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Immune Response to Pneumococcal Polysaccharides 4 and 14 in Elderly and Young Adults: Analysis of the Variable Heavy Chain Repertoire

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Streptococcus pneumoniae **is a leading cause of morbidity and mortality in both developed and developing countries. The current pneumococcal polysaccharide (PPS) vaccine is highly effective in young adults; however, vaccine efficacy is dramatically decreased in the elderly population. We hypothesized that the decreased vaccine efficacy in the elderly results from altered variable gene family usage. We have characterized the immunoglobulin G gene usage of the antibody response to PPS4 and PPS14 in 20 young and 20 elderly adults. The variable heavy (V_H) gene repertoire of human peripheral B cells was amplified by using PCR. A total of 364 heavy chain sequences with specificity for PPS4 and 305 heavy chain sequences for PPS14 were analyzed from young adults. In addition, a total of 325 sequences for PPS4 and 291 sequences for PPS14 were obtained from elderly adults. Complete sequence identity, somatic mutation frequencies, and V_H gene usage was determined in response to PPS4 and PPS14. In all volunteers, the immune response to both polysaccharides consisted predominantly of heavy chains belonging to the V_H3 gene family. There were significant differences in the variable gene repertoire between young and elderly adults. Somatic mutation occurred more frequently in sequences derived from young compared to elderly derived sequences. With aging, a loss of oligoclonality was noted in response to PPS4 and PPS14 compared to young adults. The observed differences in V_H repertoire, somatic mutation, and loss of oligoclonality may contribute to decreased vaccine efficacy in the elderly.**

Streptococcus pneumoniae is a mucosal pathogen that colonizes the human nasopharynx and causes meningitis, pneumonia, and acute otitis media (34). The organism is responsible for 500,000 cases of invasive pneumococcal disease resulting in approximately 40,000 deaths per year in the United States (20). The pneumococcal capsular polysaccharide is a major virulence factor and protects the bacterium from innate host defenses (1). The currently available pneumococcal vaccines are based on the observation that antibodies against capsular polysaccharides protect against disease by inducing complement mediated opsonophagocytic activity (41). The currently licensed pneumococcal polysaccharide (PPS) vaccine consists of 23 purified PPS serotypes, which account for 76 to 90% of the organisms isolated from adults with invasive pneumococcal disease (31). Pneumococcal vaccination is recommended for all individuals at increased risk for pneumococcal infection, including those with chronic illnesses, those living in environments with increased exposure to the pneumococcus, and all elderly aged 65 years or older (22). Even though highly effective in young adults, vaccine efficacy in the elderly is dramatically reduced, although estimates vary considerably, ranging from 48 to 81% (34). Studies designed to determine the postvaccination antibody concentrations to the pneumococcal capsular polysaccharides in the elderly indicate that these are similar to younger adults (6, 32). Romero-Steiner et al. (32), however, reported that despite adequate immunoglobulin G (IgG) antibody concentrations, the elderly have a significant reduction in opsonophagocytic activity against all serotypes tested. The reduced opsonophagocytic activity may explain the discrepancy between antibody concentration and vaccine efficacy studies. However, the mechanisms responsible for the discrepancy between antibody concentration and functional activity in the elderly immune response to PPSs remains to be elucidated.

Several studies performed in aging mice have shown a loss of antibody avidity or affinity in response to T-independent antigens with age (13, 25, 45). In addition, the antibodies produced by aged mice were not of the dominant idiotype, were not protective, and expressed different V_H and V_L gene families than those used by young mice (12, 24, 30). These changes in V gene family usage and idiotype expression appeared to occur independent of available V gene repertoire (47). Moreover, studies performed in aging mice suggest a potential correlation between V gene family usage and functional antibody activity.

Structural antibody studies of *Haemophilus influenzae* group b anti-polysaccharide antibodies have demonstrated the correlation between antibody avidity, fine specificity, protective efficacy, and the expression of particular variable regions (18, 23). Although several investigators (3, 7, 20, 49, 50) have studied the immune response to capsular polysaccharides on a molecular level, the characteristics of anti-polysaccharide antibodies that mediate protection remain to be defined. Furthermore, studies of the antibody repertoire in response to PPSs are limited to young, high responders. Our aim here was to define potential differences in V_H repertoire in response to PPSs in young and elderly adults.

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Previous studies demonstrated that the functional immune response to PPS14, as determined by opsonophagocytic activity, is well conserved in the elderly. However, the response to PPS4 shows a significant functional decline in the elderly despite normal antibody concentrations (32). We therefore studied the immune response to PPS4 and PPS14 on a molecular level by using peripheral blood lymphocytes obtained from 20 young and 20 elderly vaccinated volunteers. We report the sequence analysis of 689 PPS4- and 596 PPS14-specific heavy chains obtained from these volunteers.

MATERIALS AND METHODS

Human volunteers and vaccination. Healthy young adults (<30 years old) and elderly volunteers (>65 years old) were asked to participate in the present study. Each individual was questioned concerning prior pneumococcal vaccination, medications, previous illness, and present health. In addition, we obtained complete blood counts, comprehensive chemistry profiles (including renal and liver functions and serum albumin), total B-cell and T-cell subset counts, and total IgG, IgM, and IgA levels in all study candidates. Individuals previously immunized with the pneumococcal vaccine and any individual considered to be immunocompromised on the basis of medication (chemotherapy, steroid preparations, and immunosuppressive agents, including anti-tumor necrosis factor alpha agents), with previous and/or present illness (previous pneumococcal disease, splenectomy, autoimmune disease, end-stage renal or liver disease, human immunodeficiency virus positivity, organ transplant, or cancer), or with abnormal complete blood count, chemistries, B or T cells, or immunoglobulin levels did not qualify. All informed consent forms and questionnaires were approved by the Institutional Review Board of the Medical College of Ohio.

Participating volunteers were then immunized with the 23-valent PPS vaccine (Pneumovax-Merk & Co., Inc., West Point, PA). Blood samples were obtained preimmunization and 6 weeks postimmunization, and the responses to PPS4 and -14 were determined by enzyme-linked immunosorbent assay (ELISA) (15). In addition, B cells specific for PPS4 and PPS14 were isolated from postimmune sera.

Biotinylation of PPSs. Biotinylated polysaccharides were used to select PPS4- and PPS14-specific B cells. The biotinylation procedure was performed as previously described (20). Briefly PPS4, -14, and -23F (American Type Culture Collection) were dissolved in 0.2 M sodium bicarbonate and reacted with cyanogen bromide. Biotin hydrazide (Pierce Chemical Co., St. Louis, MO) was added to the activated polysaccharide solution and reacted for 2 h at room temperature. The solution was dialyzed overnight at 4°C against phosphate-buffered saline. Biotinylated polysaccharides were filter sterilized and stored at −80°C. Streptavidin-coated immunomagnetic beads (Dynal, Lake Success, NY) were labeled with selected biotinylated PPS. Beads were washed with 0.1% bovine serum albumin–phosphate-buffered saline and then mixed with 50 μg of biotinylated PPS overnight at 4°C. The labeled immunomagnetic beads were washed five times and used to select B cells specific for PPS4 and PPS14.

Affinity selection of specific PPS responding B cells. Peripheral blood lymphocytes obtained 6 weeks postvaccination were isolated by using Ficoll Histopaque gradient (Sigma, St. Louis, MO). Adherent cells were removed, and B cells were washed in RPMI 1640–1% newborn calf serum (Sigma). Immunomagnetic beads coated with irrelevant PPS23F were added, followed by incubation at 4°C for 1 h. Cross-reactive B cells were separated from the population and discarded. Further depletion of cross-reactive B cells was achieved by cross incubating the B cells. Cells for PPS4-specific isolation were first depleted with 25 μg of PPS14-coated beads. Likewise, cells for PPS14 isolation were depleted with PPS4 beads. A total of 25 μg of PPS4-coated immunomagnetic beads or PPS14-coated beads was added independently to each of two aliquots, followed by incubation at 37°C for 30 min with 10 μg of cell wall polysaccharide (CWPS)/ml to block nonspecific binding. Polysaccharide-specific B cells were isolated by magnetic separation and washed five times with RPMI 1640–5% newborn calf serum. RNA was extracted from the isolated B cells by using lysis binding buffer (Dynal, Inc.) with 5 μl of RNasin. cDNA was prepared as per manufacturer's instructions with oligo(dT_{25}) beads (Dynabeads mRNA Direct Kit).

Validation of selection method. Single B-cell clones with specificity for PPS14 were isolated as described above and expanded by using a cell culture system described by Lagerkvist et al. and Weitkamp et al. (16, 44). Selected B cells were diluted to 1 cell/10 μl and plated in 96-well culture plates containing 5.0×10^4 irradiated EL-4-B5 mouse thymoma cells (kindly provided by R. H. Zubler) in complete RPMI medium. After sorting, the feeder cell media was supplemented with 100 U of recombinant human interleukin-2, 5 ng of phorbol myristate acetate/ml, and 10% T-cell replacing factor (supernatant from pokeweed mitogen activated human T cells). The B cells were incubated for 7 days at 37°C in an atmosphere of 5% CO_2. After 7 days, 100 μl of supernatant was removed, and 1.0×10^4 irradiated CD40L transfected fibroblastic L cells (kindly provided by Y.-J. Liu) were added with the above media with the addition of 5 ng of recombinant human interleukin-4/ml. The B cells were cultured for a total of 3 weeks with the addition of the CD40L L cells again at day 14. B-cell colonies were tested by ELISA for the ability to secrete immunoglobulin on days 14 and 21. Nunc-Maxisorp (Nalge Nunc International, Rochester, NY) plates were coated with goat anti-human immunoglobulin (H+L; Southern Biotechnology Associates, Birmingham, AL) and blocked prior to the addition of B-cell culture supernatants. Captured immunoglobulin was detected by using goat anti-human immunoglobulin (H+L) AP (Southern Biotechnology Associates). A serial dilution of purified human immunoglobulin (Biodesign International, Kennebunk, ME) ranging from 1 ng/ml to 100 μg/ml was included as a standard control on each plate, along with supernatants from wells containing no B cells (negative control) or pooled selected B cells (positive control). All B-cell colonies found to be secreting immunoglobulin on day 14 were tested for specific anti-PPS14 antibody production on day 21 by using the standard pneumococcal ELISA method (10). Nunc-Maxisorp plates were coated with PPS14, PPS4, or PPS23F and then washed and blocked. Specific antibody detection was achieved by using the AmpliQ (DakoCytomation, Ltd., Cambridgeshire, United Kingdom) amplifying detection system according to the manufacturer's specifications. Supernatants from wells containing no B cells were included as negative controls, and supernatants from wells containing pooled selected B cells were included as positive controls. We performed the expansion experiments twice, with similar results on each occasion.

Production of heavy-chain libraries. The cDNA obtained from PPS-selected B cells was used as a template in the PCR to generate heavy-chain libraries. The primer sets used to generate heavy-chain products were as described by Welschof et al. (46). First-round PCR primers were complementary to the 5′ untranslated region of immunoglobulins and 3′ IgG constant region. Second-round PCR primers were complementary to the 5′ end of the V_H gene and 3′ JH chain. PCR amplification conditions consisted of 32 cycles of 94°C for 45 s, 65°C for 30 s, and 72°C for 45 s. PCR controls consisted of cDNA obtained from unselected B cells, to confirm that all gene families were being amplified consistently, determined by gel electrophoresis. Amplification products were purified by using the GeneClean gel extraction kit (Bio 101, La Jolla, CA) and ligated into the Zero Blunt Cloning Vector system (Invitrogen, San Diego, CA). The ligated plasmids were transformed into Top10 *Escherichia coli* by chemical transformation. Heavy-chain libraries were plated on Luria broth (LB)-kanamycin at low density and grown overnight at 37°C.

Selection and sequence analysis. Individual *E. coli* clones were selected and streaked onto a LB-kanamycin master plate and grown overnight at 37°C. These clones were lifted onto nylon filters and fixed by UV exposure for 5 min. Nylon filters were blocked in prehybridization buffer (7% sodium dodecyl sulfate, 0.272 Na_2HPO_4 [pH 7.2], 1% bovine serum albumin) for at least 1 h at 65°C prior to hybridization overnight at 65°C to a [^{32}P]ATP-labeled probe with specificity for the framework III region (46). Filters were washed twice with low-stringency wash buffer (0.1% sodium dodecyl sulfate and 1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) at 55°C for 3 min each time. Sequence analysis was performed on selected clones (MWG Biotech, High Point, NC) with primers complementary to the vector. The resultant sequences were compared to germ line sequences by using VBASE DNAPLOT (http://vbase.mrc-cpe.cam.ac.uk).

Statistical analysis. The percent gene usages of heavy chain against PPS4 and PPS14 were calculated for each age group and each volunteer. The Fisher exact test and Pearson chi-square value were used to determine significant differences in gene usage and oligoclonality; the Student *t* test was used to detect differences in mutational frequencies between age groups. A P value of ≤ 0.05 was considered significant. Statistical calculations were performed with SPSS 11.5.1.

RESULTS

Validation of selection method. We have performed studies to validate the B-cell isolation technique with PPS14-coated paramagnetic beads, by culturing selected cells in vitro. It should be emphasized that extensive negative selection was performed on isolated B cells. B cells that bound to uncoated immunomagnetic beads, streptavidin, CWPS, or cross-reactive

contaminants present in the PPS preparations were depleted by incubation with PPS23F-labeled immunomagnetic beads, followed by incubation with PPS4-labeled immunomagnetic beads. Finally, any remaining binding to CWPS was blocked by the inclusion of free (unlabeled) CWPS in the positive selection step with the PPS14-coated beads. To confirm the PPS14 specificity of the selected B cells, we cultured single B cells as described in Materials and Methods. B-cell culture supernatants were tested for total immunoglobulin production at days 14 and 21 and for anti-PPS14 production at day 21. At day 14, 44 of 576 single B-cell colonies were producing immunoglobulin. By day 21, 14 single B-cell colonies were producing immunoglobulin. All single B-cell clones that were still producing immunoglobulin at day 21 were secreting immunoglobulin specific for PPS14 and failed to bind to PPS4 or PPS23F. B cells selected and cultured on two separate occasions demonstrated similar results. The efficiency of this B-cell expansion system, indicating successful in vitro expansion of ca. 3% of single B cells, was consistent with previous work by Weitkamp et al. (43). Since all successfully cultured B cells produced anti-PPS14 antibody, these results suggest that our selection method accurately selects B cells expressing anti-PPS specific antibody.

Heavy chain analysis. PPS4- and PPS14-specific lymphocytes were obtained from 20 young (11 female, 9 male) and 20 elderly volunteers (10 female, 10 male). A total of 364 heavy chains with specificity for PPS4 were obtained from 15 immunized young adults and 325 heavy chains from 15 immunized elderly adults. In response to PPS14, 305 heavy chains were obtained from 19 young adults and 291 from 14 elderly adults. The V_H3 gene family comprised >90% of the total sequences isolated in response to PPS4 and PPS14 in both age groups. The V_H1, V_H4, and V_H5 gene families were isolated from both young and elderly in response to both polysaccharides; however, these gene families constituted <10% of the total heavy-chain repertoire. The V_H4 and V_H5 gene families were represented in numbers too small to allow for meaningful analysis. Variable heavy gene, complementarity-determining region 3 (CDR3) length/composition, D gene, J chain, percent identity to germ line sequence, and somatic mutation frequencies were all determined on all isolated sequences.

Heavy chain usage in response to PPS4 and PPS14. (i) Young adults. As shown in Fig. 1, the most commonly used heavy-chain gene loci in response to PPS4 were in the following order of usage: V_H3-07, V_H3-74, and V_H3-30, representing 50.9% of total variable heavy-chain gene usage in young adults. In all, 80 sequences were identified that utilized V_H3-07, 73 sequences utilized V_H3-74, and 32 sequences that utilized V_H3-30. Other V_H3 family genes were isolated with variations in percent usage between volunteers. Members of the V_H1 gene family were also used and represented 7.8% of the total V_H sequences isolated. The most common gene families expressed in response to PPS4, V_H3-07 (22%) and V_H3-74 (20%), were significantly overexpressed compared to gene locus expression found in unselected $CD19^+$ peripheral B cells: 4.2% and 1.4%, respectively (5).

In response to PPS14, V_H3-48 and V_H3-33 gene loci in order of use were the dominant heavy chains used and represented 48.4% of isolated sequences (Fig. 1). Other V_H3 gene loci, namely, V_H3-11, V_H3-21, and V_H3-30, accounted for 38.5% of the heavy-chain sequences obtained from young adults. The total numbers of sequences identified were 99, 49, 25, 15, 6, and 2 for gene families V_H3-48, V_H3-33, V_H3-30, V_H3-21, V_H-11 and V_H3-07, respectively. The V_H1 gene family was expressed in 6.3% of total sequences obtained from young adults. The most common gene families expressed in response to PPS14, V_H3-48 (32.4%) and V_H3-33 (16%), were significantly overexpressed compared to gene locus expression found in unselected $CD19^+$ peripheral B cells: 1.4 and 2.8%, respectively (5). A similar pattern of gene locus usage was maintained when the gene usage of individual donors was investigated (Table 1).

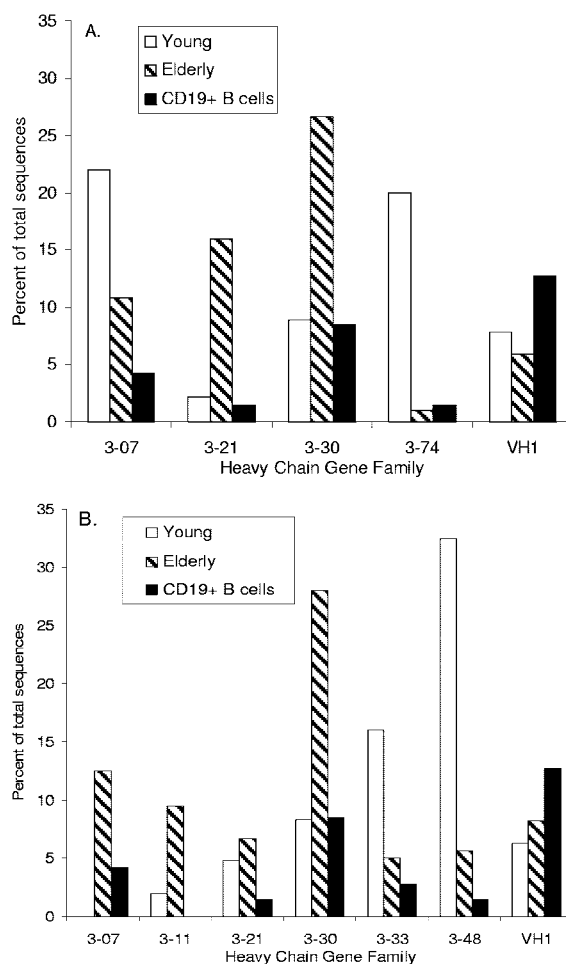

FIG. 1. Gene family and locus distribution in response to PPS4 (A) and PPS14 (B) in young and elderly donors compared to circulating $CD19^+$ B cells taken from Brezinschek et al. (5). Statistical differences between young and elderly gene expression were calculated by using the Pearson chi-square test.

Oligoclonality is defined as a response limited to one or few gene loci, with one gene locus representing at least 50% of the isolated sequences. Overall, most of the young donors demonstrated an oligoclonal response in response to PPS4 (87%) and PPS14 (84%).

(ii) Elderly adults. In elderly adults, the predominant heavy-chain gene usage in response to PPS4 and PPS14 was V_H3-30, representing 26.7 and 28%, respectively (Fig. 1) corresponding

TABLE 1. Response to PPS4 by genes from young and elderly adults[a]

Young PPS4				Elderly PPS4			
Donor	Most common locus (%)	Second most common locus (%)	Avidity	Donor	Most common locus (%)	Second most common locus (%)	Avidity
2	3-07 (77)	3-09 (20)	Medium	23	3-30 (32)	3-11 (11)	Medium
3	3-07 (62)	1-69 (38)	High	25	3-30 (30)	3-20 (15)	Medium
9	3-07 (50)	3-13 (50)	Medium	26	3-30 (41)	3-21 (14)	High
1	3-74 (57)	3-48 (25)	High	28	3-30 (33)	3-11 (25)	Low
11	3-74 (100)		High	29	3-30 (50)	3-07 (13)	Medium
14	3-74 (23)	3-30.3 (23)	High	33	3-30 (35)	3-53 (33)	Medium
8	3-11 (70)	3-07 (27)	High	38	3-30 (80)	3-11 (13)	High
17	3-11 (33)	3-49 (17)	Medium	24	3-21 (100)		High
20	3-11 (64)	3-48 (33)	Low	27	3-21 (45)	3-49 (38)	High
4	3-30 (75)	3-23 (25)	Medium	39	3-21 (54)	3-30 (23)	High
13	3-30 (82)	3-21 (9)	Low	21	3-09 (100)		High
19	3-15 (60)	3-74 (20)	High	37	3-09 (83)	3-48 (17)	High
16	3-23 (83)	1-69 (6)	Medium	22	3-23 (83)	3-49 (14)	Low
10	3-48 (83)		High	36	3-23 (100)		Low
15	3-72 (94)	3-21 (6)	High	32	3-07 (56)	5-51 (44)	High
5	NA	NA	Medium	31	1-69 (100)		Medium
6	NA	NA	Low	30	NA	NA	High
7	NA	NA	NA	34	NA	NA	Medium
12	NA	NA	High	35	NA	NA	High
18	NA	NA	High				

[a] Genes from young and elderly volunteers isolated with PPS4, the predominant locus, the percent representation within clones from an individual donor, and the second most common locus are shown. Avidity is the molarity of NaSCN necessary to inhibit 50% of the antibody binding: high, >0.5 M; low, <0.3 M; and medium, 0.3 to 0.5 M.

to 87 and 81 identified sequences. In response to PPS4, V_H3-21 and V_H3-07 were also commonly expressed at 16 and 10.8%, corresponding to 52 and 35 sequences, respectively, with the V_H1 gene family representing 5.9% of heavy-chain gene usage. The variable heavy-chain loci V_H3-07 and V_H3-11 were commonly used in response to PPS14 by the elderly and were identified in 36 and 27 of the isolated sequences, respectively. The V_H1 gene family represented a small percentage (8.2%) of the total heavy-chain sequences isolated in the elderly. In the elderly population the V_H3-30 gene locus was overexpressed in response to both PPS4 and PPS14 (26.7 and 28%, respectively) compared to unselected $CD19^+$ B cells (8.5%) (5). A similar pattern of gene locus usage was maintained when the gene usage of individual donors was investigated (Table 2). Overall, 63% of the elderly donors demonstrated oligoclonality in response to PPS4, whereas 60% of elderly donors showed oligoclonality in response to PPS14.

Heavy chain usage in response to PPS4: comparison of age groups. Comparison of young and elderly revealed significant differences in heavy chain gene usage. Both V_H3-07 ($P < 0.01$) and V_H3-74 ($P < 0.01$) were more frequently expressed in the young compared to the elderly (Fig. 1). In contrast, the V_H3-21 and V_H3-30 genes were significantly more common in elderly than young individuals ($P < 0.01$). No significant difference was found in V_H1 gene family expression between age groups.

When the immune response was investigated at the donor level (Tables 1 and 2), it was found that significantly more young donors ($P < 0.05$) utilized one predominant gene locus (87%) compared to the elderly (63%).

Heavy chain usage in response to PPS14: comparison of age groups. As shown in Fig. 1, with aging, a significant shift in gene usage occurred with decreased expression of V_H3-48 and V_H3-33. Elderly adults demonstrated a significant increase in V_H3-30, V_H3-11, and V_H3-07 heavy chain gene usage compared to young adults ($P < 0.01$ for all). Despite the predominance of the V_H3-30 locus in response to both PPS4 and PPS14 in the elderly, no identical CDR3 sequences were isolated between these polysaccharides (Tables 1 and 2). A predominant (>50%) gene locus was expressed in 84% of young adults compared to 60% of elderly adults ($P < 0.05$).

CDR3 length and composition and D gene. The CDR3 length of the isolated sequences from the diverse array of heavy chain genes ranged from 7 to 24 amino acids in response to both polysaccharides and age groups. To facilitate data comprehension a representative sample of sequences of common heavy chain gene families in response to PPS4 and PPS14 is shown in Tables 3 and 4. These tables present examples of the most commonly expressed genes from both young and elderly and do not contain all isolated sequences for logistical reasons. As shown in Tables 3 and 4, a wide variety of CDR3 sequences were identified in response to both polysaccharides. The mean CDR3 lengths are shown in Table 5. Significant differences in CDR3 length between young and elderly were noted in V_H3-30 in response to PPS4 but not PPS14. Similarly, the CDR3 lengths of the V_H1 gene loci, V_H1-69 and V_H1-02, were significantly shorter in the young versus the elderly ($P < 0.05$).

Although the CDR3 regions were overall diverse, a recurrent motif was noted in the CDR3s associated with the V_H3-07 gene locus consisting of the amino acid motif PNR in response to PPS14. The use of the 3-16 and 3-22 D genes introduced a substantial number of hydrophobic residues in the CDR3 regions associated with the V_H3-30 and V_H3-21 sequences isolated in response to PPS4 and the V_H3-30 and V_H3-07 sequences isolated in response to PPS14. In response to PPS4 (Table 5), there were significant differences ($P < 0.001$ and $P < 0.05$) in the expression of 3-16 and 3-22 D genes between young (33 and 13%, respectively) and elderly (6.8 and 29%,

TABLE 2. Response to PPS14 from young and elderly adults[a]

Young PPS14				Elderly PPS14			
Donor	Most common locus (%)	Second most common locus (%)	Avidity	Donor	Most common locus (%)	Second most common locus (%)	Avidity
2	3-48 (75)	1-18 (25)	High	32	3-30 (50)	3-23 (25)	High
1	3-48 (79)	3-07 (18)	High	28	3-30.3 (63)	3-07 (25)	High
10	3-48 (63)	3-30 (13)	Medium	39	3-30.3 (27)	3-07 (19)	Low
12	3-48 (95)	3-30 (5)	Medium	25	3-20 (86)	3-30 (9)	Medium
9	3-33 (75)	3-30 (25)	Low	26	3-20 (27)	3-21 (27)	High
11	3-33 (100)		Medium	24	3-21 (67)	3-11 (33)	Medium
15	3-33 (63)	3-53 (25)	High	38	3-07 (85)	3-30 (13)	High
4	3-30 (50)	3-21 (17)	Medium	22	3-07 (63)	3-43 (25)	High
13	3-30 (45)	3-33 (18)	Medium	23	3-11 (100)		High
6	3-30 (50)	3-21 (25)	Low	31	3-11 (59)	3-30 (12)	High
19	3-74 (100)		High	29	3-23 (33)	3-11 (33)	High
16	3-74 (71)	3-21 (29)	Medium	36	3-23 (67)	3-30 (33)	High
3	3-13 (67)	3-30 (15)	Medium	21	3-49 (47)	1-18 (27)	High
17	3-13 (38)	3-33 (25)	Medium	37	3-33 (58)	3-30 (25)	Low
8	3-49 (50)	3-30 (17)	High	27	3-66 (33)	3-30 (30)	High
18	3-53 (75)	3-13 (25)	High	30	NA	NA	High
5	3-64 (100)		High	33	NA	NA	High
7	4-39 (100)		NA	34	NA	NA	High
14	1-02 (60)	3-49 (40)	High	35	NA	NA	High
20	NA	NA	Medium				

[a] Genes from young and elderly volunteers isolated with PPS14, the predominant locus, the percent representation within clones from an individual donor, and the second most common locus are shown. Avidity is the molarity of NaSCN necessary to inhibit 50% of the antibody binding: high, >0.5 M; low, <0.3 M; and medium, 0.3 to 0.5 M.

respectively) individuals. In the response to PPS14, these two D genes were also utilized; however, in contrast to PPS4, there was no significant difference in 3-22 D gene or 3-16 D gene usage in elderly (36 and 7.2%, respectively) compared to young (35 and 5.9%, respectively) subjects. Overall, despite identical D gene usage between individuals and polysaccharides, unique CDR3 sequences were observed as a result of the recombination of V, D, and J chains; N insertions; and deletions.

J-chain usage. Analysis of J (joining)-chain sequences in all age groups demonstrated the presence of all known J chains; however, the J_H3 and J_H4 chains were predominant (data not shown). There was a significant difference ($P < 0.05$) in JH4 usage between age groups (68% young and 46% elderly) in response to PPS4 but not in response to PPS14 (63 and 59%, respectively). In contrast, there was no significant difference in JH3 usage between the age groups in response to either PPS4 or PPS14.

Percent identity to germ line and somatic mutation. All heavy chain sequences were analyzed for percent identity to germ line and mutational frequencies in the CDRs and framework regions (FR) as summarized in Table 5. Sample sequences are shown in Tables 3 and 4. Overall, the heavy chain sequences were 87.3 to 100% identical to germ line sequences. In response to PPS4, the predominant heavy chain gene in young adults (locus V_H3-74) was highly mutated (90.8%) compared to germ line (Tables 3 and 4). Although only three V_H3-74 sequences were isolated from the elderly these sequences were 99.3% identical to germ line. Mutational frequencies in locus V_H3-74 were significantly higher ($P < 0.05$) in young compared to elderly subjects in both the FR and CDRs. The predominant heavy-chain genes in the elderly, V_H3-30 and V_H3-21, demonstrated >98% identity to germ line. The mutational frequencies in heavy chain gene families V_H3-30, V_H1-69, and V_H1-02, however, were similar between age groups.

In response to PPS14, both age groups showed >95% identity to the germ line in the most commonly used heavy chain genes, V_H3-48 and V_H3-33, for the young, and V_H3-30, for the elderly. As shown in Table 4, both age groups demonstrated similar mutational frequencies in V_H1-69, V_H1-18, and V_H1-02. There was also no significant difference in mutational frequency between young and elderly in the V_H3-30 gene products, the predominant sequence expressed in the elderly. The G-to-A substitution present in the CDR1 of V_H3-30 in both young and elderly adults is a result of allelic variation. Of the 19 identified V_H3-30 alleles in the IMGT gene database (11), 11 express an adenosine at this position and 8 express a guanidine; thus, this has not been considered a mutation in our calculations. Overall, the V_H3 gene locus is polymorphic (36), with heterogeneity resulting from the diversity of haplotypes rather than from allelic variation. In young adults, the predominant heavy chain gene (locus V_H3-48) demonstrated significantly higher ($P < 0.01$) mutational frequency in the CDRs compared to elderly expressed V_H3-48. It should be noted that this was not the case for the V_H3-33 gene products (Tables 3 and 4). However, the elderly showed a significantly higher ($P < 0.05$) mutational frequency in the CDRs in V_H3-11 and V_H3-21 genes compared to young subjects. Overall, the young group showed a greater mutational frequency in CDR1 and CDR2 compared to the elderly in the response to both PPS4 and PPS14; however, there was no significant difference between the young and elderly overall. Within all gene families, mutations were more pronounced in the CDRs than in the FRs in all age groups and in response to both polysaccharides. Analysis of mutational hotspots (RGYW) demonstrated that mutation rates within the hotspots were V_H locus dependent;

TABLE 3. CDR residues and gene usage of PPS4-specific B cells from young and elderly adults

Gene locus	Age group and donor	Sequence of H chain			Accession no.
		CDR1	CDR2	CDR3	
V_H3 3-07		SYWMS	NIKQDGSEKYYVDSVKG		
	Young				
	1	-----	-----------------	CAKPVDRELLGPDNAFDI	AY909729
	2	-----	-----------------	CASDLSTDY	AY909727
	2	--Y--	-----------------	CVRDLGELQEYYFDY	AY909725
	3	RI--T	---E-----F-------	CARAPYCSSTSCPYY	AY909726
	8	N-G-R	S-K-----QF-------	CARVGGGMDV	AY909730
	9	-----	-----------------	CARARIPESHAFDI	AY909728
	14	F----	--NE---Q-S--N----	CARSNYDMR	AY909733
	14	F----	T--PG--AED-VG--R-	CARLDWFKPDY	AY909732
	16	--S--	-------KT--------	CARDGMTTGDRGSSSLPYYFDF	AY909734
	Elderly				
	22	-----	-----------------	CARFGDNWNDWVVAFDI	AY909616
	22	-----	---P-----FS-G----	CARGDYSDSGGSFIEAFDI	AY909617
	26	-----	-----------------	CARDRTYRWVMGAFDI	AY909612
	26	--A-H	-----------------	CARDDYYYVGAFDI	AY909611
	28	N----	S---G-I-------LR-	CARDIADYYYTRDDAFDI	AY909613
	28	-----	-----------------	CAKPVDRELLGPDNAFDI	AY909614
	32	Y----	T-NE---KRS-------	CVRSVARSIDS	AY909615
V_H3 3-30		SYGMH	VISYDGSNKYYADSVKG		
	Young				
	1	-----	I--S---DE------E-	CARARSSYCTSTSCYSSAFDI	AY909645
	1	-----	-----------------	CAKDQGIVVVILYAFDI	AY909646
	3	--A--	-----------------	CARAQKWNYDSSGYYYLDAFDI	AY909642
	4	-----	-----------------	CAGMISTAFDI	AY909638
	8	--A--	-----------------	CARDTAPLYDSSGYYYNDAFDI	AY909647
	9	--A--	-----------------	CARDPTSSSYYDFWSGY	AY909644
	12	--A--	-----------------	CAREKGTITEYSSLGAFDI	AY909639
	12	--A--	-----------------	CARRYYDILTGYYEPRTDAFDI	AY909643
	13	-----	-----------------	CAKDNYDSSGYYYSYWKTDAFDI	AY909648
	13	-L---	-----------------	CAKDLWKYYDSSGSFSAFDI	AY909640
	13	--A--	-----------------	CARDYSSGYSDAFDI	AY909641
	14	-----	F-R--------------	CAKVGYSSGWPTLESDY	AY909649
	17	--A--	-----------------	CARDSSRLRLGELPTKGAFDI	AY909650
	Elderly				
	23	RH---	-V-AA-ITT--------	CARDPAHVRGSYDSSGYYPDAFDI	AY909622
	23	RH---	-V-AA-ITT--------	CARDLHYYDSSGLLLGYEKPYSLGFDP	AY909625
	23	--A--	-----------------	CARAYYGSGNDY	AY909623
	23	--A--	-----------------	CARGPYYYDSSGYTY	AY909624
	25	-----	-----------------	CARVYRSSTDAFDI	AY909632
	25	-----	F-R--------------	CAKSTYSSGWPQDY	AY909627
	25	--A--	-----------------	CAKGQRVAYNGAFDI	AY909633
	25	--A--	-----------------	CARDFEAYYFDWLLPTYDAFDI	AY909634
	26	-----	-----------------	CAKDTAMATYDAFDI	AY909629
	26	-----	-----------------	CARVRGYSYGIVYYYYGMDV	AY909618
	26	-----	-----------------	CAKPSHEYSSSSGLGAFDI	AY909620
	26	-----	-----------------	CAKLYYYDSSGYWGTAFDI	AY909621
	26	-----	-----------------	CAKNLPIYDFWSGHYGAFDI	AY909637
	26	--A--	-----------------	CARDKPLYSSGWFKSGYYFDY	AY909631
	26	--A--	-----------------	CARDEVYYDFWSGYYSDY	AY909619
	26	--A--	-----------------	CAREVQDIVVVPAAIYYYYYGMDV	AY909636
	28	-----	-M-F-------------	CAKDPGGADSSGYSYGFDI	AY909630
	38	-----	-----------------	CARDSDSSGWEPLDC	AY909626
	39	-----	F-R--------------	CAKDRTLYCSSTNCYENWFDP	AY909635
	39	--A--	-----------------	CARVPPDYYDSSGFAFDI	AY909628
V_H3 3-74		SYWMH	RINSDGSSTSYADSVKG		
	Young				
	1	NY-I-	---G-----G-------	CTRGESGYGRFDP	AY909657
	3	T----	--K--------------	CARGGGTSDY	AY909654
	3	T----	--K--------------	CARGVVPCPQESLRDPPEFQHN	AY909651

Continued on facing page

TABLE 3—*Continued*

Gene locus	Age group and donor	Sequence of H chain			Accession no.
		CDR1	CDR2	CDR3	
	3	T----	--K--------------	CARGGGPLPPGESQGPPEFCRYPSH	AY909655
	11	T----	Q--N---D-K-------	CTRDCTRDCCTKPYDL	AY909658
	14	-----	-----------------	CASLMGEKVLFKEGATDWYFDL	AY909659
	14	TS---	-L-G-----AS------	CATSTDYCDSATCYTWFAS	AY909660
	16	G-G--	-----E-VKV-------	CARENRDGYDFDS	AY909661
	18	T----	--K--------------	CARDLVAAGNY	AY909652
	19	-----	----G-T-IT-------	CARGGGYSYDYFDY	AY909653
	20	-----	-----------------	CARDHPYYDFWSGYYPGPLDP	AY909656
V_H3 3-21		SYSMN	SISSSSSYIYYADSVKG		
	Elderly				
	22	-----	-----------------	CAREGHYYDSSGYYSFDY	AY909663
	23	-----	-----------------	CARDSKGGNSDLDP	AY909669
	24	-----	-----------------	CATLPQYYDSSGYQILSFDI	AY909665
	25	-----	-----------------	CARAAVAGTQYYFDY	AY909664
	25	-----	-----------------	CARDSTSTYDFWSGYYTSDAFDI	AY909662
	26	-----	-----------------	CARVSGYTDY	AY909668
	26	-----	-----------------	CARDITMPTTI	AY909667
	27	-----	-----------------	CARDGGRDGYNYGAFDI	AY909670
	39	-----	-----------------	CARVGIVGAKGPP	AY909666
	39	-----	-----------------	CARDWYDILTGWPLQAFDI	AY909671

however, there was no significant difference in mutational frequency within these regions between young and elderly.

Relationship of serum avidity to gene family usage. We have previously analyzed the PPS-specific antibody concentrations, opsonophagocytic activity, and antibody avidity in the response to PPS4 and PPS14 in all study participants (15). Given the wide variability in gene locus usage between and within individuals, a significant association between the usage of a specific gene locus and measures of serum activity, avidity, and opsonophagocytosis could not be defined. This was especially true for opsonophagocytosis, where activity is multifactorial and related to antibody concentration, isotype, and avidity. However, when we compared the dominant utilization of specific gene loci with avidity, as defined by the molarity of sodium thiocyanate required to elute 50% of bound antibody (29) on an individual level (shown in Tables 1 and 2), specific associations were apparent.

In response to PPS4, usage of the locus V_H3-74 was associated with high avidity, and usage of the locus 3-07 was associated with medium to high avidity. In the young response to PPS14 exclusive expression of V_H3-33 was associated with moderately high avidity. However, the combination of V_H3-33 (63%) with V_H3-53 (25%) resulted in low-avidity measurements in serum. Similarly, exclusive expression of V_H3-74 (100%) resulted in high avidity, whereas in combination with V_H3-21 (29%) it resulted in moderate avidity measurements. Exclusive expression of V_H3-64 was associated with high avidity in one individual.

In the elderly response to PPS4, two gene loci, V_H3-09 and V_H3-21, were clearly associated with high avidity. One gene locus, V_H3-23, was associated with low-avidity serum measurements. In the elderly response to PPS14 two gene families, V_H3-11 and V_H3-07 were consistently associated with high-avidity serum measurements. In contrast, the most common gene locus isolated from elderly, V_H3-30, was associated with multiple other gene loci and was therefore associated with low to high avidity serum measurements to both PPS4 and PPS14.

DISCUSSION

The molecular mechanisms responsible for decreased vaccine efficacy in the aged remain poorly understood, particularly in response to bacterial polysaccharides. Advances in molecular biology have enabled studies to define the molecular characteristics of the human immune response. However, techniques within this area of research are still under development, and thus there are few data available. Initial studies examining the structure-function relationship of antipneumococcal antibodies have provided valuable information concerning the human immune response to PPS (3, 19, 20, 37, 48–50). These studies have been performed with human monoclonal antibodies (3, 37, 48), combinatorial libraries (20, 49, 50), and monoclonal antibodies derived from a transgenic mouse strain reconstituted with human immunoglobulin loci (7, 35). However, these studies were not designed to investigate the in vivo antibody repertoire to PPS in all responders or in the elderly population. The majority of these studies used B cells derived solely from high responding young adult donors and thus do not reflect variable heavy and light chain gene usage of low or intermediate responding donors. Moreover, studies characterizing the in vivo variable gene repertoire in response to PPSs in the population at large have not been performed. Further investigation of the in vivo immune response in human adults, specifically the elderly population, is necessary to achieve a better understanding of variable gene usage in response to PPSs.

TABLE 4. CDR residues and gene usage of PPS14-specific B cells from young and elderly adults

Gene locus	Age group and donor	Sequence of H chain			Accession no.
		CDR1	CDR2	CDR3	
V_{H}3 3-48		SYSMN	YISSSSSTIYYADSVKG		
	Young				
	1	--E--	-----G-----------	CARDRTYCGGDCYGPYFDY	AY909680
	1	--E--	-----G-N---------	CARYGSENYYNLFYFDS	AY609681
	2	T----	---P-----H-------	CVRDLGELQEYYFDY	AY909679
	2	T----	---P-----H-------	CSRYRVRWSYSSGPDRMDV	AY909673
	8	-----	-----------------	CARDWDIVVVPAARPSAFDI	AY609682
	10	T----	---T--TA---------	CARTMIIFGGVYDIGNDY	AY909676
	10	-HN-H	----G----------R-	CAKHCTNGVCSDY	AY909672
	10	NAW-S	----G----------R-	CARGEAGVRGSYYRAFDI	AY909675
	17	--G-H	-----------------	CAKNNQRGYSYGWQLN	AY909674
	20	--E--	-----G-----------	CARQYYDFWSGYYTGHYYYYYMDV	AY909677
	20	--E--	---G-G-A---------	CARDLAVPRWYFDL	AY909678
V_{H}3 3-33		SYGMH	VIWYDGSNKYYADSVKG		
	Young				
	1	-----	-----------------	CARGKYSGSYYFDY	AY909686
	9	-----	-----------------	CARDHPGNSP	AY909685
	10	-----	-----------------	CARRAFDI	AY909683
	11	-----	-----------------	CARDYYYDSSGYYSAVQAPDDPAFDI	AY909687
	11	-----	-----------------	CARDGEGSILTYYYDSSGYYSPRSAFDI	AY909688
	13	-----	-----------------	CAMNYYDSSGYPNAFDI	AY909690
	17	-----	-----------------	CARERGTKWELLTRGAFDI	AY909689
	20	-----	-----------------	CARVSSQYSSGLSYYFDY	AY909684
V_{H}3 3-30		SYGMH	VISYDGSNKYYADSVKG		
	Elderly				
	25	-----	-----------------	CAKLADHYYGSGSYRAFDI	AY909694
	25	-----	F-R--------------	CAKDTKTSVQRLIGYYMDV	AY909701
	25	N----	F-RH---YE--------	CAKDPGEVEPGYYMDV	AY909702
	26	-----	-----------------	CAKGEWELLWAPAGAFDI	AY909705
	27	-----	F-R--------------	CAKSGDTAMVTFNWFDP	AY909707
	27	--A--	-----------------	CARGGNTAMDSDAFDI	AY909692
	27	--A--	-----------------	CARDPIGRYCSSTSCSNWFDP	AY909693
	28	--A--	F--F----Y--------	CARTMIIFGGVYDIGNDY	AY909697
	28	--A--	F--F----Y--------	CARVSVRGGGRPYYYYNMDV	AY909696
	32	-----	-----------------	CAKDNYDSSGYYYSYWKTDAFDI	AY909699
	32	-----	----------L------	CAKDPSGAFWSGYLKLNYFDS	AY909698
	37	--A--	-----------------	CARAQKWNYDSSGYYYLDAFDI	AY909706
	38	---A-	---H-------------	CARDQGSGFDDY	AY909700
	38	---A-	---H-------------	CAKDWTHSGTLSGYFDH	AY909691
	39	-----	F-R--------------	CAKVVWLQQLAHLAAFDI	AY909703
	39	--A--	-----------------	CARDILPLYDYVWGSYRYKSPFNAFDI	AY909704
	39	--A--	-----------------	CARESSGYYDSSGYLLPSYYFDY	AY909695
V_{H}3 3-07		SYWMS	NIKQDGSEKYYVDSVKG		
	Elderly				
	22	-----	---Y------C------	CARGDYYDSGGSFIDAFDI	AY909710
	22	-----	-----------------	CARIRQFAHRVVPAAIPESDAFDI	AY909711
	27	R----	---------H----M--	CARLIYYDSSGYSAGFDP	AY909716
	28	-S---	---E-------------	CARRCLGGSCYPGY	AY909709
	28	D-S-N	-----------------	CARMPDGDYTEFYFDY	AY909719
	38	T----	---H----N-C------	CARLPNRVDKFGFDI	AY909708
	38	T----	---H----N-C------	CVRLPNRLDKFGFDL	AY909717
	38	D----	-MREE-A-E--A-----	CARLPNRVDKFGFDI	AY909712
	39	-----	-----------------	CAIEYSSSSVFDY	AY909713
	39	-----	-----------------	CARENDGSGSYPDY	AY909714
	39	-----	-----------------	CAREGGAVAGAFDI	AY909715
	39	-----	-----------------	CARDEGDYYDSSGYYYNWFDP	AY909718
V_{H}3 3-11		DYYMS	YISSSGSTIYYADSVKG		
	Elderly				
	23	-----	-----S-YTN-------	CARDLSGMDV	AY909724
	23	-----	---DG--VQF-------	CARDSESYVKGSMDV	AY909723
	24	-----	-----------------	CARALVSEQQIVVKAFDI	AY909720
	26	-----	----GSI----------	CARPTGVGPINAFDI	AY909721
	31	-----	----TS-----------	CARVGRYFDQRRKGGFFDY	AY909722

TABLE 5. Comparison of selected V_H loci[a]

PPS locus	*n*	% ID	Geometric mean mutations (both R and S)/sequence (R/S ratio)					CDR3 length
			FR1	FR2	FR3	CDR1	CDR2	
PPS4								
Y 3-74	74	90.8	1.1 (2.4)	3.6 (0.6)	5.5 (1.5)	10 (3.0)	9.8 (1.1)	9.6
E 3-74	3	99.3	0.3 (0 no R)	0 (0)	0.2 (0 no R)	0.1 (0 no S)	0.05 (0 no S)	10.3
Y 3-07	80	98.2	0.6 (0.5)	0.8 (0 no S)	1.4 (0.4)	2.2 (1.5)	1.9 (2.0)	11.9
E 3-07	34	98.6	0.6 (1.3)	0.7 (0 no R)	0.8 (1.1)	1.1 (0.3)	0.9 (0 no S)	12.7
Y 3-30	32	98.5	0.7 (0.6)	0.9 (0.7)	2.1 (3.0)	1.8 (0 no S)	1.6 (0.4)	13.8
E 3-30	86	98.4	0.7 (0.7)	0.2 (0.7)	0.8 (0 no R)	1.3 (0 no S)	1.6 (3.2)	15.8
Y 3-21	8	98.9	1.5 (3.5)	0.3 (0 no S)	0.2 (0 no S)	0.8 (0 no S)	0.3 (0 no S)	12.8
E 3-21	52	99.4	0.3 (3.0)	0.04 (0 no S)	0.4 (0.4)	0.1 (0 no S)	0.04 (0 no R)	13.2
Y 1-69	13	99.7	0 (0)	0 (0)	0.3 (0 no R)	1.5 (0.9)	1.0 (1.2)	12.5
E 1-69	7	99.6	0.3 (0 no R)	0.68 (1.0)	0.44 (0 no S)	1.9 (1.6)	0.3 (0 no S)	16.1
Y 1-02	4	99.0	0.8 (0 no R)	0 (0)	0.6 (1.0)	1.2 (0 no S)	1.3 (4.0)	16.3
E 1-02	5	99.2	0.4 (1.0)	1.4 (0.75)	0.6 (0 no R)	1.3 (2.0)	0.8 (2.0)	20.2
Y 4-34	5	99.3	0 (0)	2.3 (1.75)	0 (0)	0 (0)	0 (0)	20.0
PPS14								
Y 3-48	86	95.4	0.8 (2.1)	1.8 (1.0)	3.1 (0.45)	11.7 (3.25)	4.4 (4.5)	16.3
E 3-48	16	98.4	0.9 (1.3)	0.1 (0 no R)	1.5 (4.25)	0.7 (0 no S)	1.8 (1.0)	17.2
Y 3-33	49	98.7	0.3 (0.7)	0.8 (0.3)	1.1 (0.5)	0.9 (0 no R)	0.9 (0 no R)	15.1
E 3-33	15	99.6	0.5 (1.0)	0.1 (0.5)	0.15 (0 no R)	0.15 (0 no S)	0.4 (3.5)	19.1
Y 3-30	21	99.1	0.5 (3.0)	0 (0)	0.5 (4.25)	0.1 (0 no S)	0.05 (1.0)	15.3
E 3-30	81	98.1	0.4 (2.2)	0 (0)	0.9 (1.0)	0.2 (1.0)	0.2 (3.5)	15.1
Y 3-11	5	98.8	0 (0)	0.6 (0 no R)	0.6 (1.7)	0.2 (0 no S)	0.2 (0 no R)	16.8
E 3-11	27	97.9	0.4 (0.25)	2.4 (0.5)	1.0 (0.6)	4.4 (1.0)	4.6 (7.0)	14.8
Y 3-21	9	99.2	0.2 (0 no R)	0.3 (0 no R)	0.2 (0.5)	0.4 (3.0)	0.5 (1.0)	17
E 3-21	19	96.4	1.2 (0.5)	0.9 (0.9)	1.1 (2.0)	3.1 (1.9)	5.2 (1.25)	16.6
Y 3-07	2	96.8	1.5 (1.0)	3 (2.0)	4.5 (0.7)	8 (1.7)	0 (0)	11
E 3-07	36	97.1	0.95 (0.7)	0.05 (0 no S)	1.3 (2.1)	0.6 (7.0)	0.9 (5.0)	13.9
Y 1-69	3	99.4	0 (0)	0 (0)	0.35 (0 no S)	4.4 (1.6)	0.65 (1.0)	17.3
E-1-69	2	97	0 (0)	0 (0)	0.5 (0 no R)	6.7 (1.2)	1.9 (0 no S)	17
Y 1-18	4	100	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	11
E 1-18	12	99.5	0.3 (2.0)	0.4 (1.5)	0.3 (0 no S)	1.7 (1.2)	0.65 (1.2)	16.5
Y 1-02	9	99.5	0.5 (1.0)	1.0 (2.0)	0.45 (0 no R)	2.9 (1.1)	0.6 (3.5)	14.6
E 1-02	8	99.7	0.7 (0 no R)	0.9 (2.5)	1.0 (0.6)	3.3 (1.6)	1.0 (3.0)	16.6
Y 4-39	10	87.3	2.6 (0.4)	4.7 (0.6)	7.3 (0.8)	14.3 (1.3)	8.3 (1.5)	11

[a] Selected V_H gene loci isolated from young (Y) and elderly (E) adults in response to PPS4 and PPS14 were evaluated; all available sequences within a particular gene family were analyzed. The number of sequences (*n*), the percent identity to the germ line (% ID), the mutational frequencies (i.e., the average number of mutations) in frameworks (FR) and complementarity-determining regions (CDR), and the average CDR3 lengths are shown. Overall, no significant differences between young and elderly were found when values were compared by unpaired two-tailed Student *t* test.

The ages of our volunteers, the group sizes, the numbers of sequences analyzed, and the previously reported immunological studies make the present study unique. We examined and characterized the IgG response to PPS4 and PPS14 in 20 elderly subjects and in 20 young adults. It has been clearly shown that somatic mutations continue to accumulate until at least day 42 postvaccination in response to T-dependent antigens (38). Therefore, isolation of specific B cells was performed at 6 weeks postimmunization in an effort to capture the mature immune response in vaccine-naive and potentially previously unexposed volunteers. The preimmune sera of several young volunteers lacked PPS-specific antibodies. These individuals were considered PPS naive, and their immune response was considered a primary antibody response, justifying the chosen time point of B-cell isolation. It should be recognized, however, that most adults have been preexposed to PPS or cross-reactive antigen, as demonstrated by the presence of extensive somatic mutation in V_H repertoire isolated 7 days postimmunization (20, 49, 50). Thus, in most cases, the V_H analysis of polysaccharide-specific B cells in fact represents a secondary response or expansion of preexisting immunity.

Our study was specifically designed to address potential changes in heavy chain gene usage and mutational frequency as a function of age in the response to PPS4 and PPS14. We therefore generated a V_H library using peripheral blood lymphocytes from vaccinated individuals enriched for PPS4 and PPS14-specific B cells with the use of PPS-coated paramagnetic beads. Non-PPS specific, CWPS, and other cell-wall contaminant-specific B cells were eliminated through an extensive selection process. A V_H library was generated for each individual from a pool of their selected B cells. The major caveat of our study is thus related to the method used in generating V_H libraries. Through this process, V_H/V_L pairing was lost and, more importantly, we were not able to verify PPS-specificity after the selection process. We therefore verified our selection method and demonstrated the selection of PPS-specific B cells by expanding selected B cells in culture according to the method of Weitkamp et al. (43). Furthermore, the V_H gene family distribution of the libraries were highly polarized and do not reflect the V_H gene family of usage of unselected $CD19^+$ B cells (5). By defining the heavy-chain gene repertoire of elderly adults against PPS, we established a data set that will

allow variable gene repertoires to be examined on a comparative basis in all age groups.

Previous studies on gene expression of the B-cell repertoire in aging have been limited to the total circulating population (42), specific gene families (8, 33), or germinal centers in spleen and Peyer's patches (2). Overall, a shift toward V_H4 expression was noted with V_H4-34 and V_H4-59, as well as V_H1-69, more frequently expressed in elderly than in young circulating B cells (42). Although we did see a slight increase in V_H4 gene sequences in the elderly compared to the young, the response in both age groups was dominated by the V_H3 gene family, as has been previously described in response to a wide variety of capsular polysaccharide antigens (3, 17, 19, 20, 26, 28, 49, 50). With aging, a significant shift in gene usage was noted; elderly adults demonstrated increased V_H3-30 gene expression against both polysaccharides, while the immune response to PPS4 in the young subjects was dominated by V_H3-74 and V_H3-07 genes and the response to PPS14 was dominated by V_H3-48. The shift in V_H gene usage with aging appears to be unrelated to the available repertoire since V_H3-07, V_H3-74, and V_H3-48 were detected in the elderly, albeit at a decreased frequency. Similarly, Baxendale et al. (3) found predominant use of the V_H3 gene family to all PPS serotypes studied and dominant (three of four sequences) expression of the V_H3-07 gene locus toward PPS4. Cumulatively, these data suggest that with aging there is an alteration in V_H gene expression in response to PPS4 and PPS14 that is independent of the observed shift toward V_H4 gene family expression with aging and the available repertoire.

Studies in the elderly have also noted changes in mutational frequency with age; however, each study detected different patterns of mutation. Rosner et al. (33) detected fewer hypermutated clones in B cells expressing V_H6 gene family in the elderly than in the young. The hypermutated clones present in the elderly, however, demonstrated a significant increase in replacement mutations in CDR1 and -2 in the elderly compared to the young but not a significant difference in the number of mutations per base pair. In contrast, V_H5 gene family-expressing B cells were found to have a significantly greater mutational frequency in IgG^+ cells in elderly compared to young subjects, with a concomitant increase in replacement mutations in CDR1 and -2 in the elderly (8). Moreover, in studies of B cells isolated from germinal centers in spleen and Peyer's patches, Banerjee et al. (2) found no significant difference between either the rate of hypermutation within an individual clonal response or the frequency of mutation between young and elderly individuals. Similarly, the results of our study demonstrate that some genes were more mutated in the elderly (V_H3-11 and V_H3-21) and some were less mutated (V_H3-48) than in the young. Thus, it appears that differences in mutational frequency may not only be age specific but also gene specific in that different genes show different mutational frequencies with aging. Overall, the isolated heavy chain genes were 87.3 to 100% identical to germ line sequences, with the majority of the mutations occurring in the CDRs. Previous studies demonstrated a similar range of mutations in response to polysaccharide antigens (3, 20, 27, 37, 39, 49, 50).

Most information regarding the effects of aging on the immune system has been obtained from studies performed in mice. In mice, aging is associated with a decline in the number of bone marrow pre-B cells and the entry of bone marrow emigrants into the peripheral B-cell population (40). This leads to a skewing of the B-cell compartment in favor of long-lived antigen experienced cells (14). In aged mice, the peripheral repertoire is dominated by B cells expressing characteristics of marginal zone B cells (21). It has been postulated that the reduction in naive B cells with aging is responsible for the observed shift in the expressed V_H repertoire. Accordingly, the V_H repertoire in aged mice represents products of preformed memory B cells expressing cross-reactive antibodies and not a true de novo response to antigen (14). Several studies in humans have described a similar shift toward a memory B-cell phenotype with aging (4, 9). The effect of an increased B-cell memory pool with aging in the response to PPS remains to be elucidated since repertoire analysis of PPS-specific B cells, isolated from young adults 7 days postvaccination, shows that these cells bear the characteristics of a memory response indicative of preexisting immunity (20, 49, 50). Although one could hypothesize that an increase in cross-reactive B memory cells may be responsible for loss of oligoclonality in elderly, substantiation of this theory would require comparative analysis of the pre- and postimmune repertoire.

We hypothesized that the decreased functional immune response demonstrated in the elderly in response to PPS4 but not to PPS14 (32) may be related to altered V gene family usage. When each donor was examined individually we were able to make associations between gene expression and antibody avidity. For example, a response dominated by V_H3-21, in the elderly to PPS4, was associated with high avidity while, in contrast, V_H3-30 was associated with a variable immune response. Although not conclusive, the data indicate that there may be a link between gene utilization and antibody avidity for certain loci. We are currently undertaking further studies to fully define the role of gene usage in antibody specificity and activity.

This is the first molecular investigation of the antipneumococcal polysaccharide immune response to enroll two distinct populations: young and elderly adults. In addition, our study included all donors regardless of magnitude of the immune response. We have shown a significant difference in the variable gene repertoire between young and elderly adults. Furthermore, our results demonstrated a loss of oligoclonality with aging in response to PPS4 and PPS14. In addition, somatic mutation occurred at a lower frequency in some elderly derived sequences than sequences derived from young adults. We also noted several interesting associations between variable gene usage and functional activity. To our knowledge, our findings currently represent the largest and most extensive analysis of heavy-chain gene usage in humans against polysaccharide antigens. This new and rapidly expanding area of research will be instrumental in the elucidation of the mechanisms underlying elderly immune senescence.

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