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The effect of reconstitution of an *Haemophilus influenzae* type b-tetanus toxoid conjugate (PRP-T) vaccine on the immune responses to a diphtheria–tetanus-whole cell pertussis (DTwP) vaccine: a five-year follow-up

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Abstract

Controversial results have been obtained from previous studies on the combined administration of *Haemophilus influenzae* type b-tetanus toxoid conjugate (PRP-T) and diphtheria–tetanus-whole-cell pertussis (DTwP) combination vaccines, with regard to possible reciprocal interference between the constituent antigens. To document the priming effect and possible long-term immunogenic interference of PRP-T and DTwP combination vaccines, a randomized, double-blind, controlled study was conducted in Belgium. A total of 168 healthy infants received, at 3, 4 and 5 months of age, DTwP vaccine mixed just prior to injection either with PRP-T vaccine (group A, DTwP//PRP-T, $N=85$) or with placebo (group B, DTwP//Placebo, $N=83$). At the age of 14 months, children of both groups were randomized to receive either a dose of DTwP//PRP-T vaccine (subgroups A1 and B1) or a dose of Hib polysaccharide (PRP) vaccine (subgroups A2 and B2). Those children in subgroups A1 and B1 had an additional serum sample taken at the age of 5 years (at the time of a DT booster).

The immune response to Hib polysaccharide at the age of 4, 5 and 6 months confirmed the excellent immunogenicity profile of PRP-T in infants. In addition, the vigorous anamnestic response (i.e. a 20-fold increase of GMT) to a booster dose of the plain capsular polysaccharide (PRP) reflected the efficient Hib-priming induced by the combined DTwP//PRP-T vaccine.

Reconstitution of PRP-T with DTwP did not affect the immune response to diphtheria toxoid or pertussis agglutinins. Nevertheless, at almost any time point during the five-year follow-up, the tetanus antitoxin GMT values were significantly lower in the DTwP//PRP-T group (A and A1) than in the DTwP//Placebo group (B and B1). Despite the suppressive effect on GMT values, intergroup differences in rates of seroprotection were never significant, except after doses 2 and 3 for which there were lower percentages of children in group A with antitoxin titers >0.05 IU/mL and >1.0 IU/mL. In the group primed with the combined DTwP//PRP-T vaccine, (1) a DT booster dose at the age of 5 years provoked a 150-fold increase in tetanus antitoxin GMT, (2) a high tetanus antitoxin GMT value was attained (GMT = 19.3 IU/mL) and (3) all children in this group had tetanus antitoxin titers >1.0 IU/mL, so it may be concluded that all these children will still be protected against tetanus until at least the age of the next recommended booster dose (i.e. the age of 15 years).

No differences in the occurrence of adverse events were observed between the groups who received the DTwP//PRP-T vaccine or the DTwP//Placebo vaccine, both vaccines being associated with events customarily attributable to DTwP (data not shown).

Our results indicate (1) that the combination vaccine, DTwP//PRP-T, represents a safe and effective alternative for the

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existing uncombined vaccines and (2) that the long-term effect of interference between the components of future combination vaccines should be studied with subsequent booster doses, followed by the evaluation of persistence of antibodies over several years.

Keywords: *Haemophilus influenzae* type b; Diphtheria–tetanus–pertussis; Combined vaccines, pediatric; Priming effect, anamnestic response; Follow-up of immunogenic interference

1. Introduction

Haemophilus influenzae type b (Hib) was the most common cause of bacterial meningitis in infants until the start of vaccination with conjugate vaccines in the 1990s [1]. Vaccines that chemically link a protein to the Hib polysaccharide capsule (PRP) have been found to protect against invasive Hib disease, even when administered early in infancy. This desirable property, attributable to the conversion of PRP from a T-cell-independent to a T-cell-dependent antigen, enables these conjugate vaccines to be incorporated into the primary immunizations schedule for infants.

The ability to combine the Hib conjugate vaccine with the standard diphtheria–tetanus-whole-cell pertussis (DTwP) vaccine considerably simplified the childhood immunization schedule and process and had a positive impact on the coverage of Hib vaccination. However, experience with different Hib combinations has shown that simple mixing of the products prior to injection can affect the immune response in unpredictable ways. In several studies on the combined administration of DTwP and PRP-T, depressed immunogenicity of some components of the combination product was observed [2–8]. In contrast, a full-liquid vaccine combining DTwP with the Hib–CRM conjugate (HbOC) showed some increased response to all constituent antigens compared to separate injections of the vaccines [9,10].

To judge the clinical importance during primary vaccination of the apparent antigenic suppression of combined vaccines, the priming effect of the vaccine components should be tested with subsequent booster doses, followed by the evaluation of long-term persistence of antibodies. This long-term follow-up of antibody levels is lacking in most of the studies of combined DTwP//Hib conjugate vaccines.

The present study, performed in Belgium, had two objectives: (1) to evaluate the priming capacity of the DTwP//PRP-T combination vaccine, administered at the age of 3, 4 and 5 months and (2) to follow antibody titers and record any immunogenic interference until the age of 5 years.

2. Materials and methods

2.1. Study population and plan

This randomized, double-blind, placebo-controlled, primary vaccination study was conducted in Belgium (Leuven) and was followed by a randomized, single-blind, comparative booster study including the same children. The protocols were approved by the local Ethics Committee. Rationale, risks, benefits and procedures were explained to the parents and informed written consent was obtained for each enrolled child, prior to participation. The primary vaccination study started in October 1990 and the last serum sample for long-term evaluation of antibodies was obtained in May 1996.

One hundred and sixty-eight (168) healthy Belgian infants were enrolled between October 1990 and September 1991 in 20 daycare centers, according to the following selection criteria at the time of randomization and first injection: 12–16 weeks of age (12–16 months for the booster); afebrile (rectal temperature $< 38^{\circ}C$); born at term (37 weeks or later) with a minimum birthweight of 2500 g; no history of seizures or other neurologic disorders; no family history of sudden infant death syndrome (SIDS) and not receiving medication likely to alter the immune response. Three initial vaccine doses were prescribed at intervals of 3–6 weeks.

The children were allocated to one of two study groups, according to a randomization list and following chronological order of enrollment in the trial. All infants received three doses of DTwP vaccine at the age of 3, 4 and 5 months, mixed just prior to injection with either PRP-T vaccine (DTwP//PRP-T, group A, $N=85$) or placebo (DTwP//Placebo, group B, $N=83$). At the age of 14 months (± 2 months), the children of both primary vaccination groups were divided according to a second randomization list, to receive either a dose of the combined DTwP//PRP-T vaccine (subgroup A1, $n=46$ and subgroup B1, $n=45$) or to receive a dose of unconjugated PRP vaccine, which was followed by a separate dose 3 to 6 weeks later of

DTwP (group A2, $n=39$) or DTwP//PRP-T (group B2, $n=37$). Seventy of the 91 eligible children of groups A1 and B1 received an additional diphtheria toxoid–tetanus toxoid (DT) booster dose at the age of 5 years ($\pm$1 year).

2.2. Vaccines and vaccination schedule

DTwP: each 0.5-mL dose of the vaccine (Triamer™; Pasteur Mérieux Connaught (PMC), formerly known as Pasteur Mérieux sérums et vaccins (P.M. sv.), Lyon, France; commercial lot, batches No. D1379 and E0712) contained purified diphtheria toxoid ($\geq$30 IU), purified tetanus toxoid ($\geq$60 IU) and inactivated whole-cell *Bordetella pertussis* ($\geq$4 IU). The antigens were adsorbed onto aluminium hydroxide ($\leq$1.25 mg of aluminium). The preservative was thimerosal ($\leq$0.05 mg). The vaccine was presented as an injectable suspension of DTwP in a prefilled syringe of 0.5-mL saline solution (0.9% isotonic sodium chloride solution) with a fitted 16-mm needle for intramuscular injection.

DT: each 0.5-mL dose of the vaccine (Ditemer™; Pasteur Mérieux Connaught, Lyon, France; commercial lot, batch No. 95C22) contained purified diphtheria toxoid ($\geq$30 IU) and purified tetanus toxoid ($\geq$40 IU). The methods of adsorption and preservation and the presentation were identical to those of the DTwP vaccine.

PRP-T: the vaccine (Act-HIB™; Pasteur Mérieux Connaught, Lyon, France; batch No. S2189) contained *H. influenzae* type b capsular polysaccharide (polyribosylribitol phosphate, PRP) conjugated to tetanus toxoid. The tetanus toxoid had been detoxified by formalin treatment. Each single-dose vial of lyophilized vaccine contained 10 μg of PRP conjugated to 24 μg tetanus toxoid, 0.6 mg of Tris and 42.5 mg of sucrose and was presented in a glass vial as a freeze-dried powder.

Placebo: each dose of placebo (Pasteur Mérieux Connaught, Lyon, France; batch No. S2162) contained 0.6 mg of Tris and 42.5 mg of sucrose, was presented in a single dose vial in crystalline form and was of identical appearance to PRP-T.

PRP: the vaccine (Pasteur Mérieux Connaught, Lyon, France; batch No. S2124) contained *H. influenzae* type b capsular polysaccharide (PRP). Each single-dose vial of lyophilized vaccine contained 25 μg purified PRP and 2 mg lactose and was presented in a glass vial as a freeze-dried powder; 0.5 mL of saline diluent in a 1-mL syringe with a fitted 16-mm needle for intramuscular injection was also supplied.

All materials were stored at 2–8°C.

All vaccines were administered intramuscularly in the buttock, using a 16-mm needle. Oral, trivalent, live-attenuated polio vaccine was also administered with each immunization, except at the age of 4 months. Venous blood samples were taken at the time of each dose (3, 4, 5 and 14 months) and 3–6 weeks after the third and the fourth doses. A subset of children (subgroups A1 and B1) had two additional blood samples drawn at the age of 4–6 years, i.e. immediately before and one month after the DT booster dose.

2.3. Serological testing

Global anti-PRP antibody response was assessed with a Farr-type radioimmunoassay (RIA) using ^{125}I-labeled polysaccharide [11]. The anti-PRP antibody results were expressed in μg/mL. Anti-diphtheria and anti-tetanus toxoid antibodies were measured by an RIA and results were expressed in IU/mL, with reference to an international standard [12]. Agglutination antibodies against *Bordetella pertussis* were assayed as previously described; results were expressed as reciprocal titer [13]. All assays were performed in a blinded fashion at the Pasteur Mérieux Connaught Clinical Sero-immunology Laboratories (Marcy l'Etoile, France and Val de Reuil, France).

The immune response to all antigen components, after the primary immunization and before and after the booster injections, was calculated in terms of geometric mean titer (GMT) and as the percentage of infants achieving (i) levels of anti-PRP antibodies $\geq$0.15 or $\geq$1.0 μg/mL, (ii) levels of anti-diphtheria and anti-tetanus antitoxins $>$0.01, $>$0.05, $>$0.1 or $>$1.0 IU/mL and (iii) reciprocal titers of pertussis agglutination antibodies $\geq$40, $\geq$80 or $\geq$320, or at least a four-fold increase in agglutination antibody titers as compared to preimmunization levels.

2.4. Safety

The evaluation of safety was performed in a standard manner after all injections, except for the DT booster dose at the age of 5 years. All children were monitored by the investigator for 30 min following injection. Local reactions, the rectal temperature and systematic reactions were recorded by the parents on a diary card for three days following each injection. Additionally, parents were asked to record any adverse events and intercurrent illnesses requiring medical attention that occurred between day 3 after vaccination and the following visit. Serious adverse events occurring at any time during the entire study period were to be reported, up to 30 days after the last vaccination visit.

2.5. Statistical analysis

The immunogenicity data from available blood samples were analyzed and presented on a per-protocol

Table 1
Trial design and characteristics of the study population. PRP, unconjugated *Haemophilus influenzae* type b capsular polysaccharide; PRP-T, *H. influenzae* type b capsular polysaccharide–tetanus toxoid conjugate; DTP means diphtheria–tetanus-whole-cell pertussis and DT diphtheria–tetanus

Primary immunization	Group A		Group B	
Vaccine regimen	DTwP//PRP-T[a] (3, 4, 5 months)[e]		DTwP//Placebo[a] (3, 4, 5 months)[e]	
Serum sampling	3, 4, 5, 6 months[e]		3, 4, 5, 6 months[e]	
Number of subjects	85		83	
Sex ratio M/F (n) at inclusion	41/44		48/35	
Number of samples included in analysis [b,c]	84		79	
Booster immunization (14 mo)	**Group A1**	**Group A2**	**Group B1**	**Group B2**
Vaccine regimen	DTwP//PRP-T[a] (14 mo)	PRP (14 mo); DTwP (15 mo)	DTwP//PRP-T[a] (14 mo)	PRP (14 mo); DTwP//PRP-T[a] (15 mo)
Serum sampling	14, 15 months	14, 15 months	14, 15 months	14, 15 months
Number of subjects	46	39	45	37
Sex ratio M/F (n) at inclusion	23/23	18/21	25/20	23/14
Number of samples included in analysis[b]	45	38	42	34
Mean age (months) ± S.D. at booster dose	13.4 ± 0.6	13.5 ± 0.7	13.5 ± 0.6	13.6 ± 0.6
Mean age (months) ± S.D. at postimmunization sampling	14.5 ± 0.6	14.6 ± 0.7	14.6 ± 0.6	14.6 ± 0.6
Booster immunization (5 years)	**Group A1**		**Group B1**	
Vaccine regimen	DT (5 years)		DT (5 years)	
Serum sampling	5 yr, 5 yr + 1 mo		5 yr, 5 yr + 1 mo	
Number of subjects	34		36	
Sex ratio M/F at inclusion	19/15		20/16	
Number of samples included in analysis	34[d]		36	
Mean age (years) ± S.D. at DT booster dose	5.2 ± 0.3		5.1 ± 0.2	
Mean age (years) ± S.D. at postimmunization sampling	5.3 ± 0.3		5.2 ± 0.2	

[a] DTwP//PRP-T and DTwP//Placebo: combined administration, mixed immediately prior to injection.
[b] Five samples excluded from primary analyses and eight samples excluded from booster analyses due to protocol violation.
[c] Two postdose 2 samples missing.
[d] One pre-immunization sample missing.
[e] Mean ages (months) ± S.D. were 3.2 ± 0.2 at dose 1; 4.2 ± 0.2 at dose 2; 5.3 ± 0.3 at dose 3; and 6.3 ± 0.4 at postimmunization blood sampling for both groups.

basis. Quantitative analysis was done using a two-sided Student's t-test after logarithmic transformation of antibody titers, or with Wilcoxon's rank–sum test in case of nonnormal distribution. Percentages of seroprotection and of adverse reactions were compared between groups using Pearson's chi-square test, or Fisher's exact test when appropriate.

Kinetics of the anti-PRP antibodies during primary immunization were evaluated by means of an ANOVA model of repeated measures. Since the booster analysis of PRP data included four pairwise comparisons, the appropriate α level for statistical significance was set to 0.0125 to obtain a total α risk of 0.05, according to the Bonferroni approach.

Initial sample sizes were calculated to obtain an α risk of 0.05 and a power >90% to detect a difference of 15% for the most stringent parameter, the pertussis agglutinins. Taking into account a loss to follow-up rate of 10%, it was calculated that a total of 170 infants were required.

The statistical analysis was performed using SAS software (Version 6.11; SAS Institute, Inc, Cary, NC, USA).

3. Results

3.1. Compliance to the study protocol (Table 1)

All children (n = 168) included in the study com-

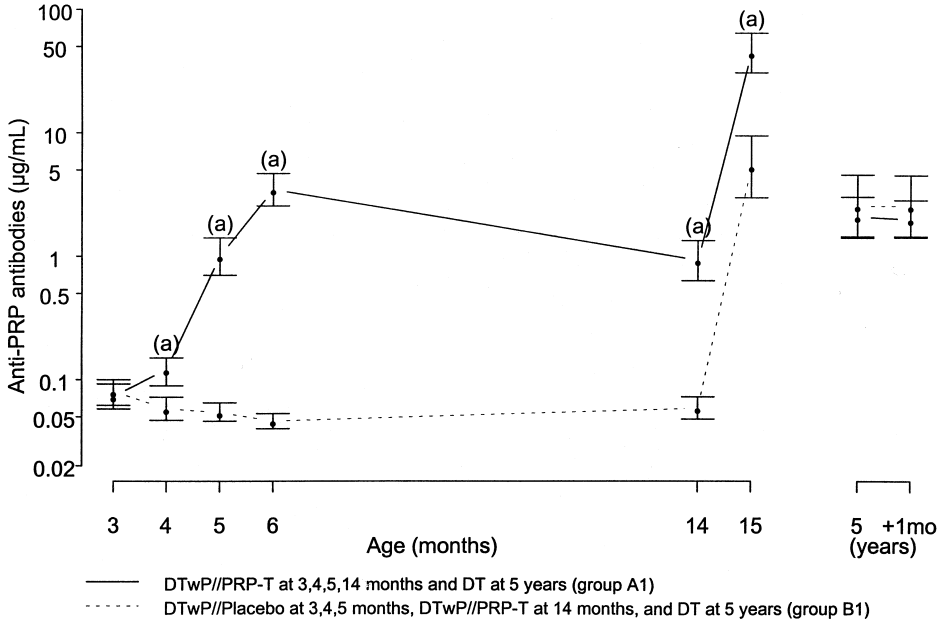

Fig. 1. Anti-PRP antibody geometric mean titers (GMT, µg/mL) and their 95% confidence intervals (95% CI) at several time points between the age of 3 months and 5 years. Comparison of GMT between primary vaccination groups; [a]$p < 0.0001$.

pleted the primary vaccination trial and only one child (in group B), diagnosed with acute lymphoblastic leukemia at 5 months of age, did not receive the booster dose (14 months). No children were lost to follow-up during primary or booster vaccination. Five infants were excluded from the primary vaccination immunogenicity analyses and three from the booster immunogenicity analyses, due to reasons considered unrelated to the vaccination.

The batch of the PRP vaccine used in this study was valid until June 1, 1992. Eighteen children who had not received their fourth dose by May 31, 1992 were given a dose of DTwP//PRP-T vaccine instead.

All 70 children included in the follow-up study at the age of 5 years received a DT booster dose and were included in the booster immunogenicity analysis.

3.2. Comparability of the study groups

The mean age at the time of the three injections during primary vaccination was comparable between groups. Sex distribution was homogeneous in both study groups, with a global sex ratio (M/F) of 1.13. Prior to vaccination, no statistically significant differences were observed in the antibody levels for any of the antigens studied, thus ensuring comparability of the study groups. At the time of booster dose (14 months) and follow-up (5 years), the mean ages and the sex distribution were also comparable between groups (Table 1).

3.3. Immunogenicity results of primary vaccination

One month after the third dose of DTwP//PRP-T vaccine (group A), a sufficiently high anti-PRP GMT value was attained (3.47 µg/mL; 95% CI, 2.57–4.69) to ensure protective antibody concentrations in most of the children (95.2% ≥ 0.15 and 83.3% ≥ 1.0 µg/mL). At the age of 14 months (prebooster), the majority of children remained seroprotected (88.0% with a titer ≥0.15 µg/mL and 49.4% with a titer ≥1.0 µg/mL), despite the nearly fourfold decrease of anti-PRP GMT values between the age of 6 months (postprimary) and 14 months (0.94 µg/mL; 95% CI, 0.64–1.36)(Fig. 1).

The anti-diphtheria and anti-tetanus toxoid immune responses during primary vaccination were vigorous and similar in the two groups. All children, except one in group A for the diphtheria antitoxins, attained a protective antibody level (>0.01 IU/mL) after the third dose in the primary vaccination series. There were no statistically significant intergroup differences at any time point between the age of 3 and 14 months, except for the GMT values and percentages of children with a tetanus antitoxin titer of more than 0.05 IU/mL or 1.0 IU/mL after the second and third doses ($p < 0.05$, for intergroup comparisons, in favor of the DTwP//Placebo group). The difference disappeared at the age of 14 months, just before the booster dose, the GMT values having fallen to near-baseline levels in both groups. Nevertheless, all children (except 5 in group A and 3 in group B) had protective antitoxin titers (>0.01 IU/mL) at this time (Table 3 and Fig. 2).

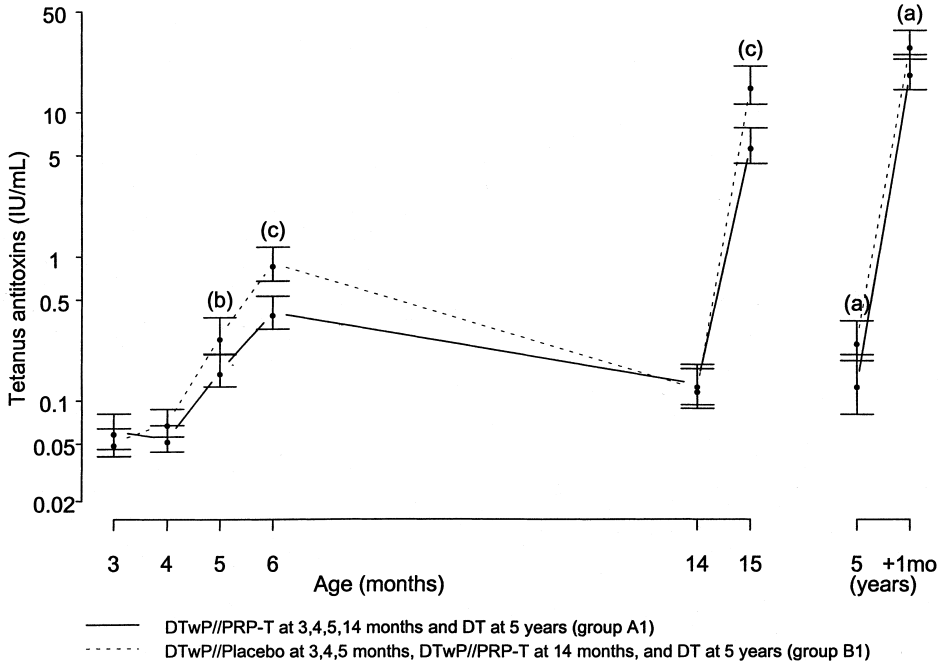

Fig. 2. Tetanus antitoxin geometric mean titers (GMT, IU/mL) and their 95% confidence intervals (95% CI) at several time points between the age of 3 months and 5 years. Comparison of GMT between primary vaccination groups; [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.0001$.

After the third injection, at least two-thirds of infants attained an pertussis anti-agglutination titer ≥320 (65.5% in group A and 70.5% in group B) and more than 85% reached a level ≥40 (86.9% and 89.7%, respectively). In group A, 85.7% had a fourfold increase of agglutinin titer between postdose 3 and baseline age, which was not significantly different from the 88.5% observed in group B (data not shown).

3.4. Immunogenicity results of booster vaccination

Prebooster GMTs and seroprotection rates were comparable between subgroups for all antigens, except for the anti-PRP seroprotection rates (≥0.15 μg/mL; A1 > A2 and B1 < B2; $p < 0.05$)(Table 2).

3.4.1. Hib capsular polysaccharide (PRP) (Table 2 and Fig. 1)

For those children who had received primary vaccination with DTwP//Placebo, the first dose of the Hib conjugate (presented as a DTwP//PRP-T vaccine) administered at the age of 14 months (group B1), induced an anti-PRP GMT value of 5.42 μg/mL (geometric mean of the individual post- to prebooster ratios, RGMT = 114.7) and high percentages of seroprotection were obtained (95.0 and 90.5% for the protective levels of ≥0.15 and 1.0 μg/mL, respectively). However, when the first Hib dose was given as unconjugated PRP vaccine (group B2) at the age of 14 months, a poor immunogenic response was observed, with a GMT of 0.15 μg/mL (RGMT = 1.9) and only 50% of children attaining a titer ≥0.15 μg/mL.

For those children who received primary vaccination with DTwP//PRP-T, a booster dose of the same vaccine (group A1) resulted in an anti-PRP GMT value of 45.80 μg/mL (RGMT = 38.4) and all children attained titers above 1.0 μg/mL. A booster dose of unconjugated polysaccharide (PRP) (group A2) resulted in an anti-PRP GMT of 14.49 μg/mL (RGMT = 20.7) and 94.7% of the children were seroprotected (≥0.15 μg/mL).

3.4.2. Diphtheria and tetanus toxoids and pertussis agglutinins

Evaluation of the immunogenicity of a DTwP booster dose was based upon the results of children who received a DTwP//PRP-T booster at the age of 14 months (groups A1 and B1). One month after the booster injection, all children attained diphtheria and tetanus antitoxin titers greater than 0.05 IU/mL and 76.6% (group A1) and 85.4% (group B1) of the children showed a fourfold increase in pertussis anti-agglutination antibody titers, as compared to prebooster concentrations. No difference between groups was observed with regard to GMT values for diphtheria antitoxin and pertussis agglutination antibody titers. The tetanus antitoxin GMT values, however, were significantly lower in group A1 (primed with DTwP// PRP-T) (GMT = 5.95 IU/mL; 95% CI, 4.47–7.93) compared to group B1 (primed with DTwP//Placebo) (GMT = 15.70 IU/mL; 95% CI, 11.6–21.3) ($p <$

Table 2
Geometric mean titers (GMT) (μg/mL), their 95% confidence intervals (95% CI) and percentage of children with protective levels of anti-PRP antibodies before and 30 days after the boosterdose at 14 months. RGMT, geometric mean of individual post- to preimmunization titer ratios

	Group A1					Group B1				
Vaccine regimen	DTwP//PRP-T at 3,4,5 and 14 months					DTwP//Placebo at 3,4,5 months and DTwP//PRP-T at 14 months				
				Seroconversion (%)					Seroconversion (%)	
	N	GMT	(95% CI)	≥0.15	≥1.0	*N*	GMT	(95% CI)	≥0.15	≥1.0
Prebooster (14 months)	45	1.19	(0.72–1.96)	95.6	53.3	42	0.047[b]	(0.037–0.060)	7.2[e]	0[e]
Post–booster (15 months)	45	45.8	(31.6–66.3)	100	100	42	5.42[b]	(3.04–9.66)	95.2	90.5[d]
RGMT (post/prebooster)	45	38.4	(21.9–67.3)	–	–	42	114.7[a]	(64.4–204.2)	–	–
	Group A2					Group B2				
Vaccine regimen:	DTwP//PRP-T at 3,4,5 months and PRP at 14 months					DTwP//Placebo at 3,4,5 months and PRP at 14 months				
				Seroconversion (%)					Seroconversion (%)	
	N	GMT	(95% CI)	≥0.15	≥1.0	N	GMT	(95% CI)	≥0.15	≥1.0
Prebooster (14 months)	38	0.70	(0.39–1.25)	79.0 [f]	44.7	34	0.078[b]	(0.054–0.11)	32.4[e,f]	0[e]
Postbooster (15 months)	38	14.5	(6.85–30.7)	94.7	86.8 [f]	34	0.15[b,c]	(0.088–0.25)	50.0[e,g]	14.7[e,g]
RGMT (post/prebooster)	38	20.7	(9.95–43.0)	–	–	34	1.91[b,c]	(1.23–3.23)	–	–

[a,b] Comparison of priming groups: A vs. B; two-sided Student's *t*-test or Wilcoxon's rank–sum test $\alpha = 0.05$ (within booster regimen for booster analysis; A1 vs. B1 and A2 vs. B2; two-sided Student's *t*-test; $\alpha = 0.0125$): [a]$p < 0.01$ and [b]$p < 0.0001$.

[c] Comparison of booster regimen within priming groups: A1 vs. A2 and B1 vs. B2 (two-sided Student's *t*-test or Wilcoxon's rank–sum test; $\alpha = 0.0125$): [c]$p < 0.0001$.

[d,e] Comparison of percentage seroconversion between priming groups: A vs. B; (within booster regimen for booster analysis; A1 vs. B1 and A2 vs. B2); Pearson's chi-square or Fisher's exact test: [d]$p < 0.05$ and [e]$p < 0.001$.

[f,g] Comparison of percentage seroconversion for booster regimen within priming groups: A1 vs. A2 and B1 vs. B2; Pearson's chi-square or Fisher's exact test: [f]$p < 0.05$ and [g]$p < 0.001$.

00.0001). Nevertheless, significant differences in the tetanus antitoxin seroprotection rates were not observed. One month after the booster dose, all infants of group A1 and all but one of group B1, had achieved tetanus antitoxin levels of at least 1.0 IU/mL (Table 3 and Fig. 2).

3.5. Persistence of antibodies

3.5.1. Hib capsular polysaccharide (PRP) (Table 2 and Fig. 1)

Before DT booster, comparable anti-PRP GMT values were observed in both groups (group A1: 2.13 μg/mL, 95% CI, 1.47–3.08 and group B1: 2.59 μg/mL, 95% CI, 1.43–4.65). All children had protective antibody concentrations (≥0.15 μg/mL), except for two children who were primed with the DTwP//Placebo combination vaccine (group B1). Antibody titers ≥1.0 μg/mL were obtained in 25 of 33 (75.8%) children in group A1 and 25 of 36 (69.4%) children in group B1.

3.5.2. Diphtheria and tetanus toxoids

Between the age of 15 months and 5 years, a striking decrease of diphtheria and tetanus antitoxins was observed. Both antitoxins attained near-baseline GMT levels by the age of 5 years. A very vigorous immune response to diphtheria toxoid was induced in both groups by administration of DT vaccine at the age of 5 years (RGMT values, 134.3 and 155.0 for groups A1 and B1, respectively). All children in both groups attained a protective diphtheria antitoxin titer of at least 1.0 IU/mL.

Pre-DT-booster GMT values for tetanus antitoxins were significantly lower in the group primed with the DTwP//PRP-T vaccine (A1) (0.13 IU/mL; 95% CI, 0.08–0.21) as compared to the group primed with DTwP//Placebo (B1) (0.26 IU/mL; 95% CI, 0.19–0.36) ($p = 0.0133$). Despite a strong booster response in both groups (RGMT values, 150.2 and 114.6 for groups A1 and B1, respectively), the significant between-group difference in GMT values still remained (19.3 IU/mL; 95% CI, 14.6–25.6 versus 29.9 IU/mL; 95% CI, 23.8–37.6) ($p = 0.0158$). Nevertheless, antitoxin titers in

Table 3
Geometric mean titers (GMT) (IU/mL), their 95% confidence intervals (95% CI) and percentage of children seroconverted for tetanus antitoxin at baseline and 30 days after each dose of vaccine. RGMT, geometric mean of individual post- to pre-immunization titer ratios

	DTwP//PRP-T at 3, 4, 5 months (group A) and DTwP//PRP-T at 14 months (group A1)						DTwP//Placebo at 3, 4, 5 months (group B) and DTwP//PRP-T at 14 months (group B1)					
				Seroconversion (%)						Seroconversion (%)		
Antibody to tetanus toxoid (IU/ml)	*N*	GMT	(95% CI)	>0.01	>0.1	>1.0	*N*	GMT	(95% CI)	>0.01	>0.1	>1.0
Baseline (3 months)	84	0.061	0.046–0.081	89.3	25.0	2.4	79	0.051	(0.041–0.064)	93.7	21.5	0
Postdose 1 (4 months)	84	0.054	0.044–0.067	97.6	22.6	0	79	0.070	(0.056–0.087)	97.5	32.9	0
Postdose 2 (5 months)	80	0.16	0.125–0.212	100	58.8	7.5	79	0.28[b]	(0.21–0.38)	100	69.6	25.3[d]
Postdose 3 (6 months)	84	0.41	0.316–0.533	100	89.3	27.4	78	0.90[c]	(0.68–1.17)	100	96.2	53.8[d]
Prebooster (14 months)	83	0.11	0.087–0.14	94.0	54.2	1.2	76	0.12	(0.096–0.15)	96.0	59.2	0
RGMT (post dose 3/baseline)	84	6.70	4.33–10.4	–	–	–	78	17.2[b]	(11.3–26.4)	–	–	–
Prebooster (14 months)	45	0.13	0.093–0.179	95.6	57.8	2.2	42	0.12	(0.088–0.167)	95.2	57.1	0
Postbooster (15 months)	44	5.95	4.47–7.93	100	100	100	41	15.7[c]	(11.6–21.3)	100	100	97.6
RGMT (post/prebooster)	44	44.6	31.0–64.1	–	–	–	41	123.4[b]	(84.4–180.3)	–	–	–
Pre booster (5 years)	32	0.13	0.08–0.21	96.9	68.8	0	36	0.26[a]	(0.19–0.36)	100	86.1	5.6
Post booster (5 years +1 month)	34	19.3	14.6–25.6	100	100	100	36	29.9[a]	(23.8–37.6)	100	100	100
RGMT (post/prebooster)	32	150.2	106.2–212.3	–	–	–	36	114.6	(87.0–150.9)	–	–	–

[a,b,c] Comparison of GMT between priming groups: A vs. B (A1 vs. B1 for booster analysis) (two-sided Student's *t*-test or Wilcoxon's rank–sum test): [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.0001$.

[d] Comparison of percentage seroconversion between priming groups: A bv. B (A1 vs. B1 for booster analysis) (Pearson's chi-square or Fisher's exact test): [d]$p < 0.01$.

both groups were high enough to ensure protective antibody titers (≥1.0 IU/mL) for all children in both groups (Table 3 and Fig. 2).

3.6. Safety (data not shown)

No immediate reactions were observed in the 30 min after each injection and no significant differences between study groups (A and B) and between doses were observed for any local or systemic reactions during primary immunization.

In contrast, rates of local and systemic reactions were higher after the DTwP//PRP-T (groups A1 and B1) than after the PRP (groups A2 and B2) booster dose ($p < 0.05$ for all local and most of the systemic reactions).

A serious adverse event was reported for one child of the DTwP//Placebo group. Shortly after the third dose, the child was hospitalized with acute lymphoblastic leukemia, which was considered to be unrelated to the vaccination.

4. Discussion

At the time of initiation of this study, there was no experience in Belgium with Hib vaccines and unconjugated PRP has never been available in Belgium; for the Hib-tetanus toxoid conjugate (PRP-T) vaccine, no data were available on the 3-, 4-, 5- and 14-month schedule, as used in Belgium. In addition, it was not clear whether a DTwP//PRP-T vaccine combination would have an immunogenicity and safety profile that was at least as good as that of the constituent components given separately.

Since that time, a number of other trials have examined the effect of mixing PRP-T with DTwP, making use of DTwP vaccines from different sources (Pasteur Mérieux sérums et vaccins, European pharmacopoeia [2–4,6,14,15] or Connaught Laboratories Inc, American pharmacopoeia [7,8,16–18]), with [6,7] or without IPV [2–4,8,14–18] included in the combination and according to different vaccination schedules (2–3–4 [15], 3–4–5 [4] or 2–4–6 [2,3,6,7,14–18] months). These trials have provided conflicting results with regard to reciprocal interference between the components of the DTwP//PRP-T combination.

4.1. Interference with PRP immune response

In two studies, serum anti-PRP antibody responses to PRP-T vaccine were reduced after mixed administration with DTwP [2,4], although the lowest levels after combined vaccination in both studies remained quite high (4.8 [2] and 7.7 μg/mL [4]). In the present trial, in the absence of a study-arm with infants receiving the PRP-T vaccine in a separate injection, we could not assess whether the DTwP vaccine had an impact on the anti-PRP response. However, the primary vaccination results in our study confirmed the excellent immunogenicity of PRP-T vaccine in infants, as was previously reported elsewhere [19]. In addition,

a vigorous anamnestic response (i.e. a 20-fold increase of anti-PRP concentration) was obtained at the age of 14 months, after injection of unconjugated polysaccharide (PRP), which is considered to be most representative for the contact with wild-type Hib bacteria. This observation could be considered as proof of efficient priming, ensuring rapidly protective immune response in case of subsequent contact with the bacterium. This was further supported by the high anti-PRP GMT values (>2.0 μg/mL) and rates of seroprotection (nearly all children with a titer ≥ 0.15 μg/mL) observed at the age of 5 years in both primary vaccination groups. The expected fall-off over time of vaccine-induced antibody concentrations to near-baseline levels was compensated completely by the potential for a vigorous anamnestic immune response, maintained by the recurrent contact with wild-type Hib or with cross-reactive antigens during the 1–5-year age period.

4.2. Interference with the DTP immune response

Equally relevant to the interference question is whether mixing with PRP-T vaccine impairs the response to any component of the DTwP vaccine. In agreement with previous reports, combination of vaccines had no effect on levels of antibodies against diphtheria toxoid in our study. The most consistent finding in previous studies has been the effect of mixing PRP-T with DTwP on responses to pertussis vaccine. Although lower levels of antibodies to various pertussis antigens have been frequently reported in those receiving the combined vaccine, statistically significant differences occurred in only three trials [3,6,7]. In our study, there was a slight indication that combination with PRP-T interfered with the serological response to pertussis agglutinins. The between-group difference was only significant after the second dose for the percentage of children attaining an anti-agglutination antibody titer ≥ 40 IU/mL, with the lower rate in the combined vaccine group (49.4% versus 65.8%, $p < 0.05$). Recently, in a large-scale efficacy trial in Chile, the clinical relevance of a statistically significant depression of anti-pertussis immune response was examined. The study revealed no evidence of increased susceptibility to pertussis, despite lower antibody titers, in those who received the combined vaccine and it was concluded that the effect of PRP-T on the immunogenicity of pertussis vaccine does not have clinical importance [20].

Although several authors have reported slightly lower levels of tetanus antitoxins after combined administration of DTwP with PRP-T vaccine [3,4,8,14], in only two studies (both with IPV in the combination) were the differences in GMT values after primary vaccination significant [6,7]. Our data demonstrate that administration of PRP-T vaccine mixed with DTwP vaccine led to diminished responses to the second and third doses of tetanus toxoid, as manifested by depressed tetanus antitoxin levels, in terms of GMTs and percentages of children attaining a titer of 0.05 or 1.0 IU/mL. As far as we are aware, this is the first published study in which combined administration of DTwP with PRP-T (without IPV in the combination) was found to depress significantly a phase of the anti-tetanus toxoid immune response, both in terms of GMT value and seroconversion rate.

4.3. Long-term follow-up of interference with DTwP immune response

Despite the fact that reciprocal interference between the components of combined DTwP//PRP-T vaccines has been observed frequently, the long-term effect of suppressed immune response to diphtheria, tetanus and pertussis antigens has only been examined in two studies. In a Chilean study [3], the significantly lower levels of anti-pertussis agglutination antibodies, which were present after primary immunization with the combined DTwP//PRP-T vaccine, had disappeared completely at the time of follow-up of antibody titers one year later (18 months of age), when antibody titers in both groups had declined to near-baseline levels. In a second, Israeli trial [6], the persistence of significant intergroup differences in tetanus antitoxin and pertussis agglutination antibody titers, which were observed after primary vaccination with DTwP//IPV, combined or not with PRP-T, was evaluated six months after booster doses of DTwP and IPV vaccines. Although a trend for lower anti-tetanus antibodies was still present at the age of 18 months, the between-group differences for both antigens were no longer significant, suggesting a good priming to all components after combined administration of the vaccines.

To judge the clinical relevance of the observed interference with the anti-tetanus immune response, we evaluated the booster response after a DTwP//PRP-T vaccine dose at the age of 14 months and after a dose of DT vaccine at the age of 5 years.

Except for the comparable GMT values prebooster (14 months), at all other time points during this five-year follow-up, anti-tetanus toxoid antibody GMT values were significantly lower in the DTwP//PRP-T group (A and A1) than in the DTwP//Placebo group (B and B1). Intergroup differences in rates of seroprotection were never significant, except for the lower rates of children in group A with regard to postdose 2 and 3 antitoxin titers >0.05 or >1.0 IU/mL. In addition, after primary vaccination and both booster doses, all infants attained protective titers of at least 0.01 IU/mL. The high rates of seroprotection in both groups at the time of DT booster (all children had a

titer >0.01 IU/mL before DT booster, except for one child in group A1) confirmed that even the lowest antitoxin GMT value measured one month after DTwP//PRP-T booster (group A1, with GMT = 5.95 IU/mL at the age of 15 months) ensured an adequate protection against tetanus until DT booster age (5 years).

A remaining point of concern might be whether the lower GMT value observed in the combined vaccine group (A1) one month after the DT booster dose could have an impact on the long-term protection and thus necessitate an earlier booster dose. (In Belgium, an adult booster dose with reduced diphtheria toxoid content, dT, is recommended every 10 years.) It is known that in adults the mean '1 log fall' of tetanus antitoxins is about 17 years [21] and that the duration of immunity of a booster given in children is longer than that obtained with a booster dose given to elderly people [22]. In children primed with DTwP//PRP-T (A1), a DT booster dose provoked a 150-fold increase in tetanus antitoxin GMT, resulting in a high GMT value (19.3 IU/mL) with all children attaining an antibody level ≥1.0 IU/ml. Furthermore, the 'absolute' difference between A1 and B1 groups was relatively small (0.2 log). These observations suggest that it is highly likely that all children primed with three doses of DTwP//PRP-T and given a booster dose at 14 months, will still be protected against tetanus until the next booster (i.e. at the age of 15 years).

Although our findings suggest that interference with immunogenicity of tetanus toxoid in the combined DTwP//PRP-T vaccine appeared to be of little clinical importance, the underlying mechanism ought to be understood in order to preclude any clinically relevant interferences in future combinations. The following possible explanations can be put forward.

The hypothesis that the tetanus toxoid carrier protein of the PRP-T conjugate is implicated in the interference is supported by the lack of such interactions after vaccination with combined DTwP and Hib–CRM conjugate (HbOC) vaccines [9,10]. Perhaps the addition of the tetanus protein of PRP-T to the tetanus toxoid of DTwP results in an antigenic load beyond an optimal dose. However, in a similarly designed study [3], such a diminishing trend with regard to the tetanus toxoid response was seen when DTwP was mixed with PRP-T, but not when the two vaccines were administered at separate sites, suggesting that the inhibition is associated with mixing rather than with overload of the tetanus antigen. This conclusion was supported by the fact that the DTwP–HbOC combination vaccine is a truly liquid combination vaccine with optimal formulation conditions for each monovalent component, whereas in the current study liquid DTwP was used as a diluent for lyophilized PRP-T. Mixing might cause the tetanus toxoid that is normally adjuvant-adsorbed to be displaced from the aluminium hydroxide, resulting in a lower immunogenicity of the antigen [23,24]. This argument does not explain why mixing did not affect the diphtheria toxoid.

High maternal tetanus antitoxin levels have been shown in some studies to suppress the immune response to tetanus toxoid [25,26]. This effect should be most prominent after the first vaccine dose and disappear over time due to waning maternal antibodies. The observation that the suppressive effect in our study only became prominent with the second and third doses of the combined vaccine, might suggest a suppressive priming effect of the first vaccine dose. By analogy with the 'carrier(epitope)-specific' suppression that accounts for interaction with the PRP immune response to Hib conjugate vaccines [27], 'non-epitope-specific' suppression might be responsible for a depressed immune response to the carrier (i.e. tetanus toxoid) of the conjugate and, by extension, to the tetanus toxoid component of the DTwP vaccine [28].

4.4. Safety

Our findings confirmed that PRP-T vaccine can be mixed with DTwP without the frequency or intensity of reactions increasing beyond that usually observed after DTwP vaccination.

5. Conclusion

The combined administration of PRP-T vaccine and DTwP vaccine is safe and highly immunogenic. The interference with the immunogenicity of the tetanus toxoid component of the combination vaccine became apparent early during primary vaccination and remained 'imprinted' in the immune system of the recipients, at least until the age of 5 years. Despite this phenomenon, the high levels of vaccine-induced antitoxins ensured protection against tetanus, persisting until the recommended age of the next booster dose. This study is a clear example that interactions between antigens of a combined vaccine are real, but not easily predictable and that the clinical importance of observed significant interactions can be tested with subsequent booster doses and with the analysis of long-term persistence of antibodies. Finally, in order to preclude clinically significant reduction in antibodies with future pediatric combination vaccines, further research is needed with regard to the underlying mechanism(s) of this type of biological interference.

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