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Relationship between Surface Accessibility for PpmA, PsaA, and PspA and Antibody-Mediated Immunity to Systemic Infection by *Streptococcus pneumoniae*

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Antibodies to capsular polysaccharide (PS) are protective against systemic infection by *Streptococcus pneumoniae*, but the large number of pneumococcal serogroups and the age-related immunogenicity of pure PS limit the utility of PS-based vaccines. In contrast, cell wall-associated proteins from different capsular serotypes can be cross-reactive and immunogenic in all age groups. Therefore, we evaluated three pneumococcal proteins with respect to relative accessibility to antibody, in the context of intact pneumococci, and their ability to elicit protection against systemic infection by encapsulated *S. pneumoniae*. Sequences encoding pneumococcal surface adhesin A (PsaA), putative protease maturation protein A (PpmA), and the N-terminal region of pneumococcal surface protein A (PspA) from *S. pneumoniae* strain A66.1 were cloned and expressed in *Escherichia coli*. The presence of genes encoding PsaA, PpmA, and PspA in 11 clinical isolates was examined by PCR, and the expression of these proteins by each strain was examined by Western blotting with antisera raised to the respective recombinant proteins. We used flow cytometry to demonstrate that PspA was readily detectable on the surface of the pneumococcal strains analyzed, whereas PsaA and PpmA were not. Consistent with these observations, mice with passively or actively acquired antibodies to PspA or type 3 PS were equivalently protected from homologous systemic challenge with type 3 pneumococci, whereas mice with passively or actively acquired antibodies to PsaA or PpmA were not effectively protected. These experiments support the hypothesis that the extent of protection against systemic pneumococcal infection is influenced by target antigen accessibility to circulating host antibodies.

Streptococcus pneumoniae is a leading cause of morbidity and mortality in developed and developing countries (38). Each year *S. pneumoniae* causes approximately 1.2 million deaths worldwide from pneumonia (43). Antibiotics are effective at controlling many cases of pneumococcal infection, but their use does not prevent mortality within the first 48 h of presentation. The effectiveness of therapeutic care is further constrained by the widespread occurrence of antibiotic-resistant pneumococcal strains (15, 16), and several retrospective studies have reported essentially no change in fatality rates due to pneumococcal bacteremia over the past 40 to 60 years (2, 26). These factors have stimulated renewed interest in the prevention of pneumococcal infections by using vaccines.

Prophylactic vaccines based on capsular polysaccharides (PS) of the pneumococcus are currently the only licensed vaccines available against *S. pneumoniae*. The 23-valent PS vaccine is not effective in children younger than 5 years (12), whereas the recently licensed 7-valent conjugate vaccine only covers a limited number of pneumococcal serotypes (18). The effectiveness of the 7-valent conjugate vaccine at reducing systemic pneumococcal disease due to vaccine serotypes and cross-reactive strains is well established (4, 50). However, this effectiveness of the conjugate vaccine is partially counterbalanced by recent reports documenting increases in pneumococcal disease due to non-vaccine-related serotypes (14, 33). This serotype replacement phenomenon has stimulated interest in developing vaccine strategies aimed at controlling pneumococcal disease in a non-serotype-restricted manner. A number of pneumococcal proteins that function as virulence factors have been identified and characterized as potential vaccine targets for inclusion in a universal pneumococcal vaccine (22). Several of these virulence factors, including PsaA (42), PpmA (36), and PspA (7), have been shown to be cell-wall-associated proteins expressed by all strains of *S. pneumoniae* analyzed to date. The genes for PsaA, PpmA, and PspA and their corresponding proteins have each been characterized in multiple pneumococcal strains. From these studies, the general observation was made that PsaA and PpmA are highly conserved, whereas PspA is relatively more variable at the DNA and protein sequence levels, among pneumococcal strains. We recently reported that immunization of mice with PsaA was only modestly protective against lethal systemic pneumococcal infection and that this relatively limited vaccine efficacy was correlated with inaccessibility of antibodies to PsaA on the surface of an intact encapsulated *S. pneumoniae* type 3 strain (17).

We undertook the present studies to increase our understanding of the relationship between accessibility to antibodies of potential vaccine targets on a diverse panel of pneumococcal strains and ability to elicit protective antibodies. We describe the accessibility of the cell-wall-associated proteins PsaA, PpmA, and PspA in 12 pneumococcal strains. We also assess the ability of active immunization with recombinant forms of PsaA, PpmA, or PspA, or passive immunization with poly-

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TABLE 1. Strains of *S. pneumoniae* used in this study

Strain	Capsule type	Comments	Source[a]
A-69	1	Clinical isolate	M. Jacobs
A-70	2	Clinical isolate	M. Jacobs
2001-169-0205	3	Sputum isolate	M. Jacobs
A66.1	3	Laboratory strain[b]	D. Briles
60161	4	Ear isolate	M. Jacobs
40206	5	Ear isolate	M. Jacobs
E-68	6B	Ear isolate	M. Jacobs
CP-0105	9V	Ear isolate	M. Jacobs
E-70	14	Ear isolate	M. Jacobs
98-105-0105	18C	Ear isolate	M. Jacobs
CP-0075	19F	Ear isolate	M. Jacobs
E-69	23F	Ear isolate	M. Jacobs

[a] Pneumococcal isolates were obtained from the collections of Michael Jacobs (Case Western Reserve University, Cleveland, Ohio) and David Briles (University of Alabama, Birmingham) as indicated.

[b] Strain A66.1 (capsular type 3) is a derivative of clinical isolate A66 (3). The virulence of this strain has been maintained by laboratory passage in mice for more than 50 years (David Briles, unpublished results).

TABLE 2. Sequences of oligonucleotide primers used for PCR amplification and cloning

Gene	Primer[a]	Sequence (5′-3′)[b]
psaA	21(F)	AATCGTCATATGG**CCATGG**GCgctagcggaaaa aaagatgcagcttc
	308(R)	ATTCCC**CTCGAG**AAGCTTGGATCCtgccaatcc ttcagcaatctt
ppmA	22(F)	AATCGTCATATGG**CCATGG**GCtcgaaagggtcag aaggtgcagac
	313(R)	ATTCCC**CTCGAG**AAGCTTGGATCCttcgtttgat gtactactgcttgagc
pspA	26(F)	AATCGTCATATGG**CCATGG**GCcctacttttgtaag agcagaagaag/tc
	409(R)	ATTCCC**CTCGAG**AAGCTTGGATCCaccgttttct tgtttccagcc

[a] Numbers represent the first amino acid encoded by the forward (F) primers or the last amino acid encoded by the reverse (R) primers, respectively. All numbering is based on pre-pro amino acid sequences for each gene.

[b] Restriction sites in each primer relevant to this study (NcoI for "F" primers and XhoI for "R" primers) are in boldface; nucleotides in lowercase are derived from sequences of each gene deposited in GenBank.

clonal antisera raised against these proteins, to protect mice against lethal systemic pneumococcal infection. The implications of our results for pneumococcal vaccine design based on highly conserved surface proteins are discussed.

MATERIALS AND METHODS

Mice. Six- to eight-week-old BALB/c mice were housed under specific-pathogen-free conditions and given sterile food and water ad libitum. The mice were purchased from Taconic Farms, Germantown, N.Y. The Case Western Reserve University Institutional Animal Care and Use Committee approved all animal experiments.

Bacteria. *Escherichia coli* DH5α (Invitrogen) was used as the host for routine plasmid cloning. Recombinant proteins were expressed in *E. coli* BL21(DE3)/pLysS (Novagen, Inc., Madison, Wis.). *E. coli* were cultured in Luria broth supplemented with antibiotics. Virulent *S. pneumoniae* strain A66.1 (3, 6) was used for challenge experiments and as a source of genomic DNA for PCR amplification experiments. Clinical isolates of *S. pneumoniae*, including serotypes responsible for the majority of pneumococcal infections in the United States (25), were selected from a library of approximately 10,000 independent isolates at the University Hospitals of Cleveland, Cleveland, and are listed in Table 1. *S. pneumoniae* were routinely grown on Trypticase soy agar plates supplemented with 5% sheep blood (blood agar) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco, Detroit, Mich.).

Production of recombinant PsaA, PpmA, and PspA. The production of recombinant PsaA, PpmA, and PspA was achieved by PCR amplification of pneumococcal genes, with subsequent cloning and expression of the genes in *E. coli*. Oligonucleotide primers used in PCR amplification experiments were all purchased from Life Technologies, Bethesda, Md., and are listed in Table 2. Pneumococcal genes used for protein expression were amplified from genomic DNA of *S. pneumoniae* strain A66.1 by using the high-fidelity thermostable DNA polymerase, Platinum *Pfx* (Life Technologies). The coding sequence for nonlipidated, mature PsaA was amplified with the primers PsaA 21(F) and PsaA 308(R); the coding sequence for nonlipidated, mature PpmA was amplified with the primers PpmA 22(F) and PpmA 313(R); and the coding sequence corresponding to the mature N-terminal region of PspA including the first of the choline-binding repeats (32) was amplified by using PspA 26(F) and PspA 409(R). The coding sequences for PsaA, PpmA, and PspA used for protein expression were cloned into plasmid pET29b+ (Novagen) at the NcoI and XhoI sites, with *E. coli* DH5α as the bacterial host. Each recombinant protein is flanked by a plasmid-encoded N-terminal S tag and a C-terminal polyhistidine tag. For recombinant protein expression, each recombinant pET29 plasmid was transcloned into the *E. coli* expression strain BL21(DE3)/pLysS. Recombinant protein expression was initiated by induction with IPTG (isopropyl-β-D-thiogalactopyranoside), and proteins were purified from the soluble fraction of recombinant *E. coli* lysates by using metal affinity chromatography resin and buffers (Novagen), according to the manufacturer's instructions. Protein concentrations were estimated by using the Bradford kit from Bio-Rad (Hercules, Calif.). The recombinant proteins were filter sterilized (Millipore) and stored at 4°C.

Detection of genes encoding pneumococcal cell-wall-associated proteins. PCR amplification was used to demonstrate the presence of genes encoding PsaA, PpmA, and PspA in clinical isolates of *S. pneumoniae*. For this purpose, genomic DNAs were prepared from 11 pneumococcal strains by using a genomic DNA isolation kit (Qiagen) and were used as templates for PCR amplification with *Taq* polymerase (Fisher) with the primers listed in Table 2. Amplification products were electrophoresed through 1% agarose gels and visualized by staining with ethidium bromide (0.5 μg/ml).

Production of hyperimmune mouse sera against pneumococcal antigens. Hyperimmune mouse sera specific for PsaA (anti-PsaA), PpmA (anti-PpmA), or PspA (anti-PspA) were generated by intraperitoneal (i.p.) immunization of mice with each recombinant protein emulsified in incomplete Freund's adjuvant (IFA) (1:1 ratio [vol/vol]). Sera specific for type 3 PS (anti-PS) were generated by inoculating mice i.p. twice at 10-day intervals with type 3 PS (obtained from the American Type Culture Collection) in phosphate-buffered saline (PBS). Pooled sera prepared from blood collected 2 weeks after the final immunization were stored at −20°C until used for assays.

Detection of antibodies to pneumococcal antigens. The levels of antibodies specific for PsaA, PpmA, or PspA in sera from immunized mice were monitored by enzyme-linked immunosorbent assay (ELISA), as previously described (17). Immulon 1 plates (Dynatech, Chantilly, Va.) were coated with recombinant PsaA, PpmA, or PspA (10 μg/ml, 100 μl per well in PBS) overnight at 4°C. Individual sera from immunized mice were tested in duplicate. The binding of antibodies to their cognate antigens was detected by using alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (κ-chain specific; Southern Biotechnologies, Birmingham, Ala.), followed by incubation in *p*-nitrophenyl phosphate (Sigma). Antibody titers were determined as the highest dilution of serum giving a detectable absorbance reading above background. Background in all of the ELISAs was defined as the mean absorbance values for sera obtained from mice immunized with mouse serum albumin (MSA) diluted 1 to 100 in PBS. These background absorbance values were close to zero throughout all of the experiments performed and were arbitrarily assigned a titer of ≤100 for each respective antigen tested by ELISA. Antibody titers specific for type 3 PS were determined in a similar fashion by using Polysorp plates (Nunc, Roskilde, Denmark) coated with type 3 PS (10 μg/ml, 100 μl per well) overnight at 4°C, as previously described (27). Serial dilutions of sera were tested in duplicate. Our observation that MSA-immunized mice exhibited low background absorbances to each of the pneumococcal antigens tested by ELISA provided additional evidence that the cohorts of mice evaluated in these experiments had not previously been exposed to *S. pneumoniae*.

Detection of pneumococcal protein expression by polyacrylamide gel electrophoresis and Western blot analysis. Recombinant PsaA, PpmA, PspA, and whole-cell lysates of *S. pneumoniae* strains [and *Salmonella enterica* serovar Typhimurium or *E. coli* BL21(DE3)pLysS as a negative control] were subjected

to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) for Western blot analysis. Individual blots were reacted with hyperimmune serum specific for either PsaA, PpmA, or PspA. The membranes were subsequently incubated in alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; γ-chain specific; Southern Biotechnologies) and developed by incubation in BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium (NBT) chromogenic phosphatase substrate (Sigma).

Detection of surface expression of pneumococcal proteins. Indirect immunofluorescence was carried out to determine the ability of antibodies raised against recombinant pneumococcal antigens to bind to the surface of intact *S. pneumoniae*, as previously described (17). Cryopreserved bacteria corresponding to 12 pneumococcal isolates were streaked individually onto blood agar plates incubated for ≤12 h at 37°C. Bacteria were harvested from the plates, washed in sterile PBS, and resuspended in staining buffer (PBS with 0.05% sodium azide and 1% bovine serum albumin). Approximately 2×10^7 bacteria were incubated with 10% serum from mice inoculated with MSA as negative controls or specific antigens (PS, PsaA, PpmA, or PspA). After incubation at 4°C, bacteria were washed in staining buffer and incubated with a 1:50 dilution in staining buffer of a $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) conjugated to Alexa 488 fluorescent dye (Molecular Probes, Inc., Eugene, Oreg.). Bacteria were then washed in PBS and subjected to flow cytometry using a Becton Dickinson benchtop flow cytometer. The data were collected and analyzed by using CellQuest software (Becton Dickinson).

PspA typing of clinical isolates. Currently available data indicate that PspAs among pneumococcal strains can be divided into three families. DNA sequence analysis has been used to assign PspAs from different isolates to family 1 (44%) and family 2 (55%) with a minority (~1%) of PspAs being assigned to family 3 (7, 19). PspAs are highly cross-reactive (10), but by analysis with well-chosen or with absorbed sera, it is possible to distinguish PspAs of family 1 and family 2 by their relative reactivities with a pair of antisera made against reference family 1 or family 2 proteins (48). In these studies, antisera relatively specific for family 1 and 2 PspA were used, and the reactivities of pneumococcal lysates with the anti-family 1 and anti-family 2 sera were determined by dot blots, as previously described (48). For dot blot analysis, serial dilutions of pneumococcal lysates were spotted onto each of two nitrocellulose membranes. After blocking of excess binding sites with blocking buffer (PBS containing 1% bovine serum albumin and 0.05% Tween 20), the membranes were incubated in 1:5,000 dilutions of pooled polyclonal rabbit antisera raised against PspA from strains Rx1 and L82016 (corresponding to family 1), or pooled polyclonal rabbit antisera raised against PspA from strains V-024 and V-032 (corresponding to family 2). After washes, the membranes were incubated sequentially with biotinylated goat-anti-rabbit IgG and streptavidin conjugated to alkaline phosphatase. Color was developed by using BCIP-NBT chromogenic phosphatase substrate.

PCR was used to confirm the PspA families by using genomic DNA of strains that reacted equally well with PspA family 1 and family 2 polyclonal rabbit antisera in the dot blot assay described above. Oligonucleotide primers LSM12 and SKH63 were used to detect family 1 PspA coding sequences, and primers LSM12 and SKH52 were used to detect family 2 PspA coding sequences, respectively, as previously described (28, 48).

Pneumococcal challenge of actively immunized mice. BALB/c mice to be used in challenge experiments were primed with 250 pmol of either PsaA or PpmA (~9.3 μg) or 100 pmol of PspA (~5 μg), each in complete Freund's adjuvant (1:1 ratio [vol/vol]) on day zero, and boosted with the same concentration of each respective antigen in IFA (1:1 ratio [vol/vol]) on day 11. The amounts of PsaA and PspA used for immunizations were based on doses used to elicit high titers of specific antibody in previous studies (17, 28), and the amount of PpmA used for immunizations was established in preliminary experiments (data not shown). We used higher doses of PsaA and PpmA, relative to PspA, in order to compensate for the higher immunogenicity of PspA, which became apparent in preliminary studies. BALB/c mice immunized with 0.5 μg of type 3 PS in sterile PBS on days 0 and 11 served as positive controls, and mice injected with 1% MSA in sterile PBS served as negative controls. The amount of PS used was based on previous studies by us demonstrating that this dose resulted in a protective type 3 PS-specific antibody response in BALB/c mice (17, 29). All vaccines were administered i.p. All mice were bled on days 10 and 21 and challenged on day 25. Individual sera from each immunized mouse were tested for the presence of specific antibodies prior to challenge with live pneumococci. Virulent type 3 *S. pneumoniae* (strain A66.1) grown to log phase was prepared for challenge via the i.p. route in actively immunized mice, as previously described (17). For challenge infections, mice were injected i.p. with approximately 500 CFU of virulent *S. pneumoniae* strain A66.1 (type 3) suspended in PBS. The actual number of CFU administered was determined retrospectively by plating serial dilutions of the inocula on blood agar. The survival of mice was monitored for 15 days, at which time the experiments were terminated.

Pneumococcal challenge of passively immunized mice. Two types of passive immunization and challenge experiments were performed. In the first series of experiments, the groups of four to five mice to be challenged were passively immunized with 100 μl of hyperimmune serum specific for PsaA, PpmA, PspA, or type 3 PS (prepared as described above) by i.p. injection. At 24 h after passive immunization, each mouse was challenged intraperitoneally with approximately 1,000 CFU of virulent A66.1 pneumococci suspended in PBS, and survival was monitored for 15 days. In a second series of experiments, groups of mice were inoculated with 1,000 CFU of A66.1 suspended in 100 μl of PBS containing 10% hyperimmune serum specific for PsaA, PpmA, PspA, or type 3 PS in PBS. Survival of mice was monitored for 15 days.

Statistical analysis. The Fisher exact test was used to compare overall survival rates for mice immunized with MSA to those of mice immunized with PsaA, PpmA, PspA, or type 3 PS. The same statistical analyses were performed to evaluate differences in overall survival rates for mice passively immunized with pooled sera from MSA-immunized mice versus mice passively immunized with pooled immune sera specific for PsaA, PpmA, PspA, or type 3 PS. Values were considered statistically significant at a P value of <0.05 (two-tailed).

RESULTS

Presence of selected pneumococcal genes in *S. pneumoniae* isolates. PCR amplification was used to demonstrate the presence of genes encoding the pneumococcal proteins PsaA, PpmA, and PspA in 12 isolates of *S. pneumoniae* (Fig. 1). The three genes demonstrated the range of variability known to exist for nucleotide sequences encoding pneumococcal surface proteins. Bands corresponding to PsaA, PpmA, and PspA were detected in all strains of *S. pneumoniae* analyzed. PCR amplification with primers specific for PsaA and PpmA exhibited single bands of identical size (ca. 900 bp for each gene) in all strains, while PCR amplification with PspA specific primers exhibited bands of different sizes from the different *S. pneumoniae* strains, although 50% of the strains showed a predominant band approximately 1.2 kb in size. These results support the notion that PsaA and PpmA are highly conserved at the DNA level, whereas the PspA locus exhibits the previously reported size variability from strain to strain (19, 48).

Expression and characterization of recombinant pneumococcal proteins. All three recombinant proteins were recovered in the soluble fraction of the *E. coli* expression strains and were purified to near homogeneity by metal affinity chromatography. Recombinant PsaA, PpmA, and PspA were characterized by SDS-PAGE (Fig. 2). PsaA and PpmA migrated in SDS-PAGE gels according to their predicted molecular masses (ca. 37 kDa for each protein). rPspA appeared to be larger than its predicted molecular mass (75 kDa versus an expected mass of 47 kDa). The reason for the lack of concordance between the apparent and actual sizes of PspA is not known but has been previously described for other PspA genes expressed by *S. pneumoniae* (53), as well as a recombinant PspA gene fragment expressed by *S. enterica* serovar Typhimurium (32). Each protein was used to prepare polyclonal mouse antisera by repeated inoculation of mice with each respective antigen emulsified in IFA for use in subsequent immunoassays.

Characterization of protein expression in pneumococcal isolates. Western blots were used to demonstrate the expression of genes encoding PsaA, PpmA, and PspA in lysates of the *S. pneumoniae* strains listed in Table 1. Antisera specific for PsaA or PpmA reacted with a single band of ca. 35 kDa in lysates of all of the strains of *S. pneumoniae* tested (Fig. 3A and B). The

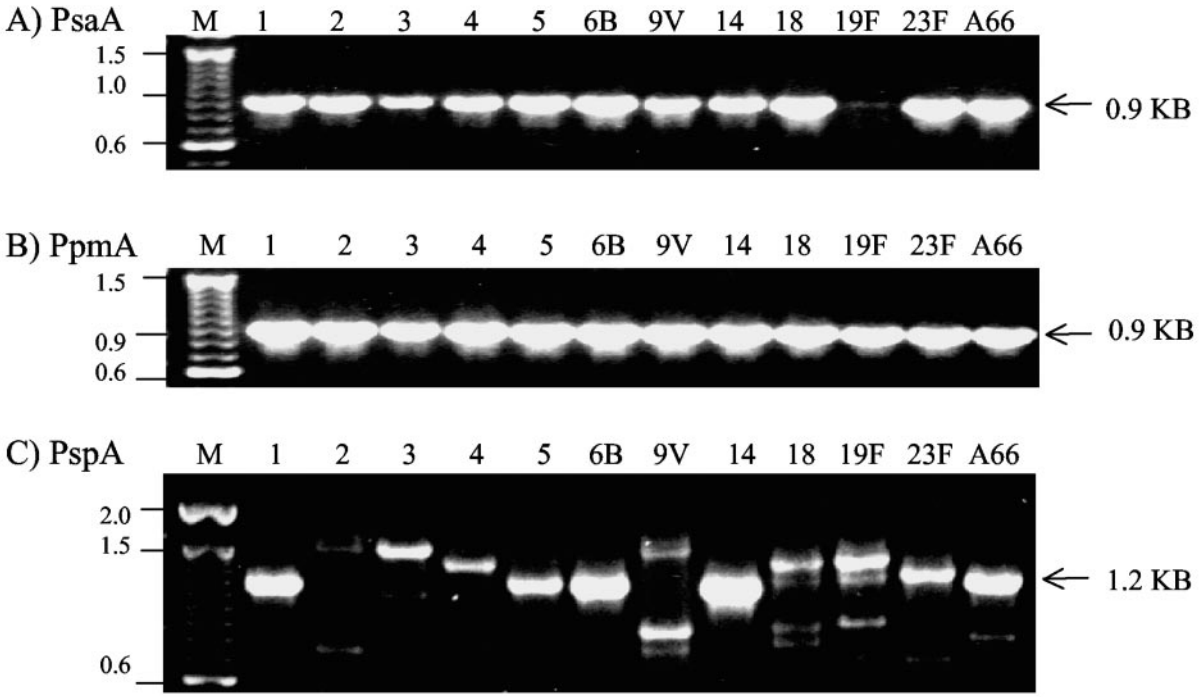

FIG. 1. PCR analysis of *S. pneumoniae* strains. Molecular weight markers are indicated at the left. Serotypes (1 through 23F) of the 11 isolates (Table 1) from which genomic DNA was amplified are indicated; A66 refers to capsular type 3 strain A66.1. All PCRs were performed by using *Taq* polymerase under identical conditions (5 min at 95°C; followed by 30 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 2 min; and finally 10 min at 72°C. M, 100-bp DNA ladder (BRL). Arrows at the right indicate the gene fragments of PsaA, PpmA, or PspA amplified from strain A66.1 by using high-fidelity *Pfx* polymerase (BRL), which were cloned and expressed in *E. coli*. KB, kilobase(s).

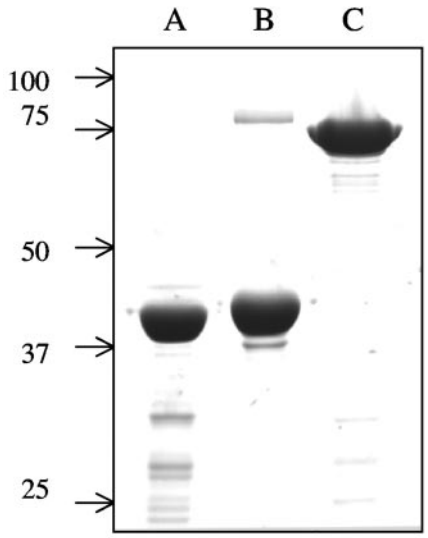

FIG. 2. Recombinant PsaA (A), PpmA (B), and PspA (C) proteins from *S. pneumoniae* strain A66.1 were cloned, expressed, and purified from *E. coli* lysates by metal affinity chromatography. The proteins (4 μg per lane) were subjected to SDS-PAGE and detected by direct staining with Coomassie brilliant blue. Apparent molecular size markers (in kilodaltons) are indicated. These purified recombinant proteins were used to prepare mouse polyclonal antisera specific for PsaA, PpmA, and PspA, respectively, for use in subsequent immunological analyses.

antisera did not react with a lysate of *S. enterica* serovar Typhimurium which was included as a negative control or with a lysate of the untransformed *E. coli* expression strain from which the recombinant proteins were purified (data not shown). It was clear that the two antisera did not cross-react with noncognate molecules since Western blots of the recombinant proteins (PsaA and PpmA) showed no cross-reactivity using the same antisera (data not shown).

The PspA-specific antiserum reacted with several bands in each *S. pneumoniae* lysate (Fig. 3C). The PspA-specific antiserum did not react with a lysate of *S. enterica* serovar Typhimurium or with a lysate of the untransformed *E. coli* expression strain from which the recombinant proteins were purified (data not shown).

Our observation that the PspAs of different strains are of different sizes is consistent with previous results (10, 49). These differences are in large part due to large differences in open reading frames of different PspAs (19). In the present study and in previous studies it has been observed that individual PspAs can yield multiple bands. These additional bands are due in part to the fact that some of the PspA molecules from some strains migrate in the SDS gel as dimers, while the rest migrate as monomers (44). The heterogeneity in the size of PspA from a single strain is also thought to result from limited proteolytic cleavage that inevitably occurs during sample preparation (44). There are also data that, under some circumstances, there can be some cross-reactivity between PspA and PspC, which may result in additional apparent heterogeneity (9). Another anomaly with PspA migration on SDS gels is that

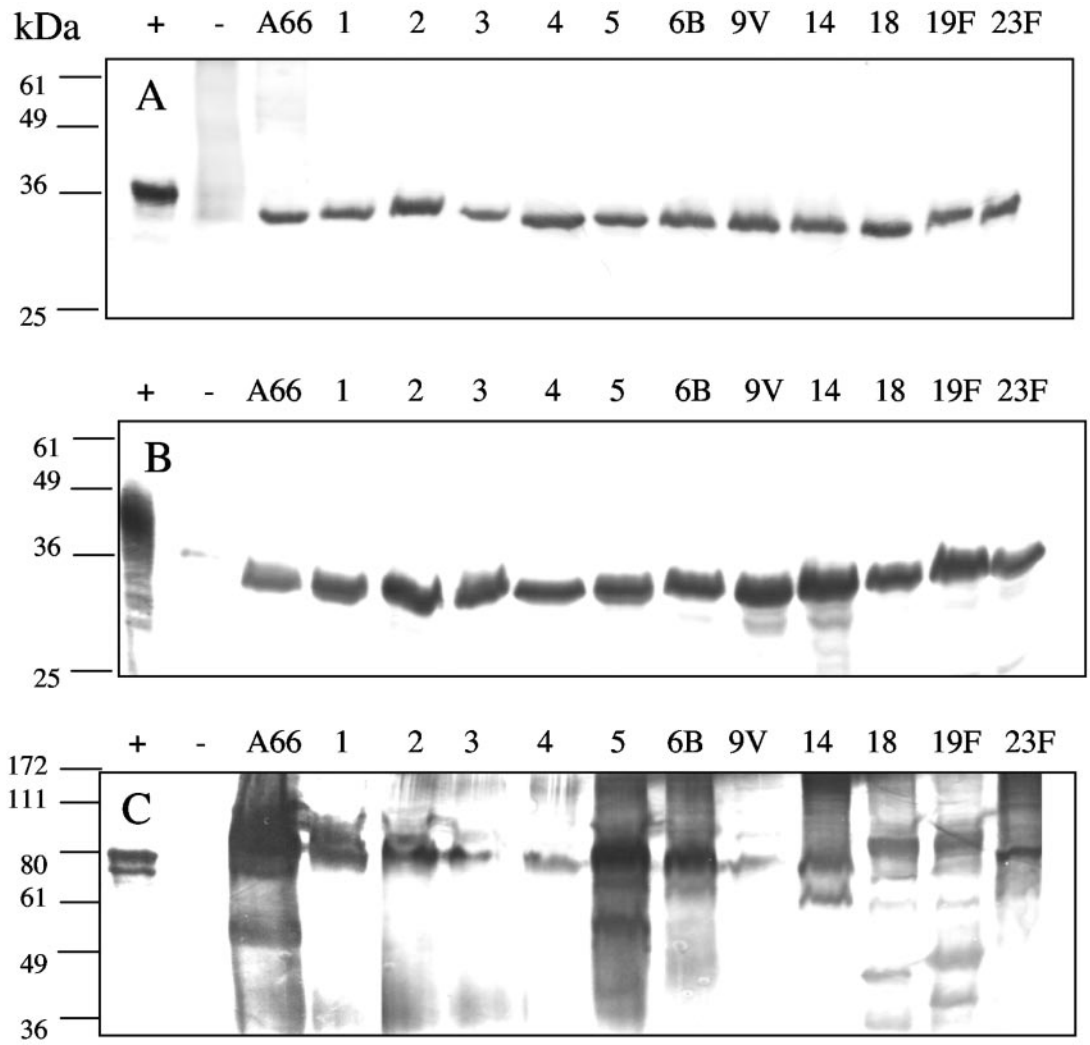

FIG. 3. Western blot analysis of *S. pneumoniae* strains. SDS–10% polacrylamide gels were loaded with rPsaA (A), rPpmA (B), or rPspA (C) (all derived from strain A66.1 coding sequences) as positive controls (+), *S. enterica* serovar Typhimurium lysate as a negative control (A to C, −), and *S. pneumoniae* lysates from strain A66 and the strains described in Table 1 and indicated by their serotypes. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes and incubated with polyclonal anti-PsaA (A), anti-PpmA (B), or anti-PspA (C). Blots were developed with alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain specific) and visualized by incubation in BCIP-NBT chromogenic substrate. Apparent molecular size markers in kilodaltons are indicated.

the PspA monomer apparently retains enough rigidity that it commonly runs somewhat larger than would be predicted by its actual molecular mass (53).

Surface expression of antigens in intact *S. pneumoniae*. We were interested in investigating the ability of sera raised against select pneumococcal surface antigens to bind to the surface of intact *S. pneumoniae*. Initial comparison of the surface binding of anti-PsaA, anti-PpmA, anti-PspA, or anti-PS to *S. pneumoniae* strain A66.1 by flow cytometry confirmed our previous finding (17) that PsaA was not detected on the surface of *S pneumoniae* strain A66.1, while the binding of anti-PS was readily detected on the surface of this strain (Fig. 4). In addition, the binding of anti-PspA to the surface of strain A66.1 was readily detected, whereas anti-PpmA did not exhibit any apparent binding to the surface of strain A66.1 (Fig. 4).

We subsequently used the same surface immunofluorescence assay to demonstrate that neither PsaA nor PpmA are accessible to antibodies on the surface of 11 clinical isolates of *S. pneumoniae* of the indicated serotypes (Fig. 4). In contrast, PspA was readily detectable on the surface of 11 of the 11 clinical isolates of *S. pneumoniae* tested (Fig. 4). The low level of binding of anti-PspA to the surfaces of the types 2 and 3 *S. pneumoniae* strains in the present study could be the result of the known heterogeneity in primary sequences of PspA that can result in a low level of cross-reactivity of some PspAs with an antiserum raised to a single PspA (45, 48) (in this case a family 1 PspA from strain A66.1). This interpretation appears to be supported by our demonstration that the PspA genes in these two strains belong to family 2 (Table 3), which is generally only weakly cross-reactive with antibodies raised against family 1 PspA (48). Taken together, these surface immunofluorescence studies confirm that PspA is highly accessible to antibodies at the surface of the intact pneumococcus (1), in

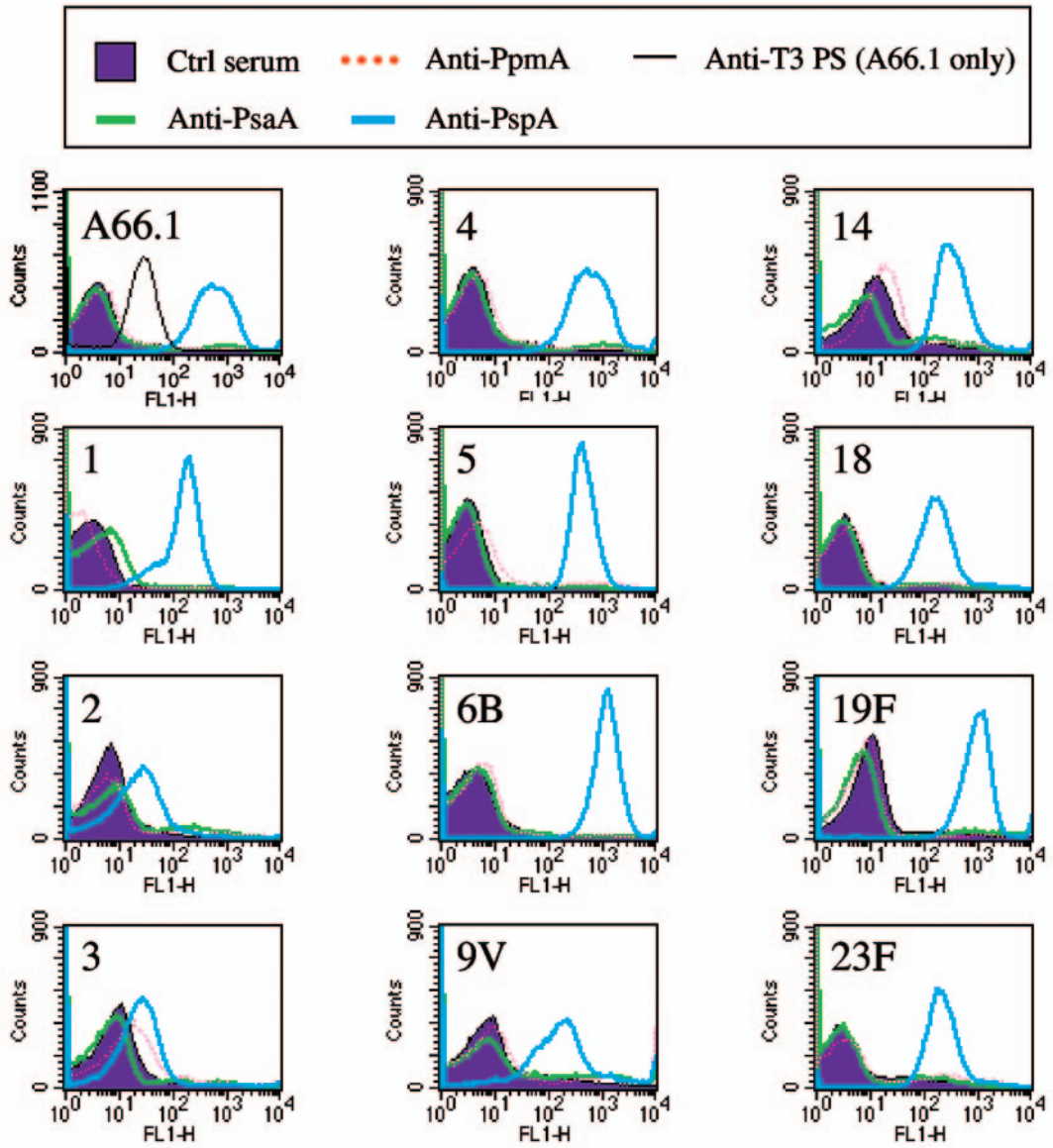

FIG. 4. Flow cytometric analysis of *S. pneumoniae* isolates. Bacteria were incubated with either control serum, anti-type 3 PS (strain A66.1 only), anti-PsaA, anti-PpmA, or anti-PspA, followed by incubation with a F(ab-)$_2$ fragment of goat anti-mouse IgG (H+L) conjugated to Alexa 488 fluorophore. Bacteria were analyzed by flow cytometry using side scatter as the threshold for detection. Specific binding by antisera is indicated as log fluorescence intensity on the *x* axis (FL1-H). Each histogram represents 100,000 events (bacterium-sized particles).

a fashion analogous to capsular PS, whereas PsaA and PpmA are not readily accessible to antibodies under similar experimental conditions.

Protection of BALB/c mice from lethal systemic infection with *S. pneumoniae*. In order to determine whether the accessibility of antigen to antibodies, as assessed by flow cytometry, predicts ability to elicit protective humoral immunity, a series of challenge experiments were performed. In the first series of experiments, mice actively immunized with pneumococcal surface antigens were challenged i.p. with ca. 500 CFU of *S. pneumoniae* strain A66.1 (type 3). Mice immunized with MSA served as negative controls, and mice immunized with type 3 PS served as positive controls. Mice immunized with either PspA or the homologous type 3 PS were significantly protected, whereas mice immunized with either PsaA or PpmA were not effectively protected from systemic challenge with virulent *S. pneumoniae* (Table 4). Sera obtained from immunized mice 3 days before challenge with live pneumococci were individually tested by ELISA for the presence of specific antibody to the respective antigens used for immunization. These data confirmed that each mouse had high titers of antibodies for each of the pneumococcal antigens administered (data not shown). To demonstrate that the observed protection was antibody mediated, groups of naive mice were passively immunized with anti-MSA, anti-PsaA, anti-PpmA, anti-PspA, or anti-PS, either 24 h prior to challenge or at the time of challenge with virulent *S. pneumoniae* strain A66.1 grown to log phase. The results were similar whether the mice received

TABLE 3. PspA typing of *S. pneumoniae* isolates used in this study

Strain[a]	Capsule type	PspA family	
		Dot blot[b]	PCR[c]
A-69	1	1	ND
A-70	2	2	ND
2001-169-0205	3	2	ND
A66.1	3	1	ND
60161	4	1, 2	2
40206	5	1	ND
E-68	6B	1	ND
CP-0105	9V	2	ND
E-70	14	1	ND
98-105-0105	18C	1, 2	2
CP-0075	19F	1, 2	2
E-69	23F	1	ND

[a] From Table 1.
[b] PspA families were assigned to the various pneumococcal isolates on the basis of reactivity with polyclonal rabbit antisera raised against prototypical family 1 and family 2 PspAs.
[c] PCR was used to confirm the PspA family for PspAs from pneumococcal strains that reacted equally well with antisera raised against prototypical family 1 and family 2 PspAs. ND, not determined.

TABLE 5. Protection of BALB/c mice after passive immunization with immune serum to pneumococcal antigens[a]

Serum specificity	Titer[c]	No. alive/ total no.[d]	% Survival	*P* (versus MSA-immunized mice)[d]
Ctrl[b]	≤100	1/10	10	NA
PsaA	218,700	0/12	0	0.45
PpmA	72,900	0/12	0	0.45
PspA	140,929	9/12	75	0.0034*
T3 PS	8,100	10/12	83	0.0009*

[a] Naive BALB/c mice were challenged with 1,000 CFU of live *S. pneumoniae* strain A66.1 (type 3) i.p. after passive immunization with pooled immune serum specific for the indicated pneumococcal antigens.
[b] Control (Ctrl) mice were given *S. pneumoniae* after passive immunization with pooled serum from mice inoculated with MSA.
[c] Antibody titers for each specific antigen in pooled immune sera were measured by ELISA. Absorbance values for MSA-immunized mice were arbitrarily assigned a relative titer for reactivity to each respective antigen of ≤100.
[d] Survival of mice was monitored for 15 days after challenge with *S. pneumoniae*. *, Statistically significant overall survival ($P < 0.05$) compared to the overall survival for control mice, as calculated by using the Fisher exact test. NA, not applicable.

serum at the time of challenge or 24 h prior to challenge and have therefore been combined in Table 5. Only mice that received anti-PspA or anti-PS were significantly protected against homologous challenge with virulent *S. pneumoniae* strain A66.1, whereas mice that received anti-PsaA, anti-PpmA, or pooled sera from MSA-immunized mice were not protected against challenge with *S. pneumoniae* strain A66.1. These passive immunization experiments suggest a direct relationship between antibody accessibility to antigens on the pneumococcal surface and protection against systemic pneumococcal infection.

DISCUSSION

Antibodies to capsular PS represent the de facto "gold standard" for vaccines against *S. pneumoniae* infection. Antibodies against capsular PS are highly protective against invasive pneumococcal disease and, when present at the mucosal surface, appear also to be effective at reducing the carriage of homologous or cross-reactive pneumococcal strains (13). The primary host protective mechanism against systemic pneumococcal infection is generally believed to be opsonophagocytosis, which is facilitated by antibodies to surface antigens (24). Based on these observations, we suggest that among suitable candidates for vaccines against pneumococcal invasive disease should be antibody-accessible antigens capable of supporting opsonization, although it is conceivable that protein antigens could elicit antibodies that protect against the pneumococcus on some other basis (e.g., antagonism of protein function or modulation of the inflammatory response). In this regard, it is worth noting that a strategy for the identification of potentially protective antigens based on antibody accessibility at the pneumococcal surface (such as the strategy used in this report) would not pick up protective pneumococcal antigens such as pneumolysin (which is released from the pneumococcus and is not attached to the pneumococcal surface), where the protection appears to be mediated by neutralization of pneumolysin function by antibodies (34, 35).

TABLE 4. Protection of BALB/c mice after active immunization with pneumococcal antigens[a]

Antigen	Amt[b]	No. alive/ total no.[c]	% Survival	*P* (versus MSA-immunized mice)[c]
Ctrl	NA	1/8	13	NA
PsaA	250 pmol	1/5	20	0.51
PpmA	250 pmol	3/13	23	0.38
PspA	100 pmol	4/5	80	0.031*
T3-PS	0.5 μg	9/9	100	0.0004*

[a] At 2 weeks after the second immunization with the indicated antigens, BALB/c mice were challenged with 500 CFU of live *S. pneumoniae* strain A66.1 (type 3) injected i.p.
[b] Mice were immunized with the indicated antigens on days 0 and 11. T3-PS is type 3 capsular PS. Control (Ctrl) mice were immunized twice with MSA.
[c] Survival of mice was monitored for 15 days after challenge with *S. pneumoniae*. *, Statistically significant overall survival ($P < 0.05$) compared to the overall survival for control mice, as calculated by using the Fisher exact test.

Throughout these experiments, we have been guided by the hypothesis that antigens being considered as non-PS pneumococcal vaccine should, after immunization, be able to elicit levels of protection against pneumococcal infection comparable to those generally observed for PS-based vaccines. As such, we used protection provided by immunization with capsular PS as the standard against which to evaluate the protective efficacy of immunization with alternative (non-PS-based) candidate pneumococcal antigens.

It is reasonable to hypothesize that the polymorphism exhibited by certain pneumococcal surface antigens is attributable to immunological selection (19, 31). This hypothesis predicts that surface antigens that exhibit variability from strain to strain are readily accessible to antibodies on the surface of intact pneumococci (such as PspA and PspC, which have been shown to interfere with complement deposition) (21, 37, 47), while highly conserved antigens are generally not readily accessible to antibodies on the surface of the intact pneumococcus. The results of the present study appear to support this hypothesis, since PspA and capsular PS (two examples of antigens that vary from strain to strain) are readily accessible to antibodies in circulation, whereas two more highly conserved antigens (PsaA and PpmA) are not. If

this notion is fundamentally correct, then the ideal third-generation pneumococcal vaccine capable of stimulating protective immunity to the pneumococcus should consist of mixtures of antibody-accessible protein variants from a single locus (such as PspA) or from different loci.

The flow cytometric assay used to assess the surface accessibility of PspA reaffirmed previous observations that although heterogeneity exists among PspAs expressed by different pneumococcal isolates, antibodies raised to a single PspA can cross-react with different PspAs (7, 8). We were able to demonstrate differences in the amounts of PspA-specific antibody that bound to different isolates. These results provide additional support for the hypothesis that the ideal PspA-based subunit vaccine should contain at least one member of each of the major PspA families in order to ensure the elicitation of protective immunity against 90% or more of pneumococci (30, 39, 40, 48).

We noted that relatively low titers of antibody to capsular PS were capable of eliciting a magnitude of protection equivalent to or slightly better than the protection elicited by much higher titers of antibody to PspA in these experiments. Although we did not perform a detailed evaluation of the minimum quantities of PS- or PspA-specific antibodies required to elicit a protective response in these experiments, the flow cytometric assay demonstrated that a larger amount of PspA-specific antibody (which had a high PspA-specific antibody titer by ELISA) bound to the challenge strain (A66.1) than did type 3 PS-specific antibodies, which had a correspondingly low type 3 PS-specific antibody titer, as measured by ELISA.

These data would appear to suggest that the development of PspA as a pneumococcal vaccine should also include strategies aimed at eliciting high titers of PspA specific antibodies. One such strategy would be the genetic fusion of PspA to cytokines, given that immunization of mice with fusion proteins consisting of PspA conjugated to interleukin-2 or granulocyte-macrophage colony-stimulating factor have been shown to dramatically enhance the immunogenicity of PspA (52). In this context, it is worth emphasizing that the advantages offered by protein vaccine antigens, such as PspA, compared to capsular PS reside not in the specific activity of the corresponding antibodies (which are probably lower) but in the prospect of broader serotype coverage and broader age-related immunogenicity.

It is important to note that, although we have demonstrated that PsaA and PpmA are poor vaccine targets for protection against systemic pneumococcal infection (at least under the present experimental conditions) on the basis of their inaccessibility to antibodies, other studies have demonstrated that mucosal immunization of mice with PsaA is highly protective against pneumococcal carriage (5, 7, 11, 23). The exact mechanisms of protection against pneumococcal carriage afforded by immunization with PsaA have not yet been elucidated. A more recent report appears to confirm the importance of immunity to PsaA as being protective against pneumococcal carriage by demonstrating that antibodies against PsaA inhibit the ability of transparent strains of *S. pneumoniae* to adhere to human nasopharyngeal epithelial cells (41).

Two groups have reported the sequencing of the entire pneumococcal genome (20, 46), and another subsequent study reported the discovery of previously unknown surface antigens based on the presence of consensus surface antigen motifs by using a genomic screening approach (51). The suitability of these new antigens as vaccine targets will depend on (among other factors) their variability across pneumococcal strains, as well as their relative accessibility to antibodies in circulation. In the present study we applied a relatively inexpensive method that can be used to screen vaccine candidate antigens, based on their accessibility to antibodies on the surface of intact *S. pneumoniae* (17). The results of these studies should provide insights regarding selection of candidate vaccine targets suitable for inclusion in a universal pneumococcal vaccine, particularly a vaccine designed to protect against systemic pneumococcal infection.

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