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B- and T-Cell Immune Responses to Pneumococcal Conjugate Vaccines: Divergence between Carrier- and Polysaccharide-Specific Immunogenicity

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Conjugation of various serotypes of pneumococcal polysaccharide (PnPS) to carrier protein enhances the magnitude of the polysaccharide-specific antibody response, presumably by eliciting T-cell help. However, variability in PnPS serotype-specific immunogenicity has been observed. CBA/J mice immunized with either 6B or 19F PnPS conjugated to the protein carrier Cross Reactive Material$_{197}$ (CRM$_{197}$) produce a strong anti-PnPS antibody response; however, when mice are immunized with 23F PnPS conjugated to CRM$_{197}$, they fail to produce a significant anti-PnPS response. In order to determine whether this difference was related to alterations in antigen processing of the carrier protein and the subsequent T-cell responses, we studied proliferation of lymphocytes from CBA/J mice immunized with CRM$_{197}$ alone or conjugated to 6B, 19F, or 23F PnPS. T-cell proliferative responses to synthetic peptides demonstrated that lymph node cells elicited by the poorly immunogenic conjugate 23F-CRM$_{197}$ recognized many, but not all, of the epitopes recognized by lymph node cells elicited by 6B- and 19F-CRM$_{197}$ as well as additional epitopes. Despite marked differences in PnPS-specific immunogenicity, all mice made high titers of CRM$_{197}$ antibodies of the immunoglobulin G$_1$ isotype. Cells from mice immunized with any of the conjugates yielded vigorous T-cell responses to whole antigen. We conclude that the serotype of PnPS can alter the peptide specificities of T-cell responses, but even a poorly immunogenic PnPS conjugate can elicit a significant T-cell response. Thus, conjugation of PnPS to a carrier protein that elicits carrier-specific T- and B-cell responses does not necessarily enhance PnPS immunogenicity.

Streptococcus pneumoniae remains a significant pathogen in children under the age of two, splenectomized individuals, and the elderly, despite the availability of a purified multivalent *S. pneumoniae* capsular polysaccharide (PnPS) vaccine (2, 5, 14, 22). Immunization with bacterial polysaccharide (PS) antigens typically induces a T-cell-independent type 2 antibody response characterized by high levels of immunoglobulin M (IgM), IgG antibodies primarily of the IgG$_3$ subclass in mice and IgG$_2$ in humans, an absent or blunted memory response, and no requirement for the direct involvement of T cells (16, 19, 20, 23, 24). Polysaccharides are thought to be unable to bind to class II major histocompatibility complex (MHC), and are thus poor inducers of T-cell responses (12, 13, 16, 23, 24). To overcome this limitation and to enhance immunogenicity, PSs have been conjugated to carrier proteins to make conjugate vaccines, an approach first reported in the 1920s and 1930s (3, 4, 9, 10). This strategy has been highly successful in the prevention of infection with *Haemophilus influenzae* type b (Hib) (1, 21).

While immunity to Hib requires antibodies to only one capsular PS serotype, there are at least 90 different *S. pneumoniae* capsular serotypes, more than 20 of which are considered clinically relevant (2). Therefore, pneumococcal conjugate vaccines will require multiple conjugates, each consisting of a different PnPS linked to a carrier protein. However, clinical trials with a heptavalent PnPS-protein conjugate vaccine, in which each PnPS was conjugated to the same carrier protein, showed that the monovalent components of the vaccine had widely varying abilities to elicit PnPS-specific antibodies (5, 7, 17). The reasons for such differences in immunogenicity are unclear, especially in instances where different PnPSs are attached by identical methods of conjugation to the same carrier protein.

In this report, we examine the immunogenicities of three PnPS-protein conjugate vaccines in a mouse model and investigate the mechanisms that might account for the significant differences observed in the magnitudes of PnPS-specific antibody responses despite linkage to the same carrier protein, Cross Reactive Material 197 (CRM$_{197}$) (25). In particular, we address the hypothesis that conjugation of different PnPSs to a carrier protein such as CRM$_{197}$ can change the T-cell response to the conjugate vaccine by altering the antigen processing of the carrier protein, thereby modifying T-cell help for B-cell production of PS-specific antibodies. *S. pneumoniae* capsular serotypes 6B, 19F, and 23F were chosen for study due to the high clinical incidence of disease caused by these three serotypes in humans and because of their inclusion as components in the new heptavalent pneumococcal conjugate vaccine undergoing clinical trials (7, 17). Our data show that conjugation of different PnPSs to the same carrier protein can alter the peptide specificity of T-cell responses. However, despite marked differences in the immunogenicity of the PnPS components of these pneumococcal conjugate vaccines in a mouse model, vigorous carrier protein-specific T-cell activation after immunization can be demonstrated with all three vaccines.

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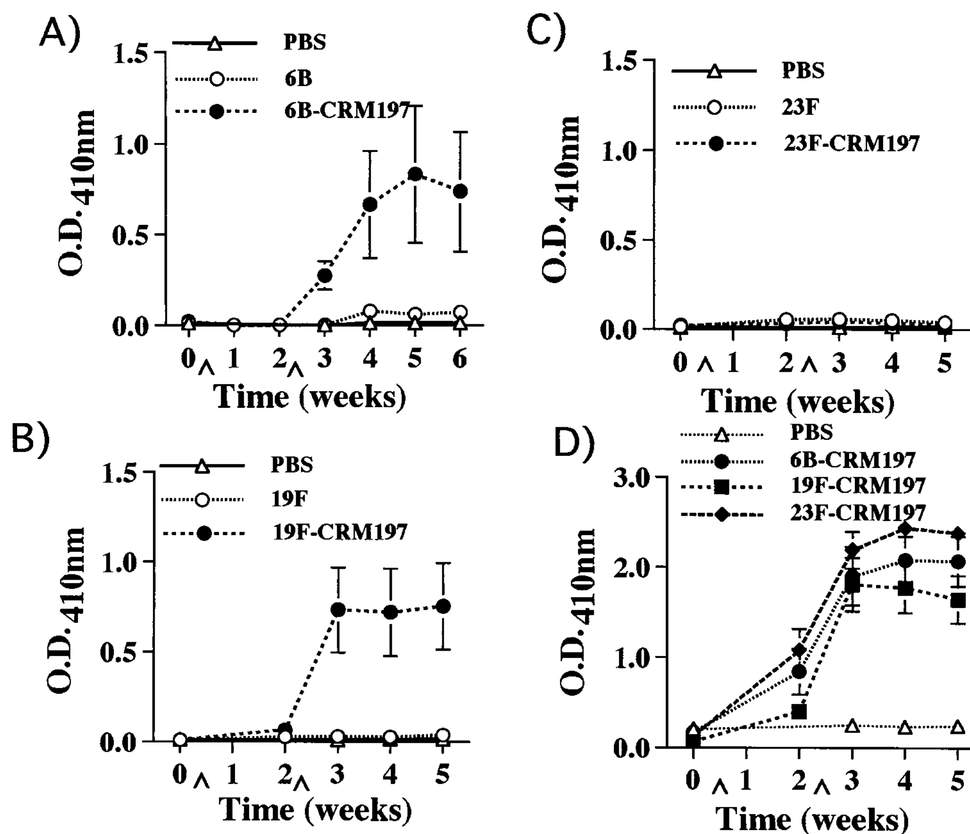

FIG. 1. Total serum Ig kappa chain reactivities of (A) 6B-specific, (B) 19F-specific, (C) 23F-specific, and (D) CRM_{197}-specific antibodies over time in CBA/J mice as detected by PnPS solid-phase ELISA. Data shown are from a representative experiment that was repeated three times with similar results. Sera were diluted 1:100, and the mean absorbances ± standard errors of the means (SEMs) of six mice per group are shown. ∧ indicates time point of immunization. O.D., optical density.

MATERIALS AND METHODS

Antigens. Experimental lots of unconjugated CRM_{197} and 6B-CRM_{197}, 19F-CRM_{197}, and 23F-CRM_{197} conjugate vaccines were the generous gift of Wyeth-Lederle Vaccines (West Henrietta, N.Y.). PnPSs were individually conjugated to CRM_{197} by reductive amination. The PS/protein ratios of the experimental vaccine lots were as follows: 6B-CRM_{197}, 0.69; 19F-CRM_{197}, 0.66; and 23F-CRM_{197}, 0.52 (8). These experimental lots did not contain any adjuvant, as is the case with the commercially available Hib-CRM_{197} conjugate vaccine HibTITER. Unconjugated 6B, 19F, and 23F PnPSs were obtained from American Type Culture Collection (Rockville, Md.). These PnPS preparations are similar to those used in the conjugation procedure. Pneumococcal cell wall polysaccharide (C-PS) was obtained from the University of Rochester (Rochester, N.Y.). A series of 16-mer CRM_{197} peptides with an overlap of 12 amino acids was produced by multipin synthesis (Chiron Technologies, Raleigh, N.C.). All conjugates and unconjugated PnPS, CRM_{197}, and peptides were determined to contain less than 0.1 U of endotoxin per ml of sample by using a *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.).

Immunization of mice. Six-week-old female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were immunized intraperitoneally (i.p.) on days 0 and 14 with 11 μg (protein content) of CRM_{197}, 10 μg (PS content) of 6B-CRM_{197}, 19F-CRM_{197}, and 23F-CRM_{197}, or 10 μg of 6B, 19F, or 23F unconjugated PnPS in phosphate-buffered saline (PBS). The dose of unconjugated CRM_{197} was approximately equivalent to the amount of CRM_{197} injected into the conjugate-immunized mice. Ten micrograms of PnPS was chosen as the amount to be injected since dose response experiments demonstrated this dose to yield optimal antibody titers. Mice immunized with sterile PBS served as negative controls. CBA/J mice were chosen based on the availability of reagents for studying murine antigen processing and T cells, specifically the H-2^k system (11), and as a result of the observed differences in the immunogenicities of the different serotypes of pneumococcal conjugate vaccines. These differences in PnPS immunogenicity are similar to those observed in humans (17). Mice were bled from the tail vein weekly for 5 weeks, and the sera were screened for anti-PnPS antibodies via enzyme-linked immunosorbent assay (ELISA) as described below.

ELISA for antibodies against polysaccharides and carrier protein. Ninety-six-well PolySorp plates (Nunc, Roskilde, Denmark) were coated with 100 μl of 6B, 19F, or 23F PnPS (obtained from the American Type Culture Collection) at 10 μg/ml of PBS. These plates were previously found to bind all of these PnPS serotypes (26). After plates were blocked with 200 μl of PBS containing 1% bovine serum albumin and 1% NaN_3, a 100-μl sample was added to each well. Sera, standards, and controls were diluted in PBS containing 1% bovine serum albumin and 1% NaN_3. Fifty micrograms of C-PS/ml of sera was added to absorb anti-C-PS antibodies (15). Serum samples diluted 1:100 were used to assess relative differences in antibody production over time. To detect CRM_{197}-specific antibodies, 96-well high-binding plates (Corning Glass Works, Corning, N.Y.) were coated with 100 μl of a solution containing 1 μg of CRM_{197} per ml of a coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6). Serum samples were prepared as for the PnPS ELISA, except that C-PS was not added. The relative titers of IgM and IgG_1 PS- or CRM_{197}-specific antibodies were determined for serum samples obtained 2 weeks after the secondary immunization. Serial dilutions of these sera were used in the ELISA. Antisera derived after hyperimmunization of BALB/c mice with 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197} in monophosphoryl lipid-A (RibiImmunoChem Research, Hamilton, Mont.) served as positive controls. Detection of total PnPS- or CRM_{197}-specific serum antibodies was performed by using goat anti-mouse kappa antibodies conjugated to alkaline phosphatase (AP) (Southern Biotech, Birmingham, Ala.). Anti-kappa antibodies were chosen since the vast majority of murine PS-specific antibodies contain kappa light chains. Serum antibodies of specific isotypes were detected by using goat anti-mouse-IgG_1–AP and -IgM–AP antibodies. The plates were washed and developed with *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) as the substrate, and absorbances were read as optical densities at 410 nm.

Lymph node proliferation assay. Female CBA/J mice were immunized in the hind footpads with 100 μl of antigen (CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, 23F-CRM_{197}, or hen egg lysozyme [HEL]) in complete Freund's adjuvant (CFA, Sigma) at a final concentration of 800 μg/ml (protein content). HEL-immunized mice served as a negative control. Nine days later, the popliteal lymph nodes were removed, and the cells were suspended in standard media (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, streptomycin, L-glutamine, and sodium pyruvate [Hyclone, Logan, Utah]) and plated at a concentration of 4×10^5 cells/well in 96-well tissue culture plates with antigen at 0, 1, 3, and 10 μg/ml. Peptide-specific lymph node cell proliferation was assessed with each CRM_{197} peptide at a concentration of 5 μM. A solution containing 10

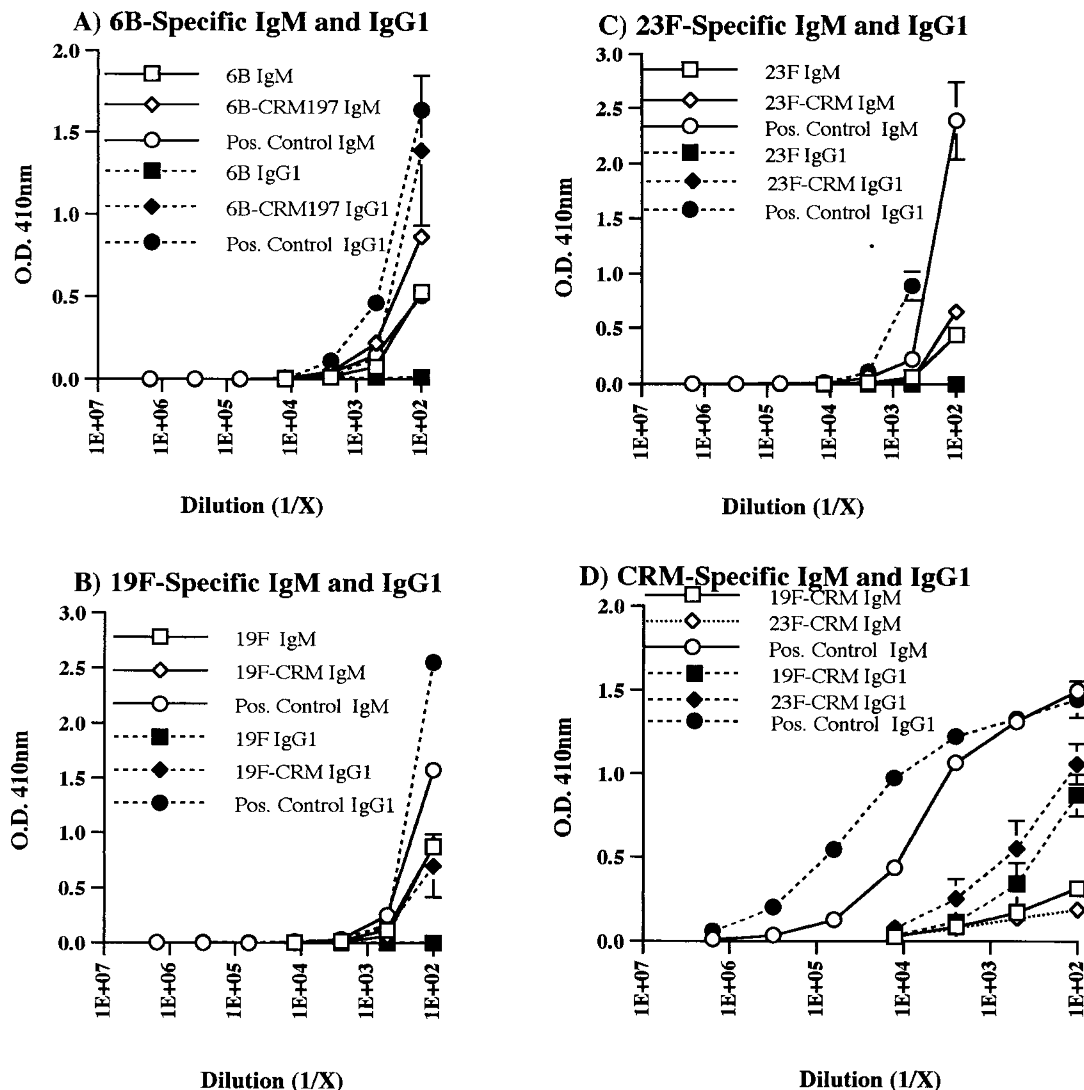

FIG. 2. Levels of (A) 6B-specific, (B) 19F-specific, (C) 23F-specific, and (D) CRM_{197}-specific IgM and IgG_1 in CBA/J mice were detected by PnPS or CRM_{197} solid-phase ELISA using isotype-specific conjugates. Reactivities of PnPS-specific IgG_1 and IgM are shown as a function of serial serum dilution 14 days after secondary immunization. Points represent means ± SEMs of six mice per group, as in Fig. 1.

μg of CRM_{197} per ml served as a positive control in these experiments. The plates were incubated at 37°C in 5% CO_2 for 4 days and then [^{3}H]thymidine (1 μCi/well) was added. The following day the cells were harvested and [^{3}H]thymidine incorporation was determined by a scintillation counter (Packard, Walkersville, Md.).

Statistical methods. An analysis of variance was used to determine statistical differences between experimental groups in the lymph node proliferation assays. Differences within groups were analyzed by the use of the Tukey multiple comparison method. The threshold for statistical significance was taken as $p \leq 0.05$.

RESULTS

Immunization with 6B-CRM_{197} and 19F-CRM_{197}, but not 23F-CRM_{197}, results in high titers of PnPS-specific antibodies. CBA/J mice were immunized i.p. with 6B, 6B-CRM_{197}, 19F, 19F-CRM_{197}, 23F, or 23F-CRM_{197}. Sera were obtained weekly for 5 weeks and then tested by ELISA for the presence of PnPS- and CRM_{197}-specific antibodies. High-titer 6B- and 19F-specific antibodies were detected in mice immunized with 6B-CRM_{197} or 19F-CRM_{197}, in contrast to the low titers of 6B- and 19F-specific antibodies detected in mice immunized with unconjugated PnPS (Fig. 1A and B). In addition, the total 6B- and 19F-specific serum antibody levels elicited by the conjugates, but not by the unconjugated PnPS, rose significantly after secondary immunization, as expected. The PnPS-specific antibodies elicited by immunization with the conjugates were primarily of the IgM and IgG_1 isotypes, in contrast to the predominantly IgM antibodies elicited by the unconjugated PnPS (Fig. 2A and B). PnPS-specific IgG_3 was not detected in any of the sera tested.

In comparison, mice immunized with either 23F or 23F-CRM_{197} had very low serum titers of 23F-specific antibodies (Fig. 1C and 2C). 23F-specific antibodies were, however, detected in sera from BALB/c mice immunized with 23F-CRM_{197}, demonstrating that the appropriate 23F PnPS B-cell epitopes were not destroyed by the conjugation process (data not shown). Finally, CBA/J mice immunized with the conjugates in CFA according to the protocol for the lymph node proliferation assays had antibody titers similar to those of mice immunized i.p. without adjuvant. This immunization protocol

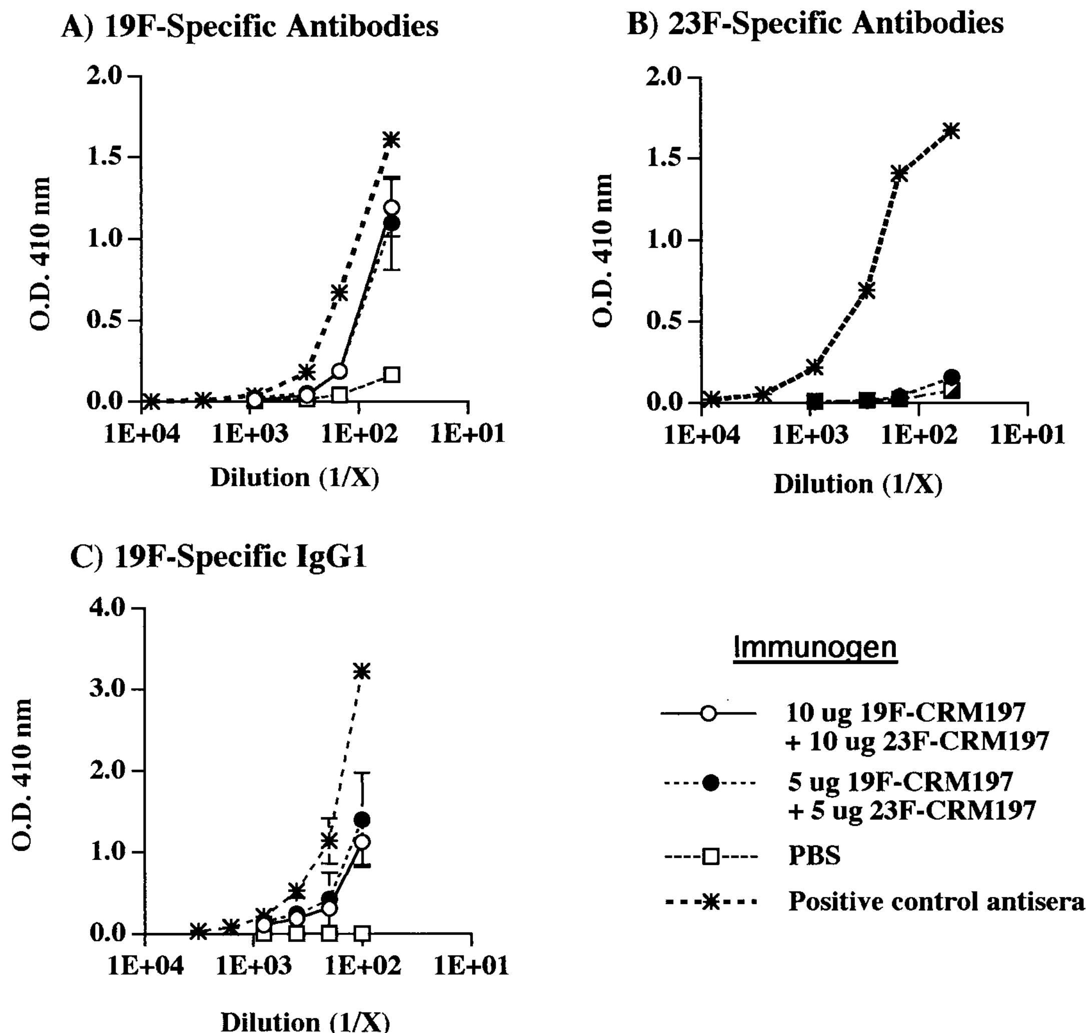

FIG. 3. Polysaccharide-specific antibody levels in mice immunized simultaneously with either 10 or 5 μg each of 19F-CRM_{197} and 23F-CRM_{197}. Total serum Ig kappa chain reactivities of (A) 19F-specific and (B) 23F-specific antibodies in CBA/J mice as detected by PnPS solid-phase ELISA. 19F-specific IgG_1 detected in the same sera is also shown (C). Reactivities of PnPS-specific antibodies are shown as a function of serum dilution. The mean absorbances ± SEMs of groups of three mice are shown. O.D., optical density.

also elicited relatively low titers of 23F-specific antibodies (data not shown).

When mice were immunized simultaneously with both 19F-CRM_{197} and 23F-CRM_{197}, the levels of 23F-specific antibody were very low (Fig. 3B). These same mice, however, produced significant levels of 19F-specific antibodies, including antibodies of the IgG_1 isotype, production of which generally indicates the presence of T-cell help (Fig. 3A and C). Thus, despite the evidence of carrier-specific T-cell help, 23F-specific antibody levels remained very low.

In contrast to PnPS-specific antibody responses, carrier-specific (CRM_{197}) antibody responses were equivalent in sera from mice immunized with 6B-CRM_{197}, 19F-CRM_{197}, and 23F-CRM_{197} (Fig. 1D). The predominant isotype of the CRM_{197}-specific antibodies, following two immunizations, was IgG_1 (Fig. 2D). These results indicate that an immunogenic form of CRM_{197} was administered to all of the mice, that CRM_{197} was processed and presented by antigen-presenting cells, and that T-cell help was elicited after immunization with any of the three conjugate vaccines used.

Conjugation of different PnPSs to CRM_{197} yields similar magnitudes of lymph node cell proliferation upon restimulation in vitro. To more directly determine whether the serotype-specific difference in the ability to elicit PnPS-specific antibodies could be due to differential T-cell activation for the various conjugates, lymph node cells were obtained from mice immunized with 6B-CRM_{197}, 19F-CRM_{197}, 23F-CRM_{197}, or unconjugated CRM_{197} in CFA. These cells were incubated in vitro with CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197}, and cellular proliferation, an indicator of antigen recognition by T cells, was measured by [^{3}H]thymidine incorporation (18). Cells from mice primed with 6B-CRM_{197} proliferated more in response to the conjugates than in response to CRM_{197} alone ($p \leq 0.0011$). Although CRM_{197} was less effective at eliciting T-cell proliferation after immunization with 19F-CRM_{197} or 23F-CRM_{197}, these differences did not reach statistical significance (Fig. 4A, B, C, and D). In addition, the responses of lymph node cells elicited by CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197} to any of the conjugates did not significantly differ between immunization groups (analysis of

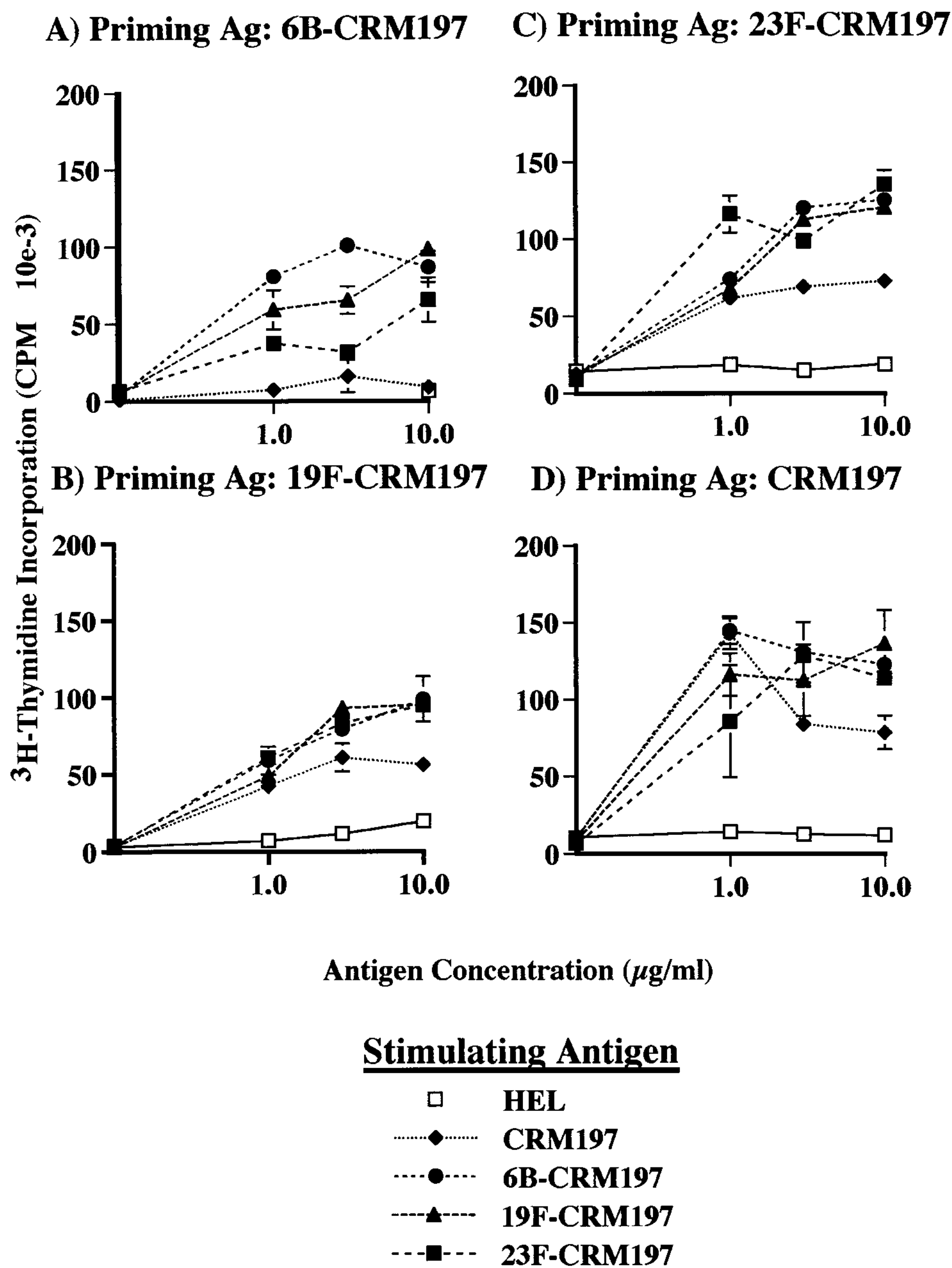

FIG. 4. Proliferation of lymph node cells from mice immunized with (A) 6B-CRM_{197}, (B) 19F-CRM_{197}, (C) 23F-CRM_{197}, or (D) CRM_{197} as determined by [^{3}H]thymidine incorporation. Lymph node cells were stimulated in vitro with whole antigen (CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197}). Data shown are derived from three mice per group and are representative of four experiments that yielded similar results. Values represent means ± standard deviations (S.D.s) from triplicate wells. Baseline proliferation in these experiments for lymph node cells stimulated in vitro with media alone was approximately 2,000 cpm.

variance, $p > 0.45$). Control lymph node cells elicited by HEL only responded to in vitro stimulation with HEL and not to any of the conjugates (data not shown), indicating the specificity of the lymph node cells and the inability of the conjugates to act as nonspecific mitogens. Similarly, the PSs alone were unable to stimulate the lymph node cells (data not shown). Finally, in vitro stimulation with a mixture of CRM_{197} and each of the conjugates did not decrease proliferation, showing that nonspecific inhibition of lymphocyte proliferation by the unconjugated CRM_{197} preparation did not occur (data not shown). The equivalent proliferation of T cells and the three PnPS-CRM_{197} conjugates used as recall antigens suggests that similar numbers of peptide-MHC II complexes were generated by antigen processing of carrier protein in the conjugates.

Lymph node cells elicited by 23F-CRM_{197} recognize different sets of CRM_{197} peptide epitopes. In order to determine if conjugation of PnPS to CRM_{197} altered the peptides recognized by carrier-specific T cells, lymph node cells from mice immunized with CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197} in CFA were challenged in vitro with a series of synthetic 16-mer peptides, overlapping by 12 amino acids and covering the entire CRM_{197} amino acid sequence. This peptide library was expected to contain all possible linear T-cell epitopes of the A and B fragments of CRM_{197}.

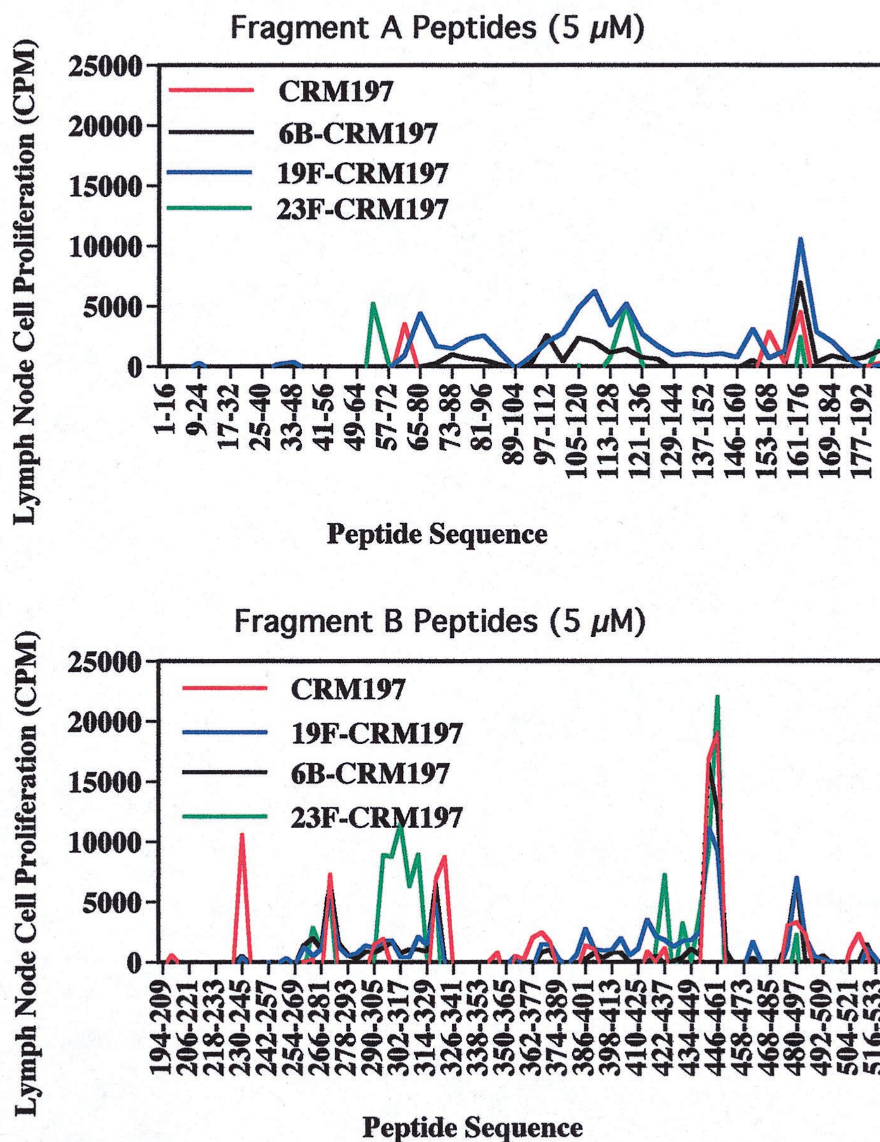

FIG. 5. Proliferation of lymph node cells from mice immunized with CRM_{197}, 6B-CRM_{197}, 19F-CRM_{197}, or 23F-CRM_{197} to 16-mer peptides derived from the CRM_{197} fragment A (A) and fragment B (B) amino acid sequences as measured by [^{3}H]thymidine incorporation. For clarity, only every third peptide in the series is marked on the *x* axis. Data shown are from a experiment that was repeated three times, yielding similar results each time. Values represent means from triplicate wells minus baseline proliferation plus two times the S.D.s. Only significant peaks are shown.

Lymph node cells elicited by CRM_{197} recognized a number of peptide sequences, including amino acids 360 to 380 of fragment B, a previously described CRM_{197} T-cell epitope in H-2^s mice (6). However, lymph node cells elicited by 23F-CRM_{197} demonstrated several major changes in peptide reactivity in comparison with lymph node cells elicited by CRM_{197}, 6B-CRM_{197}, and 19F-CRM_{197} (Fig. 5). First, there was an expansion of the sequence of amino acids 438 to 465 recognized by lymph node cells elicited by 19F-CRM_{197}, to 414 to 465 for lymph node cells elicited by 23F-CRM_{197}. Second, lymph node cells elicited by 23F-CRM_{197} shifted recognition from positions 318 to 331, as seen in the case of lymph node cells elicited by CRM_{197} or 19F-CRM_{197}, to amino acids 290 to 333 and with a different peak response. Lymph node cells elicited by 23F-CRM_{197} also recognized the sequence of amino acids 53 to 62, which was not recognized by lymph node cells elicited by the other conjugates. Lymph node cells elicited by 23F-CRM_{197} did not react to 54 other peptides that were recognized by lymph node cells elicited by 6B-CRM_{197} or 19F-CRM_{197} (Table 1). However, 38 of the 41 peptides that were recognized by lymph node cells elicited by 23F-CRM_{197} were also recognized by lymph node cells elicited by 6B-CRM_{197} and 19F-CRM_{197}. In summary, lymph node cells from mice immunized with 23F-CRM_{197} were unable to recognize some peptides derived from the CRM_{197} amino acid sequence that were recognized by cells from mice immunized with the other conjugates. In addition, cells from mice immunized with 23F-CRM_{197} recognized additional peptides that were not recognized by lymph node cells from mice immunized with CRM_{197}, 6B-CRM_{197}, or 19F-CRM_{197}. Thus, conjugation of CRM_{197} to 23F PnPS, as opposed to 6B or 19F PnPS, was associated with a different pattern of T-cell reactivity for CRM_{197}-derived peptide epitopes, although there was substantial overlap in peptide reactivities.

TABLE 1. Comparative peptide reactivities of lymph node cells[a]

Antigen	Total[b]	CRM197		6B-CRM197		19F-CRM197		23F-CRM197	
		+[c]	−[d]	+	−	+	−	+	−
CRM197	50	50	0	40	41	36	56	23	18
6B-CRM197	81	40	10	81	0	73	19	38	3
19F-CRM197	92	36	14	73	8	92	0	38	3
23F-CRM197	41	23	27	38	43	38	54	41	0

[a] Lymph node cell proliferation in response to CRM_{197} peptides from mice immunized with carrier or PnPS conjugate vaccine. Proliferation to a peptide was defined as a cpm value greater than background plus two times the S.D.

[b] Total number of peptides recognized by lymph node cells elicited by antigens listed in the first column.

[c] +, Number of peptides recognized in common by lymph node cells elicited by antigens in left and top columns.

[d] −, Number of peptides recognized by lymph node cells elicited by immunization with antigen at the top but not recognized by lymph node cells elicited by antigen at the left.

DISCUSSION

PS-protein conjugate vaccines have the potential to dramatically decrease the incidence of infection with PS-encapsulated bacteria such as pneumococcus. The Hib vaccines, the prototypes of successful conjugate vaccines, have resulted in the virtual eradication of disease due to Hib in the United States and much of the developed world. However, the Hib conjugate vaccines require multiple doses to achieve protection, and are thus costly, precluding widespread usage in the developing world. More detailed information on the properties of conjugate vaccines that contribute to greater immunogenicity may lead to improved designs for future conjugate vaccines.

Current hypotheses as to the mechanism by which the carrier protein enhances PS-specific immunogenicity envision internalization of the PS-carrier complex by the PS-specific B cells and proteolysis of the carrier protein, providing peptides able to bind noncovalently to class II MHC. The carrier confers on the PS-specific B cell the ability to activate helper T cells through the presentation of carrier-derived, class II MHC-bound peptides. The carrier-dependent boost to PS-specific immunogenicity is thus attributed primarily to carrier-dependent T-cell help. It was thus assumed that linking different PnPSs to the same immunogenic carrier protein would comparably enhance PS-specific antibody titers.

Given the success of the Hib vaccine, it would seem straightforward to create a series of PnPS-protein conjugate vaccines that consist of capsular PS from common pathogenic serotypes conjugated to the same carrier proteins successfully used in Hib conjugates. In fact, recent clinical trials with a heptavalent vaccine, using CRM_{197} as the carrier protein, showed clinical efficacy in preventing invasive pneumococcal disease from vaccine serotypes in children (7). However, it is intriguing that in clinical studies significant variations in immunogenicity of the serotype-specific PnPS components of these vaccines utilizing the same carrier protein have been observed (17). In the present study, we also observed pronounced differences in immunogenicity, in that the 23F-CRM_{197} conjugate vaccine elicited substantially less PnPS-specific antibody than the 6B-CRM_{197} and 19F-CRM_{197} vaccines in CBA/J mice. Thus, while these mice were perfectly capable of making antibodies to two of the PnPSs, one PnPS serotype was a poor immunogen even when conjugated to the same immunogenic carrier protein. We chose to explore the possibility that variation in T-cell responses to the carrier protein caused by conjugation to a PS of different structure yielded differences in antigen processing that might account for the difference in PS-specific conjugate immunogenicity. Therefore, we immunized mice with the three conjugate vaccines or carrier alone and determined the reactivity of lymph node cells following restimulation in vitro with each of the conjugates or with carrier protein. Lymph node cells from mice immunized with any of the conjugate vaccines, including 23F-CRM_{197}, proliferated similarly upon restimulation with any of the conjugates. A vigorous T-cell recall response was obtained even when the PnPS-specific antibody response was poor. Thus, these results do not provide evidence for a defect in the ability to activate carrier protein-specific T cells uniquely associated with the 23F-CRM_{197} conjugate as an explanation for the observed deficiency in 23F PnPS-specific immunogenicity.

Lymph node cells from mice immunized with 6B-CRM_{197} proliferated significantly more upon restimulation with conjugates than when stimulated with carrier alone, suggesting that antigen processing of the carrier protein linked to 6B PnPS yielded more peptide-MHC II complexes than were generated from the unconjugated carrier protein. Lymph node cells from mice immunized with 19F-CRM_{197} or 23F-CRM_{197} demonstrated a similar trend, but statistical significance was not achieved. Further studies will be required to determine if conjugation of the CRM_{197} carrier protein to PnPS directly alters the efficiency of antigen processing by PS-specific B cells.

We next carried out a higher-resolution analysis by determining lymph node cell proliferative responses to 16-mer peptides (overlapping by 12 amino acids) spanning the entire primary structures of the A and B fragments of CRM_{197}. Lymph node cells from mice immunized with CRM_{197}, 6B-CRM_{197}, or 19F-CRM_{197} exhibited similar patterns of peptide reactivity. Lymph node cells from mice immunized with 23F-CRM_{197} reacted with many of the same peptides that elicited responses from the CRM_{197}-, 6B-CRM_{197}-, or 19F-CRM_{197}-immune cells but also reacted with additional groups of peptides. In addition, lymph node cells elicited with 23F-CRM_{197} did not react to many of the peptide epitopes recognized by lymph node cells elicited by the other conjugates. Conjugation of PnPS to CRM_{197} is not site specific, and the 23F PnPS may be bound to CRM_{197} so as to change the patterns of proteolysis during processing, yielding presentation of a different set of epitopes than the other two pneumococcal conjugate vaccines containing structurally distinct PnPS. Since the 23F PnPS conjugate exhibits the lowest PS-specific immunogenicity, it is possible that 23F-CRM_{197} elicited T cells that were not optimal for stimulation of 23F PnPS-specific B cells. However, the ability of 23F-CRM_{197} to elicit a strong CRM_{197}-specific antibody response as well as strong lymph node cell recall proliferative responses to the whole vaccines suggests that the 23F-CRM_{197} vaccine activated helper T cells comparably to 19F-CRM_{197} and 6B-CRM_{197}.

Measurement of the antibody responses to the carrier protein following immunization with PnPS-CRM_{197} conjugates indicated that all three PnPS conjugate vaccines elicited approximately equivalent amounts of total carrier-specific antibody and carrier-specific IgG_1 antibody. These results imply that the failure of the 23F conjugate to induce PnPS-specific antibody or PnPS-specific IgG in this model was not due to failures of antigen administration or antigenic integrity of the carrier protein unique to that conjugate. Furthermore, since the response to the carrier protein is T cell dependent, as supported by the significant production of carrier-specific IgG_1, these results suggest that the carrier in the 23F conjugate was competent to induce helper T cells. Thus, simple measurement of antibody or T-cell responses to carrier proteins of

conjugate vaccines may not predict the immunogenicity of the PS.

In conclusion, our results suggest that variations in carrier-induced T-cell help may contribute to differences in the PS-specific immunogenicity of conjugate vaccines. However, the similarities in lymph node proliferation and CRM_{197} immunogenicity between all the conjugates studied suggest that other mechanisms may also explain the poor immunogenicity of the 23F PnPS conjugate vaccine. Differences in PnPS-specific B-cell precursor frequency or PnPS-CRM_{197}-induced patterns of cytokine production could contribute to the observed variation in the PnPS-specific immunogenicity of PnPS-CRM_{197} conjugate vaccines.

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