

Protective immunogenicity of *Haemophilus influenzae* type b polysaccharide-tetanus protein conjugate vaccine in children who failed to respond to prior invasive *H. influenzae* type b disease

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Abstract: A polyribosylribitol phosphate (polysaccharide)-tetanus protein conjugate vaccine (PRP-T) against *Haemophilus influenzae* type b (Hib) was evaluated for protective immunogenicity in 25 previously PRP-unimmunized children who had failed to develop protective PRP antibody levels (< 1 µg/ml) after prior invasive Hib disease at median age 10 months. Children under 21 months of age at time of PRP-T immunization received one, two or three doses. Serum was obtained for total PRP antibody, complement mediated bactericidal activity and specific IgG₁ and IgG₂ PRP antibodies before (n = 25), 1 to 2 months (n = 24) and > 5 months (n = 13) after completed vaccination. One to 2 months after immunization, all but one patient developed > 1 µg/ml of antibody (geometric mean level 50.7 µg/ml). The non-responder developed protective antibody levels when tested at 6 months after vaccination. Twenty out of 22 sera had detectable complement mediated bactericidal activity (median dilution titer 1:24), 1–2 months after vaccination. Three patients failed to demonstrate PRP antibodies in the IgG₁ or IgG₂ subclasses, although two of them had protective (> 1 µg/ml) total antibody levels. The second post immunization sera showed persistence of the total PRP antibody levels (geometric mean level 38.2 µg/ml) as well as of the bactericidal activity (median dilution titer 1:32). **Conclusion:** PRP-T conjugate vaccine is able to elicit a protective immune response in children who have low or unmeasurable PRP antibody levels after a systemic Hib infection.

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Four *Haemophilus influenzae* type b polyribosylribitol phosphate-protein conjugate vaccines have been evaluated extensively in the last few years for their immunogenicity in normal children as well as in children with predisposing factors to invasive *H. influenzae* type b infection (1, 2, 3, 4, 5). These vaccines are: PRP-diphtheria toxoid, PRP-outer membrane protein complex of *Neisseria meningitidis*, oligosaccharide-CRM197 and PRP-tetanus protein. The first three PRP-protein conjugate vaccines have proven immunogenicity in infants as well as in children with sickle cell anemia, malignancies, and after

systemic *H. influenzae* type b infection (Hib). The last licensed PRP-T has the advantage of being highly immunogenic in young infants (6, 7) and was reported to be immunogenic also in children with several underlying conditions associated with increased risk of Hib infection (8).

Immunogenicity per se, e.g. the production of a measurable specific antibody production after exposure to the antigen, cannot always be considered as sufficient to confer protection. The present study was designed to evaluate the protective efficacy of the PRP-T vaccine in children who failed to show

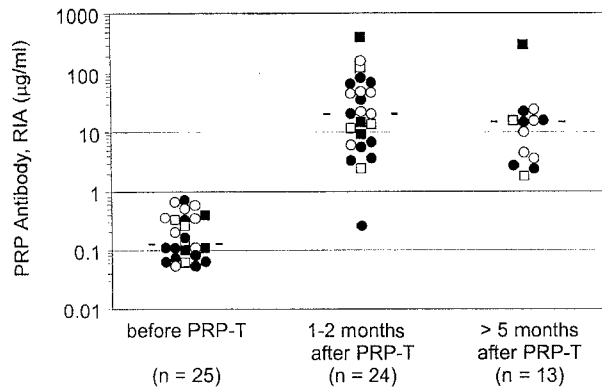


Fig. 1. Total PRP antibody (RIA method) before ($n = 25$), one to two months ($n = 24$) and more than five months ($n = 13$) after PRP-T vaccination in serum from children who failed to respond to prior invasive *H. influenzae* type b infection (empty symbols: age ≥ 21 months, filled symbols: age < 21 months, squares: IgG₂ < -2 SD for age-matched standards, lines: median levels).

protective antibody response after invasive Hib infection. The immunological pattern of their PRP antibody responses was evaluated after PRP-T vaccination using not only quantitative measurements of antibody concentration, but also functional tests of antibody activity (bactericidal assays).

Patients and methods

Vaccine

The *H. influenzae* type b PRP-T was provided by Pasteur Mérieux (Marcy l'Etoile, France). Each dose contained 10 µg purified Hib capsular polysaccharide and 20 µg purified tetanus protein derived from the detoxified tetanus toxin. Lyophilized conjugate vaccine was reconstituted with 0.4% saline solution and administered subcutaneously in the thigh.

Patients

The study was performed over a period of 4 years, immediately preceding the introduction of general Hib-immunization in Switzerland. Previously PRP-unimmunized children after systemic Hib infection (*H. influenzae* type b isolated from cultures of blood, cerebrospinal fluid, or both) who failed to show protective level for total PRP antibody at least 4 weeks after infection were eligible for this study. Patients were excluded if a pre-entry level of > 1 µg/ml serum was detected by RIA.

The protocol was approved by the ethics committee of the University Children's Hospital, Zurich. Informed parental consent was obtained before enrolment in the study.

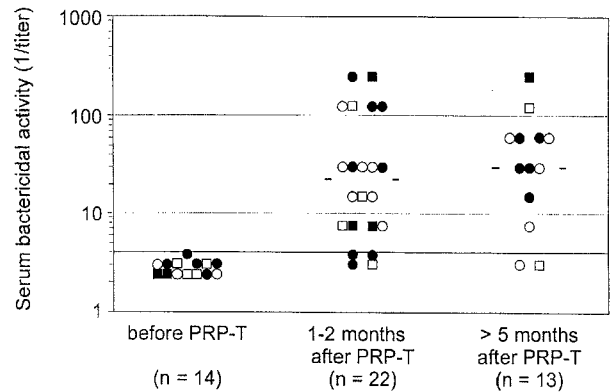


Fig. 2. Serum bactericidal activity (1/titer, see method in text) before ($n = 14$), one to two months ($n = 22$) and more than five months ($n = 13$) after PRP-T vaccination in children who failed to respond to prior invasive *H. influenzae* type b infection (empty symbols: age ≥ 21 months, filled symbols: age < 21 months, squares: IgG₂ < -2 SD for age-matched standards, lines: median values).

Vaccination schedule

Children who were 21 months of age or younger at initial immunization received one, two or three doses of PRP-T based on age according to the following schedule: the first two doses were separated by one to two months, and the last dose was given when the child had reached the minimal age of 14 months. Children who were older than 21 months of age at initial immunization received one dose of PRP-T.

Antibody assays

Total serum PRP antibody concentrations for all sera before one to two months, and five months or more after last immunization dose were measured by radio immuno assay (RIA, Farr method I²⁵) as previously described (9). The antigen was a tyramined PRP. Iodine was fixed with an oxidation-reaction with chloramine-T. Iodine-labelled PRP was titrated by competition with non-labelled PRP in order to use it at a determined concentration. The antibodies potentially present in the test serum bound with the iodine-labelled Hib polysaccharide and created antibody/antigen complexes which were then precipitated using a saturated ammonium sulphate solution. After the elimination of the supernatant (non-bound iodine-labelled polysaccharide), the radioactivity present in the sediments was measured using a gamma counter. The obtained value was proportional to the quantity of Hib antibodies present in the sample. A standard range produced with a serum of a known titer was used to determine the Hib antibody titer of the sample. We used a reference serum (OBRR) with a concentration of

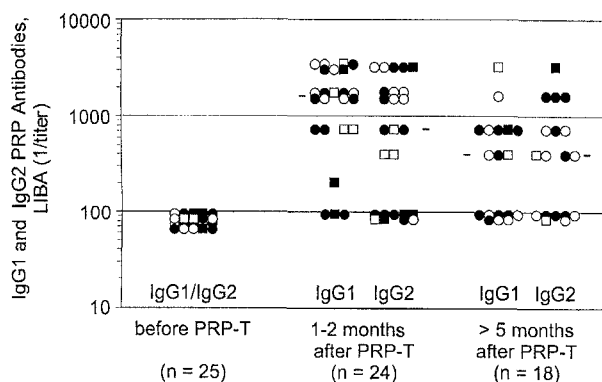


Fig. 3. IgG₁ and IgG₂ PRP antibodies (1/titer, line immuno binding assay) before (n = 25), one to two months (n = 24) and more than five months (n = 18) after PRP-T vaccination in children who failed to respond to prior invasive *H. influenzae* type b infection (empty symbols: age ≥ 21 months, filled symbols: age < 21 months, squares: IgG₂ < -2 SD for age-matched standards, lines: median values).

70 µg/ml. The results were expressed as micrograms per milliliter. The lower detection limit for the assay was 0.07 µg/ml.

Complement mediated bactericidal activity of de-complemented sera was measured as follows: sera were heated for 30 min at 56°C and serially diluted (two-fold dilutions) with Dulbecco's phosphate buffered saline containing Ca⁺⁺ and Mg⁺⁺ (final volume 200 µl) in 2 ml wells in a transparent plastic tray. 100 µl of an exponential-phase broth culture (Hib strain Madigan) diluted in the same diluent (about 1×10² organisms/well) and 100 µl of diluted complement (precolostral calf serum) were added to each well. The plastic tray was then incubated on a shaker for 30 min at 37°C, and 1 ml of supplemented Mueller-Hinton agar was added to each well. Plates were incubated overnight at 37°C and colonies were counted the next day. Controls included in each experiment consisted of organisms and the complement source without serum (complement control), organisms and heat inactivated complement with and without serum. The results were expressed as titers of the highest serum dilution which yielded a reduction of 50% in the number of CFU as compared to complement control. The lowest serum dilution tested was 1:4. The RIA and the complement mediated bactericidal activity were blindly assayed in 2 series after the collection of all sera.

Specific IgG₁ and IgG₂ PRP antibodies were determined in all sera using a semi-quantitative line immuno binding assay as previously published (10). The results were expressed as titer of the maximal serum dilution which elicited detectable specific IgG₁ and/or IgG₂ binding. The lowest serum dilution tested in this assay was a 1:100 dilution.

Immunglobulin assays

Serum levels of IgG, IgM and IgA were determined by nephelometry and IgG subclass levels by radial immunodiffusion using subclass-specific polyclonal antibodies (Central laboratory of the Netherlands Red Cross, Amsterdam). The age range normals were performed with the same assay.

Results

Patient characteristics

Twenty five children were enrolled in the study, 12 girls and 13 boys. Median age at the time of invasive Hib infection was ten months (extremes 1–52 months), site of infection was meningitis in 22 episodes in 21 patients (one patient had recurrent Hib meningitis), and septicemia in four, one of them presenting initially with pneumonia. At the time of first immunization, 12 patients were older than 21 months of age and received a single dose of PRP-T according to vaccination schedule. In the 13 patients who were younger than 21 months, four received a single dose, five received two doses and four received three doses of the vaccine.

Total serum PRP antibodies before immunization were < 1 µg/ml in all patients, according to inclusion criteria. No detectable anti-PRP levels were observed in five children and levels under 0.15 µg/ml in eight other children. Complement dependent bactericidal activity was tested in 14 pre-immunization sera and was undetectable at lowest dilution, 1:4 in all. IgG₁ and IgG₂ specific antibodies were undetectable in all pre-immunization sera at lowest dilution, 1:100.

Out of the 25 patients, 24 were available at one to two months after immunization and 13 (including the one who missed the first sample) at > 5 months for complete long lasting immunity study.

Quantitative immunoglobulins G, A, M and IgG subclasses were assayed in 23 children and were in the normal age-matched range in 16 of them. Low IgG₂ (value < -2 SD for age-matched standards) was detected in 7 patients, associated with undetectable IgG₄ in 6 of them. One patient had a complement factor 2 (C2) deficiency reported elsewhere.

Antibody response to PRP-T

Figure 1 illustrates total serum PRP antibodies before and after immunization. In the first (n = 24) and the second (n = 13) post-vaccination sera, the median level was 22 and 11 µg/ml respectively and the geometric mean level was 50.7 and 38.2 µg/ml, respectively. In all but one patient the level rose over 1 µg/ml in the first post-immunization sample. The

one who did not, however, showed levels $> 1 \mu\text{g/ml}$ in the second post-immunization sample. The final rise in the total serum PRP antibodies after one to three doses of PRP-T did not differ with age and was not influenced by low IgG₂ values in the patient's group.

Complement dependent bactericidal activity was measured in 14 pre-immunization sera, 22 first post-vaccination sera and 13 second post-vaccination sera (Fig. 2). The median titer reached in the first post-immunization sera was 1:24 and in the second ones 1:32. Two patients failed to demonstrate bactericidal activity in the first post-immunization serum. Both had total serum PRP antibodies $> 1 \mu\text{g/ml}$ (3.7 and 2.7 $\mu\text{g/ml}$, respectively). The first one showed a strong bactericidal activity (1:64) in the second post-immunization serum, without concomitant increase of his total serum PRP antibody levels; the second one did not develop serum bactericidal activity. This latter patient had IgG₂ deficiency. In the second post-immunization serum, one further patient showed decreased bactericidal activity, despite having 4 $\mu\text{g/ml}$ total PRP antibodies. The acquisition of serum bactericidal activity was not influenced by age at vaccination nor by low IgG₂ values for age.

Results of the specific IgG₁ and IgG₂ PRP antibodies are illustrated in Figure 3. Three children failed to demonstrate any IgG₁ or IgG₂ PRP antibodies in the first post-immunization sera in this assay, although all but one had total PRP antibody levels $> 1 \mu\text{g/ml}$. Six children had only detectable IgG₁ PRP antibodies, three of them being IgG₂ deficient. In the 18 second post-immunization sera, six were negative for both specific IgG₁ and IgG₂ (one of them was already negative in the first post-immunization serum), although having total PRP antibody levels $> 1 \mu\text{g/ml}$; one had only detectable IgG₁ antibodies (this one had low quantitative IgG₂) and two had only detectable IgG₂ PRP antibodies. Three of seven patients having low total IgG₂ values for age were able to elicit a PRP antibody response in the IgG₂ subclass.

Discussion

We examined the serum antibody response to PRP-T in 25 previously PRP-unimmunized children in whom Hib invasive disease had failed to induce a protective immunity. All patients but one showed the appearance of serum PRP antibody levels $> 1 \mu\text{g/ml}$ one to two months after the completed PRP-T vaccination. Thirteen children were available five months or more after the last vaccine dose, and all showed persistence of PRP antibody levels $> 1 \mu\text{g/ml}$. Even if a selection bias cannot be excluded in those follow-up patients, PRP-T can achieve a long

lasting antibody response in this group of patients. This confirms the data of a previous study which showed PRP-T immunogenicity in 17 children after Hib infection, 14 of them having PRP antibody levels $< 1 \mu\text{g/ml}$ before immunization (8).

To our knowledge, there is no study reporting that PRP-T vaccine is able to elicit production of functionally active PRP-antibodies in such a patient population. To evaluate the functional activity of the produced PRP antibodies, we studied the complement dependent bactericidal activity of the sera after immunization. This test is serum consuming, and for this reason, not all patients could be evaluated. Only one of the investigated patients had no detectable serum bactericidal activity when the serum was tested at dilutions of 1:4 or higher. Because not enough patient's serum was available to assay its bactericidal activity in lower dilution, it was not clear whether this patient was able to produce such bactericidal antibodies or not. This patient had IgG₂ deficiency, which may explain his failure to produce functionally active PRP antibodies, although six other patients with low or undetectable IgG₂ were able to elicit bactericidal activity. This study clearly demonstrates that children who failed to respond to natural Hib infection are able to elicit, after PRP-T immunization, a functionally active antibody production.

Former studies showed that other PRP conjugate vaccines (PRP-OMPC and HBOC) induce predominantly IgG₁ PRP antibody responses (11, 12). The response pattern observed in the present study is consistent with those reports and with the concept that the protein-bound PRP antigen elicits a T-cell mediated response which in turn induces preferentially an IgG₁ antibody production. Surprisingly, patients who responded clearly to the immunization (total PRP antibody level $> 1 \mu\text{g/ml}$ and detectable bactericidal activity) did not all produce detectable IgG₁ or IgG₂ PRP antibodies, or both. In the first post-immunization sera, it can be assumed that IgM was preferentially produced and accounted for the majority of the total PRP antibodies detected at this time. However, this cannot be the case in the second post-vaccinal sera, in which six out of 18 patients lacked detectable IgG₁ and IgG₂ PRP antibodies at a limit dilution of 1:100. In those patients, it may be hypothesized that functionally active IgG₃ PRP antibodies have been produced.

We conclude that the PRP-T conjugate vaccine is able to elicit a durable, functional immune response in children who failed to respond to an invasive *H. influenzae* type b infection. Based on the vast experience with anti-capsular antibody responses and their correlation with clinical efficacy in large trials, this immune response (total PRP antibody level $> 1 \mu\text{g/ml}$ and detectable bactericidal activity) will prob-

ably also be protective in vivo in most of the study children. A population who failed to respond to natural infection is at risk for a second invasive Hib infection and should be actively immunized with a conjugate vaccine (probably best done as soon as possible after the convalescent phase). The present study demonstrates that the PRP-T conjugate vaccine can reach this goal in most patients.

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Abbreviations

CFU, Colony forming unit; Hib, *Haemophilus influenzae* type b; LIBA, Line immuno binding assay; PRP, Polyribosylribitol phosphate; PRP-T, PRP-tetanus toxoid conjugate vaccine; RIA, Radio immuno assay.

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