Combinatorial Library Cloning of Human Antibodies to Streptococcus pneumoniae Capsular Polysaccharides: Variable Region Primary Structures and Evidence for Somatic Mutation of Fab Fragments Specific for Capsular Serotypes 6B, 14, and 23F

ALEXANDER H. LUCAS,* KAREN D. MOULTON, VANESSA R. TANG, AND DONALD C. REASON
Children’s Hospital Oakland Research Institute, Oakland, California 94609

Received 7 June 2000/Returned for modification 18 September 2000/Accepted 8 November 2000

Antibodies specific for capsular polysaccharides play a central role in immunity to encapsulated Streptococcus pneumoniae, but little is known about their genetics or the variable (V) region polymorphisms that affect their protective function. To begin to address these issues, we used combinatorial library cloning to isolate pneumococcal polysaccharide (PPS)-specific Fab fragments from two vaccinated adults. We determined complete V region primary structures and performed antigen binding analyses of seven Fab fragments specific for PPS serotype 6B, 14, or 23F. Fabs were of the immunoglobulin G2 or A isotype. Several V_H III gene segments (HV 3-7, 3-15, 3-23, and 3-11) were identified. V_L regions were encoded by several κ genes (KV 4-1, 3-15, 2-24, and 2D-29) and a λ gene (LV 1-51). Deviation of the V_H and V_L regions from their assigned germ line counterparts indicated that they were somatically mutated. Fabs of the same serotype specificity isolated from a single individual differed in affinity, and these differences could be accounted for either by the extent of mutation among clonal relatives or by usage of different V-region genes. Thus, functionally disparate anti-PPS antibodies can arise within individuals both by activation of independent clones and by intraclonal somatic mutation. For one pair of clonally related Fabs, the more extensively mutated V_H was associated with lower affinity for PPS 14, a result suggesting that somatic mutation could lead to diminished protective efficacy. These findings indicate that the PPS repertoire in the adult derives from memory B-cell populations that have class switched and undergone extensive hypermutation.

Streptococcus pneumoniae is a serious human bacterial pathogen causing pneumonia, bacteremia, meningitis, and acute otitis media (7). Encapsulated pneumococci are considered one of the leading causes of death worldwide (4), and in the United States approximately 500,000 cases of invasive pneumococcal disease occur per year, resulting in 40,000 deaths. Ninety or more different pneumococcal capsular polysaccharide (PPS) serotypes have been identified, but only a subset of these are responsible for the majority of invasive disease (7). Immunity to pneumococcal infection is mediated principally by opsonic PPS-specific antibodies (Abs) (17). Accordingly, efforts to develop effective pneumococcal vaccines have focused upon induction of these Ab specificities.

The vaccine presently licensed in the United States consists of a mixture of 23 purified PPS capsular serotypes (4, 51). The young and the elderly are particularly susceptible to developing pneumococcal infection and comprise the principal target groups for vaccination. The polyvalent vaccine is generally immunogenic in healthy young adults and the elderly, although efficacy estimates vary considerably (18). In contrast, the majority of the PPS serotypes are poorly immunogenic in infants, and therefore, the polyvalent pneumococcal vaccine does not provide uniform protection against invasive pneumococcal disease in this age group. The lack of an effective pediatric vaccine and the emergence of antibiotic-resistant pneumococci have prompted the development of new vaccines in which protein carriers are covalently coupled to the PPS (28, 30, 58). This design is based on that used for the development of efficacious pediatric vaccines against Haemophilus influenzae type b (Hib) (24). Unlike plain polyvalent PPS vaccines, the protein-conjugated forms of PPS are immunogenic in infants, and a recent clinical trial of a heptavalent PPS conjugate vaccine in infants has demonstrated high efficacy in preventing invasive diseases caused by pneumococci expressing the capsular serotypes contained in the vaccine (13).

Renewed interest in the serological and functional characterization of anti-PPS Ab responses has accompanied these vaccine development efforts. Although this interest stems primarily from the need to evaluate vaccine immunogenicity and to establish reliable surrogates of protection, the Ab response to PPS antigens (Ags) represents an ideal opportunity to examine the inheritance and development of protective immunity in humans. Ab responses to PPS Ags are markedly oligoclonal within individuals (31, 34, 46), and consequently variable (V) region diversity is limited. This property leads to individual variation in PPS-specific Ab fine specificity (41), avidity, and protective efficacy (52, 66). While V region polymorphism undoubtedly affects antibody protective function, little is known about the V regions encoding PPS antibodies or the structural determinants of PPS binding.

In this study we describe our initial efforts aimed at the
molecular definition of the human Ab repertoire to PPS Ags. We used combinatorial library cloning to isolate Fab fragments specific for PPS serotypes 6B, 14, and 23F. We focused on these particular serotypes because they are structurally disparate, they are components of both licensed and experimental conjugate vaccines, and the respective pneumococci are significant pathogens.

MATERIALS AND METHODS

Human subjects and vaccination. Two healthy adults, a 45-year-old Caucasian female (002) and a 24-year-old African-American male (018), received an intramuscular injection of 0.5 ml of Pneumovax vaccine (Merck & Co., Inc., West Point, Pa.). Peripheral blood samples were taken before, 7 days after, and 30 days after vaccination. The protocols were reviewed and approved by the Children's Hospital Oakland Research Committee and Institutional Review Board.

Preparation of PPS paramagnetic beads and enrichment of PPS-binding B cells. Lymphoplated PPS 6B, 14, and 23F were purchased from the American Type Culture Collection, Rockville, Md. Ten milligrams of PPS was dissolved in 1.0 ml of 0.2 M sodium bicarbonate (pH 10). Cyanogen bromide (2.5 mg dissolved in 50 µl of dimethylformamide) was added, and the mixture was stirred on ice for 10 min. Another 2.5 mg of cyanogen bromide was added, and the reaction proceeded for an additional 10 min. Biotin hydrazide (Pierce Chemical Co., St. Louis, Mo.) was added in dimethyl sulfoxide, and 14.3 mg was added to the solution of activated PPS, giving a final biotin hydrazide concentration of 5 mM. The solution was stirred at room temperature for 2 h. after which it was dialyzed extensively at 4°C against phosphate-buffered saline (PBS). The PPS-biotin was sterilized by filtration and stored at −80°C.

PPS-coated paramagnetic beads were prepared by adding 50 µg of PPS–biotin to 1.0 mg of washed avidin paramagnetic beads (Immunotech Inc., Marseille, France) in a total volume of 0.5 ml of PBS–0.2% bovine serum albumin (BSA). Beads were mixed, incubated at room temperature for 15 min, isolated with a magnet and washed several times with PBS–BSA.

Ficoll-Hypaque was used to isolate mononuclear cells (MNC) from the hemoperipheral blood sample obtained 7 days after vaccination. After a wash with RPMI 1640 medium, MNC were suspended to a concentration of 10⁷/ml in PBS containing 30% fetal calf serum (FCS) and 20 µg of pneumococcal common wall polysaccharide (PPS; Danish Statens Seruminstitut, Copenhagen, Denmark). To enrich for PPS-specific B cells, 1.0 mg of PPS-coated paramagnetic beads was added to 2 × 10⁷ MNC, and the mixture was incubated for 15 min on ice. Cells binding to beads were isolated by magnetic separation. The beads were washed two times with PBS–BSA, and RNA was extracted from the beads using RNA-STAT-60 (Tel-Test “B,” Inc., Friendswood, Tex.). RNA was stored at −80°C as an ethanol precipitate. cDNA was prepared from RNA using oligo(dT) as a primer. Reverse transcriptase, nucleotides, and buffers were purchased from Pharmacia, Inc. (Piscataway, N.J.) and were used according to instructions provided by the manufacturer.

Preparation of L chain and Fd chain libraries. The general procedures used for construction of Fab libraries have been described elsewhere (9, 30). Fd and light (L) chain cDNAs were amplified by PCR using appropriate primers. The L chain primers were those described previously (50) plus VK9a (gaatgagtcagctagctgc [all sequences are 5'-3']) VK6b (gatggtagtcacaaacatgatgct), VLAM1 (aa tttagctgacactacececc), VLAM2 (tctgagctgacactaceccgt), VLAM3 (ttctagcgctgacactacececc), VLAM4 (cttcgagctgacactacececcgct), VLAM5 (ctctcagctgacactacececegct), VLAM6 (caagctgagctgacactacececcgct), VL185 (catgctgagctgacactacececcgct), VL7 (tctcagctgacactacececcgct), VL7V (catgctgagctgacactacecece), VL7 (cagctgagctgacactacecece), VL21.1 (cagctgagctgacactacecece), and lambdacon (gacaagctgacactacecececcgtgctg). The Fd primers were those described previously (50) plus VH4.21 (5'-cagctgagctgacactacecegctg-3'). L chain and Fd chain PCR products were separately inserted into the pComb3H vector (kindly provided by Carlos Barbosa and The Script’s Research Institute [39], using the appropriate restriction sites. Phagemid DNA was electroporated into XLI-Blue cells, and transformants were selected. Combinatorial Fab libraries were prepared by inserting bulk Fd chain DNA into bulk phagemid DNA containing L chains. Purified clonal phagemid DNA was sequenced directly with Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) as previously described (30). 

Identification of PPS-specific Fabs. Fab libraries were screened for the presence of PPS-specific Fab by adding to either autoradiography of blots of clones from agar plates or radioantigen binding assay (RABA) of individual randomly picked clonal isolates. For screening by the blotting method, Fab library phage-mid clones were plated on LB-carbenicillin plates and incubated overnight at 37°C. Nitrocellulose circles were coated overnight with either anti-human kappa or anti-human lambda Ab (Biosource International, Camarillo, Calif.) at a concentration of 5 µg/ml in PBS. The nitrocellulose was blocked with PBS–1% BSA and washed five times with PBS–0.1% Tween 20. The nitrocellulose was placed directly onto the surface of the agar plate having bacterial colonies, followed by two washes with Whatman filter paper. After incubation for 1 to 2 h at 37°C, the nitrocellulose blots were lifted from the plates, washed several times with PBS-Tween, and then incubated with rotation for 1 h at room temperature with 125I-PPS (5 × 10⁶ cpm/ml) diluted in PBS–1% BSA containing 10 µg of CPS/ml. The blots were washed with PBS-Tween, air dried, and exposed to X-ray film. Following blotting, the agar plates were incubated for 4 to 8 h at 37°C to regenerate the colonies and then stored at 4°C. PPS-binding bacterial clones identified by autoradiography were picked from the agar plate and isolated by streaking onto agar plates, followed by liquid culture.

Random screening of clones was performed on supernatants of clonal bacterial lysates obtained by repetitive freezing and thawing of bacteria harvested from overnight cultures. The presence of PPS binding Fab in the supernatant was determined by RABA.

Fab purification. To facilitate purification, the carboxy-terminal region of each Fab Cγ1 domain was engineered to contain a polyhistidine region. A primer (5'-cctctgagctgatagctgatgtcgctgtgacactacecece-3') spanning the Spel site at the Cγ1-I/II junction and encoding six histidine residues was used in PCR with an upstream Vγ3 primer to generate the appropriate Fd fragment. Subsequent enzymatic digestion and ligation generated a Fab with an internal polyhistidine tag. SpeI/Nhel digestion, gel purification, and recombination of the resulting phagemid produced a Fab lacking the gH component of pComb3H and having a six-histidine tag at the carboxy terminus of the Fd chain. Enzymatic digestion with AoeI and NorI produces a cassette containing the polyhistidine region that can be subsequently used to modify other Fabs to contain the polyhistidine region. Correct insertion and lack of PCR errors was determined by sequencing the Cγ1 region, and binding studies verified that the introduced modification did not affect antigen binding.

 Cultures of Fabs were prepared by being seeded with 500 ml of LB broth with an overnight suspension culture of bacteria carrying the appropriate phagemid. The culture was shaken at 300 rpm at 37°C for ~8 h followed by overnight induction with 1 mM isopropyl β-D-thiogalactoside. Bacteria were then harvested by centrifugation. The bacterial pellet was resuspended in PBS containing protease inhibitors and was subjected to three cycles of freezing and thawing. Debris was removed by centrifugation, and the supernatant was used for immobilized metal affinity chromatography. Ni-nitriotriacetic acid-agarose (Pierce Chemical Co., St. Louis, Mo.) chromatography was performed as recommended by the manufacturer. Fabs were eluted from the absorbent with 0.2 M imidazole (pH 8.0) and were dialyzed extensively against PBS. Following dialysis, Fabs were spun at 100,000 × g for 1 h, sterilized by filtration, and stored at 4°C. Fab concentration was determined either by absorbance at 280 nm using extinction coefficients calculated from amino acid composition or by a previously described enzyme-linked immunosorbent assay (ELISA) where Fabs were captured on wells coated with anti-Fd Ab and detected using alkaline phosphatase-conjugated anti-L chain Ab (36).

RABA and PPS ELISA. The preparation of tyraminated and iodinated PPS and the RABA have been described in a previous report (34). RABA was used to determine anti-PPS Ab concentrations in pre- and postvaccination sera and to evaluate Fab binding specificity. Serum anti-PPS levels were calculated from a standard curve generated by the reference serum 89-SF as previously described (34). Prior to assay, Fab fragments were spun for 1 h at 100,000 × g. Fab fragments or sera, diluted in PBS containing 10% FCS and 10 µg of CPS/ml, were mixed with ~300,000 cpm of 125I-PPS in a total volume of 100 µl. After incubation overnight at 4°C, 100 µl of 100% saturated ammonium sulfate was added for 4 h at 4°C. Precipitates were harvested by centrifugation and washed with 200 µl of 50% saturated ammonium sulfate, and bound radioactivity was determined by counting in a gamma counter.

The ELISA was used to determine the isotypes of anti-PPS Abs in sera and in supernatants from MNC cultures. The ELISA and calibration standards are described in reference 34. Determination of Fab affinity. The affinity of Fab binding to PPS was determined by RABA. 125I-PPS binding was evaluated at various Fab concentrations, and the concentration of Fab binding 50% of added 125I-PPS was calculated. Affinity is expressed as the inverse of the Fab molar concentration binding 50% of added 125I-PPS.

GenBank accession numbers. GenBank accession numbers for Vγ cDNA sequences of Fab 6B-1, 14-1, 14-2, 14-3, 14-4, 23F-1, and 23F-2 are AF165100, AF165107, AF165109, AF165105, AF165112, AF165104, and AF165102, respectively. GenBank accession numbers for Vγ cDNA sequences of Fab 6B-1, 14-1,
H, kappa, and lambda V gene designations are according to references 45, 10, and 44, respectively.

as previously described (34) and are shown in parentheses.

...and are shown in Fig. 1, the Fabs gave specific and concentration-dependent binding to their PPS Ags. Different affinities were observed for Fabs of the same serotype specificity (Table 2). For example Fabs 14-1 and 14-2, both isolated from subject 002, differed 13.2-fold in their affinity for PPS 14.

Cloning and isolation of PPS-specific Fab fragments. To isolate PPS-specific Fab fragments, we prepared (Fd × L) chain combinatorial libraries using RNA obtained from 7-day-postvaccination MNC populations from the two subjects described in Table 1. Prior to library construction, we enriched for specific B cells using PPS-coated paramagnetic beads. Three separate (Fd × L) combinatorial libraries were prepared from subject 002: one each for PPS 6B (Fd × κ), PPS 14 (Fd × κ, λ), and PPS 23F (Fd × κ, λ). One (Fd × κ, λ) library was prepared from subject 018 using B cells enriched for PPS 14-binding cells.

Bacterial colonies carrying the relevant phagemids were screened for expression of PPS-binding Fab fragments as described in Materials and Methods. PPS-binding Fab clones were present in a frequency of approximately 1% in these libraries. For subject 002, five distinct Fabs were isolated: one specific for PPS 6B, two specific for PPS 14, and two specific for PPS 23F (Table 2). For subject 018, two distinct PPS 14-specific Fabs were isolated. Six of the Fabs were of the IgG2 isotype, and one was IgA. The L chain isotypes of the Fab fragments paralleled that observed by analysis of both culture-derived and serum Abs.

Fab specificity and affinity were analyzed using RABA. As shown in Fig. 1, the Fabs gave specific and concentration-dependent binding to their PPS Ags. Different affinities were observed for Fabs of the same serotype specificity (Table 2). For example Fabs 14-1 and 14-2, both isolated from subject 002, differed 13.2-fold in their affinity for PPS 14. They used

### RESULTS

Response to vaccination. Two adult subjects were vaccinated with polyvalent pneumococcal vaccine. Blood samples were obtained before, 7 days after, and 30 days after vaccination. Prevaccination and 30-day postvaccination serum samples were analyzed for total anti-PPS Ab, and PPS-specific κ/λ ratios were determined on the 30-day-postvaccination samples (Table 1). Also, MNC isolated from the 7-day-postvaccination blood sample were cultured for 7 days, and the anti-PPS Abs present in the culture supernatants were analyzed for heavy (H) and L chain isotypes (Table 1).

Both subjects had detectable serum PPS-specific Ab prior to vaccination, and both responded to vaccination with increased Ab levels to the PPS. In subject 002, vaccination elicited predominantly κ Abs to PPS 23F, approximately equal representation of κ and λ Abs to PPS 6B, and a λ-dominant response to PPS 14. κ Abs dominated the response of subject 018 to PPS 14. PPS-specific Abs were detectable in MNC culture supernatants, a result indicating that specific B cells were present in the peripheral circulation. The Abs secreted from MNC had L chain isotypes of the Fab Abs secreted from the MNC.

### TABLE 1. Serological characterization of subjects’ responses to pneumococcal vaccination

<table>
<thead>
<tr>
<th>Subject</th>
<th>In vitro H/L chain isotype(s)</th>
<th>Serum Ab&lt;sup&gt;c&lt;/sup&gt; concn (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6B</td>
<td>14</td>
</tr>
<tr>
<td>002</td>
<td>G/κ</td>
<td>G/κ</td>
</tr>
<tr>
<td>018</td>
<td>G/κ</td>
<td>G/κ</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two adult subjects were vaccinated with polyvalent pneumococcal vaccine. Blood was taken before and then 7 and 30 days after vaccination.

<sup>b</sup> MNC were isolated from heparinized blood obtained 7 days following vaccination and were cultured (10<sup>6</sup>/ml) in RPMI 1640 medium containing 10% FCS at 37°C in 5% CO<sub>2</sub>. Supernatants were harvested after 7 days and assayed for the presence of PPS-specific Ab by ELISA using isotype-specific secondary Abs (34).

<sup>c</sup> Serum samples obtained before (Pre) and 30 days after (Post) vaccination were assayed for total anti-PPS Abs using RABA. κ/λ ratios were calculated by ELISA as previously described (34) and are shown in parentheses.

### TABLE 2. Summary of PPS-specific Fabs

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fab</th>
<th>PPS specificity</th>
<th>C&lt;sub&gt;ε&lt;/sub&gt; C&lt;sub&gt;L&lt;/sub&gt;</th>
<th>Affinity&lt;sup&gt;b&lt;/sup&gt; (M&lt;sup&gt;−1&lt;/sup&gt;, 10&lt;sup&gt;9&lt;/sup&gt;)</th>
<th>% Identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CDR-3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>V Nucleotide</th>
<th>Amino acid</th>
<th>J V Nucleotide</th>
<th>Amino acid</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>002</td>
<td>6B-1</td>
<td>6B</td>
<td>α&lt;sub&gt;1&lt;/sub&gt;-κ</td>
<td>0.5</td>
<td>89</td>
<td>29</td>
<td>KV 4-1</td>
<td>95</td>
<td>98</td>
<td>8</td>
<td>κ2</td>
</tr>
<tr>
<td>14-1</td>
<td>14</td>
<td>14</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-λ</td>
<td>33</td>
<td>94</td>
<td>29</td>
<td>LV 1-51</td>
<td>97</td>
<td>97</td>
<td>11</td>
<td>λ2/3</td>
</tr>
<tr>
<td>14-2</td>
<td>14</td>
<td>14</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-λ</td>
<td>2.5</td>
<td>92</td>
<td>29</td>
<td>LV 1-51</td>
<td>97</td>
<td>97</td>
<td>11</td>
<td>λ2/3</td>
</tr>
<tr>
<td>018</td>
<td>14-3</td>
<td>14</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-λ</td>
<td>1.3</td>
<td>93</td>
<td>29</td>
<td>KV 3-15</td>
<td>93</td>
<td>84</td>
<td>9</td>
<td>κ4</td>
</tr>
<tr>
<td>14-4</td>
<td>14</td>
<td>14</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-κ</td>
<td>1.3</td>
<td>93</td>
<td>29</td>
<td>KV 3-15</td>
<td>94</td>
<td>84</td>
<td>9</td>
<td>κ4</td>
</tr>
<tr>
<td>002</td>
<td>23F-1</td>
<td>23F</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-κ</td>
<td>17</td>
<td>95</td>
<td>29</td>
<td>KV 2-24</td>
<td>96</td>
<td>94</td>
<td>9</td>
<td>κ2</td>
</tr>
<tr>
<td>23F-2</td>
<td>23F</td>
<td>23F</td>
<td>γ&lt;sub&gt;2&lt;/sub&gt;-κ</td>
<td>3.3</td>
<td>95</td>
<td>29</td>
<td>KV 2D-29</td>
<td>98</td>
<td>94</td>
<td>8</td>
<td>κ2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fab fragments were isolated from (Fd × L) combinatorial libraries prepared from peripheral blood MNC of subjects who were vaccinated 7 days previously with polyvalent pneumococcal vaccine.

<sup>b</sup> Inverse of the Fab concentration required to bind 50% of added 125<sup>I</sup>-PPS in the RABA.

<sup>c</sup> Percent nucleotide and amino acid sequence identity of the Fab V gene sequence to the assigned germ line gene and its formal translation product, respectively.

H, kappa, and lambda V gene designations are according to references 45, 10, and 44, respectively.

<sup>d</sup> Length in amino acid residues.
similar V regions and appeared to be intraclonal variants (see below). Fabs 23F-1 and 23F-2, also isolated from subject 002, differed fivefold in their affinity for PPS 23F. These two Fabs, however, appeared to be derived from two distinct clones, as they utilized different V<sub>H</sub> and V<sub>L</sub> regions (see below).

**V region sequences.** Complete V<sub>H</sub> (Fig. 2) and V<sub>J</sub> (Fig. 3) cDNA sequences were determined for all Fabs. The likely germ line V gene segments encoding these V regions were identified by a search of the databases, and assignments were made based on the germ line gene segment having the closest sequence identity to the PPS-specific Fab V sequence. Four distinct V<sub>H</sub> gene segments were used by these Fabs (Fig. 2 and Table 2). Fab 6B.1 utilized the HV 3-7 gene. HV 3-23 (also commonly known as V<sub>H</sub>26 and DP-47) was used by the PPS 14-specific Fabs 14-3 and 14-4 and also by the PPS 23F-specific Fab 23F-1. An amino acid insertion (aspartate) was present in the V<sub>H</sub> complementarity determining region 2 (CDR-2) of Fab 23F-1. In keeping with previously observed insertion patterns (23, 43, 70), the aspartate codon (GAT) appeared to be related to the immediately adjacent sequence (GGT). Similar insertions in the CDR-2 of the HV 3-23 gene have been described and are thought to arise during the course of somatic hypermutation (23, 43). Fabs 14-1 and 14-2 used the V<sub>H</sub> 3-15 gene or its close relative, known as LSG6.1 (2). Fab 23F-2 used the VH 3-11 gene. All of these gene segments are of the V<sub>H</sub>III family. The 3-15 gene is of the IIIb subfamily and belongs to the 1-U canonical class, whereas the 3-11, 3-23, and 3-7 genes are of the IIIa subfamily and belong to the 1-3 canonical structure class (20, 65). The increased length of V<sub>H</sub> CDR-2 in Fab 23F-1 resulting from the inserted residue is likely to alter the loop configuration and thereby change the canonical structure class (23).

The V<sub>H</sub> segments of the Fabs appeared to be mutated, as their identity to the candidate germ line nucleotide sequence ranged from 82 to 95%. As shown in Fig. 2 and summarized in Table 2, many of the nucleotide differences between the Fab V<sub>H</sub> sequence and the respective germ line gene resulted in amino acid replacements. In the absence of germ line gene sequences from the donors, the attribution of these polymorphisms to somatic hypermutation cannot be made with certainty. However, the human V<sub>H</sub> locus has been studied extensively, and the sequence of the entire V<sub>H</sub> locus was published recently (39). While some of the differences between the Fab V<sub>H</sub> sequence and the assigned germ line gene could be due to PCR errors or to individual genomic polymorphisms, we think it is likely that most of the observed differences resulted from hypermutation of germ line gene segments that are either identical or closely related to the assigned germ line gene.

V<sub>J</sub> CDR-3 regions of the PPS-specific Fab fragments are shown in Fig. 4. Candidate germ line D segments were identified for the PPS 14-specific Fabs. Lengths of the D regions varied, as did the overall length of CDR-3 (6 to 11 amino acid residues). Six Fabs used J<sub>D</sub>4b and one used J<sub>D</sub>5a. CDR-3 length was not strictly conserved between Fabs of the same serotype specificity. Fabs 14-1 and 14-2 had a CDR-3 of 10 residues, whereas Fabs 14-3 and 14-4 had a CDR-3 of 11 residues. PPS 14-specific Fabs tended to have longer CDR-3s (10 or 11 residues) compared to PPS 23F-specific Fabs (6 or 7 residues). No identical CDR-3 motifs recur in unrelated Fabs of the same serotype specificity, although the two pairs of PPS 14-specific Fabs, which used different V gene segments and D regions, had either Ser-Gly-Ser-Ser-Tyr or Thr-Gly-Thr-Thr-Phe in V<sub>H</sub> CDR-3.

V<sub>L</sub> cDNA sequences and formal translation products of the PPS-specific Fabs are shown in Fig. 3 and summarized in Table 2. The V<sub>L</sub> gene known as LV 1-51 was used by PPS 14.1 and 14.2 in combination with the λ2 or λ3 J region. The remainder of the Fabs used genes derived from either the κII, κIII, or κIV subgroup in association with either J<sub>κ</sub>α2 or J<sub>κ</sub>α3. The PPS 23F-specific Fabs used two different V<sub>κ</sub> genes, 2-24 and 2D-29. Both are from the κII subgroup and are of the 4-1-1 canonical structure class (64). With the exception of the 2D-29 gene, which is located on the portion of the κ locus distal to the telomere, the V<sub>κ</sub> genes used by the Fabs derived from the proximal cluster. The KV 4-1 gene used by the PPS 6B-specific Fab, the most proximal gene segment, is located immediately adjacent to the J regions but in opposite transcriptional orientation (32). The 2D-29 gene (also known as A2 and DPK-12) used by Fab 23F.2 is the most common V<sub>κ</sub> gene used in the human Ab response to the Hib polysaccharide (PS) (35, 55).
Similar to the V_{H} genes, V_{L} regions appeared to be somatically mutated. The sequence identity of the L chain V gene segments to their assigned germ line counterparts ranged from 93 to 98% (Table 2 and Fig. 3). L chain CDR-3 lengths were 8, 9, and 11 residues. PPS Fabs 14-3 and 14-4 have a 9-amino-acid CDR-3 resulting from the direct joining of the KV 3-15 gene to J_{k} 4, and Fabs 14-1 and 14-2 have a CDR-3 11 amino acids in length resulting from direct joining of the LV 1-51 gene segment to J_{l} 2/3 (Fig. 5). Fabs 6B-1 and 23F-2 have a CDR-3 of eight amino acids that resulted from the deletion of the proline codon at position 95. The PPS 23F-specific Fabs had CDR-3s either eight or nine residues in length. The truncation of the 2D-29 V gene of Fab 23F-2 is of note, as this gene segment is used by Abs to Hib PS, where the CDR-3 is 10 amino acids in length and contains an insertional arginine at position 95a, the V-J joint (35, 55). All the Fab L chain CDR-3s have amino acid substitutions compared to the germ line assignments. Some of these substitutions could result from mutation, while others, such as the truncations of Pro at position 95, may occur during V-J joining.
The sequence data indicate that some of the Fabs isolated from the same subject were likely clonally related. For example, Fab 14-1 and 14-2 used the same V_L region, but their V_H regions, although derived from the same rearrangement, differed at 13 amino acid residues (4 in CDR-1 and CDR-2 and 9 in the framework regions). These sequence differences, which presumably arose as a consequence of differential somatic mutation between daughter clones, conferred a 13-fold...
The more extensively mutated Fab, 14-2, had lower affinity for PPS 14 than its clonal relative 14-1. The PPS 14-specific Fabs isolated from subject 018 also appeared to be clonal variants, but the primary sequence differences between these Fabs (2 in $V_{\text{H}}$ and 15 in $V_{\text{L}}$ [Fig. 2 and 4]) did not confer a measurable difference in affinity (Table 2 and Fig. 1).

Independently derived clones present in an individual also
can have disparate functions, as shown by Fabs 23F-1 and 23F-2. These Fabs used different \( V_H \) and \( V_L \) genes, and differed fivefold their affinity for PPS 23F. Thus, affinity differences can arise in PPS Ab responses by interclonal and intraclonal variation.

**DISCUSSION**

In this study, we used combinatorial library cloning to isolate PPS-specific Fab fragments from two vaccinated adults. This approach has permitted us to identify \( V \) genes contributing to the repertoire, to assess their mutation, and to analyze the relationship between \( V \) region polymorphism and PPS binding affinity.

Before discussing the implications of these findings, it is important to consider the cloning methodology as a potential caveat to repertoire analysis. Unlike hybridomas which retain the chain combinations present in the native B cell, Fabs from combinatorial libraries derive from the recombination of bulk \( F_d \) and \( L \) chains, a process that scrambles the H-L pairs present in vivo. PPS-binding Fab fragments may therefore not represent physiological pairing configurations. Although this possibility cannot be discounted with certainty, several observations point to the likelihood that native \( V_H-V_L \) configurations are being reassembled. First, only one or two distinct Fab fragments of any single serotype specificity were isolated from an individual donor, and in some cases these appeared to be intraclonal variants. This result is consistent with previous studies showing that Ab responses to PPS Ags are markedly oligoclonal and can be dominated by the products of a single clone (31, 34, 46). Second, the MNC populations used for library construction were enriched for specific B cells using PPS-coated paramagnetic beads and were obtained 7 days after vaccination, a time when there is an increased frequency of PPS-specific B cells in the peripheral circulation (38). This increased representation of PPS-specific B cells and the relevant mRNA would increase the probability of reassembling native pairs with the appropriate PPS binding specificity. Although the use of PPS-coated beads could potentially result in affinity biases in the resulting libraries, the isolation from the same library of Fabs having greater than 10-fold differences in binding affinity for PPS14 suggests that this does not represent a significant limitation. Third, Fabs of differing serotype specificity were isolated from a single donor, and they used distinctive gene rearrangements, CDR-3 configurations, and chain pairing combinations. These features are not consistent with the promiscuous assembly of irrelevant chain combinations. Fourth, the H and L chain isotypes of the Fab fragments obtained from the combinatorial libraries resembled the isotypes expressed by serum- and MNC culture-derived PPS Abs of the donor. Fifth, previous studies of the human Ab repertoire to Hib PS have shown that combinatorial library-derived Fab fragments recapitulate \( V \) gene configurations and chain pairing of native antibodies (11, 26, 50). Thus, this collective evidence indicates that the \( V \)-specific Fabs studied here represent bona fide products of Ag-driven Ab responses.

We determined the complete primary structures of seven Fab fragments representing three PPS serotype specificities. A common pattern to emerge from this sequence analysis was the preferential usage of \( V_{HIII} \) gene segments, a result in agreement with previous studies (1, 12, 19, 34, 57, 62, 71). Biased usage of \( V_{HIII} \) genes appears to be a feature common not only to PPS Abs but to other human anti-PS Ab specificities, including Hib PS (3, 56, 59), *Cryptococcus neoformans* (49), and \( \alpha \)-galactosyl (68). The mechanism underlying the preferential usage of \( V_{HIII} \) gene segments is not understood. Structural constraints in formation of the combining site (29) and induction by \( V_{HIII} \)-specific superantigens such as staphylococcal protein A (33, 60) have been proposed as explanations. However, the demonstration of preferential expression of \( V_{HIII} \) genes in both productive and nonproductive rearrangements in peripheral blood B cells (16) suggests that this phenomenon does not involve selection at the level of the combining site but is determined by intrinsic molecular properties of \( V_{HIII} \) genes (47).

The data presented here show that the same \( V_H \) gene segment can be used by Fabs of different PPS specificity. The Fab pair 14-3-14-4 and Fab 23F-1 used HV 3-23, but their CDR-3s were distinctive, and they paired with different L chains. This promiscuity of HV 3-23 is perhaps not too surprising in light of the fact that this gene segment is commonly expressed in the peripheral repertoire (61), is used by a plethora of Abs having reactivities with either self or foreign Ags (47), and is a prominent member of the Hib PS repertoire, where it pairs with the KV 2D-29 V region to form the canonical combining site (37, 48). Table 3 summarizes our Fab results and the results of previous studies of \( V \) region gene segment usage by PPS-specific monoclonal Abs (MAbs). The recurrence of the same \( V_H \) gene segments among different PPS specificities is apparent. For example, in addition to encoding PPS 14 and PPS 23F Abs, HV 3-23 is used by PPS 6B-specific Abs; HV 3-74 is used by Abs to either PPS 3 or 6B; HV 3-15 is used by PPS 6B, 8, and 14 Abs; and the HV 3-48 gene is used by Abs to PPS 9V, 18C, and 23F. The promiscuity of \( V_{HIII} \) genes extends to other PPS specificities. The HV 3-15 pairs, which pairs with the LV 1-51 region to form a PPS 14 combining site, also is found in association with another \( V_L \) region, LV 7-43, to form a Hib PS combining site (3, 25). Similar patterns are seen with \( V_L \) usage. VK 4-1 is associated with PPS 3 and PPS 6B Abs, VK 3-20 is associated with PPS 6B and PPS 14 Abs, and VK 2(D)-28 is associated with PPS 6B and PPS 9V Abs. The usage of the 2D-29 \( V_L \) gene by the 23F-2 Fab is notable since as mentioned above this gene in conjunction with HV 3-23 is the most commonly expressed \( V_L \) gene in the Hib PS repertoire. Furthermore, the LV 1-51 gene used by PPS 14-specific Fabs is also found in association with either HV 3-23 or HV 3-7 to form a combining site specific for the capsular PS of *C. neoformans* (49). From these data, it is apparent that chain pairing and CDR-3 diversity serve as critical determinants of PPS binding specificity. Thus, it is problematic to assign a germ line specificity to any particular \( V \) gene segment.

Our study was restricted to two adult donors; therefore, this limited scope does not permit estimates of \( V \) repertoire size for any single PPS specificity. However, our findings taken with previous studies (Table 3) suggest that at the population level, the number of \( V \) gene segments contributing to a particular PPS serotype specificity may be quite large. From the present analysis we identified LV 1-51 and KV 3-15 genes as contributors to the PPS-14 \( V_L \) repertoire, and in a previous study we showed usage of the KV 3-20 gene (34). Thus, 3 different \( V_L \)
regions are used by 3 unrelated individuals to encode PPS-14 Abs. The PPS 6B repertoire is encoded by at least 10 V_{L} gene segments and 8 V_{H} gene segments (Table 3). This diversity contrasts to the Ab repertoire to Hib PS, which although encoded by 3 to 4 V_{H} genes and as many as 12 V_{L} genes, is dominated by Abs using a single V_{H}-V_{L} canonical configuration (37, 48, 55). The capacity to generate potentially complex PPS Ab repertoires at the population level suggests that unlike the Hib PS repertoire, the occurrence of canonical combining sites may be infrequent within a particular PPS serotype specificity. Despite this diversity, however, there are likely to be structural constraints on the formation of PPS-specific combining sites as indicated by the recurrence of a VH V_{H} CDR-3 motif among the PPS14-specific Fabs. Furthermore, V_{H} gene segments belonging to the 1-3 canonical class are present among our Fabs and the panel of PPS-specific MAbs described by Baxendale et al. (12). We found evidence for both interclonal and intraclonal mechanisms for generating PPS-specific combining site diversity within an individual. The two PPS 23F-specific Fabs isolated from subject 002 represent two independent clones that used different V_{H} and V_{L} combinations and whose affinities for PPS 23F differed fivefold. Since both of these Fabs had accumulated mutations, the attribution of higher affinity or fitness to one or the other germ line configuration cannot be made at present. Nonetheless, clones of independent origin coexist within an individual, and affinity differences between them could affect their ability to be selected by Ag, to be maintained within an individual, and affinity differences between them.

It is interesting that one of the PPS 23F-specific Fabs had acquired an insertion in V_{H} CDR-2. The insertion or deletion of residues into V gene segments during somatic maturation is becoming increasingly recognized as an important mechanism for generating combining site diversity (23, 43, 70). Changes in CDR length would generate a canonical loop structure entirely different from that of the original V gene (23) and could be expected to have a substantial impact on Ab affinity. The finding of an insertionally modified V region in this relatively small survey of PPS-specific V regions suggests that this mechanism may not be uncommon.

The potential for combining site functional diversity is present even when the products of a single clone dominate the expressed repertoire of an individual, as shown by the isolation of two PPS 14-specific Fab pairs from both subjects. The pair isolated from subject 018 (14-3 and 14-4) had differentially accumulated mutations, but these sequence polymorphisms did not confer measurable differences in affinity. However, the pair of clonally related Fabs isolated from subject 002 (14-1 and 14-2) varied in the extent of mutation in their V_{H} regions sufficiently to confer a 13-fold difference in affinity. The more heavily mutated V_{H} was associated with lower affinity for PPS 14, a result suggesting that hypermutation could lead to diminished anti-PPS Ab protective function. Parallel findings have been obtained in the murine response to the capsular PS of C. neoformans, where it has been shown that Abs originating from a single clone can differ in antigenic fine specificity and protective efficacy as a result of somatic mutation (40). Since the primary structures of Fabs 14-1 and 14-2 differed only in the V_{H} region (13 positions: 4 in the CDRs and 9 in the frameworks), the disparities in affinity between these Fabs could be attributed to the V_{H} region. The extent of the sequence differences precludes any precise assignment of the critical positions, but based upon our mutagenesis studies of Hib PS-specific Fabs showing that a single amino acid replacement can ablate binding (36), we might expect that only a small subset of the observed substitutions are responsible for the differences in PPS 14 binding affinity. V_{H} CDR-3 is likely to play a critical role in PPS 14 binding as the homologous sequences Ser-Gly-

---

### TABLE 3. V gene segments encoding human anti-PPS Abs

<table>
<thead>
<tr>
<th>PPS specificity</th>
<th>V_{H}</th>
<th>V_{L}</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3-74, 3-73</td>
<td>4-1, 7-46</td>
</tr>
<tr>
<td>4</td>
<td>1-46, 3-7</td>
<td>2-30, 3-20</td>
</tr>
<tr>
<td>6B</td>
<td>1-3, 2-1, 3-7, 3-15, 3-23, 3-30, 3-66, 3-74</td>
<td>1(D)-39, 2(D)-28, 2(D)-30, 3-20, 4-1</td>
</tr>
<tr>
<td>8</td>
<td>3-15, 1-27</td>
<td></td>
</tr>
<tr>
<td>9V</td>
<td>1-02, 3-48</td>
<td>2(D)-28</td>
</tr>
<tr>
<td>14</td>
<td>3-15, 3-23</td>
<td>3-15, 3-20</td>
</tr>
<tr>
<td>18C</td>
<td>3-7, 3-48</td>
<td>3-20, 7-3</td>
</tr>
<tr>
<td>23F</td>
<td>3-23, 3-11, 3-48</td>
<td>2-24, 2D-29</td>
</tr>
</tbody>
</table>

a Compiled from published reports and this study. Succeeding footnotes refer to method of V gene identification, Ab source, and reference; nomenclature is according to references 10, 44, and 45.

b cDNA sequence of hybridoma-derived MAb (57). GenBank sequences were reported by authors, but V gene assignments were made by us.

c Partial amino acid sequence of purified serum Ab (46, 62).
d cDNA sequence of combinatorial library Fab (this report).
e cDNA of hybridoma-derived MAb (62).
f cDNA sequence of hybridoma-derived MAb (57).
g cDNA sequence of hybridoma-derived MAb (62).
h cDNA sequence of hybridoma-derived MAb (12).
Ser-Ser-Tyr and Thr-Gly-Thr-Thr-Phe were present in the CDR-3s of the two PPS 14-specific Fab pairs even though they used different canonical class V\textsubscript{H} gene segments and different D regions.

The affinity variation observed among the Fabs likely has consequence with respect to their potential to mediate protective immunity in vivo. Avidity functions as a determinant of anti-PPS Ab protective efficacy, as assessed in an in vitro model of opsonophagocytosis and in a mouse model of bacteremia (52, 66). Avidity variation is present among anti-PPS Ab populations elicited in adults by the polyvalent vaccine (52, 66) and in infants following vaccination with PPS-protein conjugates (5, 6). Irrespective of whether they are generated interclonally or intraclonally, the coexistence of avidity variants implies that Ab functional capability is not equivalent between responding clones and could in principle differ within a clone as expansion generates new variants with changed avidity.

While we cannot exclude the possibility that some of the observed deviations from the assigned candidate V gene segment could be generated by PCR artifacts or by unknown germ line polymorphisms, we assume that the majority of the observed sequence polymorphisms result from the process of somatic hypermutation. The mutated nature of all of the PPS-specific Fab fragments and their expression of non-IgG isotypes (IgG2 and IgA) lead us to conclude that these Fabs originated from memory B cells. Baxendale and colleagues reached the same conclusion in their recently published sequence analysis of human PPS-specific hybridomas generated from five adults (12). Their MAAb panel, representing specificities to PPS serotypes 4, 6B, 9V, 18C, and 23F, showed a consistent pattern of mutation, and the majority were isotype switched.

Although PPSs as well as other purified PS Ags are thought to elicit minimal memory owing to their T-cell-independent nature, studies in mice have shown that some PS Ags have the capability to generate germinal centers (63, 67), regulatory T cells (8), and avidity maturation associated with hypermutation (14). Therefore, it is possible that immunization with PPS vaccine could directly induce class switch and hypermutation in primary B cells. However, we think it more likely that the B cells responding to PPS vaccination in the adult derive from a preexisting memory population. The presence of PPS-specific serum Abs to all three serotypes in the subjects prior to vaccination indicates their primed status. Furthermore, it is known that vaccination with PPS can activate memory B cells generated by prior vaccination with protein-conjugated PPS (15, 22, 42). The antigens responsible for natural priming could be the homologous pneumococci or other bacteria or food substances expressing antigenic determinants cross-reactive with PPS. Unlike purified PPS, these natural antigenic stimuli may occur on cell surfaces in a milieu of proteins or lipids, and this form of antigen might elicit T-cell-dependent activation and promote memory generation and somatic hypermutation. This process may apply generally to anti-PS repertoires in the adult, as somatically mutated V regions have been observed among a variety of human anti-PS Ab specificities, including Hib PS (3, 11, 26, 37), C. neoformans (49), α-galactosyl (68), and gangliosides (69).

Our findings together with results of previous studies indicate that Abs of a particular PPS serotype specificity can be encoded by a potentially large number of V genes, and conversely, a single V gene segment can encode combining sites of different specificity. Such degeneracy and promiscuity suggest that humoral immunity to encapsulated pneumococci has evolved not by investing a germ line specificity in a single V gene segment (21) but rather by distributing specificity (fitness) potential over a variety of genes. The high capacity for creating combining site diversity at the population level contrasts to what is seen in the individual where only one or a few V gene combinations dominate the expressed PPS repertoire. While V gene germ line content can vary among individuals (53, 54) and could limit diversity, we think it more likely, especially given the properties of V gene degeneracy and promiscuity, that repertoire restriction is determined primarily by somatic events. These events could include Ag-independent processes that control the pace of V gene assembly leading to establishment of the precursor B cell pool as well as Ag-driven processes involving memory generation, mutation, and clonal competition. For the PPS Ab repertoire in the adult, the outcome of these processes is an oligoclonal memory population that has matured under the influence of natural antigenic exposure and that has acquired a substantial mutational load. The impact this mutation exerts on the maintenance of memory and protective immunity remains to be elucidated.

ACKNOWLEDGMENTS

We thank Nancy Sweeters and Julie Simon for performing the vaccinations and phlebotomy, Charles Connolly and Adam O’Connor for technical assistance, and the volunteers for their participation in this study. This work was supported by grants AI25008, AI45250, and RR01271 from the National Institutes of Health.

REFERENCES


Editor: R. N. Moore