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Phase I clinical trial of *O*-Acetylated pectin conjugate, a plant polysaccharide based typhoid vaccine

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Abstract

Background—Typhoid fever remains an important cause of morbidity and mortality in the developing countries. Vi capsular polysaccharide conjugate vaccine demonstrated safety and efficacy in young children in high endemic regions. A novel typhoid conjugate vaccine based on plant polysaccharide pectin was studied in a phase I trial.

Methods—Fruit pectin, having the same carbohydrate backbone structure as Vi, was purified from citrus peel and used as the polysaccharide source to prepare a semi-synthetic typhoid conjugate vaccine. Pectin was chemically *O*-acetylated (*O*AcPec) to antigenically resemble Vi and conjugated to carrier protein rEPA, a recombinant exoprotein A from *Pseudomonas aeruginosa*. 25 healthy volunteers, 18–45 years old, were injected once with *O*AcPec-rEPA. Safety and IgG antibodies reactive with Vi and pectin were analyzed.

Results—No vaccine associated serious adverse reaction was reported. Six weeks after the injection of *O*AcPec-rEPA, 64% of the volunteers elicited >4 fold rise of anti-Vi IgG. At 26 weeks the level declined, but the difference between the levels at 6 and 26 weeks are not statistically significant. There is a direct correlation between the level of anti-Vi IgG before and after the injection ($R^2 = 0.96$). The anti-Vi IgG can be absorbed by Vi, but not by pectin. There was no corresponding increase of anti-pectin after the injection, indicating the antibody response to *O*AcPec-rEPA was specific to Vi. There is no Vi-rEPA data in US adults for comparison of immune responses. The *O*AcPec-rEPA elicited significantly less IgG anti-Vi in US adults than those by Vi-rEPA in Vietnamese adults.

Conclusion—The *O*-acetylated pectin conjugate, a plant based typhoid vaccine, is safe and immunogenic in adult volunteers.

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Disclosure

The authors have no conflict of interests or financial obligations to disclosure.

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Keywords

phase I pectin-based typhoid conjugate vaccine safety immunogenicity plant polysaccharide semi-synthetic

Introduction

Typhoid fever remains a common, serious and increasingly difficult to treat disease in developing countries [1, 2]. Control measures, such as safe drinking water, health screening of food handlers, and effective sewage, are not likely to be available soon in many of these countries [3, 4]. Starting in the early 1990's most *Salmonella enteric* subsp *serovar* Typhi (*S. Typhi*) from the Mekong Delta region were multiple drug resistant to first line of antibiotics such as chloramphenicol, ampicillin and treatment required newer antibiotics such as ciprofloxacin [4, 5, 6].

Vi capsular polysaccharide is both an essential virulence factor and protective antigen of *S. Typhi*. Vi vaccine licensed in more than 95 countries confers about 70% immunity in individuals >5 years of age for at least 3 years [7–9]. Its immunogenicity is lower in 2–4 years old children [10]. Vi does not elicit a booster response at any age (age-related, T-cell independent antibody response) [11]. The immunogenicity of Vi was improved by conjugation to the carrier protein, the recombinant protein A of *Pseudomonas aeruginosa* (rEPA). Vi conjugates were safe and more immunogenic than Vi in adults, in 5–14 year-olds for at least 8 year [12]. In children 2–4 year-olds, Vi-rEPA demonstrated 89% protective efficacy for 46 months [13]. Further study in infants showed Vi conjugate is safe, immunogenic and compatible with routine immunizations [14, 15].

Vi is a linear homopolymer of $\alpha(1\rightarrow4)$ -D-Gal NAcP and variably *O*-acetylated at C-3. The *N*- and *O*-acetyls dominate the surface structure and are essential for both antigenicity and immunogenicity of Vi [15–18]. However there were several technical challenges in manufacturing Vi conjugate in industrial production; for example the extremely large molecular weight of Vi made sterile filtration difficult and its chemical structure prevents it from conventional colorimetric carbohydrate analysis [16–18].

In an attempt to solve these problems we investigated a structurally similar but immunologically unrelated plant polysaccharide pectin as a replacement raw material for Vi polysaccharide. Pectin purified from fruit or plants has similar structure to Vi poly- $\alpha(1\rightarrow4)$ D-GalP [19]. Previously we demonstrated that pectin purified from citrus commonly used as food additives can be *O*-acetylated with acetic anhydride to an average of 70% acetylation at both C2 and C3 [17, 20, 21]. The *O*-acetylated pectin (OAcPec) is antigenically indistinguishable from Vi. However due to its small size, it is not immunogenic without conjugation to a carrier protein. Mice or guinea pigs injected with OAcPec conjugate elicited Vi antibodies with booster responses albeit at slightly lower levels than Vi conjugates [20, 21].

Here we evaluate the safety and immunogenicity of OAcPec conjugated to rEPA in healthy US adults.

Material and Methods

Vaccine

OAcPec-rEPA conjugate was prepared at the National Institutes of Health, Bethesda, Maryland as described [17, 20, 21]. Briefly pectin (LM-1912CSZ, Genu Pectin, Copenhagen, Denmark) was *O*-acetylated in acetic anhydride and designated as *OAcPec* [20, 21]. The degree of *O*-acetylation was determined by Hestrin reaction using the acetylcholine as the reference standard [22]. Level of endotoxin was measured by Limulus amoebocyte lysate (LAL) assay expressed in international endotoxin units.

The carrier protein rEPA was prepared at NIH following GMP guidelines [23, 24]. *OAcPec* conjugate was synthesized by carbodiimide-mediated condensation with adipic acid dihydrazide as the linker as described and designated as *OAcPec-rEPA* [21]. The formulation of *OAcPec-rEPA* used in this study was: 25 µg polysaccharide/dose in 0.5 ml of 0.2 M NaCl, 10mM sodium phosphate buffer (pH 7.2) and 0.01% thimerosal as preservative [25, 26]. The 25 µg dose is based on our experience with Vi-rEPA conjugate [12–14]. Each dose of *OAcPec-rEPA* contained 29 µg of rEPA. The vaccine was bottled by the Pharmaceutical Development Section, Clinical Center, NIH and passed safety requirements according to the code of Federal Regular Regulation, Food and Drug Administration as an Investigational New Drug (BB IND 6989).

Recruitment

The clinical study was conducted at the Clinical Center, National Institutes of Health (NIH) and approved by the Internal Review Board, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), NIH ([ClinicalTrial.gov](https://clinicaltrials.gov/ct2/show/study/NCT00277147) identifier: NCT00277147, NIH IRB protocol number OH06-CH-0070). Volunteers were recruited through the NIH Normal Volunteers Office by advertisement. Healthy adults 18 to 45 years old were recruited. Excluded were those: participating in or planning to participate in another clinical protocol during the following six months, were vaccinated against typhoid fever or had typhoid fever within the last 5 years, were regularly taking a prescription drug for chronic medical condition, had a history of allergy to citrus fruit or fruit pectin, or were pregnant or intend to become pregnant during the study period of 6 months. A consent form was read and signed by the volunteer, blood drawn for HIV, hepatitis B (HBsAg), C (HCV) tests and complete blood count, blood chemistry, and liver function performed. HIV, HBsAg, HCV and pregnancy test positives were excluded.

Vaccination

On the day of vaccination, the volunteers were asked about their recent health. No volunteer with a history of an upper respiratory infection, diarrhea (3 or more watery bowel movements per day) or fever during the preceding 3 days or who was regularly receiving medication, or with a positive pregnancy test were injected.

The volunteers were injected intramuscularly (IM) once with 0.5 ml of *OAcPec-rEPA* containing 25 µg of polysaccharide. Volunteers were examined 30 min after injections for acute hypersensitivity reactions. Each volunteer took his/her own temperature and inspected

the injection site at 6, 24, 48, 72 h and 6, 26 weeks after vaccination and measured any redness and/or swelling in 2 directions at right angles to each other according to instructions provided. Participants mailed the reaction diary forms to the Clinical Center, NIH. If fever of $>38.5^{\circ}\text{C}$ or erythema of $>2.5\text{ cm}$ or indurations of $>5\text{ cm}$ developed, participants were required to come to the clinical center and be examined by a doctor who would continue to monitor the volunteer for additional 48 h.

The study was conducted in two successive parts. Five volunteers were injected initially to evaluate the adverse reactions. If no case of fever 102.2°F (39.0°C) or a local reaction 5 cm of redness (erythema) or 2.5 cm of swelling (indurations) attributable to the vaccination within 48 h of injection, the remainder of volunteers were vaccinated subsequently.

Anti-Vi IgG serological assays

The level of serum anti-Vi IgG was measured by ELISA using Vi as the coating antigen ($0.2\mu\text{g}/\text{well}$ in PBS, pH 7.2) [12]. The ELISA unit (EU) was converted to the weight unit $\mu\text{g}/\text{ml}$ based on a human reference standard [13, 27]. Since there is no Vi-rEPA data available in US adults for comparison, levels of anti-Vi IgG were compared with the geometric mean (GM) of the antibody response in the Vi-rEPA conjugate efficacy trial in Vietnam, and $4.3\mu\text{g}/\text{ml}$ (3.5 EU) was assumed to be a conservative estimated protective level against typhoid fever [12, 13, 15, 28].

IgG antibody to pectin in volunteers before and after injection was assayed with pectin coated ELISA plates ($2.0\mu\text{g}/\text{well}$ in PBS, pH 7.2). The anti-pectin IgG human reference was a high level serum and assigned 5 EU-pectin (to distinguish from ELISA units for Vi antibodies) with initial dilution at 1:20.

To examine the specificity of the observed antibodies, 6 randomly selected post injection sera were absorbed by Vi or pectin polysaccharide. Briefly, equal volume of serum ($100\mu\text{l}$) and polysaccharide solution ($50\mu\text{g}/\text{ml}$ in saline) were mixed and incubated at 37°C for 1 hr, 4°C overnight, and the precipitate was spun down at $2,800\times g$ (Sorvall Legend RT, 3,750 rpm). The supernatant was again absorbed with equal volume of $50\mu\text{g}/\text{ml}$ polysaccharide solution and the supernatant analyzed for residual antibodies (vide supra). Controls were sera absorbed with saline and processed in parallel with samples.

Statistical analysis

Serum antibody levels were expressed individually or as GM. Comparisons were performed with the Fisher's exact unpaired or paired t-test when appropriate. Correlation coefficients R were calculated by Pearson product-moment correlation.

Results

Vaccination

The investigation vaccine *OAcPec-rEPA* conjugate had <0.1 endotoxin unit/ μg , passed pyrogenicity test and safety requirements according to the code of Federal Regular Regulations, Food and Drug Administration as Investigational New Drug.

Twenty five volunteers enrolled in the study, 14 females and 11 males, median age 29.0 yr. Two participants did not complete a 26-week blood drawn due to moving out of the area. There was no fever, swelling or redness at the site of injection or other vaccine related serious reactions. Two volunteers had moderate pain and mild general muscle ache 6 h after injection that subsided in 24 h. Another one had mild malaise 24 h after injection that subsided by 48 h. None of the side reactions required treatment or hospitalization.

Anti-Vi IgG response

There is no sex or age dependence in the serum anti-Vi IgG response before, 6 and 26 weeks after one injection of *OAcPec-rEPA* (male vs female, $P>0.2$; correlation with age $R^2<0.3$). Volunteers had low pre-immune levels of anti-Vi IgG compared with Vietnamese adults in the high endemic regions (GM 0.87 μ g/ml vs 11.9 μ g/ml, $P<0.0001$) and all except 2 had lower than the estimated protective level (4.3 μ g/ml) (Table 1) [13, 15]. Six weeks after the injection, the GM anti-Vi IgG had an 8 fold rise (7.11 vs 0.87, $P=0.045$). The GM declined to 2.94 μ g/ml at 26 weeks (2.94 vs 0.87, $P=0.09$). The difference between antibody levels at 6 and 26 weeks are not statistically significant (7.11 vs 2.94, $P=0.16$). At 6 weeks, 60% of the volunteers and at 26 weeks 39% had greater than 4-fold rise compared with the pre-immune levels. There is a strong correlation between the pre- and post-immune responses at 6 or 26 weeks, $R^2>0.96$. In particular, the 2 individuals who had high pre-immune levels, both responded with robust rises after immunization: from pre-immune level of 6.54 μ g/ml to 120.0 μ g/ml and from 19.51 μ g/ml to 474.3 μ g/ml respectively at 6 weeks interval. There was no change in the levels of anti-Vi IgG after absorption with saline or pectin and approximately $52\pm9\%$ reduction by Vi polysaccharide.

In addition to the 4-fold rise marker, the anti-Vi IgG levels were compared with the estimated protective level of 4.3 μ g/ml, a conservative value derived from our phase III clinical trial of Vi-rEPA in Vietnamese toddlers. There were 2 (8%), 16 (64%) and 6 (24%) volunteers had >4.3 μ g/ml before, 6 weeks and 26 weeks after the injection respectively.

Anti-pectin IgG

All volunteers, including the 2 individuals who had high levels of pre-immune Vi antibodies, had low but detectable levels of pre-immune anti-pectin IgG. The levels remain similar 6 or 26 weeks after one injection of *OAcPec-rEPA*; the difference between pre- and post- injection is not statistically significant (1.52 EU-pectin vs 1.66 EU-pectin, $P>0.5$). Absorption of 6 serum samples with Vi polysaccharide did not reduce the levels of the pectin antibodies. There was no correlation between the level of post immune Vi antibodies with those of pectin antibodies ($R^2<0.15$).

Discussion

In this proof of principle clinical study, a novel typhoid conjugate vaccine based on the plant polysaccharide pectin showed to be safe and induced anti-Vi IgG in healthy adults. The anti-Vi IgG elicited can be absorbed by Vi, but not by pectin [20, 21]. There is no corresponding increase of pectin antibodies after the injection of *OAcPec-rEPA*, indicating the polysaccharide antibody response is specific to Vi. A direct correlation between the Vi

antibody levels in pre-and post-injection sera was observed ($R^2=0.96$). There is no Vi-rEPA data available in US populations for comparison. The GM level of anti-Vi IgG elicited by *O*AcPec-rEPA in US adults was lower than that by Vi-rEPA in Vietnamese adults [12, 15, 28]. The difference in the pre-immune levels between the two populations might be a factor to the observed discrepancy in the immune response.

There are advantages of using a plant-derived polysaccharide as the carbohydrate source for typhoid conjugate vaccine. It does not require fermentation of the pathogenic *S. Typhi*. Pectin is abundant, inexpensive, easy to purify and requires only a simple chemical modification to prepare its *O*-acetyl derivative [19, 20]. Endotoxicity is not a concern since there is no lipopolysaccharide co-purified as in the case of Vi. Its lower molecular weight (~400 kDa) than the native Vi (> 2,000 kDa) also helps to overcome obstacles during conjugation, such as: reduced viscosity, improved yield in production and full recovery after sterile filtration of the final conjugate. Since there are no *N*-acetyl groups in *O*AcPec to hinder the carbohydrate colorimetric reactions for uronic acids, the sugar concentration in the final formulation can be easily determined [25, 26].

Because pectin is a common food additive in our diet, there is a concern that the *O*AcPec-rEPA vaccine may react with the background pectin antibody in humans or induce antibodies that cross react with pectin. We examined these possibilities and found that immunization with *O*AcPec-rEPA did not cause interference with the existing (however low) nor induce additional pectin antibodies. This is probably because by methodical addition of the bulky *O* acetyl group to pectin has transformed the specificity of the antigen epitopes [16].

There are deficiencies and limitations of the current study. First, one of the fundamental questions of Vi immunogenicity is the impact of the acetyl levels (combining both *N*- and *O*-acetyls). One report indicated that in mice the antibody response is highly related, but not proportional, to the extent of *O*-acetyl content of Vi [15]. Here only one level of *O*-acetyl content that is similar to the licensed Vi was tested. More thorough study of this important factor is needed for future improvement of *O*AcPec conjugate. Second, it is known that the *O*-acetyl groups are comparatively less stable than the *N*-acetyl group, such as in the case of native Vi. We have verified that during the 2.5 years study, there was no indication of decomposition in *O*AcPec-rEPA (unpublished data). However, ideally a pectin based synthetic Vi would consist of *N*-acetylated pectin at C-2 if technically feasible.

Due to the difficulties of recruitment, this study did not exclude volunteers had pre-existing antibodies. Under the limitation of resources the current study did not investigate the effect of booster injection and did not include a separate comparator group receiving Vi polysaccharide vaccine [7, 28]. Based on our past experience with other conjugates, usually there was no booster response in adults [29, 30]. However, we noticed that there is a direct correlation between the pre- and post-injection antibody levels and also the fact that most volunteers in this study had significantly lower background antibody levels than those in the endemic areas. Thus the injection could be treated as a primary immunization and a subsequent injection might induce a booster response.

Vaccine antigen sources other than pathogens have been explored extensively in the past 2 decades. Protein antigens derived from transgenic plants have been investigated as oral vaccine candidates or for protein antigen productions [31–34]. Carbohydrate antigens, in contrast, are more difficult to produce by genetic engineering in transgenic plants or by recombinant bioconjugates and, in term, are more commonly produced by chemical synthesis [35–37]. One synthetic carbohydrate vaccine *Haemophilus influenza* type b conjugate licensed in Cuba showed preliminary success and is still pending more clinical demonstration to prove its non-inferiority when compared with those purified from the organisms [36]. Here pectin *O*-acetylation offers another alternative by chemically modifying a plant product and by avoiding genetic manipulation or laborious chemical synthesis.

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Highlights

Pectin based vaccine
typhoid conjugate vaccine
clinical study
Vi capsular antibody
Serum IgG
Plant vaccine source

Table 1
Serum anti-Vi IgG and anti-pectin IgG in volunteers before and after immunized with *O*AcPec-rEPA conjugate

	Anti-Vi IgG ($\mu\text{g/ml}^*$)			Anti-Pectin IgG (Pectin ELISA units)	
	Pre-injection	6 weeks Post injection	26 weeks ⁺ Post injection	Pre-injection	6 weeks post injection
N=25					
GM (25–75) %	0.87 (0.31–1.87)	7.11 (1.70–10.56)	2.94 (1.14–4.03)	1.52 (0.63–4.98)	1.66 (0.69–4.82)
>4.3 $\mu\text{g/ml}^{\wedge}$ (%)	2 (8)	16 (64)	6 ⁺ (24)	NA	NA

* GM of anti-Vi IgG in adult volunteers received 1 injection of *O*AcPec-rEPA,

⁺ N=23, 2 volunteers lost to follow

[^] 4.3 $\mu\text{g/ml}$ estimated protective level (Ref. 15)

For anti-Vi IgG, 7.11 vs 0.87, $P=0.045$; 2.94 vs 0.87, $P=0.09$;

For anti-Vi IgG, correlation of pre- and 4 or 26 weeks post, $R^2>0.96$;

For anti-pectin IgG, 1.66 vs 1.52, $P=0.25$; correlation of pre and 6 wk post, $R^2=0.86$