Immunity to Cross-Reactive Serotypes Induced by Pneumococcal Conjugate Vaccines in Infants

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In infants were immunized with 1 of the 3 experimental pneumococcal conjugate vaccines that contain 6B and 19F but not 6A or 19A serotypes. Their sera were studied for the capacity to opsonize Streptococcus pneumoniae 6A, 6B, 19A, and 19F serotypes and the level of IgG antibody to the 4 serotypes. Significant increases were observed in the number of infants with detectable opsonophagocytic titers with 3 conjugate vaccines for 6B (vaccine) serotype but with only 2 vaccines for 6A (cross-reactive) serotype. Significant increases were observed with 2 conjugate vaccines for 19F serotype but with only 1 vaccine for 19A serotype. Thus, some conjugate vaccines may elicit cross-protection better than others. In addition, correlations between opsonophagocytic titers and IgG antibody levels by ELISA were high for 6B and 19F serotypes but low for 6A and 19A serotypes. Thus, ELISA may be an inadequate surrogate assay of vaccine response for cross-reactive serotypes.

Streptococcus pneumoniae is a leading cause of pneumonia, meningitis, and sepsis among young children [1]. Pneumococcal sepsis is associated with high rates of mortality even with the appropriate antibiotic treatment [2, 3], and the prevalence of antibiotic-resistant S. pneumoniae is high and increasing [4, 5]. Thus, there is a great need for effective vaccines against S. pneumoniae.

Ninety different serotypes of S. pneumoniae have been identified, based on the serologic properties of their polysaccharide (PS) capsules [6]. Because antibodies to a capsular PS can protect against S. pneumoniae expressing the homologous or cross-reactive capsule serotypes, pneumococcal vaccines are commonly designed to induce antibodies to the capsular PS. The current PS pneumococcal vaccines contain purified capsular PS from 23 common serotypes, enough to cover >90% of cases of S. pneumoniae [7]. However, many PS capsules are poorly immunogenic in young children. Under development are various PS-protein conjugate vaccines, which are formed by coupling intact pneumococcal capsular PS or oligosaccharide fragments to a “carrier” protein, as was done for the conjugate vaccines against Haemophilus influenzae type b [8]. This conjugation process converts the normally “T-independent” PS antigens to “T-dependent” antigens, and the conjugate elicits the T cell factors that enhance the antibody responses to the PS antigens [9].

The current experimental conjugate vaccines contain 7 (e.g., serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) or more serotypes [9, 10]. To increase the coverage for protection, additional serotypes (e.g., serotypes 1, 3, 5, 6A, 7F, and 19A) may be added to the conjugate vaccines in the future. However, it is commonly assumed that the 6B and 19F conjugate vaccines elicit antibodies cross-reacting with 6A and 19A serotypes, respectively (figure 1), and that the cross-reactive antibodies protect against infections of 6A and 19A serotypes [7]. To examine this assumption, we measured the opsonophagocytic capacity and the PS-specific IgG antibody concentrations to the 4 serotypes (6A, 6B, 19A, and 19F) in sera from young children who were immunized with 3 different experimental conjugate vaccines containing 6B and 19F PS. Opsonization assay was chosen because it directly measures the functional capacity of pneumococcal antibodies.

Materials and Methods

Vaccines. The 4 pneumococcal vaccines used in this study are shown in table 1. They include 3 experimental conjugate vaccines (PP, OP, and MK) and 1 currently licensed polysaccharide vaccine (PV). PV contains PS of 6B, 19A, and 19F serotypes (25 µg/dose) but does not contain 6A serotype PS (table 1). PP vaccine is a pentavalent conjugate vaccine prepared by conjugating the whole PS to a cross-reacting mutant protein of diphtheria toxin (CRM197) [13]; OP vaccine is a pentavalent conjugate vaccine prepared by coupling oligosaccharides to CRM197 protein (both from Wyeth-Lederle Vaccines, Pearl River, NY) [13]. Both conjugate vaccines were adsorbed onto alum, and each dose contained 5 µg of PS of each serotype. MK vaccine is a heptavalent conjugate vaccine pro-

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duced by conjugating PS to the outer membrane protein complex of *Neisseria meningitidis* serogroup B (NM-OMPC; Merck, West Point, PA) [14]. MK vaccine is adsorbed onto aluminum-hydroxide and each dose contained 3.5 μg of 6B and 2 μg of 19F PS [14]. None of the 3 conjugate vaccines contain 6A or 19A serotype PS.

*Human sera.* The serum samples in this study were from various clinical trials, control groups, and volunteers, as listed in table 1. The PP and OP groups each consisted of 25 infants immunized with either the PS-CRM197 or the oligosaccharide-CRM197 vaccine given at ages 2, 4, and 6 months; serum samples were obtained at age 7 months. The samples were provided by Wyeth-Leder Vaccines (Henrietta, NY) and have been described elsewhere [13]. The MK group included 53 infants vaccinated with MK vaccine by one of us (J.I.W., UCLA) at ages 2, 4, and 6 months; their postimmune serum samples were obtained at age 7 months. For comparison, we also studied serum samples from 10 7-month-old control infants who were not immunized with any pneumococcal vaccines and from 15 healthy young adult vaccinees. The adult vaccinees were recruited in a medical center (Washington University, St. Louis), and their serum samples were obtained 1 month after immunization with PV (the 23-valent pneumococcal vaccine). The samples from unimmunized children were obtained from Wyeth-Leder Vaccines. All sera were stored frozen at −20°C until analyzed.

*Opsonophagocytic killing assay.* Opsonophagocytic activities of the samples were determined by the method of Gray [15] with minor modifications as described below. The target bacteria were strains SP85 (serotype 6A), LS2016 (6B), DB18 (19A), and DS2212 (19F) from D. Bries (Birmingham, AL). The serotypes of the 4 strains were confirmed by measuring the ability of the supernatant of the bacteria strains to inhibit the binding of pneumococcal antibodies to ELISA plates coated with capsular PS of a known serotype. Also, opsonization assay for 19F serotype could be inhibited by 19F, but not 19A PS, in solution. The assay for 19A could be inhibited by 19A but not by 19F PS. The target bacteria were grown in Todd-Hewitt broth with 0.1% yeast extract and kept frozen at −70°C in aliquots in Hanks’ buffer with 15% glycerol. We incubated 10 μL of bacteria suspension (~2000 cfu) with 40 μL of appropriately diluted antibody for 30 min at room temperature. The bacteria were then incubated with 40 μL of phagocytic cell suspension (~10^5 cells) and 10 μL of baby rabbit complement (Accurate Chemical, Westbury, NY) for 1 h at 37°C with shaking. As phagocytes, we used HL60 cells (a human promyelocytic cell line; American Type Culture Collection [ATCC], Rockville, MD) differentiated in a medium containing 0.8% dimethyl formamide for 6 days. We applied 10 μL of the reaction mixture to a Todd-Hewitt agar plate with 0.5% yeast extract. After the plates were incubated overnight at 37°C in 5% CO₂, we determined the number of colonies of surviving bacteria. The opsonization titer of sera was determined as the dilution of the sample that results in a 50% reduction in viable bacteria as observed with no antisera.

*ELISA.* The amount of anti-capsular PS antibody was determined by a sandwich-type ELISA as previously described [16]. In brief, the wells of Immulon II plates (Dynatech, Chantilly, VA) were coated at 37°C with 10 or 30 μg/mL of capsular PS overnight in PBS, which was prepared fresh using water from a water purification system (Milli-Q UF; Millipore, Bedford, MA) to minimize the background signal. Pneumococcal capsular PS of serotypes 6B, 19A, and 19F were purchased from ATCC. The 6A PS was a gift from G. Schiffman (Stonybrook, NY).

After being coated with the antigen, plates were washed and blocked with PBS containing 1% bovine serum albumin. A serum pool (89-SF; C. Frasch, Food and Drug Administration, Bethesda, MD) was used as the standard in all assays. 89-SF was assigned a concentration of 16.9 mg/L IgG anti-6B and 13 mg/L IgG anti-19F antibody [17]. Our laboratory assigned 10 U/mL and 8 U/mL to 89-SF, respectively, as the IgG anti-6A and anti-19A antibody concentrations for this study. These values were chosen to make 1 U about 1 μg (unpublished data). All samples were preabsorbed with 10 μg of C-PS (Statens Serum Institut, Copenhagen) per 20 μL of serum in a total volume of 1 mL of diluent for ≥30 min at room temperature. The samples were then added to wells, titrated, and incubated for 2 h at room temperature. The wells in the plates were washed and incubated with alkaline phosphatase-conjugated goat antibody against human IgG (Sigma, St. Louis). The amount of the enzyme immobilized to the well was determined with para-nitrophenyl phosphate substrate (Sigma) in diethanolamine buffer. Optical density at 405 nm was read with a microplate reader (Cambridge Technology, Watertown, MA). The amount of antibody in the sample was determined by comparing the optical density of the samples to that of the standard using piecewise linear interpolation.

*Statistics.* Comparisons of 2 proportions were made with Fisher’s exact test using the NCSS 6.0 program (Kaysville, UT). Log mean values of the antibody concentrations and opsonization titers were compared by 2-tailed Student’s *t* test (Quattro Pro 7; Corel, Ottawa).

**Results**

*Immune responses to vaccine serotypes 6B and 19F.* To assess the immune response to vaccine serotypes, we first measured the opsonic responses to 6B serotype, a serotype included
in all of the vaccines (figure 2A). No opsonic activity (titer <30) was found in any of the 10 unimmunized infants, but it was demonstrable (titer >30) in 50% of the infants who were immunized with MK vaccine ($P = .0012$). OP and PP vaccines induced detectable titers in 70% and 80% of the infants, respectively, and these responses were higher than for MK vaccine ($P = .2$ and .013, respectively) and were comparable to that seen among the adults immunized with the 23-valent PS vaccine. Very high opsonic titers (>300) were observed in 25%–50% of infants who received any conjugate vaccine. When anti-6B ELISA antibody levels were compared (figure 2C), all 3 conjugate vaccines increased the average level of antibody by several fold over that for unimmunized infants ($P = .006$, non-immunized vs. MK vaccine), but ~10-fold less than that of adults who received PV vaccine ($P < .001$, adult PS and PP vaccine groups).

To study immune responses to another serotype included in the conjugate vaccine, we measured the capacity of the infant sera to opsonize 19F serotype (figure 2B). All unimmunized infants had undetectable (titer <30) opsonophagocytic titers to 19F serotype. Few (<10%) infants developed detectable titers after immunization with OP vaccine. Although OP vaccine elicited a small but significant increase in antibody levels (figure 2D), the data suggest that OP vaccine is poorly immunogenic for this vaccine serotype. In contrast, opsonophagocytic titers were demonstrable in about half the infants immunized with PP vaccine ($P = .0069$, PP vs. no-vaccine groups) and in all 53 infants after a course of MK vaccine. The opsonophagocytic titers induced with PP vaccine were ~10-fold less than those of adults; however, the opsonic response to MK vaccine was similar to that of adults in prevalence and magnitude of the response (figure 2B). Antibody levels determined by ELISA suggest that PP and MK vaccines elicit clear and comparable antibody responses (figure 2D). Thus, by either measure, 2 of the 3 conjugate vaccines elicit immune responses to 19F serotype in infants.

**Immune responses to cross-reactive serotypes 6A and 19A PS.** To examine the immune responses to cross-reactive serotypes, the opsonophagocytic titers and antibody levels to 6A and 19A serotypes were measured (figure 3). With respect to 6A serotype, none of the unimmunized infants demonstrated measurable titers, but all 3 conjugate vaccines elicited measurable opsonophagocytic titers (>30) in about half the infants (figure 3A; $P = .073$ and .014, respectively, for OP and PP vaccines, Fisher’s exact test). The opsonic titers were comparable in magnitude among the 3 conjugate vaccine groups but were ~10-fold less than in adults. Of interest, despite the high magnitude of their opsonic responses, adults could also be divided into 2 groups: about half the adults had detectable levels of opsonic titers, and the other half had none. To our surprise, all 3 groups of infants had levels of IgG antibody to 6A serotype that was only slightly higher than those of unimmunized infants ($P = .065$, unimmunized vs. OP vaccine group), as shown in figure 3C. Therefore, immune responses to 6A serotype were demonstrated by the opsonophagocytosis assay but not by ELISA.

When immune responses to 19A serotype were examined, none of the unimmunized infants had detectable opsonophagocytosis titers. After immunization with either PP or OP vaccines (figure 3B), only ~10% of infants developed detectable opsonophagocytosis titers. This indicated that neither vaccine stimulated the production of cross-opsionic anti-19A antibodies when compared with the unimmunized infants ($P > .58$). In contrast, the MK vaccine group had higher opsonic titers than did unimmunized infants; 60% of the infants in the group had detectable opsonic titers ($P = .0009$), and the magnitude of their opsonic responses was only ~2-fold less than that of adults ($P = .0065$) immunized with the 23-valent vaccine containing 19A PS. Thus, the MK vaccine elicited opsonophagocytic responses to 19A serotype, but OP and PP vaccines did not.

As shown above for 6A serotype, a totally different result was obtained when we measured the anti-19A antibody levels by ELISA (figure 3D). MK vaccine elicited very little anti-19A antibody responses, whereas PP and OP vaccines stimulated an increase in antibody levels of ~10-fold ($P < 2 \times 10^{-6}$ vs. no-vaccine group). The unexpected differences in results could have been explained if the serotype of pneumococci used for the opsonization assay was misidentified. However, the pneumococci had the correct serotypes. Immune responses to types 6A and 19A were observable, but the responses were dependent on the vaccines and on whether the response was measured by functional (opsonophagocytic) or quantitative (ELISA) methods.

*Young children can be divided into 2 populations by their ability to produce antibodies opsonizing cross-reactive serotypes. Opsonic responses to the cross-reactive serotypes were demonstrable in fewer infants than responses to the vaccine serotypes (figures 2, 3). This may arise because their antibodies are weakly cross-reactive or because many infants produce non-cross-reactive antibodies. To distinguish these possibilities, data shown*
Figure 2. Serum sample % (y axis) with more opsonization (Ops) titers than shown on x axis (A, B). Serum sample % (y axis) with more IgG antibody (Ab) levels than on x axis (C, D). Serotypes 6B (A, C) and 19F (B, D) are included in conjugate vaccines. Conjugate vaccines used were MK (●), OP (▲), and PP (▼) vaccines. For comparison, serum sample data from infants who did not receive any pneumococcal vaccines (■) and from adults immunized with 23-valent polysaccharide vaccine (○) are also plotted in all panels. Break in x axis indicates assay sensitivity limits for infant serum samples. Sensitivity limit for opsonophagocytosis assay for adult samples was 10, lower than that for young children (i.e., 30).

For serotypes 6B and 6A in figures 2 and 3 were replotted in figure 4. When the responses to PP and OP vaccines were examined (figure 4A), as was expected from figure 2 and figure 3, PP and OP vaccines induced opsonic titers to 6A in half the infants, and their opsonic titers to 6A were commensurate with the opsonic titers to 6B. These infants appear to produce cross-reactive antibodies in response to the 2 conjugate vaccines. The other half of the infants produced only homologous antibodies having high (>100) opsonic titers to 6B serotype with very low (or no) opsonic titers to 6A serotype. Furthermore, a zone free of data points was observed between the 2 groups of data points in figure 4A. Thus, infants can make antibodies that are either specific for the vaccine serotype or cross-reactive with other serotypes. Adults also made both types of antibodies, but most adults (12 of 15) produced antibodies cross-reactive with 6A (figure 4). This observation is not limited to 6A/6B serotypes, since the data analysis for serotypes 19F and 19A revealed similar (but less dramatic) findings (data not shown).

An interesting observation was that the cross-reactivity of antibodies is somewhat associated with the vaccines. For instance, MK vaccine appears to stimulate cross-reactive antibodies more than others, since those with measurable opsonic titers to 6B serotype generally had measurable levels of opsonic titers to serotype 6A, just like the adults (figure 4B). Similarly,
6A

19A

C

D

Figure 3. Serum sample % (y axis) with more opsonization (Ops) titers than shown on x axis (A, B). Serum sample % (y axis) with more IgG antibody (Ab) levels than indicated on x axis (C, D). Serotypes 6A (A, C) and 19A (B, D) are not included in conjugate vaccines. Conjugate vaccines used were MK (○), OP (▲), and PP (▼) vaccines. For comparison, serum sample data from infants who did not receive any pneumococcal vaccines (■) and from adults immunized with 23-valent polysaccharide vaccine (○) are also plotted in all panels. Break in x axis indicates assay sensitivity limits for infant serum samples.

PP vaccine elicited 19F-specific antibodies that did not cross-react with 19A, whereas MK vaccine elicited antibodies reacting with both 19F and 19A (figure 2, figure 3).

Correlation between opsonic titers and IgG antibody levels estimated by ELISA is weaker for cross-reactive serotypes than for vaccine serotypes. As shown in figure 3, opsonophagocytosis assays and ELISAs often showed contradictory results for cross-reactive serotypes. Less pronounced but still contradictory findings were also seen with data for the vaccine serotypes. For example, MK and PP vaccines elicited comparable anti-6B antibody responses (P = .12), but MK vaccine elicited ~10-fold lower opsonic responses than PP vaccine for this serotype (P = .0024). Conversely, MK vaccine elicited higher opsonic responses than PP vaccine, while both vaccines elicited similar levels of anti-19F antibodies. Because of the contradictory nature of the 2 assay results, the relationship between the 2 assays was investigated by directly comparing the 2 assay results obtained with MK and PP vaccines (figure 5).

For the 2 vaccine serotypes (6B, 19F), antibody levels correlated well with the opsonophagocytosis titers. The correlation coefficient for 6B serotype was 0.76 and 0.89, respectively, for MK and PP vaccines (figure 5A), and the coefficient for 19F serotype was 0.66 and 0.76, respectively, for MK and PP vaccines (figure 5C). Consistent with the observation that MK vaccine elicited more opsonic anti-19F antibodies than PP vaccine (figure 2), MK data points were generally to the left of PP vaccine data points for 19F serotype. This finding was reproducible with repeat assays of the same samples (data not
axis) plotted against those of 6B serotype (y axis). Conjugate vaccines used were OP and PP (A, ▲ and ▼, respectively) and MK (B, ■). For comparison, data for serum samples from adults immunized with 23-valent polysaccharide vaccine (○) are also plotted in all panels. Breaks in axes indicate assay sensitivity limits for infant serum samples. Sensitivity limit of opsonization assay for adult samples was 10.

Figure 4. Opsonization (Ops) titer to 6A serotype (y axis) plotted shown, suggesting that this finding was not due to any temporary bias in the assay results.

With respect to cross-reactive serotypes (6A, 19A), ELISA results correlated very poorly with opsonization titers (figure 5B, figure 5D). MK vaccines showed correlation coefficients of 0.11 and 0.46 for 6A and 19A serotypes, respectively. Similarly, for PP vaccine, the correlation coefficients were 0.38 and 0.17 for 6A and 19A serotypes, respectively. There were samples that had high levels of antibody but low opsonophagocytosis titers, and others with very high opsonophagocytosis titers but low ELISA titers. This suggests that the causes for the poor correlation are likely to be multiple and complex. Taken together, antibody concentrations determined by ELISA may be a useful measure of vaccine response for the vaccine serotypes, but not for cross-reactive serotypes.

Discussion

We studied the ability of postvaccination sera from young infants to opsonize pneumococci of 2 pairs of related serotypes (6A and 6B, 19A and 19F). We used the in vitro opsonophagocytic killing capacity of the sera as the primary measure of vaccine responses, because opsonization is the primary mechanism of protection by antibodies against S. pneumoniae [18, 19], and there are technical problems with the methods for measuring the concentration of pneumococcal antibodies by ELISA [20–22]. Although all 3 experimental conjugate vaccines varied a little, they uniformly elicited significant levels of opsonic activities to the pneumococcal serotypes in the vaccines. However, the conjugate vaccines differed greatly in their abilities to induce opsonic responses to respective cross-reactive serotypes.

At present, it is unknown why the conjugate vaccines differ in the capacities to opsonize pneumococci of cross-reactive serotypes. One possibility is that the observed differences are due to the vaccine formulations, since the vaccines evaluated differed significantly in their design. However, it is premature to conclude that one vaccine design is superior to the other in inducing cross-protection because our data are limited and are based on unmatched serum samples. An alternative explanation is that the observed differences are due to differences in the populations used in this study. MK vaccine was tested with infants in California, while PP and OP vaccines were tested with infants in several cities in the eastern half of the United States [13]. In support of this possibility, studies employing different test populations reported strikingly different H. influenzae type b vaccine efficacies [23, 24]. Also, the protection against group B streptococci induced by a pneumococcal vaccine was dependent on the characteristics of the test population [25]. To further examine these possibilities, studies using the same population must be performed.

Since the opsonic titers against the cross-reactive serotypes were smaller in magnitude and were induced less frequently than the titers against the vaccine serotypes, cross-opsonic antibodies induced in young children with conjugate vaccines may be insufficient to provide cross-protection. Although no clinical studies have investigated this issue directly, 2 groups of investigators studied the effect of conjugate vaccines on the rate of nasopharyngeal carriage of pneumococci by children. A study in South Africa reported that the carriage rate of S. pneumoniae of 6A serotype was not decreased by immunization, whereas the carriage rate of 6B serotype was [26]. In contrast, the carriage rates of both serotypes 6A and 6B in toddlers were decreased in a follow-up to an Israeli study [27]. The rate of 6A carriage was reduced later (6-17 months after the last dose), whereas the rate of 6B carriage was reduced 1 month after the last dose (R. Dagan, personal communication). There were differences in the 2 studies (e.g., population, age), and the carriage studies may reflect vaccine-induced mucosal immunity and may not predict in vivo protection against pneumococcal infections.

Measurement of opsonophagocytic capacities is tedious and technically demanding. If the opsonic capacities of the sera to either homologous or cross-reactive pneumococcal serotypes could be associated with the concentration of antibodies to the
capsular PS, the evaluation of new or modified pneumococcal vaccines would be greatly simplified. We found that the ELISA results of child sera are poorly correlated with the opsonization titers for cross-reactive serotypes even though the two correlated well for the vaccine serotypes. The observed discrepancy could have been reconciled if MK vaccine elicited more IgM antibodies than IgG antibodies compared with other vaccines. However, all child groups appeared to have comparable levels of IgM pneumococcal antibodies (data not shown).

There are several potential explanations for the discrepancy between ELISA and opsonization assay results for cross-reactive serotypes. One is that pneumococcal antibody measured by ELISA may lack assay specificity, although the neutralization of antibodies to C-PS before the assay has greatly improved the specificity of the ELISA for pneumococcal antibodies. In view of the reports describing the problems with ELISA specificity [20–22], we need to further investigate the specificity of the ELISA for measuring pneumococcal antibodies against the cross-reactive serotypes. A second possibility is that antibodies bind an epitope that is expressed on the capsular PS of live bacteria but not on PS adsorbed onto the plastic surface. We believe this possibility is remote, but we recently discovered a mouse hybridoma that binds 6A PS in solution and opsonizes \textit{S. pneumoniae} 6A well but binds poorly to the 6A PS immobilized onto the plastic surface (unpublished data). Another possibility is that children produce antibodies with very low avidity to cross-reactive serotypes. There are several reasons for cross-reactive antibodies to have low avidity. First, vaccination selected the B cells producing antibodies with high avidity to the vaccine serotypes not to the cross-reactive serotypes. Second, antibodies to PS generally have low avidities, and young children have restricted abilities to make diverse antibody V regions [28, 29]. Additional studies are in progress to develop a method of measuring avidity of pneumococcal antibodies in sera from young children.

A large number of pneumococcal serotypes are clinically important within a geographic area, and the clinically important serotypes vary among different regions of the world [7, 30]. Thus, a successful pneumococcal vaccine should provide protection against a large number of serotypes, and this will depend to some extent on providing protection against cross-reactive serotypes. Our findings suggest that some conjugate vaccines
may elicit cross-protection against related PS better than others. Until the laboratory surrogates for cross-protection are better established, the presence of the cross-protection should be directly determined in clinical trials of the pneumococcal vaccines as well as during the postlicensure monitoring surveys by serotyping the clinical isolates of pneumococci.

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References