Characterization of the Antibody Response to Pneumococcal Glycoconjugates and the Effect of Heat-Labile Enterotoxin on IgG Subclasses after Intranasal Immunization

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The antibody response to pneumococcal glycoconjugate (Pnc) was characterized by analyzing pneumococcal polysaccharide (PPS)– and protein carrier–specific IgG subclass profiles and their relationship. Mice were immunized intranasally (inl) or subcutaneously (sc) with Pnc with mutants of Escherichia coli heat-labile enterotoxin, LT-R72 and LT-K63, as mucosal adjuvants. Subcutaneous immunization with Pnc alone induced predominantly IgG1, whereas native PPS administered sc induced very low IgG titers that were exclusively of the IgG3 subclass. Compared with sc immunization with Pnc alone, inl immunization with Pnc and LT mutants induced significantly higher systemic IgG2a, IgG3, and IgA antibodies to both PPS and the carrier, whereas the IgG1 titers were comparable. There also was a significant correlation between PPS- and protein carrier–specific antibody responses for all IgG subclasses. This demonstrates that LT mutants can be used to both enhance and modulate the antibody response to the PS moiety of glycoconjugate vaccines.

Infection caused by Streptococcus pneumoniae (pneumococcus) is a major cause of morbidity and mortality worldwide. Today, pneumococcus is among the most important bacterial pathogens in infants and children and is a leading cause of bacterial pneumonia, bacteremia, meningitis, and otitis media [1–3]. The polysaccharide (PS) capsule of pneumococcus is both the main virulence factor and the target for protective immunity, which is mediated mainly by phagocytosis of bacteria opsonized by PS-specific IgG antibodies and complement [4, 5]. The 23-valent pneumococcal PS (PPS) vaccine is immunogenic and protective in healthy adults [6, 7], but PS, which are T cell–independent type 2 antigens [8, 9], are not immunogenic at early ages [10]. This is explained, in part, by the immaturity of the marginal zone B cells, which are thought to be crucial in antibody responses to PS antigens [11, 12].

Immunization with PS antigens leads to limited class switching of activated B cells, no affinity maturation, and poor induction of memory cells [8, 9]. To enhance their immunogenicity, PPS have been covalently linked to various proteins to recruit T cell help [13–15]. The immunogenicity and efficacy of pneumococcal glycoconjugate (Pnc) vaccines has been found in infants and young children [16–20]. The small amount of IgG antibodies induced by native PS antigens are primarily of IgG3 subclass in mice [21] and IgG2 in humans [22, 23]. Low levels of mouse IgG3 and human IgG2 are associated with poor antibody responses to PS antigens [23–25], but this does not seem to be absolute [26]. In contrast, PPS-specific antibodies in infants vaccinated with Pnc are almost exclusively IgG1 [27]; mice immunized with Pnc respond almost entirely with IgG1 [28, 29].

By using adjuvants, it is possible to enhance the immune response to a vaccine, to reduce the number of antigen doses that are required to induce protective immunity, and to modulate the immune response to optimize protective immunity against the specific pathogen. Escherichia coli heat-labile enterotoxin (LT) is a powerful adjuvant capable of enhancing the immune response to both mucosally and parenterally coadministered antigens [30, 31], but its toxicity makes it unacceptable for use in humans. However, mutants with reduced toxicity have been produced by site-directed mutagenesis, several of which still possess potent adjuvanticity [32, 33]. The most efficient mutants are LT-K63, which is completely nontoxic [34–36], and LT-R72, which has minimal residual toxicity [37]. LT has both direct and indirect effects on immunocompetent cells [30, 38, 39] and induces a balanced response involving both Th1- and Th2-associated cytokines and antibody subclasses to various coadministered protein antigens [40, 41], but the immunomodulatory effect of LT on glycoconjugate vaccines has not been reported.

We previously demonstrated that intranasal (inl) immunization of mice with Pnc and LT mutants protect mice against invasive pneumococcal infections. Furthermore, inl immuni-
ization with Pnc and LT mutants elicits both mucosal and systemic PPS-specific immune responses that are significantly higher than those after parenteral immunization with Pnc alone [42]. In the present study, we analyzed the antibody response to PPS of 3 different serotypes (PPS-1, PPS-3, and PPS-6B) conjugated to tetanus toxoid (TT; Pnc1-TT, Pnc3-TT, and Pnc6B-TT, respectively), in addition to the antibody response induced by native PPS of the same serotypes. We also analyzed the immunomodulatory effect of LT-K63 and LT-R72 when coadministered inl with Pnc1-TT by characterization of the IgG subclass profiles to the PPS and the protein carrier of pneumococcal glycoconjugate vaccines.

Materials and Methods

**Mice.** Outbred 6-week-old female NMRI mice were obtained from M&B. The mice were kept in cages with free access to commercial pelleted food and water and were housed under standardized conditions at the Institute of Experimental Pathology at Keldur (Reykjavik, Iceland) with regulated daylight, humidity, and temperature.

**Vaccines, adjuvants, and immunization of mice.** Experimental lots of PPS conjugated to TT were produced by Aventis Pasteur. The LT mutants LT-K63 and LT-R72 (Immunobiology Research Institute Siena/Chiron S.p.A.) were produced by site-directed mutagenesis, as described elsewhere [34, 35, 37].

Before immunization, mice were sedated lightly by subcutaneous (sc) injection of Hypnorn (Jansen Pharmaceutical), which minimizes the probability of antigen delivery into the lungs during inl immunization. On the basis of results from previous studies with the same Pnc [42, 43], each mouse was immunized with 0.5 μg of Pnc1-TT, Pnc6B-TT, and Pnc3-TT. For sc immunization, we injected 0.5 μg of Pnc or 5.0 μg of native PPS (American Type Culture Collection) in 200 μL of saline in the scapular girdle region. For inl immunization, 0.5 μg of Pnc-TT was diluted in saline or was mixed with 5.0 μg of LT-K63 or LT-R72, and 10 μL of the vaccine solution was delivered slowly into the nostrils. Mice were immunized 3 times at 2-week intervals. Unimmunized mice were used as controls. Blood was sampled before each immunization and 2 weeks after immunization 3. Serum samples were isolated and stored at −70°C until use.

**PPS-specific and TT-specific ELISA.** Anti-PPS antibodies (IgG, IgG1, IgG2a, IgG3, and IgA) were measured by ELISA, as described elsewhere [42, 43]. In brief, microtiter plates (MaxiSorp; Nunc) were coated with 10 μg of PPS (ATCC) per milliliter of PBS and were incubated for 5 h at 37°C. For neutralization of antibodies to cell wall polysaccharide (CPWS; Statens Serum Institute), serum samples and standard were diluted 1:50 (or 1:25, if needed) in PBS with 0.05% Tween 20 (Sigma Chemical) and were incubated in 500 μg/mL of CPWS for 30 min at room temperature. Neutralized serum samples were serially diluted and were incubated for 2 h in duplicate in PPS-coated microtiter plates at room temperature. Horseradish peroxidase–conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG3, or IgA antibodies (Southern Biotechnology Associates) was diluted 1:5000 in PBS-Tween and was incubated for 2 h at room temperature for the detection of bound antibodies. For development of the enzyme reaction, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories) was incubated for 10 min, according to the manufacturer’s instructions; the reaction was stopped by adding 0.18 M H2SO4. Absorbance was measured at 450 nm in an ELISA spectrophotometer (Titertek Multispec Plus MK II; ICN Flow Laboratories).

For the detection of TT-specific antibodies, we coated microtiter plates (MaxiSorp) with 5.0 μg of purified TT (Aventis Pasteur) per milliliter of 0.10 M carbonate buffer (pH 9.6) and incubated the plates overnight at 4°C. After blocking of the coated plates with PBS containing 1% bovine serum albumin (Sigma), duplicates of samples and standard were serially diluted in PBS-Tween, and added to TT-coated plates, and were incubated for 2 h at room temperature. The detection of TT-specific antibodies and the development of the reaction were done as described above.

Reference serum was included on each microtiter plate for calculation of the titers expressed in ELISA units (EU) per milliliter. The titers of the reference serum samples (EU/mL) corresponded to the inverse of the serum dilution, giving an optical density of 1.0. Assays were done at room temperature; PBS-Tween was used for dilutions and washing. We used 100-μL volumes in all incubation steps with 3 washings between each.

**Statistical analysis.** We used the Student’s t test to compare antibody titers between groups. Correlation was calculated with Pearson correlation coefficient. P < .05 was considered to be statistically significant.

**Results**

**Characterization of antibody responses to Pnc and PPS.** To characterize the antibody response to Pnc of different serotypes, mice were immunized sc with 0.5 μg of Pnc1-TT, Pnc6B-TT, or Pnc3-TT without any adjuvant. Compared with preimmune levels, the IgG response after 3 immunizations with Pnc was highly significant (P < .001) and was dominated by the IgG1 subclass for all 3 serotypes (table 1). In addition, a significant increase in PPS-specific IgG3 antibodies was induced after immunization with Pnc of all serotypes, compared with those at preimmune levels (P < .001). In contrast, IgG2a antibodies increased significantly, compared with those at preimmune levels for serotype 1 (P = .003) but not for serotypes 3 and 6B (P = .160 and P = .133, respectively). This might be explained by the high immunogenicity of pneumococcal serotype 1 in mice.

To compare the antibody response to Pnc and native PPS, mice were immunized sc with 5.0 μg of native PPS of serotypes 1, 3, and 6B, which induced significantly lower anti-PPS IgG antibodies than did immunization with Pnc (table 1). IgG3 was the only IgG subclass that was increased significantly after immunization with Pnc with PPS, compared with those at preimmune levels. This applied to all 3 serotypes used in this study (P = .030 for PPS-1, P < .001 for PPS-3, and P = .006 for PPS-6B). Significantly higher IgG3 titers were induced after immunization with Pnc than with PPS for all 3 serotypes tested (P < .001 for serotypes 3 and 6B; P = .010 for serotype 1).

**Effect of LT mutants on anti–PPS-1 antibody subclasses after inl immunization with Pnc1-TT.** To characterize the effect of
Table 1. Characterization of pneumococcal polysaccharide (PPS)-specific antibody responses to pneumococcal glycoconjugate (Pnc) or PPS of serotypes 1, 3, and 6B.

<table>
<thead>
<tr>
<th>Vaccine, when determined</th>
<th>Log anti-PPS antibodies, EU/mL (±SD)</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pnc</td>
<td>PPS</td>
<td>Pnc</td>
<td>PPS</td>
</tr>
<tr>
<td>Serotype 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.50 (0.14)</td>
<td>0.48 (0.16)</td>
<td>0.08 (0.21)</td>
<td>0.09 (0.15)</td>
<td>0.10 (0.19)</td>
</tr>
<tr>
<td>After</td>
<td>3.23 (0.23)a</td>
<td>0.81 (0.08)</td>
<td>3.17 (0.45)a</td>
<td>0.07 (0.12)</td>
<td>1.14 (0.77)b</td>
</tr>
<tr>
<td>Serotype 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.50 (0.13)</td>
<td>0.52 (0.13)</td>
<td>0.17 (0.19)</td>
<td>0.15 (0.26)</td>
<td>0.61 (0.19)</td>
</tr>
<tr>
<td>After</td>
<td>2.80 (0.25)a</td>
<td>1.49 (0.40)</td>
<td>2.77 (0.25)a</td>
<td>0.10 (0.17)</td>
<td>0.76 (0.51)</td>
</tr>
<tr>
<td>Serotype 6B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.46 (0.09)</td>
<td>0.41 (0.20)</td>
<td>0.09 (0.16)</td>
<td>0.08 (0.14)</td>
<td>0.42 (0.06)</td>
</tr>
<tr>
<td>After</td>
<td>1.50 (0.38)a</td>
<td>0.75 (0.13)</td>
<td>1.43 (0.81)a</td>
<td>0.08 (0.13)</td>
<td>0.65 (0.40)</td>
</tr>
</tbody>
</table>

NOTE: Data are log anti-PPS antibody levels in serum sampled before and 2 weeks after immunization. Mice were immunized 3 times with 0.5 μg of Pnc or 5.0 μg of PPS of serotypes 1, 3, and 6B. EU, ELISA units.

a P < .001.
b P < .05.

LT mutants on IgG subclass profiles to the PS and the protein carrier (TT) of the glycoconjugate vaccine, mice were immunized either sc or inl with 0.5 μg of Pnc1-TT; LT-R72 or LT-K63 was used as an adjuvant for inl administration. As shown in previous studies [42], inl immunization of mice with Pnc1-TT with LT-K63 or LT-R72 as adjuvants induced significantly higher systemic anti–PPS-1 IgG (P < .001; figure 1A) and IgA (P < .001; figure 1B) than inl or sc immunization with Pnc1-TT alone. No difference was found in the PPS-1–specific IgG and IgA responses between the 2 LT mutants. Intranasal immunization with Pnc1-TT alone induced a significant, but low, anti–PPS-1 IgG (P = .023) and IgA (P = .003), compared with that in unimmunized mice.

To further analyze the increase in PPS-1–specific IgG after inl immunization with Pnc1-TT and LT mutants, we measured the PPS-1–specific IgG subclasses. Figure 1C shows that the PPS-1–specific IgG1 titers were comparable in mice immunized either sc with Pnc1-TT alone or inl with Pnc1-TT and LT mutants. However, the LT mutants significantly enhanced PPS-1–specific IgG2a (figure 1D) and IgG3 antibodies (figure 1E), compared with both inl and sc immunizations with Pnc1-TT alone (P < .001). No difference was found between LT-K63 and LT-R72.

Effect of LT mutants on anti-TT antibody subclasses after inl immunization with Pnc1-TT. The TT-specific IgG (figure 2A) and IgA responses (figure 2B) were significantly higher in mice...
immunized inl with Pnc1-TT and either LT mutant, compared with those in mice immunized inl or sc with Pnc1-TT alone (P < .001), which is in agreement with antibody responses to the PS moiety of Pnc1-TT (figure 1A, 1B). Intranasal immunization with Pnc1-TT alone induced significant, but low, TT-specific IgG (P = .002) but not IgA (P = .522) responses, compared with those in unimmunized mice.

To examine whether the increase in IgG2a and IgG3 antibodies after inl immunization with Pnc1-TT and LT mutants was restricted to the PS moiety of the glycoconjugate vaccine, the TT-specific IgG subclasses were measured in the serum samples of the immunized mice. As shown in figure 2C, the TT-specific IgG1 levels were comparable in mice immunized inl with Pnc1-TT and LT mutants and sc with Pnc1-TT alone. However, a significantly higher TT-specific IgG2a (figure 2D) and IgG3 (figure 2E) response was found in mice immunized inl with Pnc1-TT and LT mutants than in mice immunized sc or inl with Pnc1-TT alone, which is similar to observations for the anti–PPS-1 antibody subclasses.

**Effect of LT mutants on anti–PPS-1 and anti-TT IgG1: IgG2a and IgG1: IgG3 ratios after inl immunization with Pnc1-TT.** To investigate further the effect of LT mutants on the relative increase of the Th1-associated subclasses IgG2a and IgG3 against the PS and protein moiety of Pnc1-TT, the PPS-1- and TT-specific IgG1: IgG2a and IgG1: IgG3 ratios were calculated for individual serum samples in the groups of mice immunized with Pnc1-TT. As shown in figure 3A and 3B, the geometric mean titers of the PPS-1–specific IgG1: IgG2a and IgG1: IgG3 ratios were significantly lower in groups immunized inl with Pnc1-TT and LT mutants, compared with those in groups immunized sc with Pnc1-TT alone (P < .001). Similarly, significantly lower TT-specific IgG1: IgG2a (figure 3C) and IgG1: IgG3 (figure 3D) ratios were found in mice immunized inl with Pnc1-TT and LT mutants, compared with those in mice immunized sc with Pnc1-TT alone. However, the increase in IgG3 was more pronounced against the PS than against the protein carrier, as clearly shown in figure 3B and 3D. This is also observed in absolute terms by comparing figure 1E and figure 2E.
The weakest correlation was for IgG3 (figure 4) antibody responses for IgG and all IgG subclasses measured. A significant correlation between the anti–PPS-1 and anti-TT responses after inl and sc immunization with Pnc1-TT. As shown in figure 4, there was a significant correlation between the anti–PPS-1 and anti-TT antibody responses for IgG and all IgG subclasses measured. The weakest correlation was for IgG3 (figure 4D). These results show that mice that responded to the protein carrier responded similarly to the PS and indicate that the T cell help to the PS-specific B cells provided by the carrier is comparable with the T cell help to the carrier-specific B cells.

Discussion

We previously demonstrated that antibodies induced by Pnc in infants show opsonic activity in vitro [27, 44] and protect mice against invasive pneumococcal infections, if passively administered before inl challenge with virulent pneumococci [45]. With the same murine pneumococcal infection model [46], it was demonstrated that inl immunization of mice with Pnc with LT-K63 and LT-R72 as adjuvants induces protective immunity against invasive pneumococcal infections [42]. A significantly higher systemic PPS-specific IgG antibody response was found in mice immunized inl with Pnc with LT mutants as adjuvants than in mice immunized sc or inl with either Pnc or PPS alone [42].

In the present study, the antibody response to Pnc and PPS was characterized in mice. Subcutaneous immunization with Pnc alone induced predominantly PPS-specific IgG1 antibodies, whereas the response to native PPS after sc immunization was dominated by IgG3 antibodies (table 1). Although the dose of Pnc was 10 times lower than the dose of PPS, Pnc elicited 10–100-fold higher IgG antibody levels, which demonstrates one advantage of glycoconjugates over native PPS. The IgG subclass profiles against PPS and TT were compared after mucosal and parenteral immunization with Pnc1-TT by use of LT-K63 or LT-R72 as mucosal adjuvants. Clearly, the increase in PPS- and TT-specific IgG after inl immunization with Pnc1-TT and LT mutants was due to an increase in IgG2a and IgG3 antibodies, whereas the IgG1 titers were comparable with those induced by sc immunization with Pnc1-TT alone. As a result, the antigen-specific IgG1:IgG2a and IgG1:IgG3 ratios were significantly higher in mice immunized sc with Pnc1-TT alone, compared with mice immunized inl with Pnc1-TT and LT mutants. These results are in agreement with those obtained when LT was coadministered mucosally with various protein antigens, in which both Th1- and Th2-associated cytokines and antibody subclasses were induced [40, 41]. Whether it is possible to use LT mutants to modulate the immune response in a similar way when administered parenterally is under investigation, but the adjuvant activity of enterotoxins may depend on immunization routes [47, 48].

When we analyzed the relationship between TT- and PPS-specific antibody responses for different IgG subclasses, we found a significant correlation (figure 4). This showed that the T cell help to the PPS-specific B cells provided by LT is comparable with the T cell help to the TT-specific B cells. Thus, individual mice that respond to TT also respond to PPS after immunization with Pnc. The weakest correlation was found for the IgG3 subclass (figure 4D). In addition, the increase in IgG3 after inl immunization with Pnc1-TT and LT mutants was more pronounced against PPS than against TT. These findings might be explained, in part, by the fact that PS antigens mainly induce IgG3 antibodies in mice [21]. However, this might also be due to a direct effect of LT on the PS-specific B cells rather than on the carrier-specific T cells, but this remains to be determined. Furthermore, LT mutants enhanced the systemic PPS-specific IgA response significantly compared with immunization with Pnc1-TT alone (figure 1B). Because IgA antibodies are capable of mediating phagocytosis of pneumococci both in vitro [49, 50] and in vivo [51], the role of IgA in immunity to pneumococcal infections might be significant.

We conclude that nontoxic mutants of E. coli LT could be used both to enhance and to modulate the PPS-specific antibody response to Pnc. The enhancement of the Th1-associated murine subclasses IgG2a and IgG3 (IgG2 in humans), in addition to IgA after mucosal immunization with Pnc, may have additional advantages in protection against pneumococcal disease.
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