INFECTION AND IMMUNITY, Aug. 1997, p. 3381–3385 Vol. 65, No. 8
0019-9567/97/$04.00+0
Copyright © 1997, American Society for Microbiology

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

CAROL O. TACKET,[1]* SANDRA M. KELLY,[2] FLORIAN SCHÖDEL,[3]† GENEVIEVE LOSONSKY,[1] JAMES P. NATARO,[1] ROBERT EDELMAN,[1] MYRON M. LEVINE,[1] AND ROY CURTISS III[2]

Center for Vaccine Development, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland[1]; Department of Biology, Washington University, St. Louis, Missouri[2]; and Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.[3]

Received 17 December 1996/Returned for modification 5 March 1997/Accepted 28 April 1997

Attenuated *Salmonella typhi* organisms which express genes encoding protective antigens of other pathogens have been developed for use as experimental oral vaccines. A Δ*asd S. typhi* strain attenuated by deletions in *cya*, *crp*, and *cdt* which contains hepatitis B core (HBc) and pre-S genes encoded on an Asd$^+$ pBR-based plasmid vector was constructed. Healthy adult volunteers ingested a single dose of 5×10^5 to 5×10^8 CFU of strain χ4073 (Δ*cya* Δ*crp* Δ*cdt S. typhi* Ty2), 6×10^7 or 1×10^9 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 3×10^7 or 7×10^8 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 2×10^7 and 5×10^8 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 *S. typhi* replicates and potentially expresses the heterologous antigen in the intestine, thereby delivering the antigen to the mucosal immune system, where local and systemic humoral and cellular responses are vigorously stimulated. Several attenuated *S. typhi* oral vaccines have been designed to serve as carriers for heterologous antigens, including antigens of *Streptococcus mutans*, *Shigella sonnei*, *Escherichia coli*, *Mycobacterium leprae*, *Vibrio cholerae*, *Plasmodium falciparum*, *Francisella tularensis*, and *Schistosoma mansoni* (3, 5, 19).

Attenuated salmonellae can also be used to deliver protective antigens of hepatitis B virus (HBV). Although parenteral hepatitis B vaccines based on serum-derived or recombinant hepatitis B surface antigen are available, they are expensive and not widely available in the developing world. A vaccine that 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 (Δ*cya* Δ*crp*) 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 catabolites (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 Δ*cya* Δ*crp 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).

* Corresponding author. Mailing address: Center for Vaccine Development, 685 West Baltimore St., Baltimore, MD 21201. Phone: (410) 706-5328. Fax: (410) 706-4171. E-mail: ctacket@umppa1.ab.umd.edu.

† Present address: Merck Research Laboratories, Lyons, France.

The attenuation of a *cya crp* mutant of *S. typhi*, χ3927 (Δ*cya* Δ*crp* Ty2), has been demonstrated in volunteers (26). However, when given a single dose of 5×10^4 or 6×10^5 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 Δ*cya* Δ*crp* 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–lethal vector–host 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 Δ*cya* Δ*crp* Δ*cdt* derivative of *S. typhi* Ty2; this strain, which was complemented with the Asd^+ hybrid HBc–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 Δ*cya* Δ*crp* Δ*cdt* 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 Δ*cya* Δ*crp* Δ*cdt* mutant, χ4073, was included to assess the safety and immunogenicity 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. Exclusion criteria included previous immunization against HBV or typhoid fever, recent antibiotic use, positive human immunodeficiency virus serology, pregnancy, and allergy to amoxicillin or ciprofloxacin. The protocol was explained in depth, 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 5×10^5 or 5×10^6 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 health, the protocol was explained and informed consent was obtained. Volunteers had negative serologic tests for hepatitis B surface antigen and antibody and for core antibody. Volunteers were randomly allocated in a double-blind fashion to six groups to ingest with bicarbonate buffer a single oral dose containing 1×10^7 to 6×10^7 CFU or 0.5×10^9 to 1×10^9 CFU of either *S. typhi* χ4073, *S. typhi* χ4632(pYA3149), or *S. typhi* χ4632(pYA3167).

The volunteers in both studies were closely observed for the next 24 days for adverse reactions (fever, headache, malaise, chills, vomiting, and diarrhea). Severe headache was defined as headache which caused the volunteer to go to bed. Fever was defined as an oral temperature of ≥38.2°C. Any volunteer who had a temperature elevation to 38.2°C had blood cultures drawn at the time observation was made. A record was kept of the number, consistency, and description of all stools passed by volunteers. Stools were graded on a five-point system: grade 1, firm stool (normal); grade 2, soft stool (normal); grade 3, thick liquid (abnormal); grade 4, opaque watery (abnormal); grade 5, rice water (abnormal). Diarrhea was defined as the passage of 2 or more stools of grade 3 or above totaling at least 200 g in 48 h or passage of one large ≥300-g grade 3 stool.

All stools were cultured, and serial blood cultures were obtained on days 3, 4, 7, 8, 10, 12, and 15 after vaccination. To determine rates of intestinal colonization with the vaccine strain, gelatin string capsules (Entero-Test) were ingested by volunteers three times during the period of hospitalization (days 7, 10, and 13). Serum for antibody measurements (days 8, 21, 28, 45, 60, and 180) and whole blood for antibody-secreting cell (ASC) assays (days 7, 10, and 14) were obtained before and after vaccination. Before and 17 days after vaccination, volunteers swallowed polyvinyl chloride intestinal tubes to a distance of 130 cm from the mouth to collect intestinal fluid for measurement of local secretory immunoglobulin A (sIgA) antibody.

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 16- by 150-mm glass 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. The vaccine inoculum was placed on ice and transported immediately to the isolation ward. Microscopic examination and slide agglutination with *S. typhi* O, H, and Vi antisera were performed before use. Replica spread plate quantitative cultures were made of the inocula before and after vaccination to confirm viability and inoculum size.

Inoculation of volunteers. The vaccines were administered by the oral route with $NaHCO_3$. Two grams of $NaHCO_3$ was dissolved in 5 oz. of distilled water. Volunteers, fasting for 90 min, drank 4 oz. of the bicarbonate water; 1 min later, the 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 string were inoculated into GN broth (BBL, Cockeysville, Md.) and plated directly on *Salmonella-Shigella* agar (Difco, Detroit, Mich.). After overnight incubation at 37°C, subcultures from GN broth were made onto *Salmonella-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-labeled anti-human IgG (Ortho) measuring the color change of *ortho*-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 peroxidase-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. Trafficking lymphocytes that secrete IgA antibody against *S. typhi* O or H antigens (8, 28) and against hepatitis pre-S1/S2 antigen were measured. 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 IgG antibody. This monoclonal antibody was used as the positive control in the ASC assay. ASCs were reported as the number per 10^6 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 microbiologic results. In the first study, strain χ4073 (Δ*cya* Δ*crp* Δ*cdt S. typhi* Ty2) was fed to 12 volunteers at a dose of either 5×10^5 or 5×10^6 CFU; the strain was

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

Strain	Dose (CFU)	No. of volunteers with indicated response/total no. tested					Geometric mean peak excretion (CFU/g)
		Fever of ≥38.2°C	Diarrhea	Severe headache	Positive blood culture	Positive stool culture	
χ4073	5×10^8	0/5	0/5	0/5	0/5	2/5	4×10^4
	2×10^7	0/5	0/5	0/5	0/5	1/5	8.4×10^3
	5×10^6	0/6	1/6	0/6	0/6	1/6	1×10^2
	5×10^5	0/6	0/6	2/6	0/6	0/6	ND[d]
χ4632(pYA3149)	1×10^9	1/4[a]	0/4	2/4	1/4[b]	0/4	ND
	6×10^7	0/4	2/4	0/4	0/4	1/4	5.1×10^5
χ4632(pYA3167)	7×10^8	0/5	1/5	0/5	0/5	3/5	9.3×10^3
	3×10^7	1/5[c]	0/5	3/5	0/5	3/5	1.1×10^3

[a] Maximum temperature, 38.7°C on day 10.
[b] Days 7 and 8.
[c] Maximum temperature, 38.7°C on day 12.
[d] ND, not detected.

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 2×10^7 or 5×10^7 CFU) or comparable doses of one of the plasmid-containing derivatives. χ4073 was well 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 doses shed the vaccine strain in their stools but only in the first 48 h after vaccination. One of 26 volunteers with adequate specimens had a positive duodenal fluid culture; this occurred on day 10 after vaccination in a volunteer who had ingested the higher dose of χ4632(pYA3149).

Four isolates recovered from volunteers given χ4632 (pYA3167) were examined for the presence of the hybrid gene. All expressed hepatitis antigens as shown by Western blotting of cell lysates reacted with anti-core and anti-pre-S antisera, demonstrating that the foreign gene was stable in the attenuated *S. typhi* in humans.

Immunologic results. In the first study, 6 of the 12 volunteers who received 5×10^5 or 5×10^6 CFU of χ4073 developed significant numbers of ASCs against *S. typhi* LPS antigen (Table 2). One of eight volunteers from whom fluid was available developed sIgA anti-LPS in jejunal fluid. Three of 12 volunteers developed IgG anti-*S. typhi* LPS in serum. In summary, at these doses, χ4073 was reasonably well tolerated but suboptimally immunogenic.

In the second study, immune responses to *S. typhi* LPS and H antigens occurred in most volunteers who received doses of 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 Δ*cya* Δ*crp* Δ*cdt* mutant tested in this study, χ4073, was generally well tolerated over a range of doses from 10^5 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 Δ*aroC* Δ*aroD* 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 Δ*cya* Δ*crp* Δ*cdt* mutants in this study stimulated <100 ASCs per 10^6 peripheral blood mononuclear cells.

Diarrhea is not frequently a part of the clinical syndrome

TABLE 2. Immune responses to *S. typhi* and hepatitis B antigens after vaccination with one of three attenuated *S. typhi* strains

Strain	Dose (CFU)	No. of volunteers with IgA ASC response/no. tested			Geometric mean peak no. of IgA ASC/10^6 mononuclear cells[a]			No. of volunteers with serum IgG response/no. tested			No. with jejunal fluid sIgA response rate to LPS, H, or HBc-pre-S/no. tested
		Anti-LPS	Anti-H	Anti-HBc-pre-S	Anti-LPS	Anti-H	Anti-HBc-pre-S	Anti-LPS[b]	Anti-H[c]	Anti-HBc-pre-S	
χ4073	4.8×10^8	4/5	2/5	0/5	13.7	56	0	4/5 (0.49)	3/5 (303)	0/5	0/5
	2.4×10^7	5/5	4/5	0/5	10.8	7.1	0	5/5 (1.0)	4/5 (348)	0/5	0/5
	5×10^6	2/6	2/6	ND[d]	3.5	4	ND	1/6 (0.12)	0/6 (100)	ND	0/5
	5×10^5	4/6	0/6	ND	5.1	0	ND	2/6 (0.13)	0/6 (79)	ND	1/3
χ4632(pYA3149)	1.2×10^9	4/4	3/4	0/4	21.4	32.9	0	4/4 (0.99)	2/4 (476)	0/4	0/4
	6.1×10^7	2/4	2/4	0/4	34.6	18.1	0	3/4 (0.39)	1/4 (100)	0/4	0/4
χ4632(pYA3167)	6.6×10^8	3/5	4/5	0/5	66.6	15	0	4/5 (1.17)	3/5 (459)	0/5	0/5
	3.3×10^7	2/5	2/5	0/5	13.4	9.5	0	1/5 (0.25)	1/5 (115)	0/5	0/5

[a] Among responders only after immunization.
[b] Numbers in parentheses are peak geometric mean optical densities among all volunteers after immunization.
[c] Numbers in parentheses are peak geometric mean titers among all volunteers after immunization.
[d] ND, not done.

caused by wild-type *S. typhi* and was not a feature of illness observed when volunteers were challenged with wild-type *S. typhi* (12). However, mild diarrhea, ranging in volume from 274 ml to 1.2 liters, occurred overall in 4 of 40 volunteers who received a Δ*cya* Δ*crp* Δ*cdt*-based *S. typhi* vaccine strain. Deletions in genes encoding important proteins in the global regulatory system could change the interaction of the organism with the host in such a way as to lead to intestinal secretion. However, diarrhea was not associated with recovery of vaccine organisms in the diarrheal stools. Moreover, Δ*cya* Δ*crp* abolishes the synthesis of at least two adhesins which *S. typhimurium* (and presumably *S. typhi*) uses for attachment to, and thus invasion of, enterocytes. The pathogenesis of diarrhea in this setting and its relation to ingestion of vaccine remain obscure.

The introduction of a deletion in the chromosomal *asd* gene and the *asd*-complementing plasmid with or without hepatitis B genes did not further attenuate χ4073 in volunteers. In fact, the febrile responses that were observed occurred in volunteers who received one of the plasmid-bearing strains. In addition, the immune responses to *S. typhi* antigens were maintained in volunteers who received a plasmid-bearing strain.

The lack of immune response to hepatitis B antigen in those who received χ4632(pYA3167) was disappointing. These data are similar to the responses of volunteers who received three doses of 3×10^8 to 3×10^9 CFU of χ4632(pYA3167) orally or rectally. In that study, only one of six volunteers developed anti-pre-S1 antibodies and only after the third rectal immunization (17). In our study, even volunteers who were colonized and excreted 10^3 CFU/g of stool and who developed serum and ASC responses to *S. typhi* antigens failed to develop anti-pre-S responses. When isolates recovered from the stools and blood of vaccinees were examined, pre-S antigen was detected, so loss of the plasmid or recombinant gene insert was not an explanation for the lack of pre-S immune response. It is possible that the genes were not expressed in vivo or that the gene product was degraded in vivo before immune priming could occur. Future studies in which the heterologous gene is controlled by a strong in vivo promoter may increase its expression and immunogenicity. Alternatively, a more hearty vector strain, which persists in the gut-associated lymphoid tissue to a greater extent than these mutants, may result in stronger responses both to *S. typhi* antigens and to cloned antigens.

ACKNOWLEDGMENTS

This work was supported by NIAID contract NO1 AI15096 and grant RO1 DE06669.

We are grateful to the excellent nursing and technical staffs of the Center for Vaccine Development and to the volunteers from our community. We thank Darrel Peterson, Virginia Commonwealth University, Richmond, Va., for providing recombinant pre-S protein, labeled and unlabeled, and Wolfram Gerlich and Dirk Sobotta, University of Giessen, Giessen, Germany, for the anti-preS assays.

REFERENCES

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133:**149–157.
2. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100–122.
3. **Clements, J. D., and S. El-Morshidy.** 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. Infect. Immun. **46:**564–569.
4. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55:**3035–3043.
5. **Curtiss, R., III, S. M. Kelly, P. A. Gulig, and K. Nakayama.** 1989. Selective delivery of antigens by recombinant bacteria. Curr. Top. Microbiol. Immunol. **146:**35–49.

6. **Curtiss, R., III, S. B. Porter, M. Munson, S. A. Tinge, J. O. Hassan, C. Gentry-Weeks, and S. M. Kelly.** 1991. Nonrecombinant and recombinant avirulent live vaccines for poultry, p. 169–198. *In* L. C. Blankenship, N. A. Cox, N. J. Stern, and R. J. Meinersmann (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, Inc., New York, N.Y.
7. **Delos, S., M. T. Villar, P. Hu, and D. L. Peterson.** 1991. Cloning, expression and characterization of the pre-S domain of hepatitis B surface antigen, devoid of the S protein. Biochem. J. **276:**411–416.
8. **Forrest, B. D.** 1988. Identification of an intestinal immune response using peripheral blood lymphocytes. Lancet **i:**81–83.
9. **Gerlich, W.** Unpublished method.
10. **Hohmann, E. L., C. A. Oletta, K. P. Killeen, and S. I. Miller.** 1996. *PhoP/phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J. Infect. Dis. **173:**1408–1414.
11. **Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine.** 1992. Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. J. Clin. Invest. **90:**412–420.
12. **Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Snyder.** 1970. Typhoid fever: pathogenesis and immunologic control. N. Engl. J. Med. **283:**686–691.
13. **Kelly, S. M., B. A. Bosecker, and R. Curtiss III.** 1992. Characterization and protective properties of attenuated mutants of *Salmonella choleraesuis*. Infect. Immun. **60:**4881–4890.
14. **Levine, M. M., J. Galen, E. Barry, F. Noriega, S. Chatfield, M. Sztein, G. Dougan, and C. Tacket.** 1996. Attenuated *Salmonella* as live oral vaccines against typhoid fever and as live vectors. J. Biotechnol. **44:**193–196.
15. **Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. Stocker.** 1987. Safety, infectivity, immunogenicity, and in vivo stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. J. Clin. Invest. **79:**888–902.
16. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an Asd^+ expression-cloning vector: stable maintenance and high-level expression of cloned genes in a Salmonella vaccine strain. Bio/Technology **6:**693–697.
17. **Nardelli-Haefliger, D., J.-P. Kraehenbuhl, R. Curtiss III, F. Schödel, A. Potts, S. Kelly, and P. De Grand.** 1996. Oral and rectal immunization of adult female volunteers with a recombinant attenuated *Salmonella typhi* vaccine strain. Infect. Immun. **64:**5219–5224.
18. **Peterson, D.** Unpublished method.
19. **Roberts, M., S. N. Chatfield, and G. Dougan.** 1994. *Salmonella* as carriers of heterologous antigens, p. 27–58. *In* D. T. O'Hagan (ed.), Novel delivery systems for oral vaccines. CRC Press, Inc., Boca Raton, Fla.
20. **Ross, E. M., and A. G. Gilman.** 1980. Biochemical properties of hormone-sensitive adenylate cyclase. Annu. Rev. Biochem. **49:**533–564.
21. **Schödel, F., S. M. Kelly, D. L. Peterson, D. R. Milich, and R. Curtiss III.** 1994. Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. Infect. Immun. **62:**1669–1676.
22. **Schödel, F., D. R. Milich, and H. Will.** 1990. Hepatitis B virus nucleosapsid/pre-S1 fusion proteins expressed in attenuated *Salmonella* for oral vaccination. J. Immunol. **145:**4317–4321.
23. **Schödel, F., D. Peterson, J. Hughes, and D. R. Milich.** 1993. Avirulent *Salmonella* expressing hybrid hepatitis B virus core/preS genes for oral vaccination. Vaccine **11:**143–148.
24. **Schödel, F., H. Will, and D. R. Milich.** 1991. Hybrid hepatitis B virus core/pre-S particles expressed in attenuated *Salmonellae* for oral immunization, p. 319–325. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 91. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Schwartzel, E. H., Jr., S. Bachman, and R. A. Levine.** 1977. Cyclic nucleotide activity in gastrointestinal tissues and fluids. Anal. Biochem. **78:**395–405.
26. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp* *Salmonella typhi* strains in adult volunteers. Infect. Immun. **60:**536–541.
27. **Tacket, C. O., D. M. Hone, G. A. Losonsky, L. Guers, R. Edelman, and M. M. Levine.** 1992. Clinical acceptability and immunogenicity of CVD 908 *Salmonella typhi* vaccine strain. Vaccine **10:**443–446.
28. **Van de Verg, L., D. A. Herrington, J. R. Murphy, S. S. Wasserman, S. B. Formal, and M. M. Levine.** 1990. Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with a bivalent *Salmonella typhi-Shigella sonnei* vaccine or infection by pathogenic *S. sonnei*. Infect. Immun. **58:**2002–2004.

Editor: J. R. McGhee