Functional Antibodies Elicited by an 11-Valent Diphtheria–Tetanus Toxoid–Conjugated Pneumococcal Vaccine

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The aluminium-adjuvanted 11-valent pneumococcal conjugate vaccine containing polysaccharides 1, 4, 5, 7F, 9V, 19F, and 23F (coupled to tetanus protein) and polysaccharides 3, 6B, 14, and 18C (coupled to diphtheria toxoid) elicits high antibody concentrations in Filipino infants when given at ages 6, 10, and 14 weeks and 9 months simultaneously with the national vaccination program. We evaluated functional activity of these antibodies by using a viable cell opsonophagocytic assay (OPA). The OPA titers correlated with the respective antibody concentrations. The geometric mean OPA titers after 3 vaccine doses were 276.9 (serotype 4), 12.3 (serotype 6B), 46.0 (serotype 14), 119.3 (serotype 19F), and 206.3 (serotype 23F). The functionality of antibodies increased after the fourth dose of vaccine (i.e., the concentration required for in vitro killing of pneumococci decreased). Both the quantity and quality of antibodies are important in the evaluation of immunogenicity of pneumococcal vaccines.

The new pneumococcal vaccines with capsular polysaccharides (PSs) from 7–11 different serotypes conjugated to various protein carriers—such as the nontoxic variant of diphtheria toxin (CRM197), meningococcal outer membrane protein complex, diphtheria–tetanus toxoid protein, or Haemophilus influenzae protein D—are immunogenic, safe, and well tolerated [1–6]. The PS antibodies elicited by the vaccines have been shown to protect against invasive pneumococcal infections, pneumonia, and acute otitis media caused by serotypes included in the vaccines [1–3].

The quantity and quality of antibodies required for protection against pneumococcal infections remain unknown and may be different for mucosal and invasive infections, as well as in different populations and epidemiological settings. Techniques such as radioantigen binding assay and EIA have been developed to measure the total binding antibody concentration. Because the host’s protection against pneumococcal infections is mediated mainly by phagocytosis, the functional activity of antibodies in killing of pneumococci, rather than just the concentration of antibodies, may correlate better with the efficacy needed to prevent infections [7, 8]. Several different opsonophagocytic assays (OPAs), using either peripheral blood leukocytes or culturable human premyelocytic leukemia cells (HL-60 cells), recently have been developed, to measure the functionality of antipneumococcal antibodies [9, 10], but, thus far, very limited data exist on the functional antibody...
responses to different pneumococcal conjugate vaccines.

We recently demonstrated that a new 11-valent diphtheria–tetanus toxoid protein–conjugated pneumococcal vaccine elicits high concentrations of antibodies against most vaccine serotypes in Filipino infants [5] and that the geometric mean concentrations (GMCs) of antibodies are considerably higher than those in Finnish and Israeli infants receiving the same vaccine [11]. The causes for this variation are not known and may include factors such as early pneumococcal carriage acquisition, priming effect of tetanus toxoid given to pregnant women, and genetic differences. It is also possible that contacts with cross-reacting bacteria, which are more common in developing countries, could result in high concentrations of non-specific antibodies with poor protective efficacy.

In this study, we used a viable cell OPA with culturable HL-60 cells to describe the functional activity of antibodies elicited by an 11-valent, mixed carrier diphtheria–tetanus toxoid protein–conjugated pneumococcal vaccine in Filipino infants. Our aim was to determine antipneumococcal PS antibody concentrations required to kill pneumococci in vitro after 3 and 4 vaccine doses. This new method for the evaluation of vaccine immunogenicity could be used with efficacy data when establishing the serologic correlates for protection against pneumococcal infections.

SUBJECTS AND METHODS

Vaccine recipients. From June 1998 to August 1999, all infants born at full term of pregnancy (≥37 weeks) who were 6–9 weeks old and who were to start receiving their national Expanded Program on Immunisation (EPI) vaccines were offered enrollment into the open, uncontrolled, descriptive study at the village health center in Cabuyao, a semirural municipality of Laguna (Island of Luzon, Philippines).

Study vaccine. The study vaccine (batch S3497; Aventis Pasteur) included pneumococcal PSs of serotypes 3, 6B, 14, and 18C coupled to diphtheria toxoid and pneumococcal PSs of serotypes 1, 4, 5, 7F, 9V, 19F, and 23F coupled to tetanus protein, adjuvanted with aluminium.

Vaccination and sampling schedule. Liquid vaccine for intramuscular injection (a prefilled 0.5-mL ready-to-use glass syringe) was administered into the anterolateral aspect of the right thigh at ages 6, 10, and 14 weeks, according to the EPI schedule, but in a separate syringe at a different site with the diphtheria, tetanus, whole-cell pertussis, and Haemophilus influenzae type b vaccine (Aventis Pasteur), oral polio vaccine (Aventis Pasteur), and plasma-derived hepatitis B virus vaccine (MedTest). The fourth vaccine dose was given simultaneously with the measles vaccine (Aventis Pasteur) at age 9 months. Three milliliters of blood was collected by venipuncture at ages 6 and 18 weeks and 10 months. All serum samples were stored at −20°C until they were transported on dry ice to the National Public Health Institute in Helsinki for analysis.

Laboratory methods. The concentration of serotype-specific antipneumococcal IgG PS antibodies was determined by using a standardized EIA method [4, 5]. The method for evaluating the killing of live pneumococci by differentiated HL-60 cells (ATCC) in the presence of serum and complement was a modification [12] of the technique originally described by Romero-Steiner et al. [9]. The pneumococcal serotypes chosen for the OPA included the most immunogenic serotypes (4 and 19F), the least immunogenic serotypes (6B and 23F), and serotype 14, against which the vaccine response was poorest [5].

Opsonophagocytic activity was expressed as a titer that is the reciprocal of the serum dilution yielding 50% killing, compared with the bacterial growth in the complement controls with no serum. If no activity was detected (titer <8), the serum was assigned an arbitrary titer of 4. Because of the serotype-specific sensitivity, the opsonophagocytic activity may not have been detectable if the respective concentration of antibodies was <1.0 µg/mL.

Statistical methods. Results are presented as GMCs of antibodies and geometric mean opsonophagocytic activity (GMOPA) titers with 95% confidence intervals (CIs). A linear regression model and Spearman’s correlation coefficient test were used to analyze the correlation between antibody GMCs and respective GMOPA titers after 3 and 4 vaccine doses. Statistical comparisons of GMCs or GMOPA titers at different time points were conducted by using paired Student’s t test in logarithmic-transformed data, with a set value of significance of P <.05.

RESULTS

Subjects and samples. In total, 50 infants were enrolled in the study. Serum samples were available from 49 infants before vaccination, from 47 infants after 3 vaccine doses, and from 45 infants before and after the fourth vaccine dose.

Antipneumococcal PS antibodies before vaccination. The individual antibody GMCs at age 6 weeks ranged from values below the limit of detection to 26.9 µg/mL for serotype 19F. The highest GMCs of antibodies were against serotypes 14 (1.54 µg/mL) and 19F (0.96 µg/mL) [5]. The prevaccination OPA was measured only for serotype 14, for which the OPA titer was 46.8, with 67% of serum samples containing antibodies with a measurable OPA titer. The correlation between the concentration of antibodies and respective OPA titer was 0.82 (data not shown).

Antibody response to 3 doses of vaccine. By age 18 weeks, 3 doses of vaccine had elicited a significant increase in GMCs of antibodies against all serotypes included in the vaccine, except against serotype 14 (P = .25), which had the highest pre-
vaccination GMC (table 1). All infants had GMCs >1.0 μg/mL against serotypes 4 and 19F, whereas 49%, 83%, and 87% of infants had GMCs >1.0 μg/mL against serotypes 6B, 14, and 23F, respectively.

Most serum samples contained antibodies with measurable opsonophagocytic activity, ranging from 43% for serotype 6B to 98% for serotype 4 (table 1). Similar to the GMCs, the GMOPA titer was highest for serotype 4 (276.9) and lowest for serotype 6B (12.3). The correlation between the antibody GMCs and GMOPA titers varied from 0.53 for serotype 19F to 0.74 for serotype 6B. The ratio between antibody concentration and OPA titer (i.e., the concentration of serotype-specific antibodies required to kill 50% of pneumococci in OPA) varied according to the serotype (table 1). A concentration of 0.016 μg/mL of antibodies against serotype 23F was as effective as the >7 times higher GMC (0.119 μg/mL) of antibodies against serotype 19F in killing of pneumococci of the respective serotype. The pre-vaccination antibodies did not inhibit responses to the vaccination (i.e., increases in antibody concentration or OPA titer) for serotypes 4, 6B, 14, or 19F. The only exception was serotype 23F; for which a positive correlation was found between the high antibody concentrations before vaccination and low OPA response after 3 vaccine doses (P = .002).

Antibody response to the fourth vaccine dose. Antibody GMCs decreased significantly before the fourth dose of vaccine at age 9 months (P < .005). GMCs ranged from 0.46 μg/mL for serotype 6B to 5.72 μg/mL for type 19F. The fourth dose increased the GMC against all serotypes (P < .001). However, compared with the GMCs at age 18 weeks (i.e., after 3 doses of vaccine), the GMCs at age 10 months were significantly higher against serotypes 19F and 23F but not against serotypes 4, 6B, and 14.

The GMOPA titer after the fourth vaccine dose was significantly higher than that after the primary series of 3 doses for all studied serotypes (table 1; P < .001). The opsonophagocytic activity was measurable in 98%–100% of samples for serotypes 4, 19F, and 23F and in 75% and 78% of samples for serotypes 6B and 14, respectively. The correlation between the antibody GMCs and GMOPA titers varied from 0.73 for serotype 23F to 0.79 for serotype 19F. The concentration of antibodies required to kill 50% of pneumococci in OPA decreased for all serotypes. A similar functional efficacy was achieved with 0.054 μg/mL of antibodies against serotype 19F and 0.004–0.01 μg/mL of antibodies against serotypes 4, 6B, 14, and 23F. The linear regression lines in figure 1 demonstrate that, regardless of antibody GMC, the functionality of antibodies was higher after the fourth vaccine dose. Moreover, for serotypes 4, 14, 19F, and 23F, the regression lines representing EIA and OPA results after 3 and 4 vaccine doses were parallel (i.e., the mean increase in OPA titer remained the same at all antibody GMCs). For example, for serotypes 4 and 14, this means that the GMOPA titers after the fourth vaccine dose were nearly 8-fold higher than those after 3 doses, regardless of the antibody GMCs. The large number of negative OPA titers obscured the analysis for the serotype 6B, but the trend was similar.

**DISCUSSION**

The 11-valent diphtheria–tetanus toxoid protein–conjugated pneumococcal vaccine elicited high concentrations of anti–PS antibodies with measurable opsonophagocytic activity against most pneumococcal serotypes studied. The concentration and functional activity of antibodies varied according to each pneumococcal serotype. The GMC of antibodies after 3 doses of vaccine was highest against serotypes 4 and 19F, but neither had EIA:OPA ratios as high as those for serotypes 23F and 14. In fact, the 4-fold higher GMC of antibodies against serotype 19F, compared with that of serotype 23F, resulted in lower killing activity of pneumococci in OPA. This is in agreement with earlier studies with other conjugate vaccines, which have reported similar low correlation between antibody concentrations and OPA titers for the serotype 19F [9, 13]. The modest

**Table 1. Antibody responses after 3 (18 weeks of age) and 4 doses (10 months of age) of 11-valent diphtheria–tetanus toxoid protein–conjugated pneumococcal vaccine.**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>GMC, μg/mL (95% CI)</th>
<th>GMOPA titer (95% CI)</th>
<th>Infants with detectable OPA activity, %</th>
<th>EIA:OPA ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3 doses (n = 47)</td>
<td>4 doses (n = 45)</td>
<td>3 doses (n = 47)</td>
<td>4 doses (n = 44–45)</td>
</tr>
<tr>
<td>4</td>
<td>23.41 (18.9–28.9)</td>
<td>30.15 (23.8–38.2)</td>
<td>277 (201–382)</td>
<td>3051 (2388–3897)</td>
</tr>
<tr>
<td>6B</td>
<td>1.12 (0.8–1.6)</td>
<td>2.02 (1.37–3.0)</td>
<td>12.3 (7.7–19.8)</td>
<td>125 (62–251)</td>
</tr>
<tr>
<td>14</td>
<td>2.18 (1.7–2.9)</td>
<td>1.65 (1.1–2.6)</td>
<td>46.0 (26.4–80.0)</td>
<td>221 (102–479)</td>
</tr>
<tr>
<td>19F</td>
<td>16.11 (12.9–20.2)</td>
<td>33.43 (25.6–43.6)</td>
<td>119 (79.7–179)</td>
<td>588 (424–818)</td>
</tr>
<tr>
<td>23F</td>
<td>3.89 (2.8–5.3)</td>
<td>8.22 (5.9–11.5)</td>
<td>206 (126–338)</td>
<td>1368 (893–2096)</td>
</tr>
</tbody>
</table>

**NOTE.** Vaccine was scheduled to be given at ages 6, 10, and 14 weeks, and 9 months. EIA:OPA (opsonophagocytic assay) ratio expresses the serotype-specific antibody concentration (in μg/mL) required to kill 50% of pneumococci in the OPA. CI, confidence interval; GMC, geometric mean concentration; GMOPA, geometric mean OPA.
Figure 1. Distribution of antibody concentrations and opsonophagocytic activity (OPA) titers after 3 (18 weeks of age) and 4 (10 months of age) doses of 11-valent diphtheria–tetanus toxoid protein–conjugated vaccine.

protection against serotype 19F, despite the good antibody response, also was noticed in the Finnish Otitis Media Vaccine Trial [2], in which the efficacy against acute otitis media caused by serotype 19F was 25% (95% CI, 14%–51%), with GMCs of 3.3 and 5.0 μg/mL after the third and fourth doses, respectively. In comparison, the efficacy for serotype 6B was 84% (95% CI, 62%–93%), with GMCs of 2.0 and 9.0 μg/mL after the third and fourth doses, respectively [2]. In the present study, the GMC was lowest against serotype 6B, and half the serum samples did not have measurable functional activity after 3 vaccine doses. However, because of the limited sensitivity of the OPA method to detect functional activity in low antibody concentrations, the negative OPA titers might not correlate with the lack of protection against pneumococcal disease. The correlation between antibody concentrations and OPA titers was similar than those reported earlier in other studies [9, 10, 12, 13].

The prevaccination antibody GMC was highest against serotype 14. A majority of these antibodies, which presumably are maternally derived, had measurable killing activity, which could provide in vivo protection against pneumococcal infections early in infancy. With the possible exception of serotype 23F, the high prevaccination antibodies did not interfere with the responses to the vaccination. This suggests that the 11-valent diphtheria–tetanus toxoid protein–conjugated pneumo-
Pneumococcal vaccine can be given according to the early EPI schedule at ages 6, 10, and 14 weeks.

The functional activity of antibodies after the fourth vaccine dose was significantly higher than that after 3 doses for all studied serotypes. This result is probably explained by the continuous maturation in the avidity of antibodies, resulting in enhanced killing activity [12], or by the lower proportion of nonfunctional antibodies at age 10 months. The increase in GMCs of antibodies after 4 vaccine doses were similar for the serotypes tested, but the OPA titers were 6–9-fold higher than those after 3 doses for serotypes 4, 6B, and 14. This suggests that both the quantity and quality of antibodies are important characteristics of the immunogenicity of pneumococcal conjugate vaccines and that the OPA may provide valuable additional information to the traditional EIA method.

The OPA method has been able to demonstrate age-dependent differences in response to pneumococcal PS vaccine in elderly persons [14], low functional activity of conjugate vaccine–induced antibodies in bone marrow transplant recipients [15], and different responses to conjugate and PS vaccines in children and adults with sickle cell disease [16]. Animal models have shown that the functionality of antibodies may correlate better than the concentration with the protection against pneumococcal infections. However, without established serologic surrogates for protection, the possible benefits of OPA, compared with those of the routine EIA method, in large-scale immunogenicity studies remain controversial. In addition, the lack of sensitivity in detecting functional activity of antibodies with low concentration still limits the usefulness of the OPA.

The results of the present study further demonstrate that the 11-valent diphtheria–tetanus toxoid–protein–conjugated pneumococcal vaccine is immunogenic in Filipino infants. The number and timing of doses required to provide sufficient protection for pneumococcal infections, as well as the actual serologic surrogates for protection, should be determined on the basis of data arising from efficacy studies.

Acknowledgments

We thank Sabine Arnoux, Beatrice Chabot, Valentine Delore, Anne Tourault, Connie Gepanyao, Kari Auranen, Pirjo H Mäkelä, Esa Ruokokoski, Lydia Sombrero, Merja Väkeväinen, the field and laboratory staff, and the families in the Cabuyao community.

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