Antigen Processing of the Heptavalent Pneumococcal Conjugate Vaccine Carrier Protein CRM$_{197}$ Differs Depending on the Serotype of the Attached Polysaccharide

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The pneumococcal (Pn) conjugate vaccine includes seven different polysaccharides (PS) conjugated to CRM$_{197}$. Utilizing antigen-processing cells and a CRM$_{197}$-specific mouse T-cell hybridoma, we found that the serotype of conjugated PnPS dramatically affected antigen processing of CRM$_{197}$. Unconjugated CRM$_{197}$ and serotype conjugates 14 and 18C were processed more efficiently.

Antibodies (Ab) against capsular polysaccharides (PS) of Streptococcus pneumoniae (Pn) are protective against disease in animal models and in humans (15, 21). PS antigens, however, are T-independent type 2 antigens and generally do not induce immunological memory or a protective Ab response in infants less than 24 months of age (3, 5, 12, 15, 20, 21). Immunization with PS conjugated to a carrier protein results in induction of protective levels of anti-PS Ab in infants as well as immunological memory (2, 21). The first effective conjugate vaccine used purified Haemophilus influenzae type b (Hib) PS linked to CRM$_{197}$ (cross-reactive material), a nontoxic mutant diphtheria toxin (22), while other Hib vaccines use different carrier proteins. The use of these conjugate vaccines has resulted in the virtual eradication of Hib infections in the United States (2).

The currently licensed heptavalent PnPS conjugate vaccine is a combination vaccine containing seven purified serotypes of PnPS (4, 6B, 9V, 14, 18C, 19F, and 23F) individually conjugated to CRM$_{197}$. This vaccine targets the Pn serotypes most frequently responsible for pediatric invasive disease in the United States (8, 9) and has been shown to have protective efficacy against invasive disease from homologous serotypes in children after four doses of vaccine (1, 17, 19).

Despite conjugation to the same carrier protein, the PnPS contained in the heptavalent conjugate vaccine elicited markedly different serotype-specific Ab titers in children and adults (1, 11, 17, 19). One potential explanation for the serotype-specific variation in immunogenicity of the components of the vaccine is that structurally different PnPS might affect antigen processing of the carrier protein, CRM$_{197}$, yielding differences in carrier protein-induced T-cell help. We studied the effect of the serotype of PnPS conjugated to CRM$_{197}$ on antigen processing of the carrier protein in cells from HLA-DR1-transgenic mice.

HLA-DR1-transgenic mice obtained from Merck Research Laboratories were added to wells containing HLA-DR1-transgenic mouse splenocytes from the mice as APC, and measuring T-cell IL-2 production. The greatest response was observed with two adjacent peptides from the B fragment of CRM$_{197}$ with the amino acid sequences PGKLDVNKSKTHISVN (CRM$_{197}$ residues 245 to 264) and DVNKSKTISVNGRKI (CRM$_{197}$ residues 249 to 264). These peptides thus contain the epitope for this hybridoma (Fig. 1). No response was observed with peptides from the A fragment of CRM$_{197}$ (data not shown).

In order to determine if the serotype of the PnPS affects processing of the carrier protein, multiple lots of PnPS-CRM$_{197}$ conjugate types 4, 6B, 9V, 14, 18C, 19F, and 23F (kindly supplied by Ronald Eby, Wyeth Vaccines, West Henrietta, N.Y.) were added to wells containing HLA-DR1-transgenic mouse splenocytes (1 $\times$ 10$^7$) or bone marrow macrophages (BMM; 1 $\times$ 10$^5$) in medium supplemented with recombinant mouse gamma interferon (R&D Systems, Inc., Minneapolis, Minn.), diluted according to the concentration of CRM$_{197}$ (0 to 16 $\mu$g/ml). Hen egg lysozyme (HEL; Sigma-Aldrich Co., St. Louis, Mo.) was used as a negative control protein, and individual free PS from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (American Type Culture Collection, Manassas, Va.) were also used as negative controls. T hybridoma cells (10$^5$) were then
FIG. 1. The epitope for CRM<sub>197</sub> T-cell hybridoma is on the B peptide of the diphtheria toxin. Splenocytes (4 × 10<sup>5</sup>) and T-hybridoma cells (1 × 10<sup>5</sup>) were incubated with 83 16-mer peptides at 5 μM concentrations. T-cell hybridomas secrete IL-2 in proportion to the amount of peptide-MHC complex presented. IL-2 production was measured by ELISA (mean optical density [O.D.]). Two adjacent B-chain peptides elicited the strongest response, as did the positive control of unconjugated intact CRM<sub>197</sub>. A-chain peptides (not shown), other B-chain peptides, and HEL did not stimulate the hybridoma.

added to each well. The degree of peptide-major histocompatibility complex (MHC) presentation to the hybridoma was determined via IL-2 ELISA of the well supernatant after incubation.

Splenocyte processing of the conjugates containing different serotypes of PnPS linked to CRM<sub>197</sub> yielded markedly different presentation of the CRM<sub>197</sub> peptide to the T-cell hybridoma (Fig. 2A). Unconjugated carrier protein was better recognized by the hybridoma than any of the PnPS-CRM<sub>197</sub> conjugates, while carrier protein from serotype 14 and 18C conjugates was more efficiently processed and presented than that from the 9V, 4, and 23F conjugates. Carrier proteins from the serotype 19F and 6B conjugates were presented least efficiently, with recognition by the hybridoma being only slightly better than that demonstrated by incubation with HEL (Fig. 2A). None of the control unconjugated PnPS serotypes elicited IL-2 production, and addition of free PS to CRM<sub>197</sub> did not affect antigen processing (data not shown). Similar results were obtained when BMM from HLA-DR1-transgenic mice were used as APC. As observed in the splenocyte experiments, unconjugated CRM<sub>197</sub> was processed most efficiently, and CRM<sub>197</sub> conjugated to serotypes 14 and 18C was presented more efficiently than that conjugated to serotypes 4 and 6B (Fig. 2B). Differences in protein content of the individual PnPS serotype-CRM<sub>197</sub> conjugates did not explain the different responses, since the components were diluted to contain identical quantities of CRM<sub>197</sub> (the protein content of the conjugates was confirmed by bicinchoninic acid protein assay). Similar results were also obtained with different lots of vaccine.

The proposed mechanism for the enhanced immunogenicity of PS-protein glycoconjugate vaccines compared to that of pure PS involves recognition and internalization of the glycoconjugate by a PS-specific B cell, endosomal proteolysis of the carrier protein, noncovalent association of peptide fragments with class II MHC molecules, and presentation of this complex at the cell surface to a CD4<sup>+</sup> T cell with receptor specificity for the carrier protein and the MHC molecule (4, 6, 23). The association of carrier-derived, MHC II-bound peptide with the T-cell receptor activates the T cell to secrete a variety of cytokines along with CD40/CD40L interactions that presumably stimulate the PS-specific B cell to proliferate, secrete Ab, undergo isotype switching, and differentiate into memory cells.

PS do not bind to class II MHC molecules and, therefore, do not directly activate T cells (7, 10). However, Ishioka et al. observed that glycosylation of a known peptide T-cell epitope affected the MHC binding and T-cell recognition of the peptide depending on the location of the carbohydrate moieties relative to the residues of the core MHC-binding regions on the peptide (10). When carbohydrate was located in the core MHC-binding region, either T-cell recognition was abolished or the antigenic determinant recognized by the T cell was altered. It seems probable that the structure of the carbohydrate conjugated to CRM<sub>197</sub> influences the variety of epitopes produced from carrier protein if proteolysis or MHC binding is affected. We also previously demonstrated that T cells from mouse lymph nodes generated by immunization with CRM<sub>197</sub> alone, 6B-CRM<sub>197</sub>, 19F-CRM<sub>197</sub>, or 23F-CRM<sub>197</sub> recognized different peptide epitopes on the carrier protein when screened with the same 16-mer peptides used to map our T-cell hybridoma (14). This finding again suggests that the serotype of the PS had influence over the T-cell epitopes produced by the APC. Since the linkage sites in the PnPS conjugates between PS and protein are random, individual PnPS serotypes are not conjugated to the same part of the CRM<sub>197</sub> molecule. Thus, the observed differences in the antigen processing of the various conjugates by APC may relate to the proximity of the PS linkage to the MHC-binding region of the carrier protein (10).

We demonstrated substantial differences in the presentation of CRM<sub>197</sub> to T-hybridoma cells by APC after processing of different serotypes of PnPS-CRM<sub>197</sub> conjugates. Other studies have shown that the individual components of the PnPS conjugate vaccine elicit different patterns of cytokine responses from carrier-specific T cells. Mice immunized with type 14-CRM<sub>197</sub> and 19F-CRM<sub>197</sub> PnPS vaccines produced markedly different cytokine responses in splenocytes upon restimulation with the conjugates (13). These cytokine profiles were associated with different immunoglobulin G subclass Ab responses against the PS.

The observed differences in the efficiency of CRM<sub>197</sub> processing based on the serotype of the attached PnPS would not likely explain the variable serotype specific anti-PS Ab responses noted in both mice and humans. Since all seven serotypes of PnPS conjugated to CRM<sub>197</sub> in the multivalent vaccine are administered simultaneously, such differences in efficiency of antigen processing may be irrelevant in immunized humans. In addition, we have recently reported that CD4<sup>+</sup> T cells taken
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