Serum Samples from Infants Vaccinated with a Pneumococcal Conjugate Vaccine, PncT, Protect Mice against Invasive Infection Caused by Streptococcus pneumoniae Serotypes 6A and 6B

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Streptococcus pneumoniae serogroup 6 is an important cause of respiratory tract disease worldwide. Vaccination with 6B polysaccharide induces antibody response to the cross-reacting serotype 6A, but the protective capacity of 6A antibodies induced in infants remains unknown. In this study, passive immunization with serum samples obtained from infants vaccinated with an octavalent polysaccharide protein conjugate vaccine, PncT, protected mice against bacteremia and/or lung infection caused by intranasal challenge with serotypes 6B and 6A. Protective infant serum samples had significantly higher serotype-specific IgG levels and opsonic activity than did nonprotective serum samples. The protective level to either serotype was ~1 μg of specific IgG antibodies injected per mouse (corresponding to ~0.3 μg/mL). The protection was strongly related to opsonophagocytic antibody levels measured in vitro. These results demonstrate that PncT induces antibodies in infants that protect mice against invasive disease caused by the homologous serotype and by the cross-reacting serotype 6A.

Streptococcus pneumoniae is a common bacterial pathogen that causes infections that range from mild mucosal to severe systemic [1–3]. Pneumococci rank among the most frequent causes of purulent otitis media in infants and young children [3–5], and the annual incidence of invasive pneumococcal disease (i.e., pneumonia, bacteremia, and meningitis) is high [6–9]. Rapid increases in antimicrobial drug resistance worldwide pose an additional threat [10, 11].

S. pneumoniae is surrounded by a polysaccharide capsule that makes the organism resistant to phagocytosis, and protection against disease is primarily mediated by capsular-specific antibodies and complement that stimulate phagocytosis [12]. Increased immunogenicity of pneumococcal polysaccharides is a major goal of vaccine development [13] because of their poor inherent immunogenicity, especially in infants [14], young children [15–17], and other risk groups [18]. Conjugation of pneumococcal polysaccharides to proteins is an effective means to stimulate polysaccharide-specific antibody responses in infants [19–25], and these conjugate vaccines are effective against invasive disease and otitis media [26]. The vaccines include the serotypes that are the predominant causes of disease; however, there is concern that the vaccines will not protect against other serotypes [13, 27, 28]. It is also not clear whether and to what extent protection can be mediated against serotypes that cross-react with the vaccine serotypes (e.g., serotypes 6A and 19A, which are not included in the vaccines, cross-react with the vaccine serotypes 6B and 19F, respectively) [2, 29–31].

In vivo and in vitro protection models provide one way to study the protective capacity of conjugate vaccines. We have created an opsonophagocytosis assay in which serum-dependent uptake of live 3H-labeled pneumococci by fresh neutrophils is measured in vitro [32–34]. We also recently developed a murine pneumonia and bacteremia protection model and demonstrated protection by passive immunization with human antibodies [35]. We previously found a good correlation between IgG levels and opsonic activity (OA) of serum samples obtained from infants vaccinated with 8-valent polysaccharide protein conjugate vaccines, PncD and PncT [22], or a monovalent vaccine, 6B-TT [25]. The aims of the present study were to determine whether antibodies elicited by PncT (containing 6B) in infants protect mice against infection caused by serotype 6B and whether antibodies elicited by the 6B polysaccharide cross-protect against infection caused by serotype 6A. We also analyzed the relationship among serotype-specific antibody levels,
isotypes, IgG subclasses, avidity, and OA in vitro and protection against lung infection and bacteremia in mice.

Materials and Methods

Infant serum samples. Infant serum samples were obtained from a clinical study on octavalent conjugate vaccines: serotypes 3, 4, 6B, 9V, 14, 18C, 19F, and 23F conjugated to tetanus toxoid (PncT; Aventis Pasteur) [22]. In brief, the infants were vaccinated at 3, 4, and 6 months of age and were boosted at 13 months of age, with either the same conjugate or a 23-valent polysaccharide vaccine (Pneumo23; Aventis Pasteur). Serum samples used in the present study were obtained from 14-month-old infants and were grouped depending on whether they received the PncT (PncT/PncT) or Pneumo23 (PncT/PS23) booster.

Antibody measurements. IgG antibodies to pneumococcal polysaccharides were measured by ELISA, according to the consensus protocol recommended by the pneumococcal ELISA workshop at the Centers for Disease Control and Prevention (CDC) in 1996 with minor modifications. In brief, ELISA plates (Maxisorp; Nunc) were coated with 10 μg/mL pneumococcal polysaccharide for 5 h at 37°C (6B; American Type Culture Collection [ATCC]; 6A; Aventis Pasteur). The international standard 89-SF (provided by C. E. Frasch, US Food and Drug Administration, Bethesda, MD) and test serum samples were diluted 1:50 and were adsorbed with 10 μg/mL cell wall polysaccharide (Statens Serum Institute) before incubation at 42°-fold dilutions for 2 h in the coated ELISA plates. Bound IgG was detected after a 2-h incubation with monoclonal antibody (MAb) to human IgG (HP-6043-HRP; Hybromida Reagent Laboratory). The reaction was developed by 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry), and the reaction was stopped by adding 0.18 M H2SO4. Absorbance was measured at an optical density (OD) of 450 nm in an ELISA spectrophotometer (TiterTek Multiscan Plus MK II; Flow Laboratories). IgG antibody levels were calculated from the international standard 89-SF and were expressed in micrograms per milliliter. Because no anti-6A IgG levels in the 89-SF standard are available, 6A IgG levels were arbitrarily assigned as 6B IgG but were expressed in arbitrary units per milliliter (AU/mL).

IgG1 and IgG2 antibodies to 6B polysaccharide were measured by the same ELISA protocol, except that the detection of biotinylated MAb to IgG1 and IgG2 (HP-6069 and HP-6002, respectively; Hybromida Reagent Laboratory) were diluted 1:200 in PBS-Tween. The reaction was developed after 2 h incubation with horseradish peroxidase-streptavidin (Research Diagnostics) diluted 1:10000 in PBS-Tween. The reaction was developed by TMB as described above, and antibody levels were calculated from the 89-SF standard by using the values assigned by Soininen et al. [36].

Avidity of IgG antibodies. Avidity of IgG antibodies was measured according to the ELISA protocol described above and included a potassium thiocyanate (KSCN) elution step [37]. Serum samples were incubated for 2 h at 1:50 dilution followed by a 15-min incubation with PBS-Tween (100% binding) or KSCN in 2-fold dilutions from 10 to 0.16 M. After being washed, bound IgG was detected after incubation for 2 h with biotin-labeled MAb HP6043-B (Hybromida Reagent Laboratory), diluted 1:500 in PBS-Tween, and incubated with avidin–alkaline phosphatase (Dako) diluted 1:2000 in PBS-Tween. The reaction was developed by p-nitrophenyl phosphate (Sigma) in diethanolamine buffer (pH 9.8), and absorbance was read at 450 nm by Titertek Multiskan spectrophotometer (Flow Laboratories). The results are expressed as an avidity index (AI), which is defined as the M KSCN needed to displace 50% of bound IgG antibody.

Opsonophagocytosis assay. OA of human serum samples was measured, as described elsewhere [33, 34], by using fresh polymorphonuclear leukocytes (PMNL) and 125I-labeled pneumococci. PMNL were isolated from donors homozygous for the FcγRIIA-H131 allele (genotyped by C. L. Anderson and J. M. Osborne, Ohio State University College of Medicine, Columbus, OH). Pneumococci were maintained in batches with known density in TrypT broth with 20% glycerol at −70°C and were cultured to midlog phase in RPMI (Life Technologies) with 10% heat-inactivated fetal calf serum (FCS; Life Technologies) and 50 μCi/mL [125I]IgG (Amersham Laboratories). Bacterial (1.5 × 107 cfu) and PMNL suspensions (1.5 × 106 cells; ratio of ~10:1) in Hank’s buffered salt solution (Life Technologies) with 2.5% heat-inactivated FCS (Life Technologies) were mixed with intact test serum samples (kept at −70°C) at 15% concentration, predetermined to be in the sensitivity range of the assay, without the addition of external complement. The total 0.5-mL volume was incubated with agitation (250 rpm) for 30 min at 37°C. Controls for nonspecific binding (NSC; all reactants except FCS instead of human serum) and the total bacterial input (TB; all reactants) were included in each assay. The reaction was stopped by adding 2 mL of PBS with 0.02% NaN3 (PBS-NaN3). The PMNL and the cell-associated bacteria (CAB) were pelleted by centrifugation at 160 g; TB was centrifuged at 2200 g. After 2 washes in PBS NaN3, cell pellets were resuspended in 0.5 mL of 1.25% deoxycholate and were transferred to 4.5 mL of scintillation liquid (Hionic Fluor; Packard). Radioactivity was measured in a liquid scintillation counter (Packard), and percentage of uptake of 125I-labeled bacteria was calculated as [(counts per minute [cpm] of CAB – cpm of NSC)/cpm of TB – cpm of NSC] × 100. OA was expressed in AU calculated from a standard curve of an adult postvaccination serum pool that was assigned 100 AU.

Mice. Outbred 8-week-old female NMRI mice (Bomholtsgard) were housed at the Institute of Pathology (Keldur, Reykjavik) and were given pelleted food and water ad libitum.

Pneumococcal challenge. We obtained serotypes 6A and 6B (strain DS 2215-94) from ATCC and CDC, respectively. Bacteria were kept in tryptose broth with 20% glycerol at −70°C until use. The day before an experiment, stocks were plated on blood agar (Difco) and were incubated overnight at 37°C in 5% CO2. Isolated colonies were transferred to a heart-infusion broth (Difco) supplemented with 10% horse serum, were incubated for 3.5 h at 37°C, and were centrifuged at 2200 g for 20 min; the pelleted was then resuspended in sterile 0.9% saline. Serial 10-fold dilutions were plated on blood agar to determine inoculum density.

The mouse infection model has been described elsewhere [35]. In brief, anesthetized NMRI mice were challenged intranasally with pneumococci in 50 μL of saline. To evaluate bacteremia, blood samples were obtained from the tail vein at various times after challenge, were diluted in sterile saline, and were plated on blood agar for life counting of pneumococci (expressed as colony-forming units per milliliter of blood). After 24 h, the mice were killed, and
were performed in which the mice were challenged with serotype 6A and boosted with Pneumo23. Two sets of experiments with PncT and 30 serum samples obtained from infants primed with PncT and 30 serum samples obtained from infants primed with PncT and 30 serum samples obtained from infants primed and boosted with Nascent vaccine (PncT/PS23). In total, we used 59 serum samples to passively immunize 2 mice each 3 h before intranasal challenge with serotype 6B. Control mice, injected intraperitoneally with saline before challenge, developed low bacteremia that was detected at 12, 18, and 24 h. At 24 h, 15 of 16 control samples were nonprotective. Thirteen serum samples protected 1 of 2 mice; these were excluded from the statistical analysis (table 1), which suggests that they were near protective levels and activity but may also have been due to experimental inaccuracy when the mice were injected.

The lungs were removed and homogenized, and then were diluted to 3 mL of sterile PBS. Serial dilutions were plated on blood agar (Difco) with selective supplement for staphylococci and streptococci containing nalidixic acid and colistin sulphate (Unipath), to determine colony-forming units per lung.

Study design. We intraperitoneally injected 59 randomly selected infant serum samples into 2 mice each (175 µL of undiluted serum per mouse) 3 h before intranasal challenge with pneumococci: 29 serum samples obtained from infants primed and boosted with PncT and 30 serum samples obtained from infants primed with PncT and boosted with Pneumococci. Two sets of experiments were performed in which the mice were challenged with serotype 6A or 6B. We used the same serum samples for passive immunization in both experiments. The inoculum dose of serotype 6B was 10^6 cfu/mouse, and mice were bled at 12, 18, and 24 h. Detection limit (1 cfu detected in the first dilution of sample x dilution factor) of 6B bacteremia was 2.26 log cfu/mL in blood. The inoculum dose of serotype 6A was 5 x 10^6 cfu/mouse, and mice were bled at 18 and 24 h. Detection limit of 6A bacteremia was 3 log cfu/mL in blood. Mice with bacteremia below the detection limit were considered to be protected. 6B-challenged mice with detectable bacteremia were considered to be not protected. Mice challenged with 6A were considered to be not protected if they had bacteremia >5 log cfu/mL; at 3–5 log cfu/mL, we considered them to have reduced bacteremia.

Serotype 6B is much less virulent than 6A [35]. Although 6A consistently caused high bacteremia (>5 log cfu/mL) in the control group, it was possible to determine reduced or undetectable bacteremia, whereas, for 6B, bacteremia was lower and variable in the control group, and we could only reliably determine whether bacteremia was present or undetectable. The detection limit for lung infection caused by either serotype was 2.48 log cfu/lung. Each serum sample was injected into 2 mice and was considered protective if both mice were protected at 24 h or nonprotective if neither mouse was protected. Serum samples that produced inconsistent results (protecting 1 of the 2 mice) were excluded from the statistical analysis.

Table 1. Protective efficacy of infant postvaccination serum samples against Streptococcus pneumoniae serotypes 6B and 6A bacteremia in mice, antibody levels, avidity, and opsonic activity (OA).

<table>
<thead>
<tr>
<th>Serotypea</th>
<th>No. (%) of serum samples (n = 59)</th>
<th>IgM µg/mouseb</th>
<th>IgG µg/mouseb</th>
<th>IgG1 µg/mouseb</th>
<th>IgG2 µg/mouseb</th>
<th>IgG AFb</th>
<th>OA AU/mousec</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B</td>
<td>Protective 31 (53)</td>
<td>0.31 (0.25–0.37)</td>
<td>1.47 (0.96–2.26)</td>
<td>0.86 (0.57–1.30)</td>
<td>0.31 (0.21–0.47)</td>
<td>2.7 (2.0–3.3)</td>
<td>17.4 (14.2–20.7)</td>
</tr>
<tr>
<td></td>
<td>Nonprotective 15 (25)</td>
<td>0.35 (0.27–0.46)</td>
<td>0.19 (0.10–0.34)</td>
<td>0.12 (0.07–0.21)</td>
<td>0.05 (0.03–0.09)</td>
<td>3.1 (1.9–4.2)</td>
<td>7.1 (4.4–9.8)</td>
</tr>
<tr>
<td></td>
<td>Inconsistent 13 (22)</td>
<td>0.28 (0.19–0.40)</td>
<td>1.45 (0.81–2.59)</td>
<td>0.85 (0.50–1.43)</td>
<td>0.20 (0.099–0.39)</td>
<td>2.3 (1.3–3.2)</td>
<td>15.3 (13.1–17.6)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>6A</td>
<td>Protective 22 (37)</td>
<td>0.51 (0.40–0.65)</td>
<td>0.81 (0.39–1.65)</td>
<td>ND</td>
<td>ND</td>
<td>2.2 (1.5–2.8)</td>
<td>13.4 (10.5–16.3)</td>
</tr>
<tr>
<td></td>
<td>Nonprotective 22 (37)</td>
<td>0.39 (0.31–0.48)</td>
<td>0.24 (0.17–0.34)</td>
<td>ND</td>
<td>ND</td>
<td>2.0 (1.4–2.5)</td>
<td>6.7 (5.3–8.1)</td>
</tr>
<tr>
<td></td>
<td>Inconsistent 15 (26)</td>
<td>0.37 (0.26–0.54)</td>
<td>0.44 (0.20–0.99)</td>
<td>ND</td>
<td>ND</td>
<td>1.9 (1.2–2.5)</td>
<td>9.3 (6.8–11.8)</td>
</tr>
<tr>
<td>P</td>
<td>.09</td>
<td>.01</td>
<td>ND</td>
<td>ND</td>
<td>.85</td>
<td>&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. AI, avidity index; AU, arbitrary units; ND, not done.

* Serum samples protecting 2 mice are considered to be protective, and serum samples protecting neither of 2 mice are considered to be nonprotective.

* Serum samples protecting 1 of 2 mice are considered to be inconsistent. Statistical comparison was done between the protective and nonprotective serum samples, and inconsistent serum samples were excluded from statistical analysis.

* Antibodies, geometric mean (95% confidence interval [CI]).

* Mean (95% CI).

Statistical analysis. We used Student’s t test to compare antibody levels (log-transformed data), AIs, and OA among groups. When the data were not normally distributed, we used the Mann-Whitney rank-sum test. We calculated correlation by Pearson’s correlation coefficient and protective efficacy by χ² analysis.

Results

Protective efficacy of infant postvaccination serum samples against serotype 6B. Serum samples were obtained from a clinical study in which infants were primed with an 8-valent polysaccharide protein conjugate vaccine, PncT, and were boosted with the same vaccine (PncT/PncT) or a 23-valent polysaccharide vaccine (PncT/PS23). In total, we used 59 serum samples to passively immunize 2 mice each 3 h before intranasal challenge with serotype 6B. Control mice, injected intraperitoneally with saline before challenge, developed low bacteremia that was detected at 12, 18, and 24 h. At 24 h, 15 of 16 control mice had 2.26–5.13 log cfu/mL in blood, and 1 mouse had undetectable bacteremia (<2.26 log cfu/mL). In general, there was good agreement between results for the 2 mice given the same serum samples. By passive immunization, 31 infant serum samples were protective against bacteremia, and 15 serum samples were nonprotective. Thirteen serum samples protected 1 of 2 mice; these were excluded from the statistical analysis (table 1). Protective infant serum samples had significantly higher IgG, IgG1, and IgG2 antibody levels and higher OA than did nonprotective serum samples, but there was no difference in IgM antibody levels or avidity of IgG antibodies (table 1). Serum samples that were inconsistent had antibody concentrations and OA between those of the protective and nonprotective serum samples (table 1), which suggests that they were near protective levels and activity but may also have been due to experimental inaccuracy when the mice were injected.

There was a correlation between IgG antibody levels and OA (r = .78; P < .0001) and between OA and IgG1 (r = .73; P <
Figure 1. Relationship among IgG levels, opsonic activity, and protection against serotype 6B (A) and 6A (B) pneumococcal infection. Each symbol represents 1 mouse injected with 175 μL of infant serum. A, Mice with undetectable bacteremia (<2.26 log cfu/mL; circles); mice with positive cultures (>2.26 log cfu/mL; black diamonds). B, Mice with undetectable bacteremia (<3 log cfu/mL; circles); mice with reduced bacteremia (3–5 log cfu/mL; gray triangles); mice with full-blown bacteremia (>5 log cfu/mL; black diamonds). AU, arbitrary units.

There was no correlation between OA and IgM levels ( ; ) or OA and IgG avidity ( ; ). There was a significant correlation between IgG levels and OA, although the correlation coefficient was low ( ; ). IgG avidity did not correlate with OA ( ; ), but there was a significant correlation between IgM and OA ( ; ). Protective serum samples from infants boosted with PS23 had significantly higher IgG levels, compared with those of protective serum samples from infants boosted with PncT (1.46 vs. 0.28 μg; ; P < .0105), although the difference in OA was not significant (15.0 vs. 10.6 AU; ; P = .125). There was no difference in IgG AIs (both 2.2; ; P > .973) or IgM antibody levels (both 0.5 μg; ; P > .973). Analysis of the relationship between IgG levels and protection revealed that 67% of serum samples containing >1 AU of IgG injected per mouse were protective, 13% were nonprotective, 20% were inconsistent, and that serum samples with lower IgG levels could be either protective or not protective (figure 1B). Of the 44 serum samples containing <1 AU of IgG, 12 (27%) were protective, and 21 (48%) were nonprotective. Statistical analysis showed comparable geometric mean IgG levels ( ; ) for the protective (0.23 AU/mL) and nonprotective (0.22 AU/mL) serum samples, but OA was higher in the pro-
Table 2. Protective efficacy of infant postvaccination serum samples against Streptococcus pneumoniae serotype 6A lung infection in mice.

<table>
<thead>
<tr>
<th>No. (%) of serum samples</th>
<th>IgM AU/mouse</th>
<th>IgG AU/mouse</th>
<th>IgG AF</th>
<th>OA AU/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protective</td>
<td>11/59 (19)</td>
<td>0.52 (0.35-0.79)</td>
<td>0.82 (0.29-2.33)</td>
<td>2.1 (1.3-2.9)</td>
</tr>
<tr>
<td>Nonprotective</td>
<td>21/59 (35)</td>
<td>0.40 (0.33-0.48)</td>
<td>0.22 (0.15-0.32)</td>
<td>1.9 (1.4-2.5)</td>
</tr>
<tr>
<td>Inconsistent</td>
<td>27/59 (46)</td>
<td>0.41 (0.31-0.53)</td>
<td>0.59 (0.32-1.07)</td>
<td>2.1 (1.5-2.6)</td>
</tr>
</tbody>
</table>

NOTE. AI, avidity index; AU, arbitrary units; OA, opsonic activity.

a Serum samples protecting 2 mice are considered to be protective, and serum samples protecting neither of 2 mice are considered to be nonprotective. Serum samples protecting 1 of 2 mice are considered to be inconsistent. Statistical comparison was done between the protective and nonprotective serum samples, and inconsistent serum samples were excluded from statistical analysis.

b Antibodies, geometric mean (95% confidence interval [CI]).

c Mean (95% CI).

Discussion

Serogroup 6 is an important cause of pneumococcal disease worldwide [4, 6, 9, 15]. The 2 cross-reactive types in this serogroup, 6A and 6B, have an identical capsular composition, except in the rhamnosyl-ribitol bonds [38]. Because of their high cross-reactivity, it was considered unnecessary to include both in the currently licensed polysaccharide vaccine. Serotype 6B was included mainly because it has greater chemical stability than serotype 6A [2, 38]. Multivalent pneumococcal conjugate vaccines in clinical trials also contain serotype 6B [19–25], and the clinical consequences of this, with respect to serotype 6A, are not known.

Good surrogate models for protection can give important information on the biological activity of antibodies. In a clinical trial, the 2 octavalent polysaccharide protein conjugate vaccines, PncT and PncD, elicited antibodies in infants that were functional against serotype 6B in an in vitro opsonophagocytosis assay, and there was a significant correlation between capsular-specific IgG antibody levels and OA [22]. Here we further demonstrate that antibodies in serum samples from infants vaccinated with PncT are functional in vitro against the cross-reactive serotype 6A and protective in mice against bacteremia caused by serotypes 6A and 6B. There was a good relationship among IgG antibody levels, OA, and protection against bacteremia. Protective serum samples had significantly higher IgG levels and OA than did nonprotective serum samples. Proportionally fewer serum samples were protective against serotype 6A (37%) than against 6B (53%), although the difference did not reach statistical significance (P = .094). However, this difference is not surprising, since the vaccine only includes serotype 6B, and since the antibodies elicited may differ in cross-reactivity to 6A. An alternative explanation could be the higher virulence of serotype 6A in mice.
OA to serotype 6A was low ($r = 0.47$), compared with that for serotype 6B ($r = 0.78$). Similarly, the difference between IgG antibody levels of protective and nonprotective serum samples was much higher for serotype 6B ($P < 0.0001$) than for serotype 6A ($P = 0.01$), whereas the difference in OA was highly significant for both serotypes ($P < 0.0001$). These observations may be because the vaccine only contains serotype 6B, and some antibodies induced by the vaccine may cross-react with serotype 6A without being functional. This is supported by the observation that, at low IgG concentrations ($<1$ AU/mouse), there was a difference in OA to type 6A between protective and nonprotective serum samples ($P = 0.03$), but there was no difference in anti-6A IgG antibody levels. IgG avidity or IgM levels did not seem to contribute to protection at such low IgG concentrations. Nahm et al. [30] similarly demonstrated that most adults vaccinated with either 6A or 6B polysaccharides produced antibodies that were functional against the cross-reacting serotype in an in vitro opsonophagocytosis assay. However, they found that $10\%$–$20\%$ of serum samples from persons vaccinated with the cross-reactive serotype had strikingly less opsonophagocytic activity than expected from the antibody concentrations [30]. In agreement with our findings, Yu et al. [39] recently demonstrated that the correlation among IgG antibody levels to serotypes 6A and 19A in vaccinated infants correlated poorly with opsonophagocytic activity, whereas the correlation was good for serotypes 6B and 19F, which were the serotypes included in the vaccines. They also demonstrated that the induction of functional antibodies to the cross-reacting serotypes varied between the conjugate vaccines used [39].

Clearance of lung infection was demonstrated against serotype 6A. Proportionally fewer serum samples protected mice against lung infection than against bacteremia, which may be explained by the lack of inflammation that is probably needed at the lung mucosa to allow for the influx of IgG antibodies and the migration of phagocytes. This process is more complicated than IgG-mediated protection in blood, and $24$ h may not be sufficient to eliminate the bacteria from the lungs. This is in agreement with our results from mice immunized parenterally or mucosally with conjugate vaccine, in which a $10$ times higher concentration of antibodies was needed to clear the lungs than to prevent bacteremia [40, 41]. In the case of serotype 6B, most of the mice had detectable, but low, pneumococcal density in the lungs, and no protection by passively immunized serum samples was observed. This was previously observed by passive immunization with adult postvaccination serum samples against this 6B strain [35]; the reason for this phenomenon is unknown.

A question that remains unanswered is what level of antibodies is needed to protect against pneumococcal disease in humans. Landesman and Schiffman [42] demonstrated that $>200$–$300$ ng of antibody nitrogen per milliliter of serum samples was needed to protect a high-risk population. Passive immunization of infant rats showed that $0.1$–$1.15 \mu g/mL$ of pneumococcal antibodies significantly reduced mortality, and that $50\%$ reduction was obtained with $0.1$–$3.5 \mu g/mL$ for $10$ serotypes tested [43]. In yet another study, $>0.05$ to $\leq 0.4 \mu g/mL$ of capsular-specific IgG antibodies (depending on serotype) eliminated bacteremia in $70\%$ of mice, but protection correlated only with opsonophagocytic activity and not with IgG levels [44]. In the current study, when mice were injected with more than $\sim 1 \mu g$ of polysaccharide-specific antibodies ($1$ AU for serotype 6A), they were protected against bacteremia caused by either serotype 6A or 6B. This corresponds to $\sim 0.3 \mu g$ of human pneumococcal IgG antibodies per milliliter of mouse serum (data not shown). Not surprisingly, some serum samples with lower concentrations of capsular-specific IgG antibodies also were protective. This was related to the OA of the serum samples in vitro, as shown for serotype 6A. It is unlikely that the discrepancy between antibody levels and OA found for some serum samples (figure 1B) was due to insufficient specificity of the ELISA, as commonly found for adult prevaccination serum samples and, to a lesser extent, for adult postvaccination serum samples [45]. Nonspecific antibodies that can be removed by adsorption with a heterologous pneumococcal polysaccharide, such as 22F [46], are rarely detected in infant serum samples after conjugate vaccination [47].

Functional activity of the serum samples may be dependent not only on IgG antibodies to pneumococcal polysaccharide but also on other factors. Avidity of IgG antibodies may influence their protective capacity [48], and antibodies of other isotypes (e.g., IgM and IgA) can be functionally relevant [49, 50]. However, it was not possible to demonstrate the contribution by IgG avidity or IgM in the current study, perhaps due to the few serum samples tested. Naturally occurring antibodies to other pneumococcal virulence factors (e.g., pneumolysin, PspA, and PsaA) may also contribute to protection [27].

Our results demonstrate that a pneumococcal polysaccharide protein conjugate vaccine, PncT, containing serotype 6B, induces functional antibodies in infants that protect mice against invasive disease caused by serotype 6B and the cross-reacting serotype 6A. This information is important, because the current pneumococcal vaccines only contain serotype 6B.

Acknowledgments

We thank Helga Erlendsdottir (Department of Microbiology, Landspitali–University Hospital, Reykjavik, Iceland) for valuable assistance and discussions. We appreciate the facilities provided by the Department of Microbiology, Landspitali–University Hospital.

References


