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Isotypes and Opsonophagocytosis of Pneumococcus Type 6B Antibodies Elicited in Infants and Adults by an Experimental Pneumococcus Type 6B-Tetanus Toxoid Vaccine

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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.**

Streptococcus pneumoniae continues to be an important cause of morbidity and mortality, particularly among elderly individuals with a variety of chronic diseases and in children younger than 5 years of age (4, 10, 14, 22, 23). In adults, the pneumococcus is the most frequent cause of community-acquired pneumonia, with a mortality of 5 to 10% despite modern antimicrobial therapy and intensive care (17). In children pneumococci are a frequent cause of meningitis, sinusitis, and bacterial pneumonia (14) and the most common cause of acute otitis media (15). The need for a pneumococcal vaccine effective in children has become urgent, especially as the incidence of penicillin-resistant pneumococci has increased worldwide (20, 21). The currently used 23-valent pneumococcal polysaccharide (PPS) vaccine represents up to 95% of the serotypes isolated from patients (19). Vaccination with PPS stimulates antibody production (5, 7, 37) and is protective in healthy adults (3, 33), but immunogenicity is low in certain groups at risk (22) and in children under 2 years of age (10, 14, 23). To increase immunogenicity, protein-conjugated PPS vaccines are being developed (1, 11, 32).

The pneumococcal polysaccharide capsule does not activate complement, and pneumococci are not susceptible to complement-mediated lysis (2, 13). Host defenses against pneumococcal infections therefore depend on opsonization of the bacteria by type-specific serum antibodies (37) and on complement, followed by phagocytosis and killing by polymorphonuclear leukocytes (PMNL) and macrophages (36, 39). The PPS are T-cell-independent antigens of type 2 (TI-2) (26), and human antibody responses to PPS in adults have been reported to be predominantly of the immunoglobulin G2 (IgG2) subclass (6, 16, 24, 27), which does not readily activate complement unless at high concentration or high epitope density (9, 25). Furthermore, the IgG Fc receptor (FcγR) most active in phagocytosis by normal PMNL, FcγRIIa, exists in two allotypes (H131 and R131) (29), and IgG2 binds efficiently only to the FcγRIIa-H131 allotype (38). This may have clinical consequences, as increased phagocytic activity by homozygous FcγRIIa-H131 PMNL has been reported (8), and increased susceptibility to respiratory infections has been demonstrated in individuals homozygous for FcγRIIa-R131 (30).

Pneumococcal serotype-specific opsonic activity of sera may be a more direct indicator of the protective potential of an experimental vaccine than serum antibodies alone. We have shown for several pneumococcal serotypes that in adults vaccinated with polysaccharide vaccine, opsonic activity of sera correlated best with IgG anti-PPS (5), while antibodies to the pneumococcal cell wall polysaccharide (CWPS) had little op-

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TABLE 1. Geometric means of Pn6B antibody levels in sera of infants and adults injected with Pn6B-TT

Ab (unit)	Result for group[a] (P^b)							
	Adults (HA)		Infant group A (age [mo])			Infant group B (age [mo])		
	Pre	Post	7	19	24	10	19	24
Total Ab (ng of Ab N/ml)	339.8 (<0.001)	1,372.5	44.3 (<0.001)	723.0 (0.054)	444.36 (<0.05)	211.4 (<0.001)	1,068.0 (0.425)	723.17 (0.267)
IgM (μg/ml)	3.20 (<0.05)	4.89	1.07 (<0.001)	2.47 (<0.05)	3.11 (<0.05)	1.87 (<0.01)	3.29 (0.123)	3.41 (0.083)
IgA (μg/ml)	0.42 (<0.001)	0.96	0.04 (<0.001)	0.09 (<0.001)	0.06 (<0.001)	0.06 (<0.001)	0.05 (<0.001)	0.07 (<0.001)
IgG (μg/ml)	2.45 (<0.001)	7.32	0.62 (<0.001)	2.46 (<0.05)	1.59 (<0.001)	1.22 (<0.001)	4.80 (0.510)	2.22 (<0.05)
IgG1 (AU/ml)	2.24 (<0.001)	15.32	4.93 (0.054)	54.48 (0.050)	15.70 (0.987)	17.70 (0.945)	84.12 (<0.05)	23.52 (0.504)
IgG2 (AU/ml)	7.15 (<0.001)	19.21	0.23 (<0.001)	0.48 (<0.001)	0.48 (<0.001)	0.34 (<0.001)	1.64 (<0.001)	0.70 (<0.001)

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

sonic activity (37). Antipneumococcal IgG subclass levels correlated well with opsonization (IgG2 = IgG3 > IgG1) (37).

We now report a comparison of vaccine-induced antibody levels and opsonic activities between sera from adults and two groups of infants vaccinated at different ages with pneumococcal polysaccharide type 6B (Pn6B) conjugated to tetanus toxoid (TT) (Pn6B-TT). We also compared the antibody responses of these adults to those of adults immunized with a 23-valent pneumococcal polysaccharide. The safety and immunogenicity of Pn6B-TT after repeated vaccinations of the infants have been reported previously (34).

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-NO19 and OH93-CH-NO24), and the U.S. Food and Drug Administration (IND 1977), according to European and U.S. regulations.

Vaccines. Pn6B-TT was prepared at the Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, Bethesda, Md. (lot 55683). Twenty-three-valent pneumococcal polysaccharide vaccine (Pneumo 23 Imovax) was obtained from Pasteur Mérieux, Lyons, France.

Vaccinees were injected with 0.5 ml of Pn6B-TT containing 12 μg of type 6B polysaccharide and 37.5 μg of TT or with 0.5 ml of Imovax. Healthy adult volunteers aged 23 to 50 (median, 28.9 years) received one injection of Pn6B-TT (HA; $n = 15$) or one dose of the control vaccine, Imovax (C; $n = 15$). Full-term healthy infants were recruited at the time of their routine health care visits. Group A ($n = 21$) received three primary Pn6B-TT injections at 3, 4, and 6 months of age, and group B ($n = 19$) received two injections at 7 and 9 months of age. Blood samples were collected before each and one month after the last injection. Both groups received a booster injection of Pn6B-TT at 18 months, and blood was collected at 19, 24, and 30 months of age (34). Sera were kept in aliquots at −20°C for antibody measurements and at −70°C for analyses of opsonic activity. Antibody responses and opsonic activities were compared between infants and the adults (group HA, except where otherwise stated).

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 Seruminstitute, Copenhagen, Denmark) per ml for 30 min at room temperature, prior to incubation in four 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, IgG1, and IgG2 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 monoclonal antibodies (IgG1, clone HP6012; IgG2, clone HP6014; ICN/Flow, Irvine, Scotland) followed by biotin-labelled rabbit-anti mouse Ig (E354; Dako) and ALP-avidin (E365; Dako). All reactions were developed by *p*-nitrophenyl phosphate (Sigma), and optical density was read at 405 nm in a Titertek Multiscan Spectrophotometer (Flow Laboratories, Irvine, Scotland).

IgG, IgM, and IgA anti-Pn6B levels are expressed in micrograms of antibody (Ab)/milliliter calculated from 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 89SF, provided by Carl E. Frasch, Food and Drug Administration, Bethesda, Md. IgG1 and IgG2 antibody levels are expressed in arbitrary units (AU) per milliliter by using the in-house standard assigned a value of 25 AU/ml.

Total anti-Pn6B antibodies were measured by radioimmunoassay (RIA) (31), and the results were expressed in nanograms of Ab N/milliliter (conversion factor for nanograms of Ab N/milliliter to antibody concentration is 6.25).

Bacteria. Freeze-dried *S. pneumoniae* serogroup 6 (by subtyping with specific monoclonal antibodies [Statens Seruminstitute]; this strain was found to be of serotype 6A, after the study had been completed) was reconstituted in Todd-Hewitt broth and subcultured on sheep blood agar (37°C, 5% CO_2). Colonies were harvested and suspended in Tryptoset broth (Difco Laboratories, Detroit, Mich.) for storage at −70°C. For radiolabelling, a culture with an initial density of 10^4 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 ^{3}H-labelled lysine (Amersham, Amersham, United Kingdom), collected in mid-log phase by centrifugation at 2,200 × *g* for 20 min, and washed in Hanks' balanced salt solution (HBSS) (GIBCO) containing 5% FCS. The labelled pneumococci were adjusted to 1.5×10^7 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 (PMN) were isolated from the peripheral blood of a healthy adult volunteer by dextran sedimentation followed by Ficoll (Histopaque; Sigma) gradient centrifugation to remove mononuclear cells. The final concentration was adjusted to 1.5×10^6 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 FcγRIII-NA1/NA2 heterozygotes (typed using fluorescence-activated cell sorter [FACS] analysis with monoclonal antibodies CLBgran11 and GRM1, 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 ^{3}H-labelled 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 reactants except heat-inactivated FCS instead of human serum) and total bacteria input (TB) (with all reactants) were included in each assay. The reaction was stopped by adding 2 ml of phosphate-buffered saline–0.02% NaN_3. 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 transferred 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 ^{3}H-labelled bacteria was calculated as (counts per minute of CAB − counts per minute of NSC)/(counts per minute of TB − counts per minute of NSC) × 100.

Serum pools. 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).

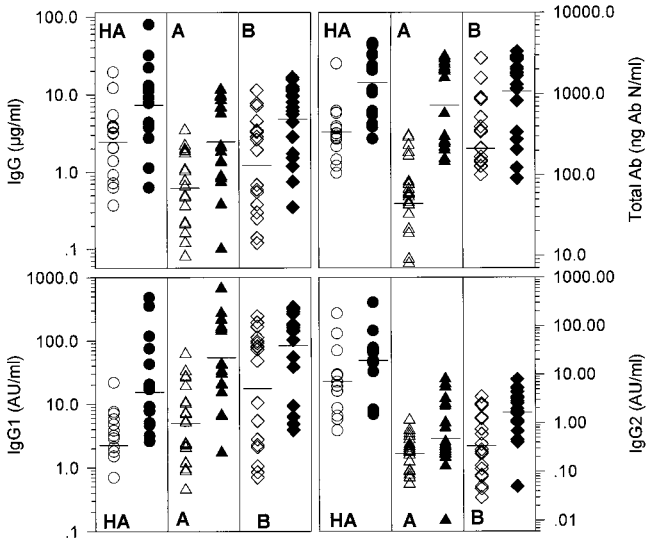


FIG. 1. Pn6B antibody levels; total, IgG, and IgG subclasses in sera of adults, before (open symbols) and after (closed symbols) injection with Pn6B-TT and in sera of infants after primary injections at 7 months in group A and at 10 months in group B (open symbols) and after booster at 19 months (closed symbols). Horizontal lines indicate the geometric means for the corresponding group.

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.

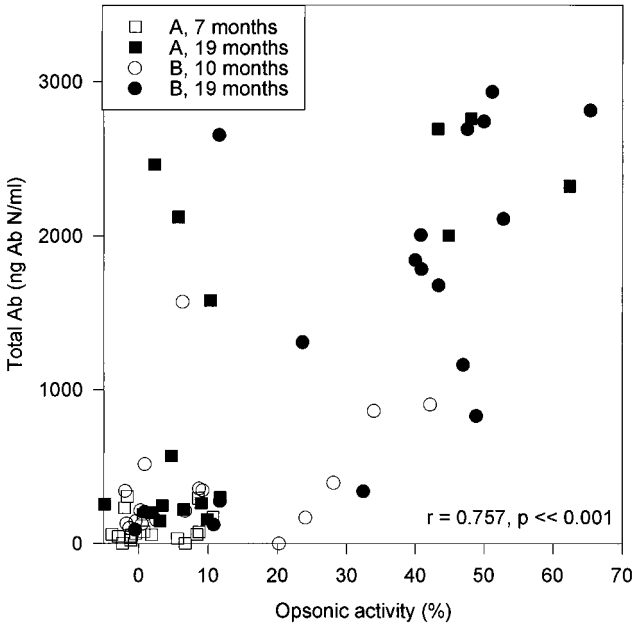
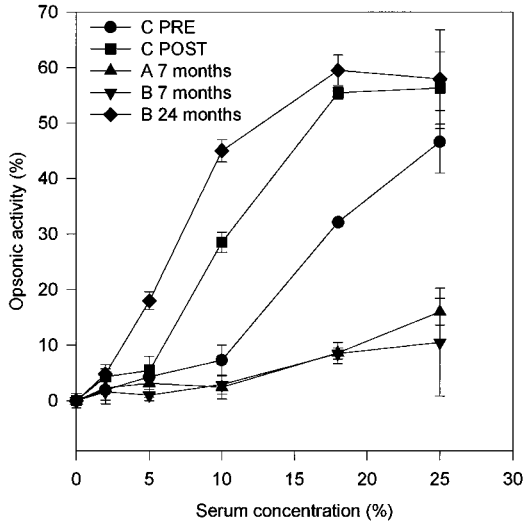


FIG. 3. Scatter graphs correlating the opsonic activities and total Pn6B antibodies in the two infant groups: group A at 7 months (open squares) and 19 months (closed squares) and group B at 10 months (open circles) and 19 months (closed circles).



	Total Ab (ng Ab N/ml)	IgG (µg/ml)	IgA (µg/ml)	IgM (µg/ml)	IgG1 (AU/ml)	IgG2 (AU/ml)
C PRE	785.6	2.3	0.42	4.86	3.68	4.67
C POST	2010.1	6.6	1.67	5.55	9.34	14.96
A 7 months	118.3	5.32	0.05	1.36	4.39	0.31
B 7 months	99.9	1.14	0.03	0.89	7.90	0.10
B 24 months	3589.5	31.78	0.48	5.38	178.63	8.14

FIG. 2. Opsonic activity of five serum pools: adults before and after vaccination with 23-valent polysaccharide vaccine and infants at 7 months (group A after primary injections with Pn6B-TT and group B unvaccinated) and group B at 24 months (6 months after booster injection). The antibody levels of each serum pool are shown in the table below.

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-

TABLE 2. Correlations between opsonic activities and antibody levels of adults and of infant sera[a]

Anti-Pn6B	Adults (C) (n = 30)		Adults (HA) (n = 30)		Infant group A (n = 36)		Infant group B (n = 35)	
	r	P	r	P	r	P	r	P
Total	0.652	<0.001	0.756	<0.001	0.758	<0.001	0.741	<0.001
IgG	0.572	<0.001	0.611	<0.001	0.741	<0.001	0.653	<0.001
IgG1	0.347	0.06	0.594	<0.001	0.711	<0.001	0.617	<0.001
IgG2	0.568	<0.001	0.334	0.071	0.771	<0.001	0.68	<0.001
IgA	0.288	0.123	0.145	0.444	0.428	<0.01	0.339	<0.05
IgM	−0.068	0.723	0.065	0.735	0.17	0.322	0.62	<0.001

[a] Adult sera were obtained before and after vaccination with Pneumo23 (C) or Pn6B-TT (HA). Infant sera were obtained after priming and booster injections with Pn6B-TT.

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 relationship 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 *Haemophilus influenzae* 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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