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Phase I Clinical Evaluation of a Synthetic Oligosaccharide-Protein Conjugate Vaccine against *Haemophilus influenzae* Type b in Human Adult Volunteers

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Since 1989, we have been involved in the development of a vaccine against *Haemophilus influenzae* type b. The new vaccine is based on the conjugation of synthetic oligosaccharides to tetanus toxoid. Our main goals have been (i) to verify the feasibility of using the synthetic antigen and (ii) to search for new production alternatives for this important infant vaccine. Overall, eight trials have already been conducted with adults, children (4 to 5 years old), and infants. We have described herein the details from the first two phase I clinical trials conducted with human adult volunteers under double blind, randomized conditions. The participants each received a single intramuscular injection to evaluate safety and initial immunogenicity. We have found an excellent safety profile and an antibody response similar to the one observed for the control vaccine.

Haemophilus influenzae type b (Hib) was the leading cause of bacterial meningitis in many parts of the world before the introduction of conjugate vaccines against Hib (1, 7). Despite the decline in the number of cases of Hib-associated disease (8, 16), the use of vaccines against Hib in developing countries is expected to be an important tool for the reduction of vaccine-preventable morbidity and mortality among children less than 5 years old. The high cost of available conjugate vaccines is among the major obstacles (1, 3, 11, 21, 24) to their introduction in the developing countries' routine immunization programs (19, 21).

Hib conjugate vaccines are produced largely by methods based on fermentation/isolation of the natural capsular Hib polysaccharide prior to conjugation. Recently, a new alternative strategy in the fight against Hib infections was proposed, with the development of a synthetic methodology amenable to the large-scale manufacture of Hib polysaccharide fragments. The resulting conjugate vaccine incorporating a synthetic bacterial antigen was demonstrated to be as safe and immunogenic for humans as already-licensed vaccines incorporating the native polysaccharide. This vaccine (Quimi-Hib) was recently approved in Cuba and is now part of the country's National Immunization Program (24).

As part of the clinical evaluation of the investigational vaccine, leading to its registration in Cuba in 2003, two phase I clinical trials were conducted in healthy adult volunteers to assess the safety and preliminary immunogenicity of the Quimi-Hib vaccine candidate. This article will discuss the major results obtained from this initial clinical evaluation.

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MATERIALS AND METHODS

Vaccines. The Quimi-Hib vaccine is composed of a synthetic polyribosylribitol phosphate (sPRP), with an average of eight repeating units (10 μg/ml) and an average sPRP-to-tetanus toxoid ratio of 1/2.6 by weight. Two different vaccine presentations were prepared and tested: N1, in a single vial, containing the conjugate in additive-free phosphate buffer solution, and N2, in two separate vials, each with a one-half volume of the same buffer solution, one with the conjugate and one with aluminum phosphate adjuvant. The contents of the vials were mixed just before use.

The two vaccine presentations were prepared by following the good manufacturing practice established at the Center for Genetic Engineering and Biotechnology and at the Center for the Study of Synthetic Antigens, Havana, Cuba (24). The following investigational lots of the test vaccine were prepared for the purpose of these trials: for N1, vaccine lots 1019E, 1016E, 1017E, and 1024E, and for N2, vaccine lots 1021E and 1022E, with aluminum phosphate AP 1003T.

A licensed, commercially available vaccine (Vaxem-Hib from Chiron) was used as a control. This vaccine is composed of oligosaccharide fragments obtained from the capsular polysaccharide by acid hydrolysis, coupled to the cross-reacting mutant 197 (CRM_{197}) carrier protein and adsorbed on either aluminum hydroxide (vaccine lot 3581) or phosphate (vaccine lot 0101).

Study design. The clinical protocol was first approved by the local Ethics Committee of the Tropical Medicine Institute Pedro Kouri (IPK) and then peer reviewed and approved by the National Regulatory Authority (CECMED) from Havana, Cuba.

Two phase I clinical trials (studies 1 and 2) were conducted on a double blind basis in accordance with good clinical practice (national regulations and ICH E6) and the principles of the Helsinki declaration. A total of 40 subjects were enrolled in each study and randomly assigned to four groups by using a standard table of random numbers. The participants were all healthy males between 20 and 35 years old and without history of chronic disease or vaccination with Hib vaccine. In study 1, the participants were admitted to the special unit at the Institute for Tropical Medicine Pedro Kouri hospital and remained there for 72 h after immunization. In study 2, the participants were asked to remain in surveillance for 3 h after immunization. All subjects provided written informed consent.

In the first trial (study 1, August to September 2001), using 40 volunteers, the

investigational vaccine N1 (groups A, B, and D) was compared to a control vaccine (group C) that was in use in Cuba at that time (Vaxem-Hib, mixed with aluminum hydroxide just before use).

In the second phase I trial (study 2, March to April 2002), using 40 volunteers, the investigational vaccine N2 (groups G and H) was compared to the control vaccine (group E; Vaxem-Hib, adsorbed on aluminum phosphate). An additional group (F) received the reference vaccine N1 for comparison.

In both studies, the volunteers were randomly assigned to one of four groups, and each group received a single dose of one of the vaccines, i.e., either the control vaccine (Vaxem-Hib) or different production batches of the Quimi-Hib candidate vaccine, adsorbed on aluminum phosphate or without adjuvant. Each subject received a single injection in the deltoid muscle with 0.5 ml of the investigational or control vaccine. Blood samples were collected for antibody measurement and clinical laboratory testing, at the time of vaccination and 1 month later. Antibody response was measured according to the methods described below. The collected sera were separated and stored at −20°C until use for analysis.

Safety and reactogenicity. Acute safety data were obtained from reports of local and systemic adverse events. The adverse events detected were erythema, inflammation, swelling, local pain on the injection site, temperature, headache, and general uneasiness. The volunteers were carefully observed for 72 h after the immunization. The temperatures of the injection sites and of the body as well as other reactions were monitored by a medically credentialed provider or nurse, before and every 2 h after injection, over a period of 12 h. Clinical laboratory tests (hematological panel, hepatic, and renal functions) were performed only during study 1.

Antibody assays. The following assays were performed.

(i) ELISA. Immunoglobulin A (IgA), IgG, and IgM anti-PRP antibodies in pre- and postvaccination sera were determined using a slightly modified version of an enzyme-linked immunosorbent assay (ELISA) previously published (17). The coating antigen used was HbOHA (supplied by NIBSC, Potters Bar, United Kingdom). In the second study, only IgG antibodies were measured. A standard curve was generated by using reference serum (lot 1983; Center for Biological Evaluation and Review, Food and Drug Administration, Bethesda, MD) with a calculated Ig antibody concentration. The immunoglobulin concentrations were logarithmically transformed, and the geometric-mean concentrations were calculated (5, 10).

(ii) Antibody specificity. The specificities of the antibodies were evaluated by inhibition studies with the same ELISA. The sera used were diluted to an optical density of up to 1 and then incubated separately overnight with a serial dilution of the natural capsular polysaccharide of Hib (PRP; supplied by NVI, Biltjoven, The Netherlands), starting at a concentration of 0.5 μg/ml. The geometric means of the PRP concentrations inhibiting 50% of the ELISA were compared for different groups.

(iii) Relative antibody avidity. The relative avidities of antibodies were measured only for serum samples from study 1, by using the above-described ELISA, with slight modifications.

The serum dilution was chosen to obtain an optical density of 1. After the plates were washed, the serum dilution was incubated with concentrations ranging from 0.1 to 1.0 M of ammonium thiocyanate. The plates were then allowed to stand for 15 min at room temperature before being washed, and the assay was continued as described above. The avidity index (AI) was calculated by the formula $AI = C \times f$, where C is the concentration of ammonium thiocyanate destabilizing 50% of the antigen-antibody interaction and f is the dilution factor of serum (4). The avidity index was logarithmically transformed, and both the geometric mean and the standard deviations were calculated.

SBA. The complement-mediated bactericidal activities of the antibodies elicited in the first study's volunteers were measured by using a serum bactericidal assay (4, 18). The final reaction mixture contained different dilutions of the test serum samples that had been heat inactivated at 56°C for 30 min, with 0.15 mmol/liter of calcium chloride, 0.50 mmol/liter of magnesium chloride, 12.5 μl of an 8×10^3/ml log-phase Hib cell suspension (strain Eagan), and 25% of agammaglobulinemic serum as the complement source. Serum bactericidal activity (SBA) titers were expressed as the reciprocal of the highest serum dilution that resulted in the killing of 50% of the initial inoculum at 30 min.

Statistical analysis. The geometric means for the groups in each study were compared using the t test. Chi-square and Fisher's exact tests were used when appropriate to compare the proportions of both adverse reactions and the proportions of volunteers who attained ≥4-fold increases in antibody titers in different groups. Overall effect measures were calculated using risk ratios for 95% confidence intervals. The statistical computer program SPSS 10.00 was used for the statistical analysis.

TABLE 1. Local and systemic responses of volunteers receiving a single dose of the vaccine under study[a]

Sign or symptom	Positive rate (%) for indicated vaccine group[b]							
	Study 1				Study 2			
	A	B	C	D	E	F	G	H
Local								
Swelling	0	0	0	0	10	10	10	0
Local pain	20	20	30	0	30	30	30	50
Inflammation	10	0	0	0	0	0	0	0
Erythema	0	0	0	0	0	0	10	0
Systemic								
Febricula (37–37.9°C)	30	60	30	80	0	0	30	0
Fever (≥38°C)	0	0	0	10	0	0	10	0
Slight headache	0	10	0	0	0	10	0	10
General uneasiness	0	0	0	10	0	0	0	0

[a] Each group consisted of 10 human adult volunteers. Groups C and E received the control vaccine Vaxem-Hib (Chiron). All other groups received the vaccine under study.

[b] Adjuvant used for each group: A, none; B, none; C, $Al(OH)_3$; D, none; E, $AlPO_4$; F, none; G, $AlPO_4$; H, $AlPO_4$. Vaccine lot used for each group: A, 1019E; B, 1016E; C, 3581; D, 1017E; E, 0101; F, 1024E; G, 1022E; H, 1021E.

RESULTS

Study population. Eighty subjects with a median age of 25 years were enrolled, and their distribution among the four groups in each study reflected the randomized nature of the trials. The resulting groups were similar with respect to race, age, and Quetelet index. All subjects were surveyed until the end of the trials, and the data collected were included in the analysis of safety, reactogenicity, and immunogenicity.

Safety and reactogenicity. There were no serious local or systemic reactions in either study. All the reactions observed were slight, transient, self-limiting in time, without lasting more than 72 h after the administration of the vaccine, and resolved without medical intervention. The adverse reactions after injection are listed in Table 1. None of the local reactions was debilitating or prolonged. There was no significant difference in symptoms between the vaccinated groups, except for group D in the first study, where only one subject presented fever above 38°C, slight headache, and general uneasiness. The subject later developed a respiratory infection that was not related to the vaccination effects. In addition, all subjects from study 1 showed normal values for hematological, hepatic, and renal parameters.

Immunogenicity. Table 2 summarizes the distribution of the IgG anti-PRP antibody responses in both clinical trials. In all study groups, the geometric means of the anti-PRP antibody levels increased significantly after vaccination, compared to the respective concentrations found in prevaccination sera. The studies did not show results for nonresponders, and most volunteers had a fourfold-or-greater rise in antibody levels over their preinjection levels. The vaccine candidate was highly immunogenic for all groups, and the immune response was consistent between the vaccine lots.

In study 1, the IgGs obtained from groups A, B, and D displayed similar behavior on average. For group C (control vaccine group), the geometric mean for the IgG anti-PRP was lower; however, there were no differences between the results for the evaluated groups ($P > 0.05$). An important postvacci-

TABLE 2. IgG anti-PRP antibody responses in adult volunteers[a]

Study	Vaccine group	Geometric mean of antibody concn (μg/ml)[b]		No. of volunteers with a ≥4-fold rise in seroconversion[c]
		Before vaccination (IgG [range])	1 mo after vaccination (IgG [range])	
1	A	4.38 (1.00–21.43)	84.64 (21.21–1009.7)	10/10
	B	3.91 (1.21–18.33)	82.75 (5.54–1009.7)	8/10
	C	2.95 (0.72–49.13)	54.95 (7.82–1644.5)	9/10
	D	5.53 (1.00–295.88)	189.97 (13.37–1575.7)	9/10
2	E	7.03 (2.69–39.89)	159.17 (33.86–1475.1)	9/10
	F	4.66 (1.16–12.07)	134.29 (12.19–941.33)	10/10
	G	5.47 (0.98–21.98)	101.49 (14.21–1239.1)	9/10
	H	3.86 (0.90–22.90)	56.83 (7.83–163.91)	9/10

[a] Each group consisted of 10 human adult volunteers. Groups C and E received the control vaccine Vaxem-Hib. All other groups received the vaccine under study.
[b] There is no significant difference between the IgG geometric mean antibody concentrations for the groups ($P > 0.05$).
[c] There is no significant difference between the groups regarding the numbers of volunteers responding with a ≥4-fold rise ($P > 0.05$).

nation increase in IgG anti-PRP titers was also obtained in the second study. Aluminum phosphate did not enhance the antibody response against the conjugate under study, as the groups receiving the investigational vaccine with aluminum phosphate showed the lowest geometric means (group H, 56.8 μg/ml; and group G, 101.4 μg/ml). As expected from this phase I study, significant differences were obtained only between pre- and postvaccination sera for each group. In both studies, the different types of vaccines elicited IgG anti-PRP antibody concentrations four times higher (seroconversion) than the corresponding prevaccination concentrations for most vaccines. The seroconversion percentages fluctuated between 90 and 100% with only one exception (group B, 8/10). The difference depended on, among other factors, prevaccination titer, which was as high as 280 μg/ml ($P > 0.05$) in some cases.

Immunoglobulin class composition. The IgA and IgM anti-PRP antibody responses from study 1 are shown in Table 3. Preimmunization levels of IgA and IgM were similar in the four vaccine groups. The different vaccines elicited increases in all three immunoglobulin classes; the highest levels and greatest increase (*n*-fold) were observed for the IgG isotype, with more moderate increases in IgM and IgA levels. There were no differences in postimmunization levels for the three immunoglobulin classes ($P > 0.05$).

TABLE 3. Immunoglobulin class composition for *Haemophilus influenzae* type b capsular polysaccharide antibodies elicited by vaccination in study 1[a]

Vaccine group	Stage of treatment	Geometric mean antibody concn (μg/ml)		
		IgM (range)[b]	IgA (range)[c]	IgG[d]
A	Preimmunization	0.31 (0–0.63)	1.29 (0–6.51)	4.38
	Postimmunization	6.83 (0–16.90)	6.31 (0–15.28)	84.64
B	Preimmunization	0.11 (0–0.63)	0.98 (0–6.51)	3.91
	Postimmunization	3.31 (0–7.70)	7.42 (0–18.48)	82.75
C	Preimmunization	0.24 (0–1.06)	0.23 (0–0.85)	2.95
	Postimmunization	3.92 (0–10.86)	5.38 (0–17.43)	54.95
D	Preimmunization	0.29 (0–2.27)	2.10 (0–19.11)	5.53
	Postimmunization	6.99 (0–26.79)	8.29 (1.09–25.32)	189.97

[a] Group C received the control vaccine Vaxem-Hib. All other groups received the vaccine under study.
[b] Number of volunteers responding with a ≥4-fold rise in seroconversion for each group: A, 8/10; B, 4/10; C, 4/10; D, 3/10.
[c] Number of volunteers responding with a ≥4-fold rise in seroconversion for each group: A, 3/10; B, 5/10; C, 4/10; D, 4/10.
[d] Number of volunteers responding with a ≥4-fold rise in seroconversion for each group: A, 10/10; B, 8/10; C, 9/10; D, 9/10. Ranges for geometric mean antibody concentration are shown in Table 2.

TABLE 4. Specificities, avidity indexes, and bactericidal activities in vitro of anti-PRP antibodies from sera in study 1[a]

Vaccine group	Serum bactericidal activity		Relative avidity		Specificity ($PRP_{50\%}$)
	Log_2 SBA	Range	Log AI	Range	
A	4.02	2.0–7.0	2.06	1.57–2.68	1.06E−04
B	5.20	3.0–9.0	2.21	1.68–3.04	8.69E−05
C	3.84	2.0–10.0	2.08	1.77–2.60	1.17E−04
D	4.42	2.0–10.0	2.10	1.38–2.68	1.02E−04

[a] Group C received the control vaccine Vaxem-Hib. All other groups received the vaccine under study. There is no significant difference between the values for the groups ($P > 0.05$). $PRP_{50\%}$, geometric mean of the PRP concentration inhibiting 50% of the ELISA.

Specificities of the antibodies. The coating antigen (HbOHA) recommended for the detection and quantification of anti-PRP antibodies is composed of oligosaccharide fragments obtained from Hib capsular polysaccharide by periodic oxidation and conjugated to human serum albumin by reductive amination. Its source is the native polysaccharide, and the modification and conjugation procedures have nothing in common with the method employed for the conjugation of the synthetic antigen. However, the ultimate proof of specificity requires the use of the native capsular polysaccharide. Table 4 shows the results obtained after inhibiting the sera of vaccines with the Hib natural capsular polysaccharide, followed by their reaction with HbOHA as a coating antigen.

Relative avidities of antibodies. The evaluation of antibody avidity provided a measure of strength for antigen-antibody interaction. Relative avidity was measured for every single serum in the first trials. As can be seen in Table 4, the average avidities for the study (A, B, and D) and control (C) groups were quite similar. The analysis showed no difference between them ($P > 0.05$).

Bactericidal activity. As can be seen in Table 4, the sera of the subjects immunized with Quimi-Hib or with the control vaccine displayed bactericidal activity. An increase in the bactericidal activities of the sera was reached after the administration of a single vaccine dose for each group of volunteers.

DISCUSSION

During the last 15 years, conjugate vaccines have become an excellent tool for the prevention of bacterial infections due to *H. influenzae* type b and are therefore recommended for use in the vaccination schedule for every child (15, 22, 23). However, the global introduction of Hib vaccines has uncovered several obstacles, including the high complexity of vaccine production. The sequence for the production of most conjugate vaccines consists of three main steps: (i) bacteria cultivation followed by extraction/purification of the capsular polysaccharide, (ii) chemical modification of the polysaccharide, and (iii) conjugation to a suitable protein carrier. The first two steps could be accomplished at least potentially by synthetic chemistry. In-

deed, several attempts have demonstrated its feasibility for Hib on a laboratory scale, but the respective products have been developed only up to the stage of testing in laboratory animals (2, 13, 14) due to the complexity associated with the synthesis.

We developed a new, improved procedure for the synthesis of *H. influenzae* type b oligosaccharide fragments, leading to additional possibilities for vaccine production (24). The synthetic antigen conjugated to tetanus toxoid was further investigated as a vaccine candidate against Hib. The initial pharmaceutical presentation that was available for testing with humans consisted of the phosphate buffer solution of the conjugate without any additive. This was tested in the first clinical trial described herein. A second pharmaceutical presentation was later developed. It consists of two separated vials, each with a one-half volume of material. One vial contained a phosphate buffer solution of the conjugate and the other one a phosphate buffer solution of the aluminum phosphate adjuvant. The contents of both vials were mixed just before use. This latter presentation was the one evaluated in the second trial.

In the first trial, a total of 30 of the 40 adult volunteers enrolled received for the first time the investigational vaccine containing the synthetic Hib antigen. Therefore, all participants were admitted to the hospital special unit during the first 72 h after injection. They were also submitted to a very rigorous follow-up including hematological, renal, and hepatic parameters. As a result, we observed an excellent safety profile, very similar to that observed for the control vaccine.

The safety profile for the pharmaceutical formulation containing aluminum phosphate was assessed in the second clinical trial. As can be seen in Table 1, a very good safety profile was observed for all groups included in the study. No significant differences were observed between the two formulations for the group receiving the investigational vaccines and the control group.

Although the adults' immune responses to the conjugate vaccine could not be extrapolated to infants, the characterization of the immune responses in both studies was an essential initial step before further vaccine testing in children and infants was allowed.

In most volunteers, antibodies to the capsular polysaccharide of Hib are regularly present in the serum before vaccination (10, 21). They are produced as a response to nasopharyngeal colonization by Hib. It is not clear to what extent cross-reacting bacteria, such as *Escherichia coli* K100, account for this natural immunization (12, 20).

The investigational and control vaccines elicited an increase in IgG anti-PRP over preinjection levels in a high number of volunteers, as shown in Table 2. Similar responses in adults injected with other conjugate vaccines have been reported (6, 9, 11, 19). A single dose of a synthetic conjugated polysaccharide vaccine against Hib also induces increases in anti-PRP IgM and IgA antibodies. The increment is more pronounced for IgG, as expected for a typical secondary immune response (Table 3).

The present study is the first evaluation of a synthetic conjugate vaccine against Hib in humans. The control vaccine used in both trials is composed of conjugated PRP oligosaccharides with similar structures but obtained from the native polysaccharide. Therefore, it was interesting to characterize the IgG anti-PRP antibodies in more detail and to compare them with the antibodies elicited by the control vaccine. Generally, the immune responses elicited by the vaccines had the same behavior regardless of the presence of the synthetic-oligosaccharide moiety attached to the protein carrier.

In a series of experiments with natural capsular Hib polysaccharide used as an inhibitor, we demonstrated the specificities of the antibodies elicited by the vaccine under study. The sera obtained from all groups were very specific, as shown in the inhibition index observed (Table 4).

Avidity is more likely than intrinsic affinity to have biologic relevance in understanding the protective capacity of antibody (5). Therefore, in the present study, we investigated the avidities of the antibodies elicited in adults after vaccination with the Quimi-Hib vaccine. An important finding was that both Quimi-Hib and the control vaccine elicited serum anti-PRP antibodies of similar average avidities.

Finally, the functional capacity of antibodies in vitro was assessed by using a bactericidal assay. Significant increases in dilutions of serum-killing Hib bacteria were observed in all groups, with no differences between them.

The excellent safety profile and the preliminary antibody responses in the adults observed during these studies were determinant factors in encouraging further clinical evaluations in children and infants (24).

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