Safety and Immunogenicity in Humans of an Attenuated Salmonella typhi Vaccine Vector Strain Expressing Plasmid-Encoded Hepatitis B Antigens Stabilized by the Asd-Balanced Lethal Vector System

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Attenuated Salmonella typhi organisms which express genes encoding protective antigens of other pathogens have been developed for use as experimental oral vaccines. A Δasd S. typhi strain attenuated by deletions in cya, crp, and cdt which contains hepatitis B core (HBc) and pre-S genes encoded on an AsdB-1 pBR-based plasmid vector was constructed. Healthy adult volunteers ingested a single dose of 5 × 103 to 5 × 108 CFU of strain χ4073 (Δcya Δcrp Δcdt S. typhi Ty2), 6 × 107 or 1 × 108 CFU of strain χ4632(pYA3149), a further derivative of χ4073 deleted in asd and containing the Asd+ vector without the HBc–pre-S fusion, or 3 × 107 or 7 × 108 CFU of strain χ4632(pYA3167), a derivative containing the vector with the HBc–pre-S fusion. χ4073 was generally well tolerated by 22 volunteers. No volunteer had fever or positive blood cultures; 4 of 22 volunteers shed vaccine organisms in the stool in the first 48 h only. Two of 18 volunteers who received one of the plasmid-containing derivatives of χ4073 developed low-grade fevers on day 10 or 12 after ingestion. One of these volunteers had positive blood cultures on days 7 and 8. Seven of these 18 volunteers had vaccine organisms detected in their stools in the first 48 h only. Most volunteers developed S. typhi-specific serum responses and developed S. typhi-specific antibody-secreting cells. However, no volunteer developed serum antibody to hepatitis pre-S or pre-S-specific antibody-secreting cells. Although the parent strain χ4073 was well tolerated, induced immunoglobulin G seroconversion to S. typhi lipopolysaccharide in 80 to 100% of vaccinees and stimulated specific IgA-secreting lymphocytes in 80 to 100% of vaccinees given a single oral dose of 2 × 107 and 5 × 108 CFU, χ4073 derivatives containing the Asd+ vector with and without sequences encoding the HBc–pre-S fusion caused occasional febrile reactions at high doses and did not stimulate detectable immune responses to hepatitis B antigens.

Attenuated Salmonella typhi has been proposed as a useful vector to carry protective antigens of other pathogens to make hybrid vaccine strains. The potential advantage of an oral typhoid vaccine vector, compared to other vaccine vectors such as vaccinia virus, is that it is easily administered, efficacious after a single dose, and inexpensive would allow mass immunization against hepatitis B and therefore against its sequelae, including chronic hepatitis, cirrhosis, and hepatic carcinoma.

Deletion of adenylate cyclase and the cyclic AMP (cAMP) receptor protein genes (Δcya Δcrp) attenuates Salmonella enterica (4, 6, 13) and the human pathogen S. typhi (26). These proteins are necessary for the transcription of many genes and operons concerned with the transport and breakdown of carbohydrates (2). Although cAMP is present in mammalian cells, the concentrations present in gastrointestinal tissues and fluids and other cells in which S. typhi invade are below that necessary to allow cya mutants to exhibit a wild-type phenotype (1, 20, 25). Furthermore, inclusion of a crp mutation abolishes any benefit to the organism that could result from uptake of cAMP in vivo by cya mutants. In animals, avirulent, recombinant salmonellae elicit immune responses against HBV when given by the oral route (21–24). Viral neutralizing epitopes of the HBV pre-S envelope genes were fused to the hepatitis B core (HBc) gene, and the hybrid HBc–pre-S1–pre-S2 fusion proteins were constitutively expressed in a Δcya Δcrp Salmonella typhimurium strain (21, 24). The recombinant salmonellae were immunogenic when administered to mice by the oral or intraperitoneal route, with a single immunization resulting in moderate to high antibody titers against salmonellae and the recombinant proteins (23, 24).
The attenuation of a cya crp mutant of S. typhi, χ3927 (Δcya Δcrp Ty2), has been demonstrated in volunteers (26). However, when given a single dose of 5 × 10⁴ or 6 × 10⁵ CFU, 1 of 12 volunteers developed a fever of 40.1°C 22 days after vaccination and 1 of 6 volunteers at each dose developed vaccine bacteremia. From this initial study, it was clear that additional attenuation of this strain was needed for safety and that larger doses could be given to elicit more vigorous immune responses. Therefore, a further derivative of the Δcya Δcrp strain containing a deletion in the cdt gene, which is responsible for colonization of deep tissue, was constructed. In animals, deletion of this gene has no effect on the ability of S. typhimurium or Salmonella choleraesuis to colonize the intestinal tract but significantly diminishes the ability to reach and persist in deep organs and tissues when compared to the parent strains (13).

A new strain attenuated by cya crp cdt deletions which carries cloned genes from HBV on a plasmid was constructed in S. typhi. This strain utilizes the Asd-balanced–lacteh vector system to allow stable, high-level expression of a hybrid fusion hepatitis B antigen by S. typhi in vivo in the absence of any drug resistance genes (16). Salmonella strains deleted in asd grow only in the presence of diaminopimelic acid or asd-complementing plasmids. When the cells lose their plasmids, they lyse and liberate their antigenic contents. In the new strain, designated χ4632, an asd deletion mutation was introduced into a Δcya Δcrp Δcdt derivative of S. typhi Ty2; this strain, which was complemented with the Asd+ hybrid HBe-pre-S PBR-based vector, stably expressed high levels of the fusion protein at >1% of the total cellular protein (21).

In this study, we evaluated the following three mutant strains in volunteers: (i) strain χ4073, a Δcya Δcrp Δcdt derivative of S. typhi Ty2, (ii) strain χ4632(pYA3149), a further derivative containing the asd+ plasmid, and (iii) strain χ4632(pYA3167), containing the asd+ plasmid carrying a gene for the HBC and fused pre-S1 and pre-S2 epitopes. The parent Δcya Δcrp Δcdt mutant, χ4073, was included to assess the safety and immuno-genicity of the background strain at the same doses to be used for the recombinant strains. The strain carrying the asd+ plasmid was included to assess the effect of the balanced lethal vector system and the plasmid itself on the attenuation of the parent strain.

MATERIALS AND METHODS

Clinical methods. In the first study, a group of 12 healthy adult volunteers were recruited and screened to determine their good medical and psychological health, the protocol was explained and informed consent was obtained. After admission to the isolation ward of the Center for Vaccine Development located in the University of Maryland Hospital, these volunteers were randomized to receive a single oral dose of either 5 × 10⁴ or 5 × 10⁵ CFU of strain χ4073 with sodium bicarbonate buffer.

In the second study, a group of 28 healthy adult volunteers were admitted to the isolation ward. Following appropriate screening to document medical and psychological status, the volunteers were enrolled into the study and randomly assigned to receive a single oral dose of either 3 × 10⁴ or 3 × 10⁵ CFU of strain χ4632(pYA3167), or 3 × 10⁴ or 3 × 10⁵ CFU of strain χ4632(pYA3149), or 3 × 10⁴ or 3 × 10⁵ CFU of strain χ4073.

Preparation of vaccine inocula. Stock cultures of the Salmonella candidate vaccine strains were stored as a cell suspension in 1% peptone–5% glycerol at −70°C until needed. To make an inoculum, this suspension was thawed and inoculated into 5 ml of Luria broth in a 15-ml screw-capped test tube and incubated at 37°C as a standing overnight culture (approximately 14 to 18 h). On the day of challenge, a 1:20 dilution was made in prewarmed Luria broth and the culture was aerated until the late log phase of growth was reached. Dilutions were made directly from this culture into phosphate-buffered saline (PBS) and standardized turbidimetrically to achieve the approximate concentration of Salmonella required.

Inoculation of volunteers. The vaccines were administered by the oral route with NaHCO₃. Two grams of NaHCO₃ was dissolved in 5 oz of distilled water. Volunteers, fasting for 90 min, drank 4 oz of the bicarbonate-water mixture, and then 10 ml of the vaccine was ingested. Volunteers ingested the vaccine suspended in the remaining 1 oz of bicarbonate water. Volunteers took no food or water for 90 min after inoculation.

Bacteriology methods. Stools, rectal swabs, and the distal 15 cm of bile-stained duodenal fluid were collected from every volunteer. Stool and duodenal fluid were cultured onto Trypticase soy agar (BBL, Cockeysville, Md.) and plated directly on Salmonella-Shigella agar (Difco, Detroit, Mich.). Over night incubation at 37°C, subcultures from GN broth were made onto Salmonel-la-Shigella agar. Suspicious colonies were transferred to triple sugar iron slants, and confirmation was made by agglutination with S. typhi Vi, O, and H antisera. Blood cultures (7 ml) were inoculated into 50-mL Septachek bottles.

Blood and stool isolates recovered from volunteers who received χ4632(pYA3167) were examined by Western blot (immunoblot) for expression of the hybrid gene.

Immunology methods. Serum and jejunal fluid specimens were tested for IgA, IgM, and IgG antibodies to S. typhi O and H antigens measured by enzyme-linked immunosorbent assay (ELISA) (15). For IgG lipopolysaccharide (LPS) antibody responses, sera were diluted to 1:100; a change in optical density from pre- to postvaccination specimens of at least 0.2 was considered seroconversion. For other Salmonella antibody measurements, a fourfold rise in titer was considered seroconversion.

Serum antibodies against HBV pre-S1 were determined by ELISA with a modification of previously published protocols (21). A recombinant pre-S protein (7) was used as the solid-phase reagent, and nonspecific binding sites on the plastic were saturated with a blocking buffer containing 1% horse serum (Sigma), 0.1% bovine serum albumin (Sigma), and 0.1% Tween 20 (Sigma) or, alternatively, with 5% Tween 10 (Sigma) in PBS as the blocking buffer. Binding IgG in a 1:100 serum sample dilution in blocking buffer was detected by horseradish peroxidase (Ortho) measuring the color change of o-phenylenediamine as a substrate at 492 nm. Alternatively, undiluted human serum samples were incubated on pre-S protein as a solid-phase reagent and bound immunoglobulins were detected by use of a directly horseradish peroxi-dase-labeled recombinant pre-S protein (18) as the probe. In addition, serum samples were screened for the presence of anti-pre-S1 antibodies in a competition binding assay with a pre-S1-specific monoclonal antibody (9). Intestinal fluid specimens were assayed for antibodies to hepatitis B pre-S antigen by ELISA (15).

Peripheral blood mononuclear cells were collected and separated for ASC assays for cells producing antibody to Salmonella and hepatitis B antigens. Traficking lymphocytes that secrete IgA antibody against S. typhi O or H antigen, or pre-S1/S2 antigen were quantified. Anti-pre-S ASCs were measured as described previously for other antigens (8, 28). Immulon II plates were coated with 0.5 μg of antigen per ml; the coating concentration was determined by checkerboard titration with mouse monoclonal IgA antibody. This monoclonal antibody was used as the positive control in the assay. ASCs were reported as the number per 10⁶ peripheral blood mononuclear cells. A rise in specific ASC number was defined as an increase over the mean number of ASCs in the prevaccination serum plus 2 standard deviations.

RESULTS

Clinical and microbiological results. In the first study, strain χ4073 (Δcya Δcrp Δcdt S. typhi Ty2) was fed to 12 volunteers to a dose of either 5 × 10⁵ or 5 × 10⁶ CFU; the strain was...
generally well tolerated (Table 1). None of 12 volunteers developed fever or typhoid-like illness, although 1 volunteer who received the larger dose had diarrhea between days 3 and 16 intermittently, totaling 1.2 liters over a 2-week period; stool cultures were negative for the vaccine strain during the period of diarrhea. None of the volunteers had positive blood cultures, and only one volunteer shed the vaccine strain in his stool; this occurred on day 0 with 100 colonies per g of stool. None of 11 volunteers who had adequate duodenal fluid specimens had a positive culture.

In the second study, volunteers received higher doses of strain χ4073 (approximately $2 \times 10^7$ or $5 \times 10^7$ CFU) or comparable doses of one of the plasmid-containing derivatives. χ4073 was weakly tolerated by all 10 volunteers who received $10^7$ to $10^8$ CFU. One volunteer who received χ4632(pYA3149) and one who received χ4632(pYA3167) developed fever; one had fever only on day 10, while the other had fever on days 12, 14, and 15 (Table 1). Severe headache occurred in five recipients of χ4632(pYA3149) and χ4632(pYA3167) in both the low and high-dose groups. Mild diarrhea occurred in three volunteers who received χ4632(pYA3149) or χ4632(pYA3167) (range of diarrheal stool volumes, 240 to 465 ml).

One volunteer who received χ4632(pYA3149) had positive blood cultures on days 7 and 8; on these days, he had maximum oral temperatures of 37.7°C and 38.0°C and severe headache, photophobia, and sore throat. Ten of the 28 volunteers who received $10^7$ to $10^9$ CFU of the three vaccine strains (Table 2). No volunteer achieved a sIgA response to S. typhi H or LPS antigen in jejunal fluid.

Finally, no volunteer who received χ4632(pYA3167) developed an immune response to hepatitis B pre-S antigen, measured either as circulating ASC IgA anti-pre-S or by serum or intestinal fluid antibody.

### DISCUSSION

The only natural hosts of S. typhi are humans. Preclinical evaluation of attenuated S. typhi relies heavily on murine models of Salmonella infections. However, the clinical experience with recombinant constructs such as those described here highlights the limitations of using animal models for assessing S. typhi strains. S. typhimurium expressing the HBC-pre-S1-pre-S2 fusion protein elicited immune responses to both Salmonella and HBV antigens after a single oral dose (21). S. typhi expressing the same HBV fusion protein elicited immune responses to both Salmonella and HBV antigens when mice were injected intraperitoneally with hog gastric mucin. However, a single oral dose of the same S. typhi recombinant construct led to no detectable immune responses to the expressed HBV antigens in humans.

Several strategies for attenuating S. typhi have been attempted, and the resultant strains have been tested in volunteers (10, 15, 26, 27). The Δcya Δtcp Δcdt mutant tested in this study, χ4073, was generally well tolerated over a range of doses from $10^7$ to $10^9$ CFU, was not detected in surveillance blood cultures, and was minimally shed in stools—all desirable characteristics. However, the magnitudes of the immune responses to χ4073 do not compare favorably with those observed after vaccinating volunteers with ΔaroC ΔaroD strains (14, 26, 27) or with phoP phoQ deletion mutants of S. typhi (10). For example, these strains, at similar doses, typically stimulated ASCs in the range of 100 to 1,000 per $10^6$ peripheral blood mononuclear cells (11, 14, 26), while the Δcya Δtcp Δcdt mutants in this study stimulated <100 ASCs per $10^6$ peripheral blood mononuclear cells.

Diarrhea is not frequently a part of the clinical syndrome

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### TABLE 1. Clinical and microbiological responses to vaccination with three attenuated S. typhi vaccine strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (CFU)</th>
<th>Fever of ≥38.2°C</th>
<th>Diarrhea</th>
<th>Severe headache</th>
<th>Positive blood culture</th>
<th>Positive stool culture</th>
<th>Geometric mean peak excretion (CFU/g)</th>
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<tr>
<td>χ4073</td>
<td>$5 \times 10^6$</td>
<td>0/5</td>
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<td>1/6</td>
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<td>ND</td>
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<td>χ4632(pYA3149)</td>
<td>$1 \times 10^6$</td>
<td>0/4</td>
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<tr>
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<td>$6 \times 10^7$</td>
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<td>3/5</td>
<td>0/5</td>
<td>3/5</td>
<td>$1.1 \times 10^5$</td>
</tr>
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</table>

* Maximum temperature, 38.7°C on day 10.
* Days 7 and 8.
* Maximum temperature, 38.7°C on day 12.
* ND, not detected.
TABLE 2. Immune responses to S. typhi and hepatitis B antigens after vaccination with one of three attenuated S. typhi strains

<table>
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<tr>
<th>Strain</th>
<th>Dose (CFU)</th>
<th>No. with jejunal fluid sIgA response rate to LPS, H, or HBc-pre-S</th>
<th>No. of volunteers with serum IgG response/No. of volunteers with IgA ASC response/Geometric mean peak no. of IgA ASC/10^6</th>
<th>Anti-LPS</th>
<th>Anti-H</th>
<th>Anti-HBc-pre-S</th>
<th>Anti-LPS</th>
<th>Anti-H</th>
<th>Anti-HBc-pre-S</th>
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<td>4632(pYA3149)</td>
<td>4.8 × 10^8</td>
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<td>0.5</td>
<td>6</td>
<td>15</td>
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<td>4632(pYA3167)</td>
<td>12.5 × 10^8</td>
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<td>6</td>
<td>15</td>
<td>9.5</td>
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**REFERENCES**


Gerlich, W. Unpublished method.


Peterson, D. Unpublished method.


