Significant Variation in Serotype-Specific Immunogenicity of the Seven-Valent \textit{Streptococcus pneumoniae} Capsular Polysaccharide–CRM$_{197}$ Conjugate Vaccine Occurs Despite Vigorous T Cell Help Induced by the Carrier Protein

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\textit{Streptococcus pneumoniae} capsular polysaccharides (PnPSs) induce protective antibodies but are T cell–independent type 2 antigens and are poorly immunogenic in infants. Conjugate vaccines of PnPSs linked to proteins like cross-reactive material (CRM$_{197}$) increase PS antibody titer and elicit immunologic memory in infants. Despite being linked to an identical carrier protein, each PS component of the 7-valent PnPS-CRM$_{197}$ vaccine has different immunogenicity. To determine whether variations in conjugate-induced memory T cell responses or PnPS-specific antibody-secreting cells (ASCs) were responsible for serotype-specific differences in immunogenicity, adults were immunized with 7-valent PnPS-CRM$_{197}$, and antibody component–specific CD4$^+$ T cell recall response, numbers of PnPS-specific ASCs, and cytokine production were measured. PnPS-CRM$_{197}$ induced significantly different serotype-specific antibody titers, despite vigorous T cell recall responses to all 7 vaccine components, and production of interleukin (IL)–2, IL-5, IL-6, IL-10, and interferon-\(\gamma\). We conclude that PnPS-CRM$_{197}$ induces variable serotype-specific antibody titers, despite induction of comparable CRM$_{197}$-specific memory T cell responses.

Antibodies against capsular polysaccharides (PSs) of encapsulated bacteria, such as \textit{Haemophilus influenzae} type b (Hib) and \textit{Streptococcus pneumoniae} (Pn), fix complement to the bacterial surface and are protective in animals and humans [1, 2]. However, capsular PSs are T cell–independent type 2 antigens and are poor inducers of immunological memory [3–7]. In addition, these PSs yield isotype- and clonotype-restricted antibodies, which mature late in ontogeny, so that infants aged <24 months do not produce adequate levels of anti–PS antibody titers to protect against infection [1, 2, 5, 6–8].

Immunization with PSs conjugated to a carrier protein results in induction of protective levels of PS antibodies in infants and immunologic memory to the PS [2, 9]. The first successful use of such conjugate vaccines linked the purified Hib PS capsule to CRM$_{197}$
(cross-reactive material), a nontoxic diphtheria toxin mutant protein. The use of this vaccine and other Hib conjugates that use different carrier proteins resulted in the virtual elimination of Hib infections in the United States [9]. However, disadvantages of these vaccines include the need for multiple doses to achieve protective antibody levels and high cost, which makes their use in the developing world problematic [10].

Development of an effective vaccine to prevent pneumococcal infections is complicated, because many different serotypes are associated with clinical disease, in contrast to the single serotype B that caused the vast majority of invasive H. influenzae disease [11]. Therefore, the use of multivalent vaccines is required to provide adequate protection against infection. The currently licensed 23-valent PS vaccine covers ∼90% of invasive pneumococcal serotypes circulating in the United States and is immunogenic and protective in most healthy adults. However, like the early, unconjugated Hib PS vaccine, the 23-valent pneumococcal PS vaccine is unable to stimulate a protective anti–PS antibody response in most children aged <24 months [6, 7]. Thus, the use of PS-protein conjugate vaccines to eliminate pneumococcal infections is complicated by the fact that many serotypes cause invasive human disease, so that each capsular serotype requires separate purification and conjugation to a protein. Recently, a 7-valent PnPS conjugate vaccine containing a capsular PS of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, all separately conjugated to CRM197, was shown to have protective efficacy against invasive pneumococcal infections of homologous serotypes and is now licensed for use in infants [12]. A 4-dose immunization series in infants induced protective levels of anti–PnPS antibodies and evidence of booster response after the initial immunization. Because all the capsular serotypes used in the vaccine were conjugated to the same carrier protein, it was assumed that each component of the vaccine would elicit similar T cell help and thus have similar enhanced immunogenicity. Interestingly, the multivalent pneumococcal conjugate vaccine induces markedly different anti–PnPS antibody titers, depending on the serotype of the PnPS component of the vaccine, in both children and adults [13, 14].

Enhanced immunogenicity of conjugate vaccines is thought to occur as a result of binding of the PS component of the conjugate vaccine to PS- or carrier-specific B cells, processing of the carrier protein, and presentation of carrier-derived T cell epitopes via major histocompatibility class II molecules to CD4+ T helper (Th) cells. These cells then produce cytokines that presumably stimulate PS-specific B cell clones and modulate the isotype and quantity of anti–PS antibody produced [15–17]. In practice, repeated doses of these vaccines in infants yield a booster response and T and B cell memory that are more consistent with T cell–dependent antigens than T cell–independent antigens. However, it is unclear whether conjugate vaccines yield a similar advantage in adults, in which repeated immunization with the CRM197 pneumococcal conjugate vaccine does not necessarily yield enhanced anti-PnPS antibody titers, compared with unconjugated PnPS vaccines, especially in the elderly population [18]. The mechanisms responsible for increased immunogenicity of conjugate vaccines are still poorly understood, and the duration of immunity to invasive disease after immunization with the pneumococcal conjugate vaccine, particularly to those serotypes that are poorly immunogenic, remains unknown.

Because each of the components of the 7-valent PnPS conjugate vaccine are linked to the same carrier protein, it seemed possible that linkage of the carrier protein to different capsular serotypes might affect carrier protein structure and antigen processing and presentation of the carrier protein. Such alterations might change subsequent T cell help and cytokine production, causing variation in the serotype-specific antibody titer. Alternatively, certain serotypes might elicit low antibody titers as a result of restricted B cell repertoire and decreased frequency of serotype-specific B cells.

In this report, we used single cell–resolution ELISPOT analysis, ELISA, and proliferation assays to measure pneumococcal PSs and carrier protein ASC frequency, carrier protein–specific CD4+ T cell recall responses, cytokine production, and antibody titer after pneumococcal conjugate vaccine immunization of adults. Despite marked variation in serotype-specific anti–PnPS IgG antibody titers similar to previous reports, all the individual vaccine components elicited a strong carrier protein–specific CD4+ T cell recall response after immunization, which suggests that serotype-specific variation in immunogenicity is not caused by reduced carrier protein–specific T cell help.

SUBJECTS, MATERIALS, AND METHODS

Subjects and sample collection. Twenty-four healthy adults were enrolled in the study. Fifteen volunteers (6 men and 9 women; mean age, 30 years; age range, 24–35 years) were vaccinated subcutaneously with 1 dose of the 7-valent PnPS-CRM197 conjugate vaccine containing 2 µg of capsular PS from each of 6 serotypes (4, 9V, 14, 18C, 19F, and 23F) and 4 µg of capsular PS from serotype 6B covalently linked to a total of 20 µg of CRM197 (a nontoxic, single–amino acid mutant of diphtheria toxin) [19]. Administration of multiple doses of conjugate vaccine was not permitted under our institutional review board and investigational new drug protocols. Nine volunteers (5 men and 4 women; mean age, 26 years; age range, 22–30 years) were immunized with the unconjugated 23-valent pneumococcal PS vaccine (Pneumovax 23; Merck) containing 25 µg of each of 23 capsular PSs, so that we could include control individuals who were immunized with Pn PS but not with the carrier protein. Blood samples (50 mL) were collected before vaccination and at 1, 2, 4, and 6 weeks after vaccination. None of the volunteers
had previously been vaccinated with any pneumococcal vaccine or with any Hib conjugate vaccine. History of immunological abnormalities, vaccination with diphtheria vaccine within 2 years, current use of prescription medication, age $\geq$55 years, and pregnancy were criteria for exclusion from the study.

**Anti-PnPS and anti–carrier protein antibody assays.** Anti-PnPS antibodies (total immunoglobulin [Igs], IgG, IgM, and IgA) were measured by ELISA, as described elsewhere [20]. In brief, microtiter plates (Polysorp; Nunc) were coated with 100 mL of a solution containing 10 mg of serotype-specific PS (ATCC)/mL of PBS. Antibodies to cell-wall PS (C-PS; kindly provided by Wyeth Lederle Biologicals) were neutralized in serum samples via incubation with 50 $\mu$g/mL C-PS. Serum samples were serially diluted and were incubated overnight in PS-coated microtiter plates at 4°C. Plates were washed, and alkaline phosphatase–conjugated goat anti–human Ig, IgG, IgM, and IgA antibodies (Southern Biotechnology Associates) diluted in PBS-Tween containing 1% bovine serum albumin (BSA) then was added. $p$-nitrophenyl phosphate disodium (pNPP; Sigma) then was added to each well, and absorbance was measured at 415 nm in a Microplate ELISA reader (model 550; BioRad). Quantitation was performed via interpolation from a standard curve generated with pneumococcal antibody reference serum (serum 89-SF; provided by Dr. Karl Frasch, US Food and Drug Administration, Center for Biologic Evaluation and Research, Bethesda, MD).

To detect CRM$_{197}$–specific antibodies, microtiter plates (Costar) were coated with 2 $\mu$g/mL CRM$_{197}$ (kindly provided by Dr. Ronald Eby; Wyeth-Lederle Vaccines), as we have described elsewhere [17]. After blocking, serum samples were added to the plates, and anti-CRM$_{197}$ antibodies bound to the plates were detected via alkaline phosphatase–conjugated secondary antibodies, as described above. Because standard sera were not available, antibody titer was determined via a linear fit for optical density values of 8 dilutions, and the antibody level was expressed in arbitrary units by calculating the reciprocal dilutions that gave 50% of the maximum absorbance response.

**Isolation of peripheral blood mononuclear cells (PBMCs).** PBMCs were isolated from peripheral venous blood by centrifugation on a ficoll-hypaque gradient (Pharmacia LKB Biotechnology). The cell suspension was washed and suspended in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin and streptomycin, and l-glutamine, as described elsewhere [17, 21]. Magnetic beads coated with anti-CD4 were used to remove CD4$^+$ T cells from PBMCs from selected patients, as described elsewhere [17, 22], to demonstrate that proliferation and cytokine production were primarily by CD4$^+$ T cells.

**Carrier protein–specific T cell recall responses after immunization.** T cell recall responses to CRM$_{197}$ were measured via a proliferation assay that we have described in detail elsewhere [17, 23]. PBMCs, isolated as described above, were added to microtiter wells containing different concentrations (5–20 $\mu$g/mL) of CRM$_{197}$, PnPS CRM$_{197}$ of each specific serotype or control antigens of unconjugated PnPS, or hen egg lysozyme (HEL), and the cells then were pulsed with tritiated thymidine. DNA-incorporated radioactivity then was measured, and results were expressed as the percentage of maximal stimulation derived from pokeweed mitogen to minimize intradonor variation.

**Single-cell resolution ELISPOT to determine the frequency of anti-PnPS and anti-CRM$_{197}$ ASCs.** B cells secreting anti-CRM$_{197}$ or anti-PnPS IgG, IgM, or IgA antibodies were enumerated by a modification of the ELISPOT, as described elsewhere [17, 21]. Nitrocellulose-based microtiter plates (Millipore) were coated individually with each of the 7 unconjugated PnPSs or CRM$_{197}$. The PBMCs were separated by ficoll-hypaque density gradient, as described above, and adjusted to a concentration of 2 $\times$ 10$^5$ cells/mL in RPMI 1640 medium. Cells were cultured in 12-well plates for 4 days, because preliminary experiments established that this time period yielded maximal numbers of ASCs. Comparable ASC responses were obtained in cultures stimulated with CRM$_{197}$, PS, and no antigen; thus, we did not stimulate cultures with antigens during this incubation. On day 4, 10$^6$ cells/mL were resuspended in fresh RPMI 1640 medium, and C-PS was added to prevent cells secreting antibodies against C-PS to bind to the plate. Goat anti–human IgG, IgM, and IgA antibodies conjugated to alkaline phosphatase (AKP; Southern Biotech) then were added to the cells in a final concentration of 1 $\mu$g/mL. The cells were transferred to duplicate wells of the PS antigen–coated plates, as described above, incubated at 37°C in 5% CO$_2$ washed again, and developed by adding 100 $\mu$L of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT$^{PLUS}$; Southern Biotech). Plates were dried, and the number of spots, representing antibody-producing cells per 10$^5$ total cells, were determined by use of an automated ImmunoSpot series I Image Analyzer (Cellular Technology) [21]. Digitized images were analyzed for the presence of areas in which color density exceeds background by a factor calculated from comparing the control wells (coated with nonspecific antigen) to experimental wells (coated with specific antigen). After separating the spots, additional criteria of spot size and circularity were applied to gate out noise caused by artifacts of substrate precipitation and nonspecific antibody binding.

**Determination of cytokine production by CD4$^+$ T cells by ELISPOT and ELISA.** ELISPOT analysis also was performed on a subset of 16 randomly chosen subjects (10 immunized with the 7-valent PnPS-CRM$_{197}$ vaccine and 6 control subjects immunized with the 23-valent unconjugated PnPS vaccine) to assess cytokine production, as described elsewhere [17, 21]. In brief, study subject PBMCs were isolated from heparinized blood, as described above, and were placed in culture with carrier protein (CRM$_{197}$), PnPS, and anti-CD3 (controls) at
IgM, day before or after immunization
IgA, day before or after immunization

after stimulation with PnPS-CRM197 and CRM 197 were simi-
larly enumerated using software specifically designed for the
spots. The ELISPOT plates were read at 450 nm.

...tively, and images of wells were acquired and saved on compact
disk for further analysis.

...tivated overnight at 4°C (OptEIA; Pharmingen; linear range,
7.8-500 pg/mL) and were incubated overnight. Streptavidin-
A KP conjugate (Southern Biotech) was added to each
well. Plates were washed, and spot color was developed, as
described above. Plates were then dried, and images of wells
were acquired and saved on compact disk by use of an
automated ImmunoSpot Series I plate reader, and the
spots were enumerated on an ImmunoSpot Satellite Analy-
zer (Cellular Technology) using software specifically
developed for the ELISPOT assay, as described above. Be-
cause the results after stimulation with PnPS-CRM_197 and
CRM_197 were similar, cytokine results and statistical analyses used data from
CRM_197-stimulated PBMCs.

ELISA also was performed on supernatants of cells from the
same subset of subjects to measure cytokines secreted by
PBMCs. Supernatants from unstimulated and CRM_197-
stimulated cells were collected after 48 h of incubation.
Microtiter plates (Maxisorp) were coated with anti–IL-2, –IL-4,
–IL-5, –IL-6, –IL-10, and –IL-12 and –IFN-γ antibodies
(OptEIA; Pharmingen; linear range, 7.8-500 pg/mL) and
were incubated overnight at 4°C. Plates were washed and
blocked with 10% FCS in PBS, and then supernatants and
standards were added. The wells were then washed again and incubated
with biotinylated anti–IL-2, –IL-4, –IL-5, –IL-6, –IL-10, and
–IL-12 and –IFN-γ antibodies and streptavidin conjugated to
horseradish peroxidase. After extensive washing, 100 μL of
tetramethylbenzidine (BD Pharmingen) substrate solution was
added, followed by stop solution (2NH_2SO_4), and absorbance
was read at 450 nm.

Statistical analysis. The association of serum antibody
concentration with lymphocyte proliferation and cytokine
production was determined by use of Spearman’s rank correlation.

To adjust the significance level for multiple testing, the Bonferroni correction formula, \( \alpha' = \alpha/k \), was used, where \( \alpha = .05 \) (overall significance level) and \( k \) is the number of corre-
lations performed. Here, we were able to test correlations in
groups of 21, yielding an adjusted significance level of \( \alpha' = .0024 \). The comparison of lymphocyte proliferation and cyto-
kine production across serotypes was determined by use of
Friedman’s test for comparing multiple related groups. In the
event that the test was significant, post hoc comparisons of
pairwise means by the Bonferroni correction also was per-
formed. Specifically, in these data, there were 28 multiple com-
parisons at each week (\( \alpha' = .0018 \)), whereas, for cytokine pro-
duction, there were 21 multiple comparisons at each week
(\( \alpha' = .0024 \)).

Table 1. Serum capsular polysaccharide (PS) antibody concentration before (day 0) and after immunization (days 7, 14, 28, and 42) with 7-valent Streptococcus pneumoniae capsular PS (PnPS-CRM_197) conjugate vaccine.

<table>
<thead>
<tr>
<th>PS</th>
<th>IgG day before or after immunization</th>
<th>IgM day before or after immunization</th>
<th>IgA day before or after immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PnPS</td>
<td>0 7 28 42</td>
<td>0 7 28 42</td>
<td>0 7 28 42</td>
</tr>
</tbody>
</table>

NOTE. Data are geometric mean antibody concentrations (μg/mL).

a Type 4 PS IgG antibody titer was lower than 18C PS IgG antibody titer 4 and 6 weeks after immunization (P < .0024).
b Type 6B PS IgM antibody titer was higher than types 14 and 18C PS IgM antibody titer 2 weeks after immunization (P < .0001) and higher than types 19F, 23F, and 18C PS IgM antibody titer 2 and 6 weeks after immunization (P < .0003).
c Type 23F PS IgG antibody titer was lower than types 6B, 19F, 4, and 9V IgM antibody titer 2 weeks after immunization (P < .0001).
d Type 4 PS IgA antibody titer was higher than types 19F, 23F, and 18C PS IgA antibody 1 week after immunization and higher than types 19F and 18C PS IgA antibody titer 6 weeks after immunization (P < .0024).
e Type 6B PS IgG antibody titer was higher than type 18C PS IgA antibody 2 and 6 weeks after immunization (P < .0024).

RESULTS

Anti-PnPS and -CRM_197 antibodies. Serum geometric mean
IgG, IgA, and IgM anti-PnPS antibody levels increased for all
serotypes after immunization with the PnPS conjugate vaccine
(table 1). However, there were marked differences in PnPS-
specific antibody levels, depending on serotype. Types 4 and 9V consistently yielded the lowest anti-PnPS Ig antibody levels.
Type 4, in particular, elicited significantly lower total Ig and

2 × 10^6 cells/mL in complete RPMI 1640 medium. Anti-in-
terleukin (IL)–2, –IL-4, –IL-5, –IL-6, –IL-10, and –IL-12 (2 μg/
ml) and –interferon (IFN)–γ (5 μg/mL) antibody (BD Phar-
mingen)–coated ELISPOT plates were washed with PBS,
blocked with PBS containing 10% FCS, and washed again. Cells
from the cultures were resuspended in fresh medium containing
biotinylated anti–IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 (2 μg/
ml) and IFN-γ (5 μg/mL) antibodies (BD Pharmingen) and
were transferred to these coated ELISPOT plates at a concen-
tration of 10^5 cells/well. After incubation of the plates at 37°C
in 5% CO_2 overnight, streptavidin-A KP conjugate (Southern
Biotech) was added to each well. Plates were washed, and spot
color was developed, as described above. Plates then were dried,
and images of wells were acquired and saved on compact disk
by use of an automated ImmunoSpot Series I plate reader, and
the spots were enumerated on an ImmunoSpot Satellite Analy-
zer (Cellular Technology) using software specifically designed
for the ELISPOT assay, as described above. Because the results
after stimulation with PnPS-CRM_197 and CRM_197 were similar,
cytokine results and statistical analyses used data from
CRM_197-stimulated PBMCs.

ELISA also was performed on supernatants of cells from the
same subset of subjects to measure cytokines secreted by
PBMCs. Supernatants from unstimulated and CRM_197-
stimulated cells were collected after 48 h of incubation.

...
IgG titers, compared with serotypes such as 18C, after immunization (type 4 anti–PS IgG geometric mean antibody titer 4 weeks after immunization, 5.22 vs. 17.92 μg/mL for type 18C; P < .0024; and 6 weeks after immunization, 6.93 vs. 25.36 μg/mL; P < .0024; table 1).

Although the conjugate vaccine tended to elicit higher anti–PnPS IgG antibody titers than the unconjugated PnPS vaccine, these differences were not significant for any serotype (tables 1 and 2). Because the study was not designed to directly compare antibody responses to unconjugated with those to conjugated vaccines, it is possible that the IgG antibody titer differences between the 2 groups would have been significant if more study subjects had been included in the unconjugated vaccine group. In addition, types 4, 9V, and 23F elicited lower anti–PnPS IgG antibody titers than did other serotypes after immunization with the unconjugated PnPS vaccine (table 2). However, because of the small sample size of the unconjugated PS vaccine group, these differences did not reach statistical significance.

The anti–PnPS IgM antibody response after immunization with the conjugate vaccine also varied by serotype. Serotype 6B in particular, elicited anti–PnPS IgM titers that were significantly higher than those induced by other serotypes, such as types 14 and 18C 2 weeks after immunization (P < .0001; table 1) and types 19F, 23F, and 18C 6 weeks after immunization (P < .0003; table 1). In contrast, type 23F IgM titers were significantly lower than those of serotypes 6B, 19F, 4, and 9V 2 weeks after immunization (P < .0001; table 1).

Serum anti–PnPS IgA antibody levels also increased significantly after immunization with the conjugate vaccine, with most serotypes having a 2–4-fold increase in anti–PnPS IgA levels with conjugate vaccine immunization (table 1). Interestingly, type 18C, which induced the highest level of anti–PnPS IgG antibodies, elicited the lowest level of anti–PnPS IgA antibodies, whereas type 4–CRM197 elicited significantly higher levels of anti–PnPS IgA antibodies than did most other serotypes 1 week after immunization (compared with types 19F, 23F, and 18C; P < .0024; table 1). Similar results were seen after immunization of control volunteers with unconjugated 23-valent PnPS vaccine (table 2). Geometric mean CRM197 IgG titers increased >50-fold after immunization of volunteers with the conjugate vaccine containing CRM197 as a carrier protein but did not increase as expected in subjects after immunization with the unconjugated PS vaccine (data not shown).

**Frequency of circulating PnPS- and CRM197-specific ASCs.** There were few PnPS-specific ASCs detected before immunization. One week after immunization, there was a 29-fold increase in type 6B, a 33-fold increase in type 18C, a 29-fold increase in type 23F, and a 22-fold increase in type 14 PnPS secreting ASCs, respectively (figure 1A). All these serotypes induced high peak geometric mean levels of anti–PnPS IgG antibodies (>15 μg/mL; table 1). In contrast, there was only a 9-fold increase in type 4 and an 8-fold increase in type 9V PnPS–secreting ASCs 1 week after immunization, and both of these serotypes elicited low peak geometric mean IgG levels (<7 μg/mL). In addition, there was a significantly higher number of type 18C IgM–secreting ASCs 2 weeks after immunization, compared with numbers of “low responder” types 4 and 9V IgM–secreting ASCs (figure 1B; P < .0024). Despite low numbers of IgG- and IgM-secreting ASCs, serotype 4 induced more IgA PS-specific ASCs than any other serotype within 7 days after immunization and significantly more IgA-secreting ASCs than types 19F and 18C (figure 1C; P < .0005). Finally, all conjugate vaccine-immunized individuals had a substantial increase in CRM197-specific ASCs (28-fold increase in IgG anti-CRM197 ASCs; figure 1A) at 7 days after immunization, whereas control unconjugated PnPS vaccine recipients had no increase in CRM197 ASCs (data not shown).

**CRM197 carrier protein–specific CD4+ T cell recall responses are induced by all serotype components of the conjugate vaccine.** There was a vigorous CRM197–specific proliferative response by PBMCs 1 week after immunization that decreased rapidly over the course of the 6-week experiment. Despite marked differences in the ability of each component of the 7-valent vaccine to induce anti–PS antibodies, there was no sig-

### Table 2. Serum capsular polysaccharide (PS) antibody concentration before (day 0) and after immunization (days 7, 14, 28, and 42) with unconjugated Streptococcus pneumoniae capsular PS vaccine.

<table>
<thead>
<tr>
<th>PnPS</th>
<th>IgG, day before or after immunization</th>
<th>IgM, day before or after immunization</th>
<th>IgA, day before or after immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 7 14 28 42</td>
<td>0 7 14 28 42</td>
<td>0 7 14 28 42</td>
</tr>
<tr>
<td>6B</td>
<td>1.39 1.64 11.06 11.67 5.02</td>
<td>3.17 4.84 6.41 6.25 4.76</td>
<td>0.19 0.57 3.46 3.31 3.25</td>
</tr>
<tr>
<td>19F</td>
<td>3.43 2.67 14.71 15.09 8.37</td>
<td>4.61 4.81 7.26 6.63 5.01</td>
<td>0.29 0.46 3.05 2.56 1.88</td>
</tr>
<tr>
<td>23F</td>
<td>0.93 0.91 5.68 6.16 3.09</td>
<td>1.14 1.23 1.8 1.73 1.25</td>
<td>0.15 0.13 0.99 0.86 0.36</td>
</tr>
<tr>
<td>4</td>
<td>0.98 0.96 6.77 7.21 5.38</td>
<td>1.01 2.56 3.77 3.67 3.32</td>
<td>0.11 0.81 5.63 4.81 2.63</td>
</tr>
<tr>
<td>14</td>
<td>2.05 2.58 12.68 12.81 14.1</td>
<td>1.65 3.56 3.9 3.09 3.7</td>
<td>0.3 0.44 2.43 1.9 1.67</td>
</tr>
<tr>
<td>9V</td>
<td>1.82 3.07 7.77 9.35 8.74</td>
<td>1.9 3.78 5.16 5.1 5.44</td>
<td>0.26 1.11 4.97 4.35 3.67</td>
</tr>
<tr>
<td>18C</td>
<td>1.74 2.94 10.6 10.63 10.96</td>
<td>1.33 1.85 2.73 2.77 2.08</td>
<td>0.11 0.14 1.04 0.91 0.44</td>
</tr>
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</table>

**NOTE.** Data are GM antibody concentrations (μg/mL). There was no significant difference between the antibody titer induced by unconjugated PS vaccine and that induced by conjugated vaccine.
Figure 1. IgG (A), IgM (B), and IgA (C) polysaccharide-specific antibody-secreting cells (ASCs) to the 7 pneumococcal serotypes included in the conjugate vaccine were measured before and after immunization by use of computer-assisted single-cell resolution ELISpot analysis. Data are mean ± SEM ASCs/10⁵ cells.
significant difference in the ability of any of the individual components of the vaccine to induce proliferation in PBMCs after immunization (figure 2). Unconjugated PnPs alone and HEL were unable to stimulate proliferation in PBMCs, whereas PBMCs from unconjugated PS-vaccinated individuals also did not proliferate when stimulated with carrier protein (data not shown). PBMC proliferation was caused by the activation of CRM$_{197}$-specific CD4$^+$ T cells, because depletion of these cells from the PBMCs using anti-CD4 monoclonal antibody–labeled magnetic beads abrogated PBMC proliferation induced by CRM$_{197}$ (data not shown).

**Immunization with the pneumococcal conjugate vaccine induces a mixed Th1/Th2 CD4$^+$ T cell cytokine response.** Immunization with the 7-valent pneumococcal conjugate vaccine yielded an increase in PBMCs that secreted IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ within 7 days after immunization (table 3). In contrast, PBMCs from control subjects immunized with unconjugated PnPs vaccine produced only small quantities of IL-6 (data not shown). There were strong positive associations between type 18C–CRM$_{197}$–specific lymphocyte proliferation and T cell IL-10 production ($r = 0.89; P < .0005$) and between type 4–CRM$_{197}$–specific lymphocyte proliferation and IL-4 production ($r = 0.89; P < .0005$; table 3). The control antigen HEL did not induce cytokine production by T cells, and depletion of CD4$^+$ T cells via magnetic beads coated with anti-CD4 monoclonal antibody abrogated the cytokine responses (data not shown), which indicates that cytokine-producing cells were predominantly CRM$_{197}$-specific CD4$^+$ T cells.

**DISCUSSION** Despite linkage to an identical carrier protein, we found that the 7-valent PnPs-CRM$_{197}$ conjugate vaccine elicited strong serotype-specific differences in magnitude of IgG, IgM, and IgA anti–PnPs antibody responses in adults immunized with a single dose. These data are similar to previous reports that found variation in serotype-specific immunogenicity of the 7-valent PnPs-CRM$_{197}$ vaccine, as well as with other experimental pneumococcal conjugate vaccines [12–14, 24]. In fact, types 4 and 9V, even when conjugated to another carrier protein (tetanus toxoid), were found to be modestly immunogenic in terms of inducing IgG, compared with other conjugated serotypes, such as types 14, 18C, and 6B, when given to adult volunteers [25].

Despite the modest immunogenicity of type 4–CRM$_{197}$ for induction of anti–PnPs IgG antibodies, this component of the multivalent vaccine elicited a strong anti–PnPs IgA antibody response, as well as large numbers of PnPs-specific IgA ASCs. These data suggest that the characteristics of the B cells that bind type 4 Pss may be different from those of B cells specific for other serotypes of pneumococcus. Recent data have sug-

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**Figure 2.** Vigorous CD4$^+$ T cell recall responses to the carrier protein CRM$_{197}$ occurs after immunization of adults with 7-valent *Streptococcus pneumoniae* capsular polysaccharide–CRM$_{197}$ conjugate vaccine, as indicated by the proliferation of postimmunization peripheral blood mononuclear cells (PBMCs) stimulated in vitro with CRM$_{197}$ or individual serotype–specific components of the 7-valent vaccine. There were no significant differences in stimulation between any of the different serotypes conjugated to CRM$_{197}$. Stimulation of preimmunization PBMCs with CRM$_{197}$ or vaccine components did not elicit proliferation. In addition, stimulation of PBMCs with unconjugated pneumococcal polysaccharides or the control protein hen egg lysozyme did not elicit any carrier protein–specific recall responses (data not shown). Data are percentage of maximal [H]-thymidine incorporation elicited by pokeweed mitogen.
Table 3. Cytokine production by carrier protein–stimulated CD4+ T cells before and after immunization with 7-valent Streptococcus pneumoniae capsular polysaccharide–CRM197 conjugate vaccine.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mean cytokine concentration, pg/mL</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-4</td>
<td>4.74</td>
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<tr>
<td>IL-5</td>
<td>11.80</td>
</tr>
<tr>
<td>IL-6</td>
<td>19.70</td>
</tr>
<tr>
<td>IL-10</td>
<td>12.67</td>
</tr>
<tr>
<td>IL-12</td>
<td>7.44</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>12.31</td>
</tr>
</tbody>
</table>

NOTE. IFN, interferon; IL, interleukin.

a IL-4 production correlated with type 4–CRM197-induced lymphocyte proliferation 2 weeks after immunization ($r = 0.89$; $P = .0005$).
b IL-10 production correlated with type 18C–CRM197–induced lymphocyte proliferation 2 weeks after immunization ($r = 0.89$; $P = .0005$).

Suggested that adults immunized for the first time with bacterial PSs, such as Hib PS, may respond with expansion of highly selected memory B cells that have intracutaneous isotype switching to IgA2 and may have been elicited via colonization with bacteria that have cross-reactive PSs [26]. Because the frequency of colonization with the various pneumococcal serotypes or other bacteria with cross-reactive PSs may differ, the presence of serotype-specific PS memory B cells may be variable. In addition, previous studies also have shown that the adult human B cell responses to pneumococcal PS types 6B, 14, and 23F are severely restricted, are oligoclonal in character both on the individual and population levels, and probably arise from a very small number of B cell clones [27, 28]. It seems possible that B cells specific for type 4 and other low-response serotypes across the population are severely restricted, leading to lower PnPS-specific IgG antibody titers than are seen with other, less restricted serotypes.

Cytokines induced after immunization with the conjugate vaccine may have different effects on different subsets of B cells specific for PS, allowing some B cell lines, such as those specific for type 4 PnPS, to preferentially differentiate and produce IgA. The Hib conjugate vaccines, as well as the pneumococcal conjugate vaccine, have been found to eliminate nasopharyngeal carriage of the pathogens, and anti–PS IgA and IgG antibodies have been found in saliva after immunization [14, 29, 30]. Interestingly, much of the serum IgA produced after immunization with PnPS vaccines in adults is polymeric and therefore is capable of transferring across epithelial cells via the polymeric immunoglobulin receptor, binding to secretory component, and entering mucosal secretions [31]. Polymeric antipneumococcal IgA has recently been found to opsonize pneumococcus in the presence of complement for uptake and killing by phagocytes [32]. Therefore, because the PnPS conjugate vaccine has good efficacy for the prevention of infection with homologous pneumococcal serotypes [12], including low-response IgG-inducer serotypes, such as type 4, which generate high anti–PnPS IgA antibody titers, it seems possible that the serum anti–PnPS IgA produced is relevant to protection. Further studies are needed to determine the full role of serum and mucosal IgA in protection from pneumococcal infections after immunization.

We found that all the individual components of the 7-valent pneumococcal conjugate vaccine were capable of inducing vigorous CD4+ T cell proliferative responses after immunization and that these responses did not differ depending on the serotype of the PS component of the vaccine. These data suggest that any variations in immunogenicity of the PS component of the vaccine are not caused by gross serotype-specific alterations of the carrier protein due to the conjugation process. Indeed, individuals receive all 7 components of the vaccine at once, including serotypes that were highly immunogenic that induced vigorous carrier protein–specific recall responses in vivo. Thus, it seems unlikely that certain serotypes of the vaccine were modestly immunogenic because of poor carrier protein–specific T cell help after immunization, even if that particular vaccine component had altered antigen processing because of the particular structure of the PnPS linked to the carrier. We have previously developed a model in which type 23F PnPS was a poor immunogen in CBA/J mice, even when conjugated to CRM197 [23]. Although we found that the serotype of the PnPS seemed to influence the peptides made by antigen-processing cells and subsequently recognized by T cells, there was vigorous induction of carrier protein–specific T cells after immunization with any of the serotypes [23]. These data suggested that, although PS of the PnPS conjugate vaccine could influence antigen processing of the carrier protein, vigorous T cell help was induced in any case. Further experiments showed that type 23F–specific ASC frequency was significantly decreased, compared with that of other serotype-specific B cells, in these mice after immunization [33]. Thus, it was likely that restricted 23F–specific B cell repertoire was the cause of the decreased 23F PnPS antibody response in this model, rather than lack of T cell help. It seems possible that PnPS-specific B cell frequency also plays a role in the differences in serotype-specific antibody production after immunization with this conjugate vaccine in humans. Because the mouse experiments used splenic B cells to generate numbers of PnPS-specific ASCs, we found good correlation with PnPS-specific ASC numbers and antibody titer. Measurement of numbers of peripheral serum ASCs in our human volunteers showed some correlation between fold increase in PnPS-specific ASCs and antibody titer, but this correlation did not reach statistical significance, presumably because these circulating ASCs do not represent the total number of PnPS-specific ASCs available in lymph nodes and spleen.

Large doses of carrier protein have been associated with tol-
erance and/or epitope suppression and decreased PS-specific antibody titers in animal and human experiments [34, 35], and all components of the pneumococcal vaccine were linked to the same carrier protein. However, the total CRM197 dose in the multivalent pneumococcal vaccine is 20 μg, similar to the dose present in the highly immunogenic Hib-CRM197 vaccine (25 μg). Thus, there is no reason to suspect that carrier protein–induced epitope suppression played a role in the selective variations in serotype-specific antibody responses.

Immunization of our subjects with multiple doses of the pneumococcal conjugate vaccine might have yielded PnPS-specific IgG antibody titers superior to titers induced by unconjugated PnPS vaccine and improved the IgG response to low-frequency serotypes. One dose of the PnPS-CRM197 vaccine in children elicits low titers of antibodies, and 3 booster doses are required to reliably elicit IgG titers that are considered to be protective [13]. However, some serotypes are mediocre immunogens, even in children receiving 4 doses of this conjugate vaccine, and small studies of adults receiving a second dose of a similar pneumococcal conjugate vaccine did not demonstrate a marked improvement in antibody titers [18]. Furthermore, even if a multiple-dose regimen were found to be superior to single-dose PnPS conjugate immunization in adults, such a frequency of immunizations would be impractical and costly in the adult population.

The PnPS-CRM197 vaccine induced a rapid increase in T cell cytokine production, as measured by ELISPOT and ELISA. The cytokines produced were a direct result of recall responses by carrier protein–specific T cells after reexposure to carrier protein, because only CRM197, not PS or a control protein, induced T cell cytokine production. There was a substantial increase in both Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-5, and IL-10) cytokines within 7 days after immunization, similar to the cytokine profile induced by the Hib-CRM197 vaccine in adults (table 3) [17]. In contrast to adults immunized with Hib-CRM197 in whom IL-2 and IL-5 production correlated with anti–PS IgA production, we were unable to correlate the production of these cytokines with a quantity of anti–PnPS IgA antibody because of the multivalent nature of the vaccine (in contrast to the single serotype B in the Hib vaccine) and the requirement of statistical adjustment due to multiple comparisons. However, we did find that levels of IL-10 and IL-4 were positively correlated with type 18C–CRM197– and type 4–CRM197–induced CD4+ T cell proliferation, presumably reflecting increased cytokine production by antigen-stimulated memory T cells. The study of larger numbers of individuals immunized with PnPS conjugate vaccine may reveal further patterns of cytokine influence on anti–PnPS antibody titers and the postimmunization immune response.

PnPS conjugate vaccines using carrier proteins other than CRM197 will soon be available for clinical use. Hib conjugate vaccines with different carrier proteins exhibit different immunogenicities [36]. In particular, the Hib vaccine that uses outer-membrane protein from meningococcus (OMP) as a carrier protein is immunogenic after 1 dose in neonates, who have very low numbers of Hib-specific B cells. OMP is known to increase B cell proliferation and surface expression of T cell costimulatory molecules, such as B7-2, which induces an augmented T cell cytokine response [37, 38]. Because we have found that there is variation in serotype-specific pneumococcal antibody titer after immunization that may be related to B cell repertoire, carrier proteins, such as OMP, that also are B cell mitogens may be useful to enhance the antibody response to pneumococcal serotypes that are poorly immunogenic.

We conclude that this multivalent pneumococcal conjugate vaccine induces anti–PnPS antibody responses that vary by the serotype of the PnPS attached to the carrier protein, despite evidence of vigorous carrier protein–specific recall responses, including proliferation and cytokine production by CD4+ T cells. These data suggest that B cell repertoire may be an important factor in the adult response to PnPS antigens, even when in a T cell–dependent form, such as a glycoconjugate vaccine. It is possible that expansion of small numbers of B cell clones with adjuvant and/or carrier proteins that are mitogenic for B cells may provide increased PnPS antibody titers for serotypes of pneumococcus that are modest immunogens, even when conjugated to proteins. Further data will be required to determine whether similar findings are present in infants receiving several doses of conjugate vaccine.

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References


