

Construction of a Recombinant Oral Vaccine against *Salmonella typhi* and *Salmonella typhimurium*

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The *viaB* gene coding for the Vi antigen of *Salmonella typhi* Ty2 was subcloned into expression vector pYA248. The recombinant plasmid was termed SMM202 and transformed into *Salmonella typhimurium* χ 4072, an attenuated Δ *cya* Δ *crp* mutant. Recombinant *S. typhimurium* Vi4072 had the ability to produce Vi capsular polysaccharide and also to invade and colonize the small intestine, mesenteric lymph nodes, and spleen of BALB/c mice. Mice orally immunized with Vi4072 developed serum and secretory antibody responses to the Vi antigen, as measured by a passive hemagglutination assay. Mice developed a delayed-type hypersensitivity following a footpad injection with Vi antigen after being sensitized orally with a suitable dose of Vi4072. Immunization of mice with Vi4072 afforded complete protection against fatal infection with virulent *S. typhi* Ty2. All data indicate that this route of antigen delivery is effective for stimulating antibody-mediated immunity and for inducing a cell-mediated immune response in BALB/c mice. Thus, *S. typhimurium* Vi4072 may serve as a vaccine for protection against typhoid fever and salmonellosis caused by *S. typhimurium*.

Typhoid fever remains an important public health problem in many developing countries, where the incidence is usually highest in school-aged children. It is estimated that the worldwide incidence of typhoid fever exceeds 50 million cases per year, with 500,000 deaths occurring annually (7). Typhoid fever has not been controlled by vaccination because the two existing vaccines have limitations. The first vaccine, composed of inactivated *Salmonella typhi* cells, requires two injections and confers 60 to 70% protection, but the notable adverse reactions occur at such high frequency (2, 10, 13) that the killed whole-cell vaccines are not satisfactory immunizing agents for use either in school-aged children in areas where typhoid fever is endemic or in travelers. The second vaccine, an attenuated strain of *S. typhi* Ty2, formulated as an enteric-coated tablet, requires at least three doses to achieve 60 to 70% protection (17). The mode of action of the mutant Ty21a strain has not been identified, and the lack of this information has prevented precise standardization.

The drawbacks of the present vaccines and the persistence of typhoid fever as a public health problem have encouraged efforts to develop new and improved typhoid vaccines. The new immunizing agents include several attenuated *S. typhi* strains used as live oral vaccines and the purified Vi antigen, alone or conjugated to a protein carrier, used as a parenteral vaccine (17, 28).

Recently, two randomized field trials have been conducted to determine the efficacy of purified Vi antigen. The results demonstrate the importance of the Vi capsule as a protective antigen (1, 16). Previous studies have revealed that Vi antigen expression in *S. typhi* is controlled by two physically separate genetic loci, *viaA* and *viaB*. *Escherichia coli* and *Salmonella typhimurium* strains contain a functional *viaA* gene but do not contain the *viaB* region and therefore do not express the Vi antigen (4, 23). In this study, we constructed a plasmid containing the *viaB* gene and introduced it into an attenuated *S. typhimurium* Δ *cya* Δ *crp* mutant (9). This

recombinant *S. typhimurium* mutant expressing the Vi capsule should serve as an antityphoid and anti-*S. typhimurium* salmonellosis oral vaccine. Since *S. typhimurium* infection is less frequent than *S. typhi* infection in all cases of salmonellosis (24), this new candidate vaccine strain may be valuable.

MATERIALS AND METHODS

Strains, plasmids, and culture media. pWR127 (26), containing an 18-kilobase *viaB* fragment, was propagated in *E. coli* HB101 (kindly provided by L. S. Baron, Walter Reed Army Institute of Research, Washington, D.C.). pYA248, containing an *asd*⁺ gene, was used as the expression vector (21). *S. typhimurium* χ 3730 (a restriction-negative, modification-positive strain) containing the *asd* mutation was used as an intermediate transformation recipient of the plasmid DNA, and an avirulent *S. typhimurium* SR-11 double mutant (Δ *cya* Δ *crp*), χ 4072, containing the *asd* mutation was used for delivery of Vi antigen in vivo (21). All these plasmids and strains were kindly provided by R. Curtiss III, Washington University, St. Louis, Mo. A P22 (HT, Int⁻) transducing phage lysate was routinely produced from cultures of *S. typhimurium* LT2 (kindly provided by the Beijing Institute of Microbiology, Chinese Academy of Science). Virulent *S. typhi* Ty2 was used as the challenge strain (kindly provided by the Shanghai Institute of Biological Products, Ministry of Public Health).

The *E. coli* strains and *S. typhimurium* LT2 were grown in LB medium at 37°C. *S. typhimurium* strains χ 3730 and χ 4072 were cultured in LB or BHI medium containing 50 μ g of DL- α -diaminopimelic acid per ml. χ 4072 has a *gyrA1816* mutation and can be selected with nalidixic acid. The medium contained nalidixic acid (50 μ g/ml) for selecting *S. typhimurium* χ 4072 and its derivatives.

Mice. Female BALB/c mice (Laboratory Animal Resource Facility, Second Military Medical College) were bred and maintained in the laboratory animal room at the Institute of Medical Biotechnology and Molecular Genetics. Mice were housed in cages and given food and water ad libitum. All mice used in these experiments were 5 to 8 weeks of age.

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DNA techniques. Plasmid pWR127 DNA was isolated and partially digested with *EcoRI* restriction endonuclease to generate seven DNA fragments. The 18-kb Vi gene fragment was isolated by low-melting-temperature agarose gel electrophoresis and ligated to completely *EcoRI*-digested pYA248 to result in recombinant plasmid SMM202. These procedures were performed as described previously (19).

Genetic transformation and transduction. Because of restriction barriers, it was necessary to first transform plasmids into a restriction-negative, modification-positive rough *Salmonella* strain (χ 3730), and successful transformation was confirmed by a rapid screening technique (19). The plasmids were transduced into *S. typhimurium* χ 4072 (Δ *cya* Δ *crp*) by using P22 (HT, Int⁻) (11) or transformed into χ 4072 by electroporation. Electroporation at high voltage was used for transforming smooth-type *S. typhimurium* χ 4072 with plasmid DNA isolated from χ 3730. χ 4072 recipient cells were prepared as described previously (3). For transformation, the pulse field strength was set at 15 kV/cm and the pulse time was set to 10 ms.

Screening of transformants and transductants. Both *S. typhimurium* χ 3730 and χ 4072 are *asd* mutant strains, unable to grow in LB medium or brain heart infusion (BHI) medium. When the plasmid containing the *asd*⁺ gene was transformed or transduced into χ 3730 or χ 4072, both strains attained the ability to grow in LB or BHI medium (21). The presence of the plasmids in colonies of transformants and transductants was demonstrated by a rapid screening technique (19). For primary identification of bacteria with and without the Vi antigen, bacterial colonies were readily identified visually under oblique lighting. Vi⁺ colonies appear bright and orange-hued, and Vi⁻ colonies exhibit an opaque grayish appearance. For further confirmation, slide agglutination tests with *Staphylococcus aureus* Cowan 1 cells coated with rabbit anti-*S. typhi* Ty2 Vi serum (25). The successful transformant of Vi-positive strain χ 4072 (SMM202) was named Vi4072.

Serological tests. The method of Rockhill et al. (25) was used for the slide coagglutination test. The passive hemagglutination assay was used to measure serum antibodies to the Vi antigen. Human O-type erythrocytes were sensitized with Vi antigen from *S. typhi* Ty2 (Shanghai Institute of Biological Products, Ministry of Public Health), and the method used has been described previously (22). Vi antigen was purified by the procedure of Wong et al. (30). The gel diffusion test was that described by Wong et al. (31).

Immunization of mice with *S. typhimurium*. Inocula for oral immunizations were prepared from log-phase cultures of recombinant *S. typhimurium* Vi4072 and χ 4072(pYA248). BHI broth cultures (6 ml per tube) were inoculated with cells from frozen stocks (-40°C) and incubated overnight at 37°C without shaking. These cultures were diluted 1:20 in BHI broth and incubated for 4 h with shaking (260 rpm at 37°C) to obtain log-phase growth. Cultures were then put in ice to inhibit further replication. The cell suspension was pelleted (8,000 × *g* for 10 min at 4°C) and then suspended in buffered saline with gelatin (BSG) (9) to 1/50 the original volume. Food and water were removed from each cage 4 h prior to oral infection and returned 30 min after oral infection. Stomach acidity was neutralized with 30 μ l of 10% sodium bicarbonate administered orally 5 min prior to a 20- μ l oral dose of *S. typhimurium* (9).

Colonization study. Groups of three mice were killed on days 2, 7, 14, 21, 28, 35, and 42 post-oral inoculation with Vi4072 (4 × 10⁸ CFU per mouse). The spleen and mesenteric lymph nodes were removed from each animal and homoge-

nized in 5 ml of sterile water with a glass homogenizer. Homogenates were plated on LB agar containing 50 μ g of nalidixic acid per ml and incubated for 24 h at 37°C. Nalidixic acid-resistant colonies were identified as Vi4072 by the presence of Vi antigen.

DTH reaction. To test for delayed-type hypersensitivity (DTH), groups of five mice orally immunized with 4 × 10⁸ CFU of *S. typhimurium* Vi4072 or χ 4072(pYA248) on day 0 received a 5 × 10⁶-CFU booster inoculation intraperitoneally on day 45. After 3 weeks, the mice immunized with *S. typhimurium* and unimmunized controls (orally fed BSG and boosted with BSG) were injected in the right hind footpad with 5 to 10 μ g of soluble Vi antigen isolated from *S. typhi* Ty2 in 20 μ l of phosphate-buffered saline (PBS). The left foot was injected with PBS as a negative control. Footpad swelling (mean difference in thickness, in 0.1-mm units, between PBS-injected and antigen-injected footpads) was measured after 24 and 48 h with a vernier caliper. *P* values were calculated between groups (Vi antigen reaction in unimmunized controls and in mice immunized with *S. typhimurium*) by Student's one-tailed *t* test.

Evaluation of protective immunity. Mice were immunized orally with 5 × 10⁷ recombinant Vi4072. As controls, groups of mice were inoculated orally with 5 × 10⁷ χ 4072(pYA248) or BSG. At 60 days postimmunization, mice were challenged with virulent *S. typhi* Ty2. The surviving mice were counted 2 weeks later.

RESULTS

Construction of recombinant vector containing the *viaB* gene. The 18-kb *viaB* fragment was inserted into the *EcoRI* site of pYA248, resulting in plasmid SMM202. Strain χ 3730 carrying plasmid SMM202, containing the *asd*⁺ and *viaB* genes, was able to grow in LB medium and to produce Vi capsule.

Transforming χ 4072 with plasmid SMM202 by electroporation. By using bacteriophage P22 (HT, Int⁻), we have successfully transduced plasmid pYA248 from χ 3730 (pYA248) into χ 4072, but we failed to transduce plasmid SMM202 from χ 3730(SMM202) into χ 4072, probably because strain χ 3730(SMM202) has the ability to produce Vi capsule and therefore bacteriophage P22 cannot attach to the lipopolysaccharide of or infect χ 3730(SMM202) to produce transducing phages. By electroporation, we successfully transformed χ 4072 with plasmid SMM202 isolated from χ 3730(SMM202). We obtained approximately 5 × 10⁴ bacterial transformants with 50 ng of plasmid SMM202. All of the χ 4072 transformant colonies appearing in BHI agar carry plasmid SMM202 and have the ability to produce Vi capsule.

Colonization of mice with Vi4072 and analysis of serum Vi antibodies for mice immunized with Vi4072. The insertion of the plasmid and expression of Vi capsule did not make Vi4072 lose its ability to invade and colonize the small intestine, mesenteric lymph nodes, and spleen of BALB/c mice. *S. typhimurium* Vi4072 colonization of the small intestine and mesenteric lymph nodes occurred rapidly, within the first week after oral immunization. Colonization of the spleen occurred more gradually, with much lower cell numbers. By day 28 postinoculation, *S. typhimurium* Vi4072 was virtually undetectable in all tissues (Table 1).

Mice orally immunized once with *S. typhimurium* Vi4072 developed a Vi-specific serum antibody. The Vi antibody serological response varied with the dose used for inoculation. Mice orally immunized with 5 × 10⁸ *S. typhimurium*

TABLE 1. Colonization of spleens and mesenteric lymph nodes of BALB/c mice after oral infection with 4×10^8 CFU of Vi4072^a

Tissue	Mean CFU \pm 2 SE ($n = 3$) on day postinfection:				
	2	7	14	21	28 35
Peyer's patches	>100	ND ^b	ND	ND	0 0
Mesenteric lymph nodes	38 ± 12	434 ± 77	200 ± 54	24 ± 15	0 0
Spleen	0	36 ± 13	52 ± 21	4 ± 3	0 0

^a Groups of three mice were killed on days 2, 7, 14, 21, 28, 35, and 42 postinfection. The spleen and mesenteric lymph nodes were removed from each animal and homogenized.

^b ND, not determined.

Vi4072 elicited more Vi antibodies than those immunized with 5×10^6 organisms (Table 2).

Analyses of the Vi antigen nature of Vi4072. The Vi capsule of Vi4072 was compared with that of *S. typhi* Ty2. Vi antigen was purified from both strains and analyzed by immunodiffusion against specific anti-Vi type serum (Shanghai Institute of Biological Products, Ministry of Public Health). A single band of identity was observed for two Vi antigen preparations. Vi antigen from *S. typhi* Ty2-sensitized human erythrocytes gave positive hemagglutination with Vi antiserum against Vi4072. *S. aureus* Cowan 1 cells coated with anti-Vi4072 rabbit serum (rabbit immunized with Vi4072) gave positive slide agglutination with *S. typhi* Ty2 Vi antigen (Shanghai Institute of Biological Products, Ministry of Public Health). All of these results indicated that Vi4072 produced typical Vi capsular antigen immunologically identical to the Vi capsular antigen of *S. typhi* Ty2.

DTH responses following immunization. Mice immunized with *S. typhimurium* Vi-negative strain χ 4072(pYA248) did not respond to Vi antigen (mean increase in footpad thickness \pm standard error [$n = 8$], 0.06 ± 0.15 mm), but the group immunized with Vi-positive strain Vi4072 gave a clear response to a subcutaneous footpad DTH-stimulatory dose of soluble Vi antigen (mean increase, 1.22 ± 0.17 mm), indicating that mice immunized with Vi4072 developed a cell-mediated DTH response to the *S. typhi* Vi antigen. The value for the BSG control was 0.02 ± 0.16 mm. The difference between the BSG and Vi4072 groups was significant ($P < 0.002$).

Effectiveness of immunization with Vi4072. Table 3 presents data on the ability of *S. typhimurium* Vi4072 to induce

TABLE 2. Passive hemagglutination analysis of Vi antigen-specific serum in mice orally immunized with Vi4072^a

Day after infection	Geometric mean serum dilution showing positive hemagglutination		
	Mice immunized with Vi4072		Mice immunized with χ 4072(pYA248) (5×10^8 CFU)
	5×10^6 CFU	5×10^8 CFU	
7	7.3	51.0	— ^b
14	36.1	100.5	—
21	13.2	151.3	—
28	8.3	189.8	—
35	4.8	126.0	—
45	—	54.7	—

^a Serum was collected and pooled from five mice in each group on days 7, 14, 21, 28, 35, and 45 postinfection and assayed by the passive hemagglutination test.

^b —, no hemagglutination.

TABLE 3. Effectiveness of oral immunization with recombinant Vi4072 in protecting against intraperitoneal challenge with wild-type virulent *S. typhi* Ty2^a

Group	Dose (CFU)	No. of mice in group	No. ill	No. surviving
Vi4072	5×10^7	19	3	19
χ 4072(pYA248)	5×10^7	7	7	0
BSG		16	16	0

^a Mice were challenged with *S. typhi* Ty2 on day 60 postimmunization.

immunity to subsequent intraperitoneal challenge with 55 times the 50% lethal dose of virulent *S. typhi* Vi-positive strain Ty2 cells. All of the mice orally immunized with Vi4072 were completely protected; a few of them displayed illness.

DISCUSSION

Plasmid SMM202, containing *viaB* gene, was transformed into *S. typhimurium* χ 4072 (Δ *cya* Δ *crp*) by electroporation. The *viaB* gene, in cooperation with the native *viaA* gene, gave *S. typhimurium* χ 4072 the ability to express Vi capsule. From previous reports (8, 27), even in nonselective conditions, the plasmids in Δ *cya* Δ *crp* mutant strains are maintained stably, probably owing to the reduced growth rate of Δ *cya* Δ *crp* strains compared with wild-type strains. Plasmid SMM202 carried a complementing *asd*⁺ gene, so SMM202 was stable in vitro and in vivo (data not shown). This allowed Vi4072 to express Vi antigen stably.

Colonization studies determined that between 7 and 14 days postinfection, recombinant *S. typhimurium* Vi4072 effectively invades and colonizes the small intestine, mesenteric lymph nodes, and spleen. When mice were orally fed *S. typhimurium*, all of the Vi4072 cells were cleared by day 28. The report by Stabel et al. (27) revealed that Δ *cya* Δ *crp* strains can be isolated from the small intestines and spleens of infected mice 10 weeks post-primary oral immunization, and in our experiments, we also isolated χ 4072(pYA248) from mice 2 months post-primary oral immunization, but we could not isolate any Vi4072 from mice at the same time. Quite possibly, the extremely high levels of Vi capsule (data not shown) are a burden to *S. typhimurium*, and after infection, the mice quickly developed specific antibodies against the Vi capsule, so the Vi-positive *S. typhimurium* cells were quickly cleared from the tissues of the mice. Thus, the organisms of strain Vi4072 can establish a limited infection in the host after oral inoculation, leading to an immune response to the Vi antigen and the antigens of *S. typhimurium*.

By the passive hemagglutination test, we found a strong humoral antibody response of orally infected mice against the Vi antigen, and the antibodies were detectable by 7 days post-oral inoculation with *S. typhimurium* Vi4072 (5×10^6 or 5×10^8 CFU). The DTH response indicates that the Vi antigen of *S. typhimurium* Vi4072 can stimulate cellular immunity in BALB/c mice. Oral delivery of antigen via an attenuated *Salmonella* strain is an effective method for stimulation of localized secretory immunity (20), and the oral route of immunization with live recombinant Vi4072 was also intended to stimulate a localized secretory antibody response to Vi antigen. In our primary experiments, intestinal homogenates contained high-titer immunoglobulin A directed against *S. typhi* Vi antigen (data not shown).

The oral vaccine Vi4072 can afford a high level of protec-

tion in BALB/c mice against intraperitoneal challenge with virulent *S. typhi* Ty2. This also demonstrates that the Vi antigen is an important protective antigen of *S. typhi* and indicates that Vi4072 can effectively elicit an immune response when administered orally.

Infection of mice with the natural mouse pathogen *S. typhimurium* is the most widely accepted model for studying immunity to typhoid fever. Mouse typhoid model studies indicated that the use of nonviable vaccine strains and of passively transferred serum to protect against infection with *Salmonella* species shows a marked difference in different mouse strains (12). Serum antibodies were shown to protect inherently resistant mice but not inherently susceptible mouse strains (12). But mice that are not protected by nonviable vaccines are highly protected by live *Salmonella* organisms, either avirulent strains or sublethal doses of virulent organisms (5, 6, 14, 18, 29). The general view is that salmonellae are intracellular pathogens and that adequate host defense requires cellular immunity (6), and only living salmonellae are believed to induce cellular immunity, although there is a question whether salmonellae are intracellular pathogens (15). Human susceptibility and immunity to *S. typhi* may bear close resemblance to murine susceptibility and immunity. If humans exhibit variability in innate susceptibility similar to that of different mouse strains, some people may be protected by serum antibodies to *S. typhi*, but some susceptible people may not and may require cell-mediated immunity. Thus, a live vaccine would be advantageous, as it can elicit both humoral immunity and cell-mediated immunity and might have a greater chance of being universally effective.

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