvested by centrifugation at 1,500 x *g* for 15 min and suspended in 60 mM phosphate-buffered saline (PBS, pH 7.2). The bacterial concentration was estimated by measuring absorbance at 600 nm and confirmed by viable counts on blood agar plates.

TABLE 1. Bacterial strains and plasmids

Strain	Characteristic	Source/reference
S. pneumoniae		
WU2	Wild type, capsule type 3	8
EF3296	Wild type, capsule type 4	5
TIGR4	Wild type, capsule type 4	1,31
BR93.1	WU2 *pspA*::pBR93	Ren B, A. Szalai, and D.E. Briles. In preparation
BR6.1	TIGR4 *pspA*::pBR6	Ren B, A. Szalai, and D.E. Briles. In preparation

PspA constructs for insertion-duplication. Constructs made for insertion-duplication mutagenesis have been described in detail elsewhere (Ren B, A. Szalai, and D.E. Briles. manuscript in preparation).

Pneumococcal transformation. Plasmid preparations were used to transform *S. pneumoniae* strains WU2 and TIGR4. Strains were grown in competence media (THY with the addition of 0.2% BSA, 0.2% glucose, and 0.02% $CaCl_2$, made fresh) until turbidity became visible. Bacteria were then diluted 1:30 in fresh competence media, and 200 ng/ml of competence-stimulating peptide 1 (WU2) or 500 ng/ml of competence-stimulating peptide 2 (TIGR4) (15, 18) was added, together with 100 ng of plasmid DNA. The bacteria were grown at 37°C for an additional 2 h, after which the culture was plated on blood agar containing 0.3 μg/ml of erythromycin. Resistant transformants were saved and analyzed for insert ional mutagenesis of the genes.

Western blot. Bacterial cells were grown to optical density at 600 nm of 0.6. The cells were washed twice in PBS and treated with 2 mg/ml of hen egg lysozyme for 2 h at 37°C. Sodium dodecyl sulfate (1%) was added and the suspension was vortexed until clear. The lysates were stored at –20°C until further use.

Whole cell lysates were run on polyacrylamide gels (Bio-Rad Ready gels, Bio-Rad Laboratories) and were then electroblotted to a 0.45μm nitrocellulose membrane (Bio-Rad Laboratories) in Tris-glycine buffer (20% methanol, 25 mM Tris and 192 mM glycine [pH 8.1-8.4]) at 100 V for 1 h at 4°C. The blotted membrane was blocked with 1% BSA in PBS-T (PBS containing 0.05% Tween-20) for 1 h at room temperature and washed three times (5 min each) with PBS-T. The membranes were overlaid with the monoclonal antibodies to PspA (antibody PC3.1, for detection of PspA/TIGR4, or Xi126, for detection of PspA/WU2 and PspA/Rx1) for 30 min at 37°C and washed three times in PBS-T. The membrane was further incubated with a mix of biotinylated goat anti-mouse

antibody (1:1,000 in PBS-T) and alkaline phosphatase conjugated streptavidin (1:500 dilution in PBS-T) for 30 min at 37°C. After washing, the membrane was developed by using 0.1 mg/ml NBT and 0.5 mg/ml of BCIP in 0.15 M Tris-HCl (pH 8.8).

Binding of anti-PspA serum antibodies to the bacterial surface. Bacteria were grown on blood agar plates or in THY medium and were suspended in PBS at a concentration of approximately 1 x 10^8 bacteria/ml. A bacterial pellet of 10^7 bacteria was suspended in monoclonal anti-PspA PC3.1 antibodies (20 μg/ml) or anti-PspA Xi126 (undiluted hybridoma supernatant) for 30 min at room temperature and washed by centrifugation at 1500 x *g* for 5 min in PBS. Biotinylated goat anti-mouse antibodies (1:100 dilution in PBS) were added for an additional 30 min at room temperature. After a second wash in PBS, Alexa Fluor-conjugated streptavidin was added (1:100 dilution) for 30 min at room temperature, and the cells were washed and inspected by epi-fluorescence microscopy using a Leitz upright microscope (Leitz, Wetzlar, Germany). The binding was quantitated by flow cytometry with a FACSCalibur Flow Cytometer (Becton-Dickinson Biosciences, Rutherford, N.J.).

PspA fragments for immunization. PspA/Rx1 fragment JAS218 was constructed and expressed as described previously (14). Fragment SW102 of PspA/EF3296 constituting amino acids 1-478 was kindly provided by Aventis Inc. (Toronto, Canada). Fragment HR108 was constructed and expressed as described previously (Roche, H., A. Håkansson, S.K. Hollingshead and D.E. Briles, manuscript in preparation).

Mouse immunization and challenge. Five- to 8-week-old CBA/N mice or BALBc/ByJ mice (Jackson Laboratories, Bar Harbor, Me.) were immunized subcutaneously with purified recombinant PspA fragments. The procedure and fragments have been described elsewhere (14; and Roche, H., S.K. Hollingshead, A.Håkansson and D.E. Briles, manuscript in preparation). Mice were immunized subcutaneously with 5 μg of SW102 (amino acids 1-478, PspA/EF3296), HR107 (amino acids 75-490, PspA/EF3296), HR108 (amino acids 314-418, PspA/EF3296), and JAS218 (amino acids 170-288, PspA/Rx1), with alum as adjuvant (100 μg/ml) conjugated in alum. The mice were boosted subcutaneously 2 weeks later with the same dose of immunogen. A fortnight after the boost the mice were challenged with the recombinant strains BR93.1 or BR6.1 or with wild-type strains WU2 or TIGR4 at a dose that was two logs higher than the LD_{50}. The mice were then monitored for death over 21 days, and mice that survived the challenge were scored as protected against infection by the respective strain.

RESULTS

Characterization of the strains. *S. pneumoniae* TIGR4 was chosen as a representative for type 4 strains. This strain was chosen partly because it is easy to transform, and partly because it expresses a PspA with an α-helical region identical to that of PspA/EF3296. The only difference between PspA/EF3296 and PspA/TIGR4 is a phenyl -alanine to leucine replacement at amino acid position 16 of the leader (16, 31). However, the mature proteins PspA proteins are identical. For comparison, *S. pneumoniae* WU2 (capsule type 3) was chosen to represent strains that are easy to protect against with PspA immunization. Two mutants were made to determine whether the PspA structure

or the genetic background was responsible for the difficulty in protecting capsular type 4 strains by immunization with PspA. In the first recombinant strain (BR93.1) the *pspA* sequence of WU2 was replaced by *pspA*/TIGR4, resulting in a WU2 strain with PspA/TIGR4 as the only expressed PspA. In the second recombinant strain (BR6.1), the *pspA* sequence in TIGR4 was replaced with *pspA*/Rx1, resulting in a TIGR4 strain expressing only PspA/Rx1.

The mutant strains expressed the correct PspAs as shown by Western blot analysis (Fig. 1). TIGR4 reacted strongly with the monoclonal antibodies PC3.1 specific for PspA/EF3296 and PspA/TIGR4, and WU2 reacted strongly with the monoclonal antibodies Xi126 specific for PspA/Rx1. No binding was seen when antibodies for strains were switched, thus confirming the specificity of the monoclonal antibodies.

The BR93.1 mutant (WU2 with PspA/TIGR4) expressed capsule type 3, but lysates of BR93.1 no longer reacted with the Xi126 antibodies. Instead, it reacted strongly with the PC3.1 antibodies, indicating that the bacteria expressed only PspA/TIGR4. The strain BR 6.1 (TIGR4 with PspA/Rx1) expressed capsule 4, but its lysate no longer reacted with the PC3.1 antibodies. Instead, the lysate reacted strongly with the Xi126 antibodies, indicating that the bacteria expressed only PspA/Rx1 (Fig. 1). When the blots were analyzed, no obvious difference in expression levels was detected in the WU2 and BR6.1 strains or in the TIGR4 and BR93.1 strains.

To verify that the PspAs of the parental and mutant strains were expressed on the surface, binding of antibodies to the bacterial surface was quantitated by flow cytometry (Fig. 2). These results verified the lysate studies above and demonstrated the mutant and parental strains expressed the correct PspAs. The findings also showed that in each case,

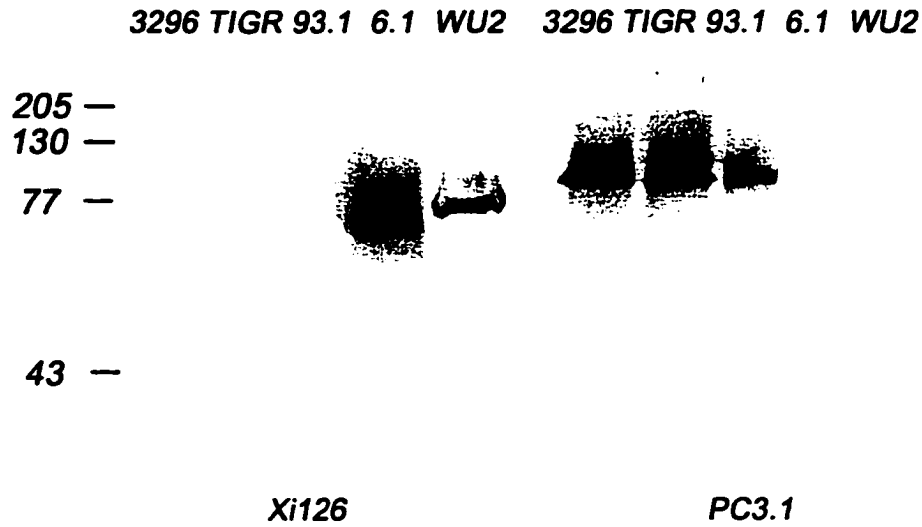

FIG. 1. PspA reactivity in pneumococcal strains. Bacterial lysates were run in 12% SDS-polyacrylamide gels and subjected to Western blot by using monoclonal anti-PspA antibodies (MAbs) reactive with PspA/Rx1 (Xi126) or PspA/EF3296 (PC3.1). These two MAbs were chosen because neither reacts with non-homologous PspA. The samples run were 3296 = EF3296 lysate, TIGR = TIGR4 lysate, 93.1 = BR93.1 lysate, 6.1 = BR6.1 lysate, and WU2 = WU2 lysate. The left panel shows the pattern of reactivity for the strains with Xi126 antibodies, and the right panel shows reactivity with PC3.1. EF3296, TIGR4, and BR93.1 (WU2 expressing PspA/TIGR4) lysates all showed reactivity with PC3.1 antibodies but did not react with Xi126, showing that the strains only expressed family 2 PspA. WU2 and BR6.1 (TIGR4 expressing PspA/Rx1) lysates reacted only with Xi126 antibodies, and no reactivity with PC3.1 antibodies was detected, showing that these strains only expressed the family 1 PspA.

the respective PspAs were expressed at the pneumococcal surface. BR93.1 did not bind Xi126 antibodies, and BR6.1 did not bind PC3.1 antibodies. TIGR4 and BR93.1 bound equal amounts of PC3.1 antibody (21.6 and 23.8 times their respective streptavidin-alone -treated controls), indicating that they expressed similar levels of antibody-accessible PspA on the surface (Fig. 2). The same was true for WU2 and BR6.1, showing binding of 8.1 and 9.3 times their respective streptavidin-alone-treated controls (Fig. 2).

Relative ability of family 2 PspA fragments to protect against challenge strains expressing either cognate with varied backgrounds or non-cognate PspAs. To investigate the role of PspA/EF3296 to elicit protection against fatal infection, CBA/N and BALBc/ByJ mice were immunized with the recombinant fragments PspA/SW102 and HR108. Recombinant PspA/SW102 encodes the entire α-helical domain of PspA/EF3296 and a portion of the proline-rich region (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation). HR108 is a 104 amino acid (314-418) fragment (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation) corresponding to the so-called clade-defining region (CDR) of EF3296 (16). Prior studies have shown that the homologous CDR of PspA/Rx1 is important for elicitation of cross-protection (20), as was recently shown also for PspA/EF3296 (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation). After immunization with the two different EF3296 PspA fragments, the mice were challenged with WU2, TIGR4 and BR93.1 (WU2 background expressing PspA/TIGR4); protection was compared with non-immunized mice challenged with the same strains (Fig. 3).

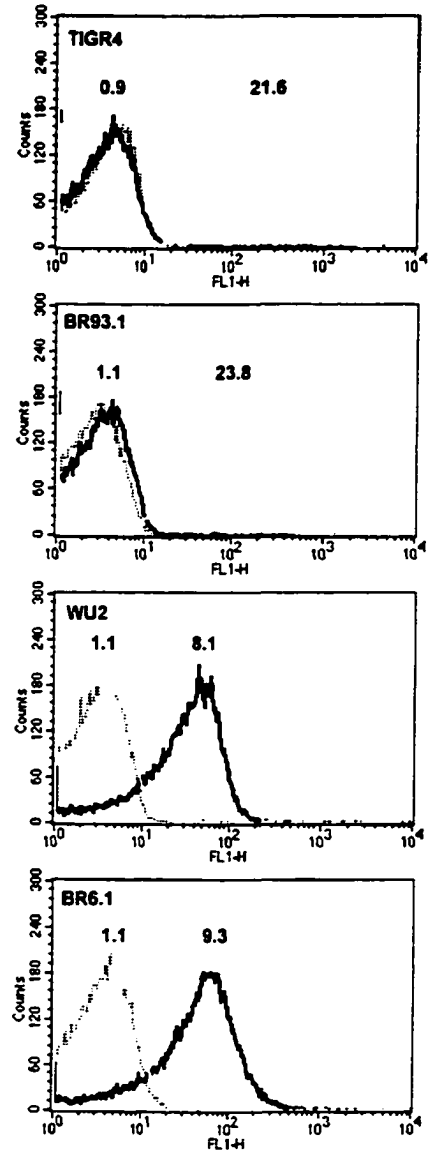

FIG. 2. Flow cytometric analysis of PspA expression. *S. pneumoniae* TIGR4, BR93.1, WU2, and BR6.1 were treated with PspA antibodies Xi126 or PC3.1 antibodies and counterstained with biotinylated anti-mouse antibodies and AlexaFluor-conjugated streptavidin and binding was quantitated by flow cytometry. Dotted trace represents streptavidin-alone treated bacteria, gray trace represents binding of Xi126 antibodies, and black trace represents binding of PC3.1 antibodies. Numbers above the black and gray traces represents the levels of binding in fluorescent signal of sample divided by fluorescent signal of the streptavidin control. TIGR4 and BR93.1 (WU2 expressing PspA/TIGR4) showed no binding of Xi126 antibodies, but showed strong and similar levels of binding of PC3.1, indicating that the strains only expressed PspA/TIGR4. WU2 and BR6.1 (TIGR4 expressing PspA/Rx1) showed no binding with PC3.1 antibodies but showed strong and similar levels of binding with Xi126, indicating that they only expressed family 1 PspA.

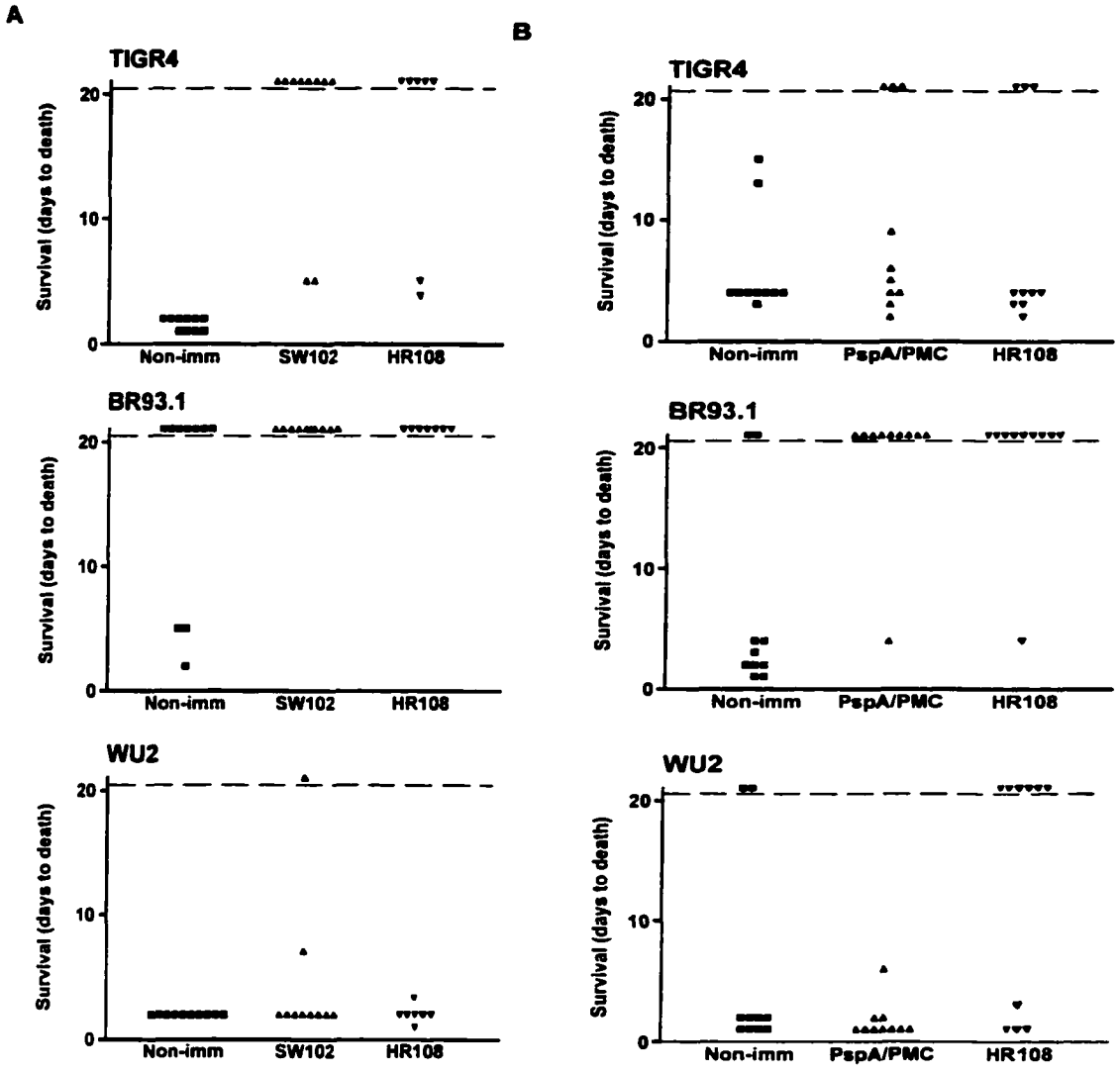

FIG. 3. Protection against pneumococcal infection after immunization with PspA/EF3296 fragments. (A) CBA/N mice were immunized with either SW102 or HR108; after a booster immunization the mice were infected with TIGR4, BR93.1, or WU2. Survival of the mice was recorded, and mice surviving 21 days were considered protected. B. BALBc/ByJ mice were immunized with either SW102 or HR108 and after a booster immunization were infected with TIGR4, BR93.1, or WU2. Survival of the mice was recorded, and mice surviving 21 days were considered protected.

Immunization with SW102 resulted in no protection in either CBA/N or BALBc/ByJ mice against WU2, suggesting that no clade2/clade3 cross-protective antibodies were formed. Almost complete protection of CBA/N mice was seen against TIGR4 (8 of 10 mice survived the challenge; $P < 0.001$), and complete protection was seen after challenge with BR93.1 (10 of 10 surviving mice; $P < 0.001$) (Fig. 3A). Both of the latter challenge strains express a PspA cognate to the immunizing PspA.

The apparently better protection against the strains BR93.1 on the WU2 background as opposed to the TIGR4, with its own background, was not statistically significant in CBA/N mice. In similar studies with BALBc/ByJ mice, the trend for better protection against BR93.1 when compared with TIGR4 was more pronounced and statistically significant (Fig 3B). Immunization of BALBc/ByJ mice with SW102 resulted in rather poor protection against TIGR4, with only 3 of 10 mice surviving. On the other hand, protection was very good against BR93.1, with 9 of 10 mice surviving the challenge ($P = 0.003$). When the ability to protect against TIGR4 after immunization with SW102 was compared with the ability to protect against BR93.1, the protection was significantly better against BR93.1 ($P = 0.023$ and $P = 0.02$ when median days to death and alive:dead ratios were compared, respectively).

The protection pattern after immunization with HR108 was similar to that seen for SW102 for challenge with TIGR4 and BR93.1 (Fig. 3). SW102 failed to protect either mouse strain against WU2. HR108, unlike SW102 was able to elicit cross protection against WU2 in BALBc/ByJ, but not in CBA/N mice.

Taken together, these results indicate that for immunization with the clade 3 PspA expressed by the capsular type 4 challenge strains, better protection was observed against

the PspA clade 3 challenge strain that expressed capsular type 4. The study also demonstrated that recombinant clade 3 PspA provided better protection against pneumococci expressing clade 3 than did recombinant clade 2 PspA.

Relative ability of family 1 PspA fragments to protect against challenge strains expressing either cognate with varied backgrounds or non-cognate PspAs. To examine the role of strain background on the protective effects of clade 2/family 1 PspA immunization, CBA/N and BALBc/ByJ mice were immunized with the PspA/Rx1 fragment JAS218,amino acids 169-288 and challenged with TIGR4, WU2, and BR6.1 (TIGR4 expressing clade 2 PspA/Rx1). The JAS218 fragment constitutes the B-region of PspA/Rx1 and is homologous to fragment HR108 from PspA/TIGR4.

Immunization of CBA/N mice with JAS218 protected against both WU2 ($P <$ 0.001 for both median days to death and alive:dead ratios) and BR6.1 ($P = 0.003$ and $P =$ 0.011 when median days to death and alive:dead ratios were compared, respectively) (Fig. 4A). However, the protection seen for homologous strains WU2 was significantly better than for BR6.1 ($P = 0.063$ and $P = 0.02$ when comparing median days to death and alive:dead ratios, respectively). This finding supported the earlier finding that it was easier to protect against infections on the type 3 than on the type 4 background. Immunization with JAS 218 did not confer any protection against TIGR4 in either CBA/N mice, although this immunization had protected against strain BR6.1, which is capsular type 4 but expresses the immunizing clade 2 PspA. This finding indicated that insufficient cross-protective antibodies were formed that could be detected on the TIGR4 background (Fig. 4).

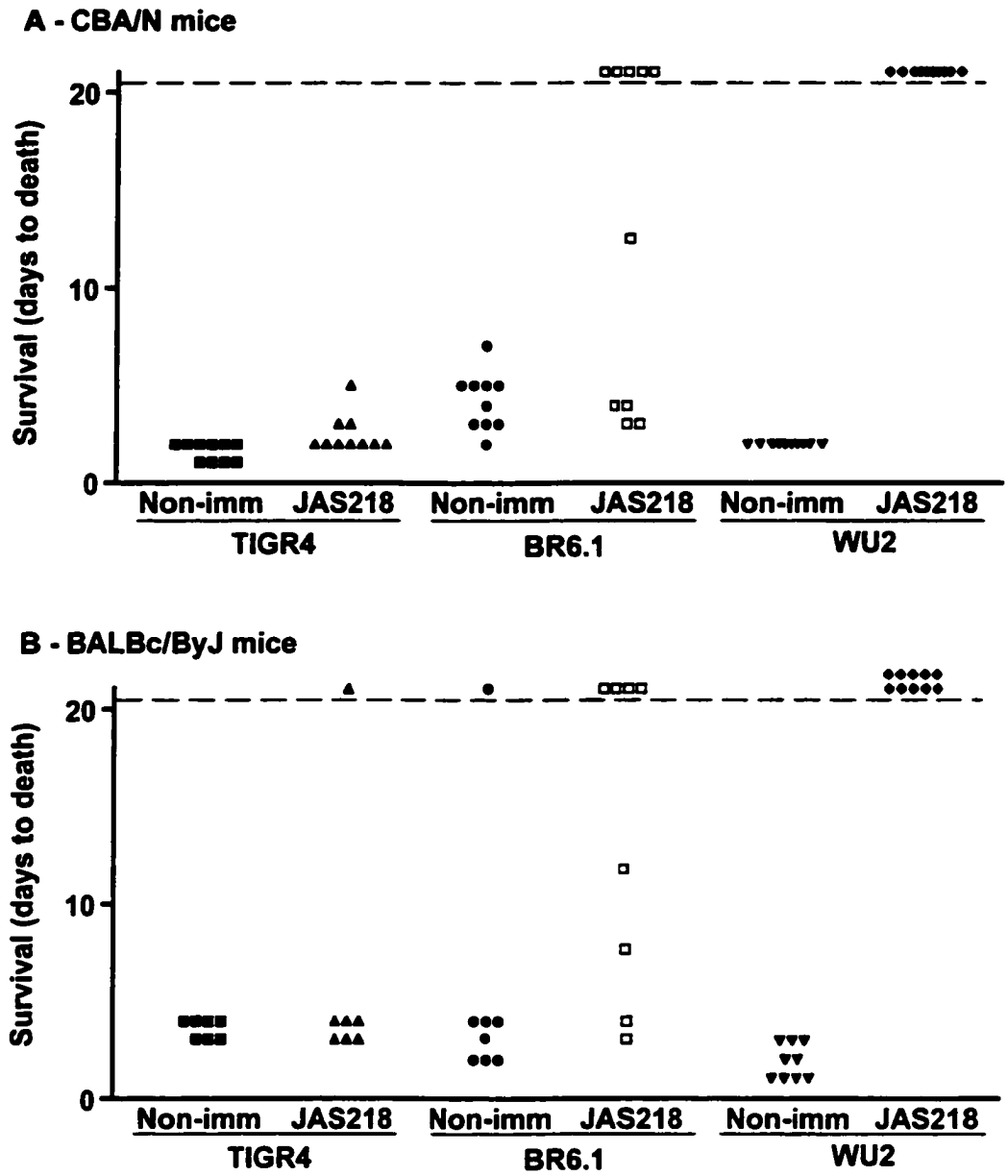

FIG. 4. Protection against pneumococcal infection after immunization with PspA/Rx1 fragment JAS218. (A) CBA/N mice were immunized with either SW102 or HR108; after a booster immunization, the mice were infected with TIGR4, WU2, or BR6.1. Survival of the mice was recorded and mice surviving 21 days were considered protected. (B) BALBc/ByJ mice were immunized with either SW102 or HR108 and after a booster immunization, the mice were infected with TIGR4, WU2, or BR6.1. Survival of the mice was recorded, and mice surviving 21 days were considered protected.

Similar data were obtained when immunized and non-immunized BALBc/ByJ mice were challenged (Fig. 4B). JAS218 immunization resulted in complete protection against WU2 ($P < 0.001$ for both median days to death and alive:dead ratios), whereas only 4 of 8 mice survived challenge with BR6.1 ($P = 0.28$). When these protective effects are compared, JAS218 protected better against WU2 than against BR6.1 ($P = 0.054$ and $P = 0.023$ when median days to death and alive:dead ratios were compared, respectively).

Compiled, these data indicate that when PspA/Rx1 clade 2 is expressed in a type 4 pneumococcal background, PspA immunization is less effective than when expressed in the WU2 background. These data support the results above and suggest that the background of the strain determines the ability of immunization to elicit protection against PspA-expressing strains. The results using both immunogens also strongly indicate that better protection is observed when the immunizing and challenge PspAs are of the same family/clade.

DISCUSSION

This study investigated the role of PspA and strain background for effective protection against bacterial challenge after immunization with PspA. PspA is one of several pneumococcal immunogenic proteins that have shown promising results in mouse protection experiments (3, 6, 26, 30, 33). The PspA molecule is highly diverse between strains but is still cross-reactive and confers cross-protection in murine infection models (16, 20, 23). So far, the PspA structure has been classified according to the CDR. This classification makes sense for vaccine purposes because, with both a family 1 and family

2 PspA, it has not been shown that the CDR is of primary importance for eliciting cross-protective antibody (20, 21) (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation).

Despite the fact that strains of different families can differ in their B-region sequences by 60% or more, it has also been observed that when the challenge strains are of capsular type 3, 6A, or 6B backgrounds, immunization with a single PspA can protect against strains of diverse clades and families. Most of the original studies were done by using native or recombinant PspA from clade 2 family 1 as immunogens (6, 7); however, a recent study using recombinant clade 3 family 2 PspA as immunogens has shown similar but slightly less cross-protection than the studies with the clade 2 immunogens (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation).

During the studies of protection-eliciting responses to PspA, it has been noticed that certain capsular serotypes, namely types 2, 4, and 5, are harder than others to protect against with PspA immunization (27, 30). The reason for this is unknown but has been speculated to be related either to the PspA these strains express or to other factors associated with the different genetic backgrounds. This question was investigated in the present study.

S. pneumoniae of capsular type 3 (WU2) and capsular type 4 were chosen for comparison. WU2 is a capsule type 3 strain expressing clade 2 family 1 PspA , which is easily protected against by immunization with PspA/Rx1. TIGR4 is a capsule type 4 strain that is easily transformable and expresses a clade 3 family 2 PspA. Since the mature expressed PspA/TIGR4 is identical in its α-helical sequence (Roche, H., A. Håkans-

son, S. K. Hollingshead, and D. E. Briles, manuscript in preparation), we have been able to immunize with the recombinant EF3296 fragments that we have already produced (Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles, manuscript in preparation). From these two parental wild-type strains, two new strains were produced: a WU2 strain in which the native PspA was replaced with PspA/TIGR4 and a TIGR4 strain in which the native PspA was replaced with PspA/Rx1. PspA/Rx1, rather than WU2 PspA, was chosen because of the existence of molecular fragments of PspA/Rx1 and because PspA/Rx1 was known to elicit protection against infection with WU2 (20). The α-helical domain of PspA/Rx1 is 86% identical to PspA/WU2 at the amino acid level (16). The recombinant strains made by switching PspAs between the two parental types were each of similar virulence to the parental background strain and were not affected by the fact that they were expressing a different PspA (Ren, B., A.Szalai and D.E Briles, manuscript in preparation).

Western blot and flow cytometry analysis verified that the mutant strains no longer expressed their native PspA but only expressed the inserted PspA. The flow cytometry analysis also indicated that the PspA molecules were indeed expressed on the bacterial surface and suggested that the quantities of PspA on the surface were equal in the different background strains expressing the same PspA. Capsular typing revealed no differences in capsule expression between the mutants and the parental strains.

PspA fragments from PspA/Rx1 and PspA/TIGR4 were used to immunize immunologically competent BALBc/ByJ mice or CBA/N mice carrying the XID trait (4, 8, 22). The CBA/N mice are several logs more susceptible to the challenge strains than

BALBc/ByJ mice. As a result, the challenge doses used with CBA/N mice were about 4 logs less than that used in BALBc/ByJ.

A general observation from these studies was that when immunizations were conducted with either the Rx1 or EF3296 PspA fragments, there was some evidence of PspA family/clade specificity of the protection. This observation is consistent with the known divergence in the α-helical sequence of PspA but surprisingly had not been observed in our earlier studies in which we immunized with recombinant fragments of Rx1 PspA. As a result, this study provides the first protection data supporting the hypothesis that the design of a PspA vaccine should include more than one PspA and the choice of PspAs for the vaccine should be made at least in part based on the family/clade diversity based on B- region structure.

The study also confirmed earlier findings that suggested that pneumococci with some genetic backgrounds were more difficult to protect against than others. The data show that this problem is independent of the PspA expressed by the challenge strain. In previous studies, several different capsular type 4 strains were used as challenge strains; and all appeared more difficult to protect against than most strains of capsular types 3, 6A, and 6B. The present results make it clear that this difference derives not from the PspA of the challenge strain but from something else in the genetic background. The role that the different capsular type plays in this is not known. Because of recent data indicating that strains of the same capsular type are sometimes clonally related, the association of "capsular type" with ease of protection with antibody to PspA may instead be dependent on other genes shared by strains with the same capsular type.

It is also possible, for example, that because of the capsular material or other genetic factors common within the serotype, PspA expressed in a type 4 or type 5 strain may be presented on the surface in a way that makes the protein less accessible to protective antibodies. This possibility seems less likely in light of our flow cytometry assays that showed equal antibody binding to both strains carrying the same PspA. Still, there is a possibility that host factors or environmental signals in the host change the surface of the bacteria and thus the exposure of PspA.

There is also a possibility that other factors associated with the genetic background of the type 4 and 5 strains are involved. These strains may have systems that interfere downstream of the antibody activation. Types 4 and 5 capsular material may enable the bacteria to avoid the complement system or phagocytes more easily. These factors will be investigated in future studies.

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CONCLUSIONS

Despite the presence of antibiotics and vaccines, *S. pneumoniae* is still a major cause of morbidity and mortality, both in the US and worldwide. For the year 1999, statistics collected by the Centers for Disease Control show that *S. pneumoniae* infections resulted in 100,000 to 135,000 hospitalizations for pneumonia, 6 million cases of otitis media, and over 60,000 cases of invasive disease, including 3,300 cases of meningitis in the US (4). *S. pneumoniae* is the sixth leading cause of death in the US and is a major killer in the developing world.

In these times of emerging antibiotic-resistant strains, preventive vaccines have become more important than ever. A polysaccharide vaccine developed in the 1980s is readily available, but children below the age of 2 years and the elderly have not benefited significantly from the vaccine (160). This lack of benefit has mainly been attributed to the inability of young children to elicit immune responses effectively against polysaccharide structures and to tolerance in the elderly, leading to the same result. The current strategy for enhancing the efficacy of polysaccharide structures to elicit immunity stems from the successful implication of protein conjugation seen for the vaccine against Hib (8, 147). Pneumococcal protein conjugate vaccines have been more successful than the polysaccharide vaccine in preventing infection in the main target populations (25, 71, 162) but problems of increasing the number of capsule types to yield complete protection will not be easily solved. Also, the prohibitive cost of these vaccines makes them inaccessible to children in developing countries, where the need is most acute.

Recent research for new vaccines has focused heavily on protein vaccine candidates that can be employed to overcome the lack of immune responsiveness of infants to the polysaccharide vaccine. Of the proteins evaluated so far, PspA, alone or in conjunction with other protein molecules such as PsaA and/or pneumolysin, has resulted in promising protection in a variety of model systems (34, 112, 121, 125, 134, 135, 173).

Although PspA is a highly variable molecule based on both serological data and gene sequencing, the structure still contains cross-reactive epitopes. Antibodies reacting with these epitopes have also been shown to have the highest level of cross-protection against strains expressing other PspAs (39, 40). It has been speculated that these epitopes may be involved in performing the functional aspects of PspA during colonization and infection of the host. Studies with isogenic mutants have provided evidence that PspA is involved in protecting pneumococci against complement attachment, and this involvement has been attributed to the ability of PspA to inhibit complement deposition and/or binding of lactoferrin (81, 82, 179). Therefore, studies have investigated the variability of the molecule and attempts have been made to identify the cross-reactive and cross-protective epitopes.

Sequence homology studies of one region of the molecule, the CDR, indicated groupings of sequences within the family structure and resulted in the definition of 5 clades comprising two major families. The PspA sequences within each of these families show greater than 60% sequence identity (90). This CDR corresponds to the localization of protective and cross-protective epitopes of the family 1, clade 2 PspA from *S. pneumoniae* Rx1 (120). Using six monoclonal antibodies, McDaniel et al. showed that anti-

bodies that reacted with epitopes within amino acids 192 to 260 of PspA/Rx1 could protect against infection with both homologous and heterologous strains (120).

In the current dissertation, we obtained information to support these earlier observations with regard to protective epitopes and extended their significance to both family 1 and family 2 PspAs. Active immunization with the CDR from PspA/Rx1 totally protected immuno-competent and XID mice against infection (dissertation, Paper 2), supporting the earlier data using passive immunization with monoclonals (120) and active immunization with recombinant molecules expressing the CDR, proline region, and choline-binding region (173).

Because all of the existing information about protective regions of PspA was obtained with family 1 PspA (Rx1) and whether protection seen with immunization with this PspA could be extended to family 2 PspAs has not been elucidated, we investigated protective regions of a family 2 PspA. It is estimated that roughly 50% of disease-causing strains belongs to each of families 1 and 2 (54); and as the variability of the sequences between families is almost 40% in some instances, studies of the protective epitopes of family 2 proteins are merited to ensure that the families do not show structural or functional diversity. This is important lest we make incorrect decisions about what epitopes of the PspAs of the different families should be included in a potential vaccine.

The strain chosen for our study was a clade 3, type 4 strain, EF3296. The strain was chosen in part because it expressed a family 2 PspA and in part because it was one of several capsular type 4 strains that were particularly difficult to protect against with family 1 PspA (119). Moreover, in studies in which we immunized with isolated PspAs, we also observed some trouble getting complete protection (37); however, we also had very

low yields of isolated PspA from this strain, raising the possibility that the isolated material was not representative (in conformation) of the bulk of the PspA expressed by the strain.

The study was designed to characterize the protective epitopes by using overlapping fragments from the PspA/EF3296 sequence and to identify potential differences in location of protective epitopes from what had been observed with family 1 PspA/Rx1. We also hoped that if we had fragments with different abilities to elicit protective antibody responses, that the fargments might be useful in producing antisera that help us to develop laboratory correlates of protection.

Six fragments of EF3296 were produced and expressed by recombinant technology and used for immunization. All fragments except one elicited a high degree of protection in immuno-competent mice, and four of the fragments contained the CDR that is clade defining and has been shown to contain cross-protective epitopes. The only protective fragment that did not contain the CDR (amino acids 314-418) was the N-terminal fragment HR101 (amino acids 1-115). This finding confirms much less robust results, indicating that pneumococcal supernatants expressing the N-terminal 110 amino acids of PspA/Rx1 were able to elicit protection against infection with WU2 but that comparable supernatants lacking the fragments did not (36).

Interestingly, HR101 did not elicit a substantial antibody response, as detected in ELISA against either the immunizing fragment or a recombinant fragment expressing the full α-helical region and a portion of the proline-rich region. Immune sera from the immunized mice also contained such low antibody concentrations that they failed to exhibit detectable staining on the surface of the bacteria.

The fragment that did not elicit protection, HR102, covered amino acids 75 to 305 and thus included the sequence located between the N-terminal region and the B-region. From these studies we could conclude that major protective regions are located in the N-terminal domain and in the CDR of the protein but not in the region in between.

The protective ability of these protein fragments differed when XID mice were used for immunization and challenge. For all the fragments containing the CDR, protection was generally better in this mouse model. For the N-terminal region, the situation was reversed with no protection seen after immunization with this fragment.

The ability of these fragments to protect against strains expressing PspA belonging to other clades was addressed next. The fragments containing the CDR showed significant protection against strains expressing PspAs of other PspA clades, with the full-length α-helical fragment conferring best protection overall. Our findings regarding this are consistent with earlier studies examining the cross-protection elicited by PspA/Rx1 and suggested that the structure and antigenicity patterns of PspA of the different families are similar. The cross-protection was better in the XID mouse background than in the immuno-competent BALB/c mice. The N-terminal fragment showed some cross-protection in BALB/c mice but did not protect against pneumococcal infection in the XID background.

The present study concluded that the major protective and cross-protective epitopes reside in the clade-defining (or B) region and also indicated that other parts of the molecule can confer protection against pneumococcal challenge. The finding that the full-length α-helical domain conferred the highest degree of cross-protection suggests that this fragment constitutes a promising candidate for inclusion in a future vaccine. An

even better candidate might be one that included the N-terminal 75 amino acids fused to the amino-acids 314-418. Such a fragment might have the advantage of not eliciting non-protective antibody to the epitopes expressed by amino-acids 76-213. Additional studies with additional fragments might provide insight into the construction of an even better fragment.

In studies of the cross-protective ability of PspA, it was noted that protection against some strains was weaker than that against others. In particular, strains of capsule types 2, 4, and 5 generally are less protected against by PspA immunization than are other serotypes. As an example, Tart et al showed that when mice were immunized with PspA from the type 4 strains L81905 and EF5668, no protection was seen against the same homologous donor strains; however, cross-protection was observed against strains expressing other capsule types and other PspAs (173). To explore the reasons behind this observation, we chose to swap the PspA from a strain (WU2, type 3, family 1) that was relatively easy to protect against with PspA immunization with PspA from a type 4 strain (family 2) that was hard to protect against, and vice versa.

When mice were immunized with family 1 PspA or family 2 PspA and then challenged with the strains expressing family 1 (WU2) or family 2 (TIGR4) PspA, the immunizations conferred clade-specific immunity upon challenge with pneumococci. When we then compared the ability of strains of a type 3 background (WU2) expressing family 1 or family 2 PspA with the matching strains of a type 4 background (TIGR4), the former were protected against death by immunization with PspA much more readily than the latter. Strains with the WU2 genetic background were easier to protect against with family 1 PspA immunization than strains on the TIGR4 genetic background. Similarly, the

WU2 strain carrying family 2 PspA was easier to protect against with family 2 PspA immunization than was TIGR4, suggesting that the type 4 background contributes to the increased virulence of the type 4 strain.

Overall, this dissertation provides evidence that PspA molecules, despite their variability, contain structures that confer cross-protective immunity in mice. This evidence is encouraging as a high level of protection against pneumococcal infection may be provided using only a few PspA molecules. This study also provides evidence that the major cross-protective epitopes in both major families of PspA molecules reside in the CDR. We have also shown that immunization with recombinant PspA fragments can cause a significant protection, even against strains that are generally hard to protect against by using PspA immunization. The protection seen here against type 4 strains is better than what has been reported earlier; and even if the protection was not always complete, these fragments have provided promising results. We have finally shown that the reason a capsule 4 strain is harder to protect against than a type 3 strain is mainly attributed to the genetic background of the type 4 strain rather than the PspA molecule the strain expressed.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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