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Recurrent Variable Region Gene Usage and Somatic Mutation in the Human Antibody Response to the Capsular Polysaccharide of *Streptococcus pneumoniae* Type 23F

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Combinatorial cloning and expression library analysis were used to isolate human antibody Fab fragments specific for the capsular polysaccharide of *Streptococcus pneumoniae* serotype 23F. Thirty 23F-specific Fabs were isolated from seven vaccinated donors, and the sequences of the heavy (H)- and light (L)-chain variable regions were determined. All individuals utilized either the Vκ A23 L chain, the Vκ L6 L chain, or both chains in forming the 23F-specific combining site. Vκ A23 L chains paired primarily with VH3-23 H chains. Vκ L6 L chains were more promiscuous in heavy-chain usage between individuals. Both H and L chains were mutated, primarily in the complementarity-determining regions, compared to their closest germ line counterpart, suggesting a recall response that has undergone affinity maturation. H-chain isotypes were reflective of those found in the serum. Shared somatic modifications demonstrated that immunoglobulin G2 (IgG2) and IgA antibodies arose from the same somatically matured B cell. Our results indicate that the response to the serotype 23F pneumococcal capsular polysaccharide is oligoclonal within the individual, with one or two paratope families accounting for the majority of expressed antibody. We also determined that, in spite of the combinatorial diversity available to the immune system, the 23F-specific response is highly restricted at the population level, with the same two L-chain-determined paratope families recurring in all individuals. Lastly, analysis of the isolated Fabs indicate all have undergone extensive somatic mutation, as well as class switch, maturational events that presumably require the participation of T cells.

Streptococcus pneumoniae is a significant human pathogen causing pneumonia, bacteremia, meningitis, and otitis media. The pathogenic pneumococci are surrounded by a complex capsule composed of polymeric sugars, C polysaccharide, peptidoglycan, and surface proteins. The pneumococcal capsular polysaccharides (PPS), are heterogeneous in structure, with at least 90 different serotypes occurring within the species *S. pneumoniae.* PPS epitopes are immunogenic in adults and elicit antibodies that protect against infection. A vaccine containing capsular polysaccharides from 23 pneumococcal serotypes (23-valent) is available and is currently recommended for persons over 65 years of age and for other adults considered to be at increased risk of developing pneumococcal disease. Purified capsular polysaccharides do not, however, induce a protective antibody response in infants, who comprise one of the primary populations at risk. Consequently, a 7-valent polysaccharide-protein conjugate vaccine has been developed and shown to be efficacious in infants and has recently been licensed for use in this age group.

Capsular polysaccharides are defined as TI-2 (T-cell-independent) antigens based on their repetitive structure and lack of immunogenicity in *xid* mice and human infants. The serum response to these polysaccharides in adults is oligoclonal and restricted in isotype, with immunoglobulin G2 (IgG2) and IgA antibodies predominating after vaccination or infection. Little is known about differing immunoglobulin gene usage in antibodies specific for different PPS serotypes or in antibodies specific for the same serotype in different individuals, due primarily to the difficulty in establishing human hybridomas. Combinatorial cloning circumvents this limitation and provides a means to analyze antibody repertoires without the necessity of generating hybridomas.

In this report we examine the expressed repertoire of human antibodies specific for the capsular polysaccharide of *S. pneumoniae* serotype 23F. Heavy (H)- and light (L)-chain sequences are reported for 30 PPS 23F-specific Fabs isolated from seven individuals. We demonstrate that the majority of individuals use the same H- and L-chain pairs to form PPS 23F-specific paratopes. We confirm the oligoclonality of PPS 23F-specific response at the level of immunoglobulin gene expression within the individual. Lastly, we show this response to be complex in terms of somatic mutation and class switch, maturational events thought to require T-cell participation.

MATERIALS AND METHODS

Subjects. Adult volunteers were randomly assigned to receive either the licensed 23-valent polysaccharide vaccine (Pnu-Immune; Wyeth-Lederle) or a 9-valent polysaccharide-protein conjugate vaccine consisting of PPS from serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F conjugated to the mutant diphtheria toxin CRM_{197} (Wyeth-Lederle). Blood was collected on the day of vaccination and at 30 days after vaccination to determine serum antibody response. A 100-ml blood sample was also collected 7 days after vaccination for the isolation of mononuclear cells (MNC). Human subject protocols were reviewed and approved by the Institutional Review Boards at both Children's Hospital Oakland and St. Louis University School of Medicine.

Affinity selection of cells. The enrichment of PPS-specific B cells has been previously described in detail (17). Briefly, MNC were isolated from the 7 day

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postvaccination blood sample by using Ficoll-Hypaque. An aliquot (10^6 cells) was placed into culture for 7 days in 1 ml of RPMI 1640 supplemented with 5% fetal calf serum, and the supernatant was assayed for PPS 23F-specific antibody production. PPS 23F was biotinylated as previously described and used to "arm" avidin-coated paramagnetic beads (Immunotech, Inc., Marseilles, France). These PPS 23F-coated beads were washed and then added to 2×10^7 MNC (preabsorbed with avidin-coated magnetic beads), and the mixture was incubated on ice for 30 min. C-polysaccharide (C-PS) (10 μg/ml), a bacterial product that copurifies with all serotype-specific polysaccharides, was included in the incubation buffer to inhibit the binding of C-PS specific cells. PPS 23F-binding cells were then isolated with a magnet. Positively selected cells were washed twice with cold phosphate-buffered saline–0.5% bovine serum albumin and used for RNA extraction.

Production of Fab expression libraries. The procedures for the construction of Fab libraries has been previously described in detail (3, 17, 19,). Briefly, total RNA was prepared from affinity-isolated cells (RNeasy; Qiagen, Valencia, Calif.) and cDNA prepared by using the Thermoscript RT-RCR System (Gibco-BRL, Carlsbad, Calif.) according to the manufacturer's instructions. cDNA was used as a template in the PCR to generate H-chain Fd fragments and total kappa L chains for insertion into the expression vector pComb 3H (3). The primer sets used to generate the H and L fragments were the same as those listed elsewhere (17), with the exclusion of the lambda L-chain primers and the IgM downstream primers. L-chain fragments were inserted into the *Sac*I/*Xba*I site of the pComb 3H vector, and the resulting L-chain library was electroporated into XL1-Blue *E. coli* cells. An aliquot was plated to determine the transformation efficiency, and the balance was expanded for 8 h. Plasmid DNA was purified from the expanded culture and digested with *Xho*I and *Spe*I, and the purified H-chain Fd fragments were ligated into the *Xho*I/*Spe*I site of L-chain library plasmid DNA. The Fd×L library was electroporated into XL1-Blue cells, plated at low density on Luria broth-carbenicillin plates, and grown overnight, and individual colonies were selected for analysis.

Identification of PPS 23F-specific Fabs. Individual transfected *E. coli* colonies were selected, mastered onto an LB-carbenicillin agar plate, and grown in 1-ml overnight cultures under antibiotic selection. Bacteria were pelleted by centrifugation, resuspended in 100 μl of lysis buffer (PBS plus protease inhibitor cocktail [Complete; Roche Molecular Biochemicals, Indianapolis, Ind.]), and rapidly frozen and thawed three times by using liquid nitrogen, and the cellular debris was pelleted by centrifugation. Next, 50 μl of the lysate was assayed for 23F-specific binding activity by the facilitated radioantigen-binding assay (RABA) described below. Clones that scored positive (2× background) for PPS 23F binding were restreaked for single-colony isolation from the master plate, individual clones picked and processed as described above to verify binding, and the VH- and VL-chain DNA sequence of positive clones was determined.

Sequencing and sequence analysis. Plasmids containing H- and L-chain genes were submitted to Davis Sequencing, LLC (Davis, Calif.), for VH- and VL-chain sequence determination. The L-chain sequencing primer LSEQ (5′-GCTTCCG GCTCGTATGTTGTGTGG-3′) and H-chain sequencing primer HSEQ (5′-GC AGCCGCTGGATTGTTATTACTC-3′) both bind to the vector. Initial sequence analysis utilized the NCBI IgBlast server (http://www.ncbi.nlm.nih.gov /igblast/) to identify candidate germ line gene (2). Subsequent analysis, alignments, and translations were performed by using MacVector (Accelrys, Inc., Princeton, N.J.). The kappa V region gene nomenclature is as described by Schable and Zachau (20). H-chain V region gene nomenclature is described in the ImMunoGeneTics database (13, 18). The complementarity-determining regions (CDRs) were as defined previously (12).

Fab purification. Representatives of each of the two dominant L-chain families were chosen for large-scale purification and binding analysis. The H-chain C_H1 domain of each of these Fabs were engineered to contain a C-terminal polyhistidine region as previously described (17). These were grown in 1-liter cultures, induced with IPTG (isopropyl-β-D-thiogalactopyranoside), and periplasmic proteins extracted with B-Per (Pierce Chemical Co., St. Louis, Mo.). Fab protein in the crude extracts was then purified by means of metal-chelate chromatography (Ni-NTA; Qiagen).

Isolation and protein sequence determination of serum antibody. PPS 23F-specific antibody was purified from donor 023 as described by Lucas et al. (15). Briefly, sera collected 30 days postvaccination were purified by a combination of affinity chromatography on PPS 23F-coupled Sepharose and immunoprecipitation with PPS 23F. Purified antibody was separated into H and L chains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, transferred onto polyvinylidene difluoride membranes, stained with Coomassie blue, washed, and dried, and H and L protein bands were excised and sent to the Protein Structure Laboratory of the University of California, Davis, for N-terminal amino acid sequence determination.

TABLE 1. Characteristics of MNC donors

Donor	Vaccine[a]	Serum anti-PPS23F titer[b] (μg/ml)		Serum isotype	No. of Fabs/no. of clones[c]	No. of unique Fabs[d]
		Pre	Post			
001	Conjugate	0.9	112.5	IgG2/IgA	0/800	0
002	Conjugate	0.17	16.9	IgG2/IgA	13/800	3
004	Conjugate	1.43	10.95	IgG2/IgA	0/500	0
008	Conjugate	2.6	34.3	IgG2/IgA	11/400	10
014	Conjugate	1.34	15.6	IgG2/IgA	3/800	3
018	PPS	11.9	22.07	IgG2/IgA	3/1200	1
023	PPS	11.0	75.8	IgG2/IgA	8/300	3
025	PPS	0.3	10.98	IgG2/IgA	7/700	6
027	PPS	5.6	24.32	IgG2/IgA	4/500	4

[a] Conjugate vaccine (PPS types 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F conjugated to CRM_{197}) or PPS vaccine (23-valent PPS vaccine) was administered 7 days prior to the collection of MNC.

[b] Pre- and postvaccination PPS 23F-specific serum titers were determined for sera collected before and 30 days after vaccination.

[c] The number of PPS 23F-specific Fabs isolated divided by the number of individual colonies screened.

[d] The number of Fabs unique in L-chain sequence, H-chain sequence, or both.

Antigen-binding and Fab concentration assays. The ability of Fabs and serum samples to bind PPS 23F was determined by a modified RABA. The preparation of radiolabeled PPS and the RABA has been described elsewhere (15). Serum anti-PPS 23F levels were calculated by comparison to the reference serum 89-SF as previously described (15). In the analyses of Fab samples, 5 μg of affinity-purified goat anti-human kappa antisera (Biosource International, Camarillo, Calif.)/ml was included in the reaction mixture to increase avidity and facilitate precipitation. Fab concentration was determined by a capture enzyme-linked immunosorbent assay in which goat anti-human Fd (The Binding Site, Birmingham, United Kingdom) or goat anti-IgA (Sigma, St. Louis, Mo.) immobilized on a microtiter plate, captures Fab, which is then detected by alkaline phosphatase-labeled goat anti-human kappa L chain (Biosource International). This assay is standardized with a purified Fab standard whose concentration was calculated from UV absorbance at 280 nm.

Accession numbers. Clone (VL/VH) accession numbers were as follows: 023.102 (AF485420/AF485421), 023.115 (AF485422/AF485423), 023.125 (AF485424/AF485425), 027.064 (AF485426/AF485427), 027.242 (AF485428/AF485429), 027.304 (AF485430/AF485431), 027.343 (AF485432/AF485433), 018.P6G5 (AF485434/AF485435), 025.P1C6 (AF485436/AF485437), 025.P1H10 (AF485438/AF485439), 025.P4E12 (AF485440/AF485441), 025.P5D3 (AF485442/AF485443), 025.P7A6 (AF485444/AF485445), 025.P7F5 (AF485446/AF485447), 002.P3B11 (AF485448/AF485449), 002.P7F3 (AF485450/AF485451), 002.P8E11(AF485454/AF485455), 008.1F9 (AF485456/AF485457), 008.3A2 (AF485458/AF485459), 008.3C12 (AF485460/AF485461), 008.3D7 (AF485462/AF485463), 008.4A3 (AF485464/AF485465), 008.4D1 (AF485466/AF485467), 008.4B7 (AF485468/AF485469), 008.4B12 (AF485470/AF485471), 008.2E7 (AF485472/AF485473), 008.2F7 (AF485474/AF485475), 014.7A2 (AF485476/AF485477), 014.6G9 (AF485478/AF485479), and 014.2B6 (AF485480/AF485481)

RESULTS

Isolation of recombinant PPS 23F-specific Fabs. A total of 30 adults, aged 24 to 45 years, were immunized with either the licensed 23-valent polysaccharide vaccine Pnu-Immune or a 9-valent conjugate vaccine consisting of serotype 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F PPS individually conjugated to the mutant diphtheria toxin CRM_{197}. MNC were isolated from each individual 7 days after vaccination. An aliquot of cells was placed into culture to verify specific antibody secretion, and PPS 23F-specific cells isolated from the remaining MNC with PPS 23F-armed magnetic beads. Based primarily on the production of 23F-specific antibody in vitro and the magnitude of serum antibody response after vaccination, nine donors were selected as cloning candidates (Table 1). From these, Fd-chain,

TABLE 2. Characteristics of the unique PPS 23F-specific Fabs

Donor	Clone	Vκ	% Identity[a]	JL	L3 (aa)[b]	VH	% Identity[a]	Isotype	JH	H3 (aa)[b]
002	3B11	L6	98.2	JK4	10	VH3-64	96.1	IgG2	JH6	14
002	7F3	L6	100.0	JK1	10	VH3-64	96.1	IgG2	JH6	14
002	8E11	L6	100.0	JK1	10	VH3-64	95.7	IgG2	JH6	14
008	1F9	A23	95.8	JK2	9	VH3-23	90.7	IgG1	JK4	6
008	2E7	A23	96.1	JK2	9	VH3-23	92.1	IgG2	JH4	6
008	2F7	L6	96.7	JK4	7	VH3-48	93.5	IgG2	JH2	16
008	3A2	L6	95.3	JK4	7	VH3-48	93.5	IgA2	JH2	15
008	3C12	A23	95.4	JK3	9	VH3-23	93.2	IgG2	JH1	6
008	3D7	A23	96.5	JK3	9	VH3-23	91.1	IgG2	JH6	6
008	4A3	L6	96.7	JK4	7	VH3-48	91.7	IgG2	JH2	16
008	4B12	A23	95.5	JK2	9	VH3-23	94.3	IgG2	JH1	6
008	4B7	L5	93.7	JK2	9	VH4-4	92.1*	IgG2	JH5	13
008	4D1	L6	96.6	JK4	7	VH3-48	93.2	IgG2	JH2	16
014	2B6	L6	98.2	JK4	10	VH3-23	94.6	IgG2	JH4	13
014	6G9	L6	98.2	JK4	10	VH3-23	94.3	IgA1	JH4	13
014	7A2	L6	97.1	JK2	11	VH3-30	95.7	IgG2	JH4	11
018	6G5	A23	96.5	JK2	9	VH3-7	90.1*	IgG2	JH4	8
023	102	L6	98.2	JK1	11	VH3-30	95.3	IgG2	JH4	11
023	115	A2	96.9	JK1	8	VH4-59	93.1	IgA2	JH5	7
023	125	L6	96.4	JK1	11	VH3-30	95.3	IgG2	JH4	11
025	1C6	A23	97.7	JK1	9	VH3-23	95.6	IgA2	JH5	3
025	1H10	L6	97.9	JK3	11	VH3-30	93.1	IgG2	JH4	15
025	4E12	A23	93.8	JK1	9	VH3-23	94.9	IgA2	JH5	3
025	5D3	L6	97.9	JK3	11	VH3-30	88.2	IgA1	JH4	15
025	7A6	L6	97.5	JK3	11	VH3-30	93.1	IgA2	JH4	15
025	7F5	A23	97.6	JK1	9	VH3-23	95.7	IgA2	JH5	3
027	064	A23	95.8	JK2	9	VH3-23	93.0*	IgG2	JH5	6
027	242	A23	95.1	JK2	9	VH3-23	89.6	IgA2	JH5	6
027	304	A23	95.1	JK2	9	VH3-23	89.6	IgA1	JH5	6
027	343	A23	94.1	JK2	9	VH3-23	84.9	IgA2	JH5	6

[a] Percent nucleotide identy over the entire V region compared to the corresponding germ line gene.
[b] The number of residues comprising the Vκ (L3) and VH (H3) CDR3 regions. Fabs that contain in-frame insertions in the VH region (two residues in CDR1 of 008.4B7, two residues in framework 1 in 018.6G5, and one residue in CDR2 of 027.064) that were not considered in calculating the percent homology are marked by an asterisk.

kappa L-chain, and Fd×L Fab expression libraries were constructed as described in the Materials and Methods. A total of 300 to 1,200 individual clones from each expression library were assayed for the production of PPS 23F-specific Fabs. Positive clones (those precipitating twice that of the negative control) were restreaked for single-colony isolation and recloned, and the binding and specificity were verified. H- and L-chain sequences were determined for each positive clone. A total of 49 PPS 23F-specific Fabs were isolated from seven of the nine donors assayed, 30 of which were unique in L-chain sequence, H-chain sequence, or both (Table 2). None of the isolated Fabs precipitated radiolabeled PPS 14, and none were inhibited by C-PS (10 μg/ml), indicating specificity for PPS 23F. Inspection of the sequences revealed that paratopes utilizing either the Vκ L6 or Vκ A23 L-chain genes accounted for most of the Fabs isolated from all donors. A representative Fab from each of these major families was produced in quantity and purified, and the specificity and relative affinity for PPS 23F were determined (Fig. 1). Both the Vκ A23 Fab (027.242) and the Vκ L6 Fab (023.102) bound to radiolabeled PPS 23F in a specific and concentration-dependent manner under both monovalent and polyvalent conditions.

Two L-chain V genes dominate the response to PPS 23F. All individuals we examined utilized either the Vκ A23 L chain, the Vκ L6 L chain, or both in forming 23F-specific combining sites. Vκ A23 L chains were, with a single exception, paired with VH3-23 H chains to form the paratope. Vκ L6 L chains were more promiscuous in H-chain pairing between individuals but utilized a single H chain within any single individual. The VH3-23 gene has previously been demonstrated to be expressed at high frequency (25%) in randomly selected IgM^+ B cells (6). Normal IgM^+ B cells express Vκ A23 (1%) and Vκ L6 (6%) at frequencies in line with their expected occurrence in the genome (9).

In addition to these two recurring L-chain families, Fabs were isolated in which a Vκ L5 L chain was paired with a VH4-4 H chain (008.4B7), and a Vκ A2 L chain was paired with a VH4-59 H chain (023.115). We have previously reported two additional PPS 23F-specific Fabs, one utilizing Vκ A23/VH3-23 gene products, and one that utilizes Vκ A2 paired with VH3-11 (17).

Vκ A23 Fab family. Four of the seven individuals we analyzed utilized Vκ A23-derived L chains in forming PPS 23F-specific binding domains. All Vκ A23 L chains were rearranged with a 9-amino-acid CDR3 and were mutated (as compared to the germ line) in the CDRs (Tables 3 and 4). In some individuals (donor 027, for example) the isolated L chains appeared to derive from a single rearrangement event, as indicated by the pattern of mutation and shared Jκ segment usage. In others (i.e., donor 008) sequences indicative of at least two rearrangement events were isolated. In several cases identical substitutions were found in different individuals (Asp^{28} to Asn^{28} in CDR1, Asn^{53} to Lys^{53} in CDR2, and Met^{89} to Thr^{89} in CDR3, for example), suggesting antigen-driven selection for these par-

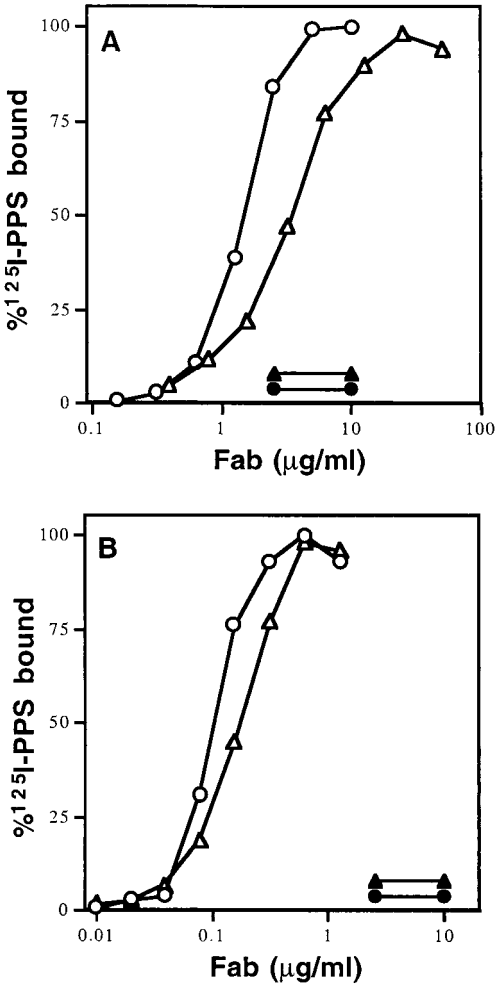

FIG. 1. RABA analysis of PPS binding by representative Fabs 023.102 and 027.242 under monovalent (A) and polyvalent (B) conditions. Symbols: ○, Fab 023.102 binding PPS 23F; ●, Fab 023.102 binding PPS 14; △, Fab 027.242 binding PPS 23F; ▲, Fab 027.242 binding PPS 14.

ticular residues at these locations during affinity maturation. Alternately, these residues may indicate the use of a previously undescribed allele of Vκ A23. The Vκ A23 L chain isolated from donor 018 differed in its mutation pattern from the others in that substitutions were primarily in CDR3, with an almost germ line configuration in CDRs 1 and 2. Notably, this was the only Vκ A23 L chain isolated paired with an H chain other than VH3-23.

The VH3-23-derived H chains paired with the A23 L chains were extensively mutated throughout CDRs 1 and 2 (Tables 3 and 4). Sequences from donor 027 probably represent at least two rearrangement events. In addition to a different pattern of substitutions, clone 027.064 has a single amino acid insertion in CDR2 that differentiates it from the other VH3-23-derived chains isolated from that individual. All isolates from donor 025 appear to be derivative of a single rearrangement event. Variations in CDR3 and JH usage indicates at least three rearrangements events occurred in donor 008. As in the L chains, there were identical substitutions seen in VH3-23 H chains from different individuals (Ala^{33} to Thr^{33} in CDR1, Ser^{35} to Thr^{35} in CDR1, and Ala^{50} to Ser^{50} in CDR2, for example) again implying antigen-driven selection. The VH3-23 locus is more heterogeneous than that of Vκ A23, however, and the assignment of such shared differences to mutation as opposed to common allele usage is somewhat less certain.

L6 Fab family. The paratope family defined by the Vκ L6 gene is much more diverse, both in VH gene usage and L-chain CDR3 structure (Tables 5 and 6). Five of the seven donors utilized the Vκ L6 L chain in forming PPS 23F specific paratopes. The most common pairing (three of five donors) was a Vκ L6 L chain with an 11-amino-acid CDR3 paired with a VH3-30-derived H chain. The unusually long CDR3 results from the addition of six nontemplated bases at the Vκ-Jκ junction. Donor 008 utilizes an equally uncommon 7-amino-acid L6 CDR3 (apparently generated by the deletion of the first two residues of the J region during rearrangement) paired with a VH3-48 H chain. Substitutions shared between individuals in L-chain CDRs 1 and 2 are less common than in the Vκ

TABLE 3. Vκ A23 Fab family: L chains[a]

Fab	Sequence of L chain			Jκ	VH
	CDR1	CDR2	CDR3		
Vκ A23	RSSQSLVH**SDGN**TYL**S**	**KISNRFS**	**MQATQFP**		
008.1F9	T-------RN-Q-F--	Q--K---	T------ YT	Jκ2	VH3–23
008.3C12	---------N-D---T	G--K---	------- FT	Jκ3	VH3–23
008.3D7	---------N-D---T	E--K---	--TS--- FT	Jκ3	VH3–23
008.4B12	--------RT-E-F--	K----V-	T------ YT	Jκ2	VH3–23
008.2E7	--------GN-E-F--	K------	T----Y- YT	Jκ2	VH3–23
025.1C6	S------R--------	E--K---	------- WT	Jκ1	VH3–23
025.4E12	S-G---------I--N	R----L-	---S-Y- WT	Jκ1	VH3–23
025.7F5	---------NRD----	E--K---	------- WT	Jκ1	VH3–23
027.064	---------N------	QV-D---	T------ YT	Jκ2	VH3–23
027.242	--------RN------	E--K-L-	T------ YT	Jκ2	VH3–23
027.304	------------A---	Q--K-V-	T------ YT	Jκ2	VH3–23
027.343	------------A--N	Q--K-V-	T------ YT	Jκ2	VH3–23
018.6G5	----------------	-----L-	-HG--L- RT	Jκ2	VH3–7

[a] CDR residues and gene usage of the A23 family of PPS 23F-specific Fabs. Germ line positions where identical substitutions were isolated from different individuals are shown in boldface. Fab 027.064 has a single residue insertion between Ser^{52} and Gly^{52a} in the H-chain CDR2; a space was inserted at the appropriate location of the germ line sequence for purposes of alignment (see asterisks, Table 4). Donors 008 received the conjugate vaccine. Donors 018, 025, and 027 received the 23-valent PPS vaccine.

TABLE 4. Vκ A23 Fab family: H chains[a]

Fab	Sequence of H chain			JH	Isotype
	CDR1	CDR2	CDR3		
VH3–23	**SYAMS**	**AIS*GSGGST**YYADSVKG			
008.1F9	-HT-T	SLN*EP-DV-R-------	VHGSAY	JH4	IgG1
008.3C12	NNT-T	S-N*E--DR-H-------	VHGSAH	JH1	IgG2
008.3D7	DNT-T	S-I*DGD-PP--------	VHGVPD	JH6	IgG2
008.4B12	INT-S	S--*E--DR-R-------	VHGSAH	JH1	IgG2
008.2E7	-HT-T	S-N*EN-DT-H-------	VHGDAY	JH4	IgG2
025.1C6	-D---	G--*---TI-------R-	STY	JH5	IgA2
025.4E12	-D---	G--*---TI-------R-	STF	JH5	IgA2
025.7F5	ND---	G-G*---DV---------	STF	JH5	IgA2
027.064	A-T-T	S-GD-G--K---------	VHGVPV	JH5	IgG2
027.242	R-T-T	S-V*D-NNRP--------	VHGEAY	JH5	IgA2
027.304	R-T-T	S-V*DGNNRP--------	VHGEAY	JH5	IgA1
027.343	R-T-T	S-V*NGNNRP--------	VHGEPS	JH5	IgA2
VH3–7	SYWMS	NIK*QDGSEKYYVDSVKG			
018.6G5	-S--G	---*H----QS-------	DWYRTFDY	JH4	IgG2

[a] See Table 3, footnote *a*.

A23 chains. Residues 95a and 95b, the nontemplated additions, do tend to be conserved between individuals that utilize extended CDR3s, however. Although there is no conservation in Jκ region usage between individuals, residue 96, the only varying residue contributed by the Jκ region to CDR3, has been mutated in all cases, most commonly to Ala in the 11-amino-acid CDR3s. These facts, taken together, suggest that the majority of antigen-specific selection pressure on the L chains contribution to the paratope is exerted on the CDR3 loop.

H-chain usage in the Vκ L6 family is more diverse than in the Vκ A23 Fabs (Tables 5 and 6). There is a conservation of VH and JH gene usage, however, in Vκ L6 Fabs of a given CDR3 length. All Fabs (from three different donors) that utilized a Vκ L6 L chain with an 11-amino-acid CDR3 used VH3-30 and JH4 to generate the paratope. All but 1 of the 10-amino-acid CDR3 Vκ L6 Fabs utilized VH3-64 and JH6, and all of the 7-amino-acid CDR3 Fabs used VH3-48 and JH2. All VH chains were mutated in the CDRs, and there were identical substitutions at several residues in H-chain CDR2 in the VH3-30 Fabs (Ile^{51} to Val^{51}, Asn^{56} to Thr^{56}, and Lys^{57} to Thr^{57}, for example). The VH3-30 locus is highly polymorphic, however (13), and although none of the shared substitutions appears to correspond to any of the known alleles, the possibility of an undescribed allele cannot be excluded.

Postvaccination sera obtained from donor 023 appeared to contain a single predominant clonotype when analyzed by isoelectric focusing (data not shown). Affinity-purified antibody from this donor was reduced and separated into H and L chains, and the separated chains were submitted for N-terminal protein sequence determination. Data was obtained for residues 1 to 21 of the light chain. The determined sequence (EIVLTQSPATLSLSPGEXATL) was 100% identical to Vκ L6, Vκ L20, and Vκ A11 and therefore consistent with the predominant L chain isolated from this individual by combinatorial cloning. Our attempts to obtain sequence data for the H chain were not successful.

In addition to the Vκ A23 and Vκ L6 Fabs isolated from all

TABLE 5. Vκ L6 Fab family: L chains[a]

Fab	Sequence of L chain			Jκ	VH
	CDR1	CDR2	CDR3		
Vκ L6	RASQSV**SSY**LA	DASNRAT	**QQRS**NWP		
014.7A2	K------RS--	-------	-H-N--- PG AT	Jκ2	VH3–30
023.102	------TN---	G------	---D--- PD AT	Jκ1	VH3–30
023.125	------G-Q--	-------	---K--- PD GT	Jκ1	VH3–30
025.1H10	-------R---	-------	-H-G--- PG AT	Jκ3	VH3–30
025.5D3	-------R---	-------	-H----- PG AT	Jκ3	VH3–30
002.3B11	------G-F--	-------	------- P LT	Jκ4	VH3–64
002.7F3	----------	-------	------- P VT	Jκ4	VH3–64
002.P8E11	----------	-------	------- P VA	Jκ4	VH3–64
014.6G9	-------I---	------P	---L--- P LT	Jκ4	VH3–23
008.2F7	---E---T---	---H---	---TD-G	Jκ4	VH3–48
008.3A2	---E---TF--	---D---	---T--G	Jκ4	VH3–48
008.4A3	---E---T---	---D---	---T--G	Jκ4	VH3–48
008.4D1	-----IN----	---Y---	------G	Jκ4	VH3–48

[a] CDR residues and gene usage of the L6 family of PPS 23F-specific Fabs. Germ line positions where identical substitutions were isolated from different individuals are shown in boldface. Grouping is by L-chain CDR3 length. Fabs isolated from donor 008 have 7-amino-acid L-chain CDR3 region resulting from the deletion of the first two residues of Jκ4. Donors 002, 008, and 014 received the conjugate vaccine. Donors 023 and 025 received the 23-valent PPS vaccine.

TABLE 6. Vκ L6 Fab family: H chains[a]

Fab	Sequence of H chain			JH	Isotype
	CDR1	CDR2	CDR3		
VH3–30	SYGMH	**VISYDGSNK**YYADSVKG			
014.7A2	-F-V-	-V-AG-TTT-------D	EGSGSKWCFDY	JH4	IgG2
023.102	R----	-V-S--RTT--------	EGGDNKFSFDY	JH4	IgG2
025.1H10	N----	-V-SH-NT---------	EGHGSDMTWRADFDY	JH4	IgG2
025.5D3	N----	-LGA--TTT--------	EGHASGMTWRADFDY	JH4	IgA1
025.7A6	H----	-V-SG-ET---------	EGHSSGMTWRADFDY	JH4	IgA2
VH3–64	SYAMH	AISSNGGSTYYADSVKG			
002.7F3	G-S--	--N---D-----V----	DIYDVSSAYYGMDV	JH6	IgG2
VH3–48	SYSMN	YISSSSSTIYYADSVKG			
008.3A2	T--A-	---GD-G----------	SK QQVSGGFYWYFDL	JH2	IgA2
008.4A3	-N-V-	----T-DI---------	SRGQQSPLGFYWYFDL	JH2	IgG2
008.4D1	-N-V-	---NTG-I----N----	SKQQQSTAGFYWYFDL	JH2	IgG2
008.2F7	TN-V-	----T-TI---------	SKAQQATAGFYWYFDL	JH2	IgG2
VH3–23	SYAMS	AISGSGGSTYYADSVKG			
014.6G9	N----	SVF--ADN-H-------	VLSPGGSNMVWDY	JH4	IgA1
014.2B6	N----	SVF--ADN-H-------	VLSPGGSNMVWDY	JH4	IgG2

[a] See Table 5, footnote *a*.

donors, a Fab was isolated that used a Vκ L5 L chain paired with VH4-4, and one that used a Vκ A2 L chain paired with VH4-59 (Tables 7 and 8). Both were mutated in the CDRs of both H and L chains. The H chain of Fab 008.4B7 (Vκ L5/VH4-4) had a two-residue insertion into CDR1. The A2 L chain of Fab 023.115 is noteworthy in that the Lys^{30}-to-Arg^{30} and Tyr^{34}-to-His^{34} substitutions in CDR1, as well as the deletion of Pro^{95} in CDR3, are shared with our previously reported A2-utilizing PPS 23F specific Fab (17).

The H-chain isotypes of the isolated Fabs were IgG2 and IgA (with one exception) reflecting what is usually found in the serum (Tables 3 to 6). There were several examples of different isotypes associated with the same H-chain V region sequence. Fabs 027.304 (IgA1) and 027.343 (IgA2) are similar in their CDRs (Tables 3 and 4) and clearly share the same postrearrangement somatic maturational history. An IgG2 Fab from this same individual (027.064) shares some substitutions but has an insertion in CDR2 and may have arisen from a separate rearrangement event. The three VH3-30 isolates from donor 025 differ in isotype (Tables 5 and 6) but share significant homology in CDR3, as well as some substitutions in CDR2, and probably arose from the same initial rearrangement. The two VH3-23 H chains paired with L6 from donor 014 (IgA1 and IgG2) are identical in their CDRs and undoubtedly arose from the same rearrangement event.

DISCUSSION

The molecular and genetic diversity inherent in an antibody response to a particular antigen is difficult to study in detail. Mouse models are unsuitable for such analysis since members of genetically identical inbred strains are not expected to produce the variability observed in outbred populations. Repertoire analysis is intrinsically difficult in humans. Stable antigen-specific human hybridomas are difficult to establish, and in only a few cases has it been possible to generate hybridomas specific for a single antigen from multiple donors (1, 4). Protein sequencing of purified serum antibodies has provided repertoire information for some specificities (22, 23) but is technically challenging, labor-intensive, and expensive when applied at a population level. Idiotypic analysis can be applied to large numbers of donors (14) but gives more limited information and is severely compromised by the necessity of generating and characterizing specific reagents for each response being studied.

Reverse transcription-PCR is a convenient and standardized methodology for deriving sequence information concerning expressed immunoglobulin genes. The two relevant approaches for repertoire analysis are to either amplify and express the products of single antigen-specific cells or to construct combinatorial expression libraries from donor-derived cells that are then screened for the production of specific Fabs. Single-cell PCR (8) retains native pairing, but the necessity of identifying antigen-specific cells and the multitude of manipulations that must be carried out on each cell, a small fraction of which are antigen specific even in enriched populations, together with the necessity of protein expression and specificity determinations for each isolated Fab, make it unsuitable for the analysis of repertoires where, by definition, the response of

TABLE 7. Non-A23/L6 Fabs: L chains[a]

Fab	Sequence of L chain			Jκ
	CDR1	CDR2	CDR3	
Vκ L5	RASQGISSWLA	AASSLQS	QQANSFP	
008.4B7	-----V-Q--G	DVF--HG	--TK--- HT	Jκ2
Vκ A2	KSSQSLLHSDGKTYLY	EVSNRFS	MQSIQLP	
023.115	R----------R--FH	-------	---M-Y RT	Jκ1

[a] H- and L-chain CDRs and isotypes of the two non-A23/L6 Fabs isolated in this study. Fab 008.4B7 has a two-residue insertion in H-chain CDR1; a space was inserted at the appropriate location of the germ line sequence for purposes of alignment (as indicated by asterisks, see Table 8). Donor 008 received the conjugate vaccine. Donor 023 received the 23-valent PPS vaccine.

TABLE 8. Non-A23/L6 Fabs: H chains[a]

Fab	Sequence of H chain			JH	Isotype
	CDR1	CDR2	CDR3		
VH4-4	S**YYWS	RIYTSGSTNYNPSLKS			
008.4B7	NSNF---	H--A--T---------	GAYASGNYYPPGE	JH5	IgG2
VH4-59	SYYWS	YIYYSGSTNYNPSLKS			
023.115	DHS--	-FHF--T---------	GSAMMGS	JH5	IgA2

[a] See Table 7, footnote *a*.

multiple individuals must be examined. The combinatorial approach we have taken in cloning and screening expression libraries is more adaptable to repertoire analysis but suffers from the fact that native H- and L-chain pairing is lost. It is not the case, however, that de novo specific paratopes are created by this methodology. In the few cases in which immunoglobulin protein sequence is available from purified serum antibody, gene usage inferred from protein sequence corresponds to that identified by combinatorial Fabs specific for the same antigen. Protein sequence obtained from purified *Haemophilus influenzae* type B capsular polysaccharide (Hib PS) antibodies (22) identify the same canonical H and L chains as are obtained from combinatorial cloning (19). In the current study, amino-terminal L-chain sequence (21 residues) obtained from purified donor 023 PPS 23F-specific antibody was identical to Vκ L6, Vκ L20 (the distal homolog of L6), and Vκ A11, a closely related Vκ III V gene. Most Fabs isolated from this donor were members of the Vκ L6 family. The relatively high frequency of Fabs in these libraries, as well as the isolation of intraclonal variants of the same H and L chains, also suggests that these chains are representative of those involved in forming specific paratopes in vivo. In addition to the polysaccharide antigens cited above, others (7) have shown that influenza virus hemagglutinin-specific Fabs isolated by combinatorial cloning recapitulate naturally occurring H- and L-chain pairs. Fabs isolated by cloning procedures also reflect serum antibodies of the donor in both H- and L-chain isotype (17, 19; the present study). Together, these findings strongly suggest that Fabs obtained by combinatorial cloning are representative of the paratopes actually used in the response. The information that is lost by using a combinatorial approach relates primarily to the effect individual mutations have on binding affinities by specific H- and L-chain pairs. Although laborious, this information can be inferred from H×L recombination or "back crossing" experiments where all relevant chain pairs from a given individual are recreated. Although the somatic history of the individual chain pairs is lost, combinatorial cloning allows the investigator to determine the repertoire of H- and L-chain gene products which combine to form antigen-specific paratopes. When library complexity is limited by antigen-specific cell selection prior to mRNA extraction, combinatorial cloning provides a powerful methodology for repertoire analysis in humans.

Our analysis of the PPS 23F-specific repertoire has revealed several points. We have demonstrated that the 23F-specific repertoire utilized by the individual is limited. Only one of the donors we examined (008) used more than two paratope families in response to vaccination. This finding concurs with serological analysis that has shown the 23F specific-response to be oligoclonal (15), with at most two or three clonotypes accounting for the majority of the antibody in serum. Oligoclonality is believed to be a characteristic of antibody responses to polysaccharide antigens in general and has been demonstrated serologically for several other polysaccharide antigens including Hib PS (11), PPS 6B, and PPS 14 (15). A more surprising finding is our determination that most individuals use the same H- and L-chain pairings in forming PPS 23F-specific antibodies. These results do not necessarily follow from oligoclonality itself and, given the combinatorial diversity available to the immune system, it is quite remarkable that such a limited diversity is seen between individuals. A similar recurrence in gene usage is seen in the response to Hib PS, where most individuals utilize an A2 L chain paired with a VH3-23 H chain to form Hib PS-specific Fabs (16). It has been suggested that the high-molecular-weight capsular polysaccharides, which are polymers of smaller repeating subunits, lack epitope diversity and that this results in the observed oligoclonality of the response. Our findings suggest not only a paucity of antigenic epitopes associated with the PPS 23F polysaccharide but also that, for any given epitope, only a few members of the germ line repertoire are normally used to form the corresponding paratope.

The genetic elements utilized by the Fabs isolated in this study have clearly undergone extensive somatic modification. These mutations are preferentially found in the CDR loops and, together with the observation that identical substitutions occur in different individuals, strongly suggest an antigen-driven mechanism of somatic mutation. Studies of type 2 TI antigen responses in mice (5) have also determined that identical substitutions arise in independently derived clones and are directly linked to increases in affinity. Such "affinity maturation" is thought to be T cell dependent. Somatically modified antibodies have been reported for both Hib PS and other PPS antigens (see below), and our findings suggest a role for T cells in the response to the 23F polysaccharide as well. The presence at 7 days of such extensively mutated paratopes implies that our findings after vaccination reflects a recall response generated from preexisting memory B cells. We found no significant correlation between the degree of mutation and the T-cell-dependent or T-cell-independent form of the vaccine. A possible explanation for our findings is that these individuals had previously encountered the antigen through natural exposure and that the 23F polysaccharide was originally encountered in a T-cell-dependent form, perhaps as consequence of being associated with the intact bacteria.

We have previously reported the isolation of two PPS 23F-specific Fabs from a single donor immunized as part of a separate study (17). One of these (23F.2) utilized an Vκ A2 L

chain paired with a VH3-11 H chain to form the paratope. As mentioned above, the 23F.2 L chain shares two substitutions in CDR1, as well as the shortened CDR3, with the Vκ A2 isolate in this study. The Vκ A23/VH3-23 Fab previously reported (23F.1) is also highly homologous to the members of that family presented here. The 23F.1 L chain shares a substitution in CDR1 and all three substitutions in CDR2 with 027.242 L chain reported in this study. The 23F.1 H chain and the 027.064 H chain in this study shares two substitutions in CDR1, as well as two substitutions and the insertion of an Asp residue between Ser^{52} and Gly^{53} in CDR2. In all cases sufficient differences exist between the isolates to rule out laboratory cross-contamination. The only other reported human antibody specific for PPS 23F utilized a VH3-48 H chain that shares two CDR2 substitutions with the 008.2F7 H-chain sequence we report here (4). No L-chain sequence for this hybridoma was reported. The isolation of the same gene products in different studies and from different laboratories and with similar substitutions supports our conclusion that this antibody response arises from a limited V gene repertoire and is subjected to antigen-driven somatic modification.

There is a substantial body of evidence suggesting that antibody responses to capsular polysaccharides undergo the same maturational processes as do responses directed toward protein determinants. In humans, non-Vκ A2 Hib PS-specific antibodies are mutated (10, 21, 22), and even the "canonical" Vκ A2 antibodies often undergo some degree of somatic modification. PPS-specific paratopes isolated either by combinatorial cloning (17) or traditional hybridoma technology (4) also show evidence of somatic mutation. Partial protein sequence obtained from purified PPS 6B antibodies show differences from the most likely germ line V genes, and the reported V gene sequences of a PPS 6B-specific human hybridoma are mutated in the V region of both the L and H chain (23). In murine models, PS antigens induce germinal center formation (24), affinity maturation, and class switch (5). These maturational events are generally thought to be T cell dependent. Although PPS antigens do not undergo intracellular processing and major histocompatibility complex-associated display as normally required for T-cell activation, the evidence supports a substantial role for T cells (or possibly NK cells) acting in a regulatory manner. Taken together, these findings suggest that the inability to respond to "T-cell-independent" polysaccharide antigens early in life may actually result from a maturational delay in the T-cell compartment of the immune system.

Our isolation of Fabs whose H chains are the same IgG2/IgA isotypes as PPS 23F-specific serum antibody supports our conclusion that these combinatorial Fabs are representative of those actually used in the response. Interestingly, our findings suggest both that PPS-specific secretory IgA and serum IgG derive from the same original somatically matured B cell and that class switching can occur independent of concomitant somatic mutation. The two VH3-23 H chains paired with L6 from donor 014 (IgA1 and IgG2) are identical in their CDRs, share several silent mutations, and undoubtedly arose from the same rearrangement event. The VH regions of Fabs 027.304 (IgA1) and 027.343 (IgA2) are both significantly mutated compared to the germ line but highly homologous to each other and clearly share the same postrearrangement somatic maturational history. Our data do not allow us to determine whether somatic mutation occurred prior to the switch from IgM to IgG or IgA, nor can we rule out further mutational events affecting only the L chain. It does appear, however, that little further somatic modification of the VH region takes place between the time of isotype differentiation and our sampling point at 7 days postvaccination.

Our determination of gene family usage may have been biased by the selection criteria we employed in selecting donors for library construction and by certain aspects of the cloning methodology. We preferentially selected donors whose MNC produced PPS 23F-specific antibody in vitro and who demonstrated a significant rise in serum antibody titer after vaccination. This may have introduced a bias toward individuals utilizing higher-affinity paratopes that would be reflected in restricted gene usage in the isolated Fabs. We have also restricted our analysis to kappa L chain Fabs. This was based on our observation that the 23F-specific response is highly skewed toward antibodies utilizing this L chain (15). There are, however, individuals that have lambda antibodies in their serum, and we are currently assessing the contribution of this L-chain isotype to the 23F-specific repertoire in those individuals. Also, the cloning methodology we employ utilizes restriction enzymes whose recognition sequences, although very rare in human germ line V genes, may arise as a result of somatic mutation, leading to the elimination of these gene products from a given library. This usually can be detected during library construction and a solution engineered, but the loss of these sequences from analysis cannot be entirely ruled out. It should also be pointed out that our analysis was restricted to specific B cells circulating 7 days after vaccination. B cells present in other compartments (bone marrow or spleen) or that circulate at other time points might utilize other gene products to make PPS 23F-specific antibodies.

Our findings represent one of the most extensive analyses, at the molecular level, of a specific antibody response in humans. The somatic history of the response to the "T-cell-independent" PPS 23F capsular polysaccharide provides compelling evidence of T-cell participation in the maturation of the adult repertoire. Given the combinatorial diversity available to the immune system, it is remarkable to find not only the same H- and L-chain pairing in different individuals, but the sharing of postrearrangement somatic modification as well. The two L-chain defined paratope families we describe suggest the existence of two distinct antigenic determinants associated with the polysaccharide. If so, this would imply that for each antigenic epitope a limited number of L- and H-chain genes can pair to form the specific paratope.

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