Streptococcus pneumoniae is a major respiratory pathogen of infants, children, and the elderly. Polysaccharide vaccines have been useful in adult populations but do not elicit protective immunity in infants and young children. To enhance their immunogenicity, vaccines of pneumococcal polysaccharides conjugated to proteins are being developed. In this study antibody levels and opsonic activities were compared in sera of infants and adults injected with pneumococcal polysaccharide type 6B (Pn6B) conjugated to tetanus toxoid (TT) (Pn6B-TT). Healthy infants were injected with Pn6B-TT; group A was injected at 3, 4, and 6 months of age, and group B was injected at 7 and 9 months of age. A booster injection was given at 18 months. Adults were injected once. Antibodies were measured by enzyme-linked immunosorbent assay and radioimmunoassay, and their functional activities were measured by opsonophagocytosis of radiolabelled pneumococci. In adults, increases in immunoglobulin M (IgM), IgG, IgA, IgG1, and IgG2 to Pn6B were observed. Infants reached adult levels of IgG1 anti-Pn6B after the primary injections. After the booster injection the infant groups had total IgG- and IgM-Pn6B antibody levels similar to those of adults. After the booster injection, IgG1 was the dominant infant anti-Pn6B isotype and at a level higher than in vaccinated adults, but IgA and IgG2 antibodies remained at very low levels. Opsonic activity increased significantly after Pn6B-TT injections; the highest infant sera showed opsonic activity comparable to that of vaccinated adults. Overall, opsonic activity correlated best with total and IgG anti-Pn6B antibodies ($r = 0.741$, $r = 0.653$, respectively; $n = 35$) and was highest in sera with high levels of all Pn6B antibody isotypes. The results indicate the protective potential of a pneumococcal 6B polysaccharide protein conjugate vaccine for young infants.
### TABLE 1. Geometric means of Pn6B antibody levels in sera of infants and adults injected with Pn6B-TT

<table>
<thead>
<tr>
<th>Ab (unit)</th>
<th>Adults (HA)</th>
<th>Infant group A (age [mo])</th>
<th>Infant group B (age [mo])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>7</td>
</tr>
<tr>
<td>Ab N/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM (µg/ml)</td>
<td>3.20 (0.05)</td>
<td>4.89 (1.07)</td>
<td>2.47 (0.05)</td>
</tr>
<tr>
<td>IgA (µg/ml)</td>
<td>0.048 (0.001)</td>
<td>0.096 (0.004)</td>
<td>0.09 (0.001)</td>
</tr>
<tr>
<td>IgG (µg/ml)</td>
<td>2.45 (0.001)</td>
<td>7.32 (0.62)</td>
<td>2.46 (0.05)</td>
</tr>
<tr>
<td>IgG1 (AU/ml)</td>
<td>2.24 (0.001)</td>
<td>15.32 (4.93)</td>
<td>54.48 (0.505)</td>
</tr>
<tr>
<td>IgG2 (AU/ml)</td>
<td>7.15 (0.001)</td>
<td>19.21 (0.23)</td>
<td>0.48 (0.001)</td>
</tr>
</tbody>
</table>

* Seven months (group A) and 10 months (group B), 1 month after primary injections with Pn6B-TT. Nineteen and 24 months, 1 and 6 months after booster injections.

* P value for statistical comparison with adult (group HA) post-Pn6B-TT vaccination levels.

### MATERIALS AND METHODS

Informed consent was obtained from the parents, and the protocol was reviewed and approved by the Ethics Committees of the National University Hospital and Reykjavik Hospital in Reykjavik, Iceland (assurance no. S-8172-01), the Medical Board of the National Institutes of Health, Bethesda, Md. (protocols OH93-CH-N019 and OH93-CH-N024), and the U.S. Food and Drug Administration (IND 1977), according to European and U.S. regulations.

**Antibodies.** IgG anti-Pn6B was measured by enzyme-linked immunosorbent assay (ELISA), according to the protocol recommended by the Pneumococcal Workshop at Centers for Disease Control, Atlanta, Ga., October 1994, with minor modifications (34). In brief, ELISA plates (Costar, Cambridge, Mass.) were coated with 10 µg of Pn6B polysaccharide (American Type Culture Collection, Rockville, Md.) per ml for 5 h at 37°C. Standard and test sera were diluted 1/25 and adsorbed with 50 µg of CWPS (Statens Seruminstitut, Copenhagen, Denmark) per ml for 30 min at room temperature, prior to incubation in two twofold dilutions for 2 h in the Pn6B-coated plates. Pn6B-IgG was detected by incubation with biotin-labelled monoclonal antibody HP-6043 (Hybridoma Reagent Laboratory, Baltimore, Md.) at 1/500 dilution, followed by incubation with alkaline phosphatase (ALP)-labelled avidin (Dako, Glostrup, Denmark) at 1/2,000 dilution for 1 h. Anti-Pn6B IgM, IgA, IgG, and IgE were measured by ELISA as previously described (37). Before incubation of standard and test sera, CWPS antibodies were adsorbed at 37°C for 2 h and 4°C overnight. Pn6B antibodies were detected by ALP conjugated monoclonal antibody to IgM (clone MB-11; Sigma, St. Louis, Mo.) or purified rabbit anti-human IgA (D338; Dako). IgG subclass antibodies were detected by ALP-conjugated monoclonal antibody to IgM (clone MB-11; Sigma, St. Louis, Mo.) or purified rabbit anti-human IgA (D338; Dako). IgG subclass antibodies were measured by ELISA as previously described (37). Before incubation of standard and test sera, CWPS antibodies were adsorbed at 37°C for 2 h and 4°C overnight. Pn6B antibodies were detected by a curve generated by serial dilutions of an in-house standard prepared from an adult post-23-valent PPS vaccination pool calibrated against reference serum 89SP, provided by Carl E. Frasch, Food and Drug Administration, Bethesda, Md. IgG1 and IgG2 antibody levels were expressed in micrograms of antibody (Ab)/milliliter calculated from a curve generated by serial dilutions of an in-house standard assigned a value of 25 AU/ml.

**Bacteria.** Freeze-dried S. pneumoniae serogroup 6 (by subtyping with specific monoclonal antibodies [Statens Seruminstitut]; this strain was found to be of serotype 6A, after the study was completed) was reconstituted in Todd-Hewitt broth and subcultured on sheep blood agar (37°C. 5% CO₂). Colonies were harvested and suspended in Tryptose broth (Difco Laboratories, Detroit, Mich.) for storage at ~70°C. For radiolabelling, a culture with an initial density of 10⁶ CFU/ml was started in 5 ml of RPMI 1640 (GIBCO; Life Technologies GIBCO BRL, Paisley, Scotland), supplemented with 10% fetal calf serum (FCS) (GIBCO) and 500 µCi of [H]-labelled lysine (Amersham, Amersham, United Kingdom), collected in mid-log phase by centrifugation at 2,200 × g for 20 min, and washed in Hank’s balanced salt solution (HBSS) (GIBCO) containing 5% FCS. The labelled pneumococci were adjusted to 1.5 × 10⁹ bacteria/ml in HBSS with 5% FCS and used immediately. The viability and density were confirmed by plate colony counts for each experiment.

**Phagocytes.** Fresh polymorphonuclear cells (PMNs) were isolated from the peripheral blood of a healthy adult volunteer by dextran sedimentation followed by Ficol (Histopaque; Sigma) gradient centrifugation to remove mononuclear cells. The final concentration was adjusted to 1.5 × 10⁶ PMN/ml of HBSS. Blood donors were FcγRIIa-H131 homozygotes (kindly genotyped by Clark L. Anderson and Jeanne M. Osborne, Ohio State University College of Medicine, Columbus) and FcγRIIa-H132 homozygotes (typed using fluorescence-activated cell sorter [FACS] analysis with monoclonal antibodies CBGlram11 and GMR1, a kind gift from M. de Haas and A. E. G. K. von dem Borne, CLB, The Netherlands).

**Opsonophagocytosis.** Sera were assayed as described previously (37) with minor modifications, by using fresh PMN and 16H-labeled Pn6B without added complement. Bacterial and PMN suspensions (150 µl of each, ratio of approximately 10:1) were mixed with test sera at a concentration (15% for infants, 5% for adults) predetermined to be in the sensitivity range of the assay (5, 37). The total volume of 0.5 ml was incubated with rotation (250 rpm) for 30 min at 37°C. Controls for nonspecific binding (NSC) (with all reagents except heat-inactivated FCS instead of human serum) and total bacteria input (TB) (with all reagents) were included in each assay. The reaction was stopped by adding 2 ml of phosphate-buffered saline-0.02% NaN₃. The PMN and the cell-associated bacteria (CAB) were pelleted by centrifugation at 160 × g, except that TB was centrifuged at 2,200 × g. After washing, cell pellets were resuspended in 0.5 ml of 1.25% deoxycholate and centrifuged to 4.5 ml of scintillation liquid (Hionic-fluor; Packard, Greve, Denmark). The radioactivity (range, 500 to 10,000 cpm) was measured in a liquid scintillation counter (Packard) and percent uptake of H-labeled bacteria was calculated as (counts per minute of CAB—counts per minute of NSC) (counts per minute of TB) × 100. The results were expressed as 100 percent uptake if the scintillation liquid did not contain medium.

**Serum groups.** Serum pools were prepared from sera of five infants in group B at 24 months, selected by their high anti-Pn6B levels, from the same infants before vaccination at 7 months, from five infants in group A at 7 months (after three injections), and from pre- and postvaccination sera from five adults (group C; vaccinated with Pneumo23 Imovax).
Statistical analysis. A paired \( t \) test was used on log-transformed values for comparison within groups, and a nonparametric signed rank test was used when normal distribution was not obtained. For comparison between groups a \( t \) test was used except when normality failed or variance was unequal, in which case the Mann-Whitney rank sum test was used. The Pearson correlation was used to evaluate the relationship between opsonic activity and antibody concentration. A \( P \) value of <0.05 was considered significant.

RESULTS
Adults injected with Pn6B-TT (HA) responded with significant rises in all isotypes measured (Table 1), similar to those vaccinated with the 23-valent pneumococcal vaccine (C). Pn6B-TT tended to induce higher IgG1 levels than the polysaccharide vaccine but not significantly higher (\( P < 0.114 \)) (data not shown).

Before vaccination, total Pn6B antibody levels in infant sera were at the lower detection level (34). Figure 1 shows the distribution and geometric mean (GM) of Pn6B-antibody levels in adults before and after injections with Pn6B-TT and in the infant groups A and B after priming and booster injections. Table 1 shows the GM and statistical comparisons with adult postvaccination levels. Both infant groups responded to Pn6B-TT with measurable antibody levels after the primary vaccinations. These levels were significantly lower than those of vaccinated adults, except for IgG1 anti-Pn6B levels that reached or exceeded adult postvaccination level in both groups (Table 1 and Fig. 1). After the booster at 19 months, both infant groups were not significantly different from the vaccinated adults, except for IgG1 anti-Pn6B levels that were significantly higher (\( P < 0.05 \)) and IgG2 and IgA levels that were lower (\( P < 0.001 \)) in infants.

Figure 2 shows titration curves for opsonic activity of infant and adult serum pools (upper panel) and their Pn6B-antibody profiles (lower panel). Serum pools obtained at 7 months of age from vaccinated (group A) and unvaccinated (group B) infants had negligible opsonic activity, in agreement with their low overall Pn6B-antibody levels. The adult postvaccination pool had increased opsonic activity compared to the prevaccination pool. The pool obtained from group B infants at 24 months had higher opsonic activity than that of the adult post-
vaccination pool, consistent with its Pn6B antibody profiles of higher total, IgG, and IgG1 anti-Pn6B levels.

The sensitivity range of the opsonization assay is narrow, and opsonization reaches a plateau of approximately 60% uptake at high antibody concentrations in serum. The 15% serum concentration was in the sensitivity range of the assay (Fig. 2) and was chosen for measurements of opsonic activity of individual infant sera. Figure 3 shows that there was a significant correlation between opsonic activity and total Pn6B antibody levels in both infant groups. The relationship between opsonic activity and Pn6B antibody isotypes in adult and infant sera is shown in Table 2. In adults the opsonic activity correlated with total and IgG anti-Pn6B, with IgG1 in those injected with Pn6B-TT, but with IgG2 in those injected with the polysaccharide vaccine. In both infant groups there was a highly significant correlation between the opsonic activities and each of total, IgG, IgG1, and IgG2 anti-Pn6B.

IgG1 anti-Pn6B correlated significantly with IgG anti-Pn6B in infants \( (r = 0.929, p < 0.001) \) but less so in adults \( (HA, r = 0.318, P = 0.086; C, r = 0.647, P < 0.001) \). IgG2 anti-Pn6B correlated with IgG-Pn6B antibodies in infants \( (r = 0.704, P < 0.001) \) and even better in adults \( (HA, r = 0.870, P < 0.001; C, r = 0.913, P < 0.001) \).

Opsonic activity was measured in serial samples from several infants. The kinetics followed closely that of Pn6B antibodies measured by ELISA and RIA, in particular total and IgG antibodies (data not shown).

DISCUSSION

Children younger than 2 years of age do not respond to most polysaccharide antigens \( (10, 14, 26) \). Covalent binding of polysaccharides to proteins has rendered the polysaccharides immunogenic and successful vaccines for infants as demonstrated by the 

\[ H. influenzae \text{ type b conjugates} \ (12, 18, 35) \]

and pneumococcal polysaccharide conjugates \( (32, 34) \). In this study, we have shown that infants injected from 3 months of age reached adult postvaccination levels in total, IgG1, and IgM Pn6B antibodies after booster vaccination at 18 months. Infants responded preferentially with IgG1 but to a small extent with IgG2 (Fig. 1 and Table 1).

As judged by fold increases, correlations with IgG and, comparison with adult levels, IgG1 was the major antibody produced by the infants in response to the Pn6B-TT vaccine.

Opsonic activity may be considered as an in vitro correlate of protection from infection. The antibodies elicited by Pn6B-TT in infants as well as in adults were functionally active in vitro. The infants had achieved adult levels of IgG1 anti-Pn6B, but few had achieved adult antibody levels of IgG2 and IgA. They had opsonic activities comparable to those of adults postvaccination (Fig. 2).

Both in infant and adult sera opsonic activity correlated well with IgG, IgG1, and IgG2 anti-Pn6B measured by ELISA. In infant group B, opsonic activity also correlated with IgM. This may be a coincidence, due to C3b/C3d deposition by IgM anti-Pn6B, or secondary to correlation between IgM and IgG2 \( (r = 0.471, P = 0.004) \) or IgA anti-Pn6B \( (r = 0.334, P = 0.050) \). Overall the best correlation was found between opsonic activity and total Pn6B antibodies measured by RIA. Potential contribution of CWPS antibodies was eliminated by CWPS adsorption before ELISA measurements \( (37) \). The specificity of the RIA for type-specific PPS \( (31) \) was recently confirmed by a modified Farr assay \( (28) \). It has been estimated that the serum antibody level of 300 ng of Ab N/ml is protective against type 6 pneumococci in adults \( (22) \). Interestingly, opsonic activity was low or undetectable in sera with antibody levels below 300 ng of Ab N/ml (Fig. 3).

Contrary to our previous experience with a Pn6A \( (32) \), the adults’ response to the polysaccharide and the Pn6B-TT conjugate was comparable. This prompted us to reanalyze this vaccine lot. We did not detect disintegration of the conjugate but found that the concentration of the conjugate was only half of the original concentration (the rest was found attached to the vials). This could explain, partly, the lesser antibody response.

Although there were qualitative and quantitative differences between the antibody responses of the adults and the infants after injections with Pn6B-TT, we demonstrated that the vaccine could induce antibody levels in serum and opsonic activities in infants comparable to those of adults and that opsonic activity correlated with antibody levels. This indicates a protective potential of a protein-conjugated pneumococcal polysaccharide vaccine in young infants. Considering that Pn6B is one of the two least immunogenic pneumococcal polysaccharides, it is anticipated that the response to the other types will be better, and such vaccines will hopefully prove to be effective against pneumococcal disease.

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