

Pattern of functional antibody activity against *Haemophilus influenzae* type b (Hib) in infants immunized with diphtheria-tetanus-pertussis/Hib Brazilian combination vaccine

D.C.S. Matos¹, A.M.V. Silva¹, P.C.C. Neves¹, R.M. Martins²,
A. Homma³ and R. Marcovistz¹

¹Laboratório de Tecnologia Imunológica, ²Assessoria Clínica, Bio-Manguinhos,
³Diretoria de Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brasil

Abstract

We evaluated the functional activity of *Haemophilus influenzae* B (Hib) antibodies elicited in a group of infants immunized with the diphtheria-tetanus-pertussis vaccine combined with an Hib vaccine produced totally in Brazil after technological transfer of Hib vaccine production from Glaxo SmithKline, Belgium. Blood samples from immunized infants (N = 985) were collected for the determination of Hib antibodies. Total Ig and IgM and IgG subclasses of antibodies against polyribosyl ribitol phosphate (PRP) were analyzed by ELISA. Almost all vaccinees (97.56%, 961/985) developed a strong anti-PRP IgG antibody response (≥ 1.0 $\mu\text{g/mL}$), while an anti-PRP IgM response was observed in 64.24% (634/985) of them (≥ 0.15 $\mu\text{g/mL}$). Only 18.88% (186/985) of the infants in the group with high PRP antibody IgG concentrations (≥ 1.0 $\mu\text{g/mL}$) developed a high IgM antibody response. Anti-PRP IgG antibody levels were significantly higher than anti-PRP IgM. These results demonstrate the predominance of IgG antibodies over IgM antibodies in response to PRP, with a ratio of 17:1. IgG antibodies were predominantly of the IgG1 subclass. An increase in IgG avidity was also observed during the course of immunization.

Key words: DTP-Hib vaccine; *Haemophilus influenzae* type b; Antibody avidity; PRP immune response

Introduction

Since 1998 the WHO has recommended that *Haemophilus influenzae* type b (Hib) vaccination be included in routine infant immunization programs, according to country capacities, preferably as a diphtheria-tetanus-pertussis (DTP)-based combination (1). However, the immunological response, which insures the improved immunogenicity and priming induced by conjugated vaccines, is still only partially understood. It is known that IgG and IgM antibodies against the capsular polysaccharide polyribosyl ribitol phosphate (PRP) are very important for the protective immunity to Hib, and that the covalent linkage of PRP to any protein is also important for the enhanced immunogenicity of glycoconjugated vaccines (2-4).

The response to conjugated vaccine is known to be thymus dependent (T cell dependent), thus permitting memory responses even in infants. In contrast, molecules such as

PRP, a thymus-independent class 2 antigen (TI-2 antigen), can activate only mature B cells, preventing infants from producing antibodies against polysaccharide epitopes in an efficient manner. The immune systems of young children are immature and do not respond effectively to carbohydrate antigens before about 5 years of age. On the other hand, most physiological TI-2 responses occur in the spleen, the marginal zone B cells, which are rare at birth and which accumulate with age (5-11). Thus, the development of vaccines with PRP conjugated with a protein carrier was very important and has reduced the recommended minimum age of 5 years to 6 months for this vaccination (5).

Over the years, new technologies for combined vaccines have been developed and several different Hib vaccines have been licensed after extensive safety and immunogenicity evaluation. At least four different PRP-conjugated vac-

Correspondence: R. Marcovistz, Bio-Manguinhos, Fiocruz, Av. Brasil, 4365, Pavilhão Rocha Lima, 21040-360 Rio de Janeiro, RJ, Brasil. Fax: +55-21-2260-4727. E-mail: rugimar@bio.fiocruz.br

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cines are available, according to the carrier protein: conjugation with diphtheria toxoid (PRP-D), or an avirulent mutant of the diphtheric toxin (PRP-CRM), PRP conjugated with tetanus toxoid (PRP-T), and PRP conjugated with the outer membrane protein of *Neisseria meningitidis* (PRP-OMP) (12-14).

Conjugated vaccines have not only been able to induce an immune response in young infants, but have also been able to induce a memory immune response and to promote a secondary boost response with either conjugated or unconjugated PRP vaccines. Vaccine trials have shown the ability of the conjugated vaccines to activate and prime the previously immature, non-responsive immunity of infants (15,16).

In 2006, a randomized, double-blind study was designed to evaluate the immunogenicity, safety and consistency of production of a combined DTP-Hib vaccine totally produced in Brazil (DTP/Hib-BM) after a technological transfer of Hib production from Glaxo SmithKline (GSK), Belgium.

Overall, 1000 infants were recruited and received vaccine doses at 2, 4, and 6 months of age. Regarding the Hib component, there was 100% seroprotection ($\geq 0.15 \mu\text{g/mL}$), and there was no clinically relevant difference in reactogenicity between the 3 lots of Brazilian DTP/Hib studied and the reference vaccine lot with Hib produced by GSK (17).

The objective of the present study was to analyze the classes and subclasses as well as the functional activity of antibodies to Hib elicited in infants immunized with DTP/Hib-BM or DTP/Hib-GSK vaccines.

Subjects and Methods

Subjects and vaccines

This study is a follow-up of a previously reported clinical trial in which the immunogenicity, reactivity and consistency of production of DTP/Hib-BM was assessed (17). One thousand infants were immunized according to the immunization program of the Brazilian Health Ministry in a double-blind and randomized study approved by the National Research Ethics Committee, the Ethics Committee of Fundação Oswaldo Cruz and of the Municipal Health Secretary of Rio de Janeiro, and by the National Regulatory Agency. The protocol was registered under Standard Randomized Clinical Trial Number 67676353 (17).

Serum samples

Blood samples from 985 infants were collected for evaluation of Ig, IgG, and IgM PRP antibodies. In a subset of 300 infants, randomly chosen sera collected before and 1 month after the 3rd vaccine dose were used in comparative studies. Serum samples from the same infant were tested in parallel.

Antibody assay

IgG, IgM, and IgG subclasses of antibodies against PRP were analyzed by ELISA (3-6). Briefly, each well of a microplate (Maxisorb; Nunc, USA) was coated with 0.4 $\mu\text{g/}$

mL PRP-tyramine antigen in 0.1 mL 0.05 M carbonate buffer, pH 9.6, overnight at 4°C. Plates were washed with PBS plus 0.05% Tween 20 (PBS-Tween), and blocked with 0.1 mL PBS containing 0.5% bovine serum albumin (BSA) for 1 h at 37°C. Sera were serially diluted 2-fold in 0.5% PBS-Tween-BSA starting at 1:10 dilution and up to 1:1280 dilution and the plates were incubated for 1 h at 37°C. After washing three times with PBS-Tween, 0.1 mL/well of a 1/500 dilution of mouse anti-human IgG conjugated with horseradish peroxidase (HRP, BD-Pharmingen, USA) or a 1/30,000 dilution of biotinylated mouse anti-human IgM (BD-Pharmingen) in PBS-Tween-BSA was added and the plates were incubated again for 1 h at 37°C. For IgM antibody detection, a streptavidin HRP-conjugate (BD-Pharmingen) was added to the plates for 1 h at 37°C. IgG1 and IgG2 anti-PRP antibody titers were determined using specific monoclonal antibodies (BD-Pharmingen). Next, the tetramethylbenzidine substrate (Sigma, USA) was added and the reaction stopped with 2 M NaOH 15 min later. Absorbance was measured at 450 nm with an ELISA microreader (Sunrise; Tecan, Germany). Standard curves were generated using human IgG, IgG1, and IgG2 and IgM immunoglobulins. The ELISA antibody titer curves for each serum specimen were analyzed by a four-parameter logistic curve-fitting technique (Softmax Pro version 5, Molecular Devices, USA). The minimum level of detection of this assay was estimated to be 0.10 $\mu\text{g/mL}$ Ig at a 1:10 dilution of the tested serum.

IgG antibody avidity assay

Anti-PRP IgG avidity was measured by ELISA by the method of Agbarakwe et al. (16) with few modifications (6). Briefly, plates were coated with PRP and saturated as explained above, duplicate serum samples were then diluted to 1:20 in PBS-Tween-BSA and plates were incubated at 37°C for 1 h followed by three washes with PBS-Tween. Next, 100 μL 3.0 M urea was added to only one of the serum dilution rows, while 100 μL PBS-Tween was added to the other serum dilutions, corresponding to the control without urea. After 15 min of incubation, plates were washed with PBS-Tween for removal of both the unbound antibodies and the chaotropic agent by washing. The remaining bound IgG antibodies were detected with peroxidase-conjugated anti-human IgG in a manner similar to that described above.

The avidity index (AI) was calculated as the mean absorbance of reactions in which the antibodies were exposed to urea divided by the mean absorbance of reactions in which the antibodies were not exposed to urea, and expressed as percentage. The avidity assay could not be evaluated for sera with $< 0.5 \mu\text{g/mL}$ IgG anti-PRP antibody. Samples with an AI below 49% were considered to have low avidity and samples with an AI of 50 to 79% and of more than 80% were considered to have medium and high avidity, respectively.

Statistical analyses

The paired nonparametric Wilcoxon test was used to

compare anti-PRP antibody titers and AI between vaccination time points (before and 1 month after the 3rd dose). A P value of <0.05 was considered to be statistically significant. Spearman's correlation coefficient was used to evaluate the relationship between IgG subclasses and IgG avidity.

Results

Anti-PRP classes and subclass antibodies

Anti-PRP Ig total, IgG and IgM antibody titers were evaluated in serum samples obtained from children 1 month after the 3rd dose of DTP-Hib vaccine and are summarized in Table 1. All infants were seroprotected against PRP-T after the complete vaccination schedule, and 97.25% (958/985) of them presented high concentrations of total anti-PRP Ig (≥ 1.0 $\mu\text{g/mL}$). Almost all vaccinees (97.56%, 961/985) developed a strong IgG anti-PRP antibody response (≥ 1.0 $\mu\text{g/mL}$), while an anti-PRP IgM response was observed in 64.24% (634/985) of them (≥ 0.15 $\mu\text{g/mL}$). Only 18.88% (186/985) of the infants developed high levels of IgM antibody in the group with high PRP antibody IgG concentrations (≥ 1.0 $\mu\text{g/mL}$). The levels of anti-PRP IgG antibodies were significantly higher than the levels of anti-PRP IgM antibodies ($P < 0.0001$), with IgG levels ranging from 0.17 to 163 $\mu\text{g/mL}$ (mean = 16.91 ± 0.62) and IgM levels ranging from 0.10 to 18 $\mu\text{g/mL}$ (mean = 0.88 ± 0.05 ; Figure 1A). These results demonstrate the predominance of IgG antibodies over IgM antibodies in response to PRP, with a ratio of 17:1.

Figure 1B shows the geometric means (GM) of specific IgG levels before and one month after the 3rd vaccine dose in all volunteers. As expected, a significant increase ($P < 0.0001$) of PRP IgG levels was observed one month after the 3rd vaccine dose (GM = 10.16 $\mu\text{g/mL}$, 95%CI = 15.6-18.12

$\mu\text{g/mL}$) compared to PRP IgG levels before the last dose of vaccine (GM = 5.03 $\mu\text{g/mL}$, 95%CI = 5.87-6.63 $\mu\text{g/mL}$).

The DTP-Hib vaccine induced a definitely predominant IgG1 response since 90% of IgG antibodies belonged to the IgG1 subclass. Anti-PRP IgG1 levels were significantly higher than anti-PRP IgG2 levels (11.13 ± 0.51 vs 4.23 ± 0.24 $\mu\text{g/mL}$, $P < 0.0001$) as shown in Figure 1C.

IgG antibody avidity

There was an increase in IgG antibody avidity during the course of immunization. In fact, the IgG AI were significantly higher in sera obtained from infants one month after the complete schedule of vaccination than in sera obtained before the 3rd vaccine dose ($P < 0.0001$), as shown in Figure 2A. The avidity percentage of absorbance values in sera, following 2 doses of DTP-Hib vaccine was 70% (95%CI = 68-73%). After the 3rd dose, the mean IgG AI in the group was 80% (95%CI = 78-83%), indicating a significant increase ($P < 0.0001$).

The pattern antibody avidity distribution induced against Hib during the immunization process presented different

Table 1. Anti-PRP antibodies in infants one month after the third DTP/Hib vaccine dose.

Anti-PRP antibody concentration ($\mu\text{g/mL}$)	Ig (%)	IgG (%)	IgM (%)
≤ 0.14	0.00	0.20	16.75
0.15-0.99	2.75	2.24	64.37
≥ 1.00	97.25	97.56	18.88

PRP = polyribosyl ribitol phosphate; DTP/Hib = diphtheria-tetanus-pertussis/*Haemophilus influenzae* B.

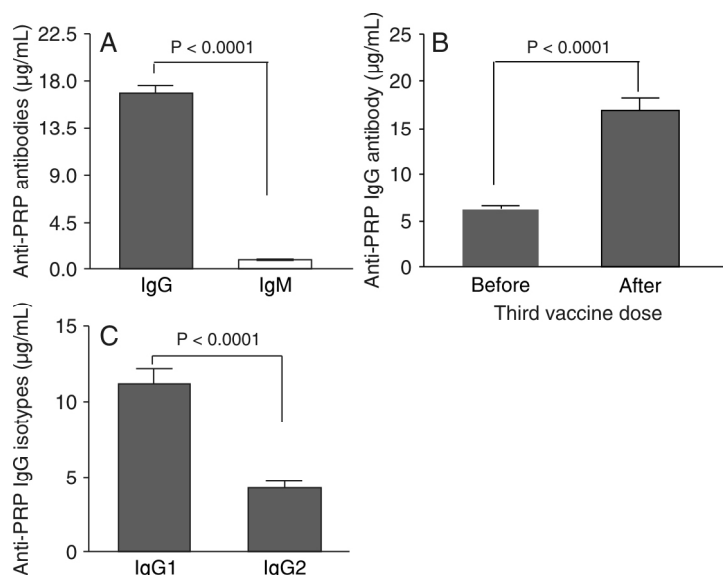


Figure 1. Concentration of anti-PRP antibodies in sera of immunized children. The bars indicate the geometric mean of antibody levels and 95% confidence intervals. Statistical comparisons were made with the Wilcoxon test. A, Comparison of anti-PRP IgG and IgM concentrations in the sera one month after the 3rd DTP/Hib vaccine dose. B, Comparison of anti-PRP IgG concentration before and one month after the 3rd DTP/Hib vaccine dose. C, IgG subclasses one month after the 3rd DTP/Hib vaccine dose. PRP = polyribosyl ribitol phosphate; DTP/Hib = Diphtheria-tetanus-pertussis/*Haemophilus influenzae* B.

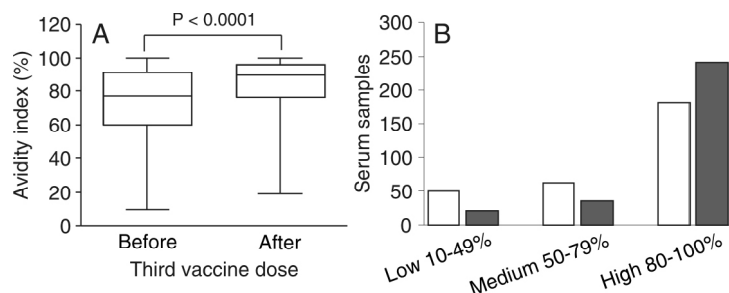


Figure 2. Avidity index and distribution of PRP IgG antibodies in serum samples from children receiving DTP-Hib vaccination. **A.** Avidity index before and 1 month after the 3rd DTP-Hib vaccine dose in 300 infants. Statistical comparison was made with the Wilcoxon test. **B.** Distribution of the avidity of anti-PRP IgG antibodies in a group of 300 infants immunized with DTP/Hib. Open and filled bars represent blood sampling before and 1 month after the 3rd dose of the DTP/Hib vaccine, respectively. PRP = polyribosyl ribitol phosphate; DTP/Hib = diphtheria-tetanus-pertussis/*Haemophilus influenzae* B.

profiles of avidity intensity before and after the 3rd dose (Figure 2B).

Discussion

In this study, we quantitatively and qualitatively evaluated the specific anti-Hib response induced by the Hib PRP-T conjugated vaccine during a clinical trial of the DTP-Hib vaccine produced by Bio-Manguinhos/Fiocruz, Brazil, after technological transfer from Glaxo-SmithKline.

High-affinity antibodies are required to neutralize the infectivity of most pathogens. Therefore, effective vaccines against these microorganisms must induce affinity maturation and memory B cell formation, and both these events will occur only if the vaccines are able to activate T helper cells. This concept has been applied to the design of vaccines against some bacterial infections in which the target antigen is a capsular polysaccharide, which is incapable of stimulating T cells. This is also the case of the polysaccharide from Hib which, when covalently linked to tetanus toxoid, forms the equivalent of an hapten-carrier conjugate, which does activate T helper cells. Thus, the antibody response induced by this conjugate vaccine requires the participation of T cells that may consequently stimulate the subclass switching, affinity maturation and memory with long-lived protective immunity in B lymphocytes. In contrast, the antibodies produced in the absence of T helper cells by polysaccharide vaccines not conjugated to proteins are generally of low affinity and consist mainly of IgM and IgG2 (18).

The humoral immune response to Hib conjugated vaccines is usually evaluated by measuring serum anti-PRP antibodies and by the AI. Avidity of anti-PRP IgG has been tested by ELISA-based elution assays (4,19-24), which have demonstrated that PRP conjugates elicit high-avidity antibodies, with increased functional activity (4,18).

We observed here that 97.25% of the infants immunized with DTP-Hib produced high levels ($\geq 1.0 \mu\text{g/mL}$) of PRP IgG antibodies, considered to be a serological surrogate of long-term protection. Some researchers have already established that protection from Hib disease is correlated with the presence of antibodies to PRP, and have considered $0.15 \mu\text{g/mL}$ of anti-PRP to be the minimum level of antibodies for short-term protection against Hib disease and $1 \mu\text{g/mL}$ to be the minimum level for long-term protection (25,26). Regarding the IgM antibody, our results showed that almost all vaccinees produced anti-PRP IgM, but at low levels compared to anti-IgG antibodies. The production of IgM antibodies against PRP induced by DTP-Hib, although small, plays an important role in opsonization and phagocytosis by activation of the classical complement pathway, an important defense mechanism against bacteria with polysaccharide-rich capsules, such as *Haemophilus*. The IgM antibody, due to its pentameric structure, is a more efficient complement-binding antibody than IgG.

When we assessed the subclasses of IgG we observed that the vaccinees responded with IgG anti-PRP of both IgG1 and IgG2 subclasses, but with a significant predominance of anti-PRP IgG1. We observed a good relationship between IgG and IgG1 antibody levels, but no correlation was found with IgG2 levels. Our results agree with those obtained with different PRP conjugates by Granoff et al. (27), who also reported that infants immunized with PRP-OMP respond predominantly with anti-PRP IgG1, while adults immunized with unconjugated PRP or PRP-D respond with anti-PRP of both IgG1 and IgG2 subclasses, as is the case for the DTP-Hib vaccine. Hetherington et al. (28) have also suggested that the IgG subclass response to PRP protein conjugate is age independent. Other investigators have also reported that IgG1 antibodies are more effective than IgG2 antibodies for Hib in an opsonization test and in activating complement-mediated bacteriolysis (29-32).

The present results demonstrate that antibody avidity was increased after the third immunization with the DTP-Hib vaccine. The increased AI between the 4th and 7th month of age indicates that memory responses must have occurred during DTP-Hib vaccination in Brazilian infants. These results might be due to the vaccine reactivation of earlier induced memory B cells, as reported by Hougs et al. (33). Goldblatt et al. (18) have demonstrated that antibody avidity was lower in infants with anti-PRP antibody concentrations below $1.0 \mu\text{g/mL}$, indicating the absence of priming when antibody concentrations are low. Amir et al. (29) also evaluated the functional antibody activity of vaccines induced by Hib and found the avidity of antibodies to be an essential factor for serum bactericidal activities.

The increase in avidity reflects the process of somatic

hypermutation of immunoglobulin genes and the subsequent selection resulting in high affinity B cells that occurs in the germinal center following a T cell-dependent response (34). Thus, the AI has been used as a surrogate marker of immunological priming since it also relies on a T cell-dependent response (18).

The present study shows that the Brazilian DTP/Hib vaccine demonstrated strong immunogenicity after 2 doses, with most infants producing high levels of IgG antibodies, but the results were even better one month after the 3rd vaccine dose. When analyzing the antibody AI, we were able to demonstrate that high avidity PRP antibodies were found after the 2nd DTP/Hib vaccine dose, and avidity had improved after the third dose. Although two vaccine doses induce antibodies with a protection level above 1 µg/mL, the 3rd dose significantly improves the functional activity of

antibodies against *Haemophilus influenzae*, thus ensuring effective long-term protection against Hib.

Finally, we emphasize that the combined DTP/Hib vaccine allowed a reduction in the number of required injections, thus improving compliance with the vaccination schedule and higher vaccination coverage. Moreover, the combined vaccine reduced the logistic costs related to factors such as number of visits to health care centers.

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