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Constitutive Expression of the Vi Polysaccharide Capsular Antigen in Attenuated *Salmonella enterica* Serovar Typhi Oral Vaccine Strain CVD 909

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Live oral Ty21a and parenteral Vi polysaccharide vaccines provide significant protection against typhoid fever, albeit by distinct immune mechanisms. Vi stimulates serum immunoglobulin G Vi antibodies, whereas Ty21a, which does not express Vi, elicits humoral and cell-mediated immune responses other than Vi antibodies. Protection may be enhanced if serum Vi antibody as well as cell-mediated and humoral responses can be stimulated. Disappointingly, several new attenuated *Salmonella enterica* serovar Typhi oral vaccines (e.g., CVD 908-*htrA* and Ty800) that elicit serum O and H antibody and cell-mediated responses following a single dose do not stimulate serum Vi antibody. Vi expression is regulated in response to environmental signals such as osmolarity by controlling the transcription of *tviA* in the *viaB* locus. To investigate if Vi antibodies can be stimulated if Vi expression is rendered constitutive, we replaced P_{tviA} in serovar Typhi vaccine CVD 908-*htrA* with the constitutive promoter P_{tac}, resulting in CVD 909. CVD 909 expresses Vi even under high-osmolarity conditions and is less invasive for Henle 407 cells. In mice immunized with a single intranasal dose, CVD 909 was more immunogenic than CVD 908-*htrA* in eliciting serum Vi antibodies (geometric mean titer of 160 versus 49, $P = 0.0007$), whereas O antibody responses were virtually identical (geometric mean titer of 87 versus 80). In mice challenged intraperitoneally with wild-type serovar Typhi 4 weeks after a single intranasal immunization, the mortality of those immunized with CVD 909 (3 of 8) was significantly lower than that of control mice (10 of 10, $P = 0.043$) or mice given CVD 908-*htrA* (9 of 10, $P = 0.0065$).

Virtually all *Salmonella enterica* serovar Typhi strains isolated from the blood or bone marrow of patients with acute typhoid fever and from the bile or feces of those who carry serovar Typhi in the gallbladder are found to express Vi capsular polysaccharide when tested in clinical microbiology laboratories (30). Indeed, sometimes agglutination with *Salmonella* group D antiserum cannot be demonstrated until the bacterial cells are boiled to remove the Vi capsule, which blocks access of the antibodies to the underlying O polysaccharide (7). In a mouse model originally described by Felix and Pitt (8, 9), Vi was found to be a virulence antigen. Immunization with purified Vi polysaccharide was shown to protect mice against intraperitoneal challenge with virulent serovar Typhi administered with gastric mucin (29, 46, 62). More important, in controlled human field trials, parenteral immunization with nondenatured purified Vi polysaccharide, which elicits serum immunoglobulin G (IgG) Vi antibody (25, 49), has conferred a moderate level of protection against typhoid fever (1, 25, 26). Due to clinical data demonstrating safety, immunogenicity, and efficacy, purified Vi polysaccharide is currently a licensed parenteral typhoid vaccine.

Circa 90% of chronic carriers (in the gallbladder) of serovar Typhi manifest elevated titers of serum Vi antibody (27, 37, 41). In contrast, only 20% of patients with acute typhoid fever exhibit elevated titers; in those patients, the elevated titers are usually short-lived unless the patients become chronic carriers (27, 37). For these reasons, whereas Vi serology is not helpful in the diagnosis of acute typhoid fever, the detection of elevated serum anti-Vi antibodies is very useful in screening for chronic typhoid carriers, even in areas of endemicity (14, 27, 28, 36).

Prior to its licensure as a live oral typhoid vaccine, the efficacy of attenuated serovar Typhi strain Ty21a in preventing typhoid fever was demonstrated in multiple randomized, placebo-controlled, double-blind field trials in Latin America (3, 31, 32, 34), Africa (60), and Asia (47). Ty21a stimulates an array of humoral and cell-mediated immune responses to various serovar Typhi antigens but neither expresses Vi capsular polysaccharide (17) nor elicits serum Vi antibody (6, 15, 24, 38–40, 56). Thus, immune responses other than the elicitation of Vi antibody account for the protection provided by this live oral vaccine. Based on these observations, it has been hypothesized that it may be possible to achieve a higher level of protection against typhoid fever if one could simultaneously elicit serum IgG Vi antibodies in addition to the other immune responses stimulated by live oral vaccines such as Ty21a (33). An early attempt to harness the protective effects of these other immune responses and serum IgG Vi antibodies was pursued by inserting a native *viaB* locus into the chromosome of Ty21a, resulting in strain WR4103, a Vi-expressing variant of Ty21a (5). However, this strain did not induce anti-Vi antibodies in subjects who ingested doses as high as 10^{10} CFU (53). More disappointing, several modern, engineered serovar Typhi vaccine strains that express Vi in vitro and that elicit high titers of O and H antibodies following ingestion of a single

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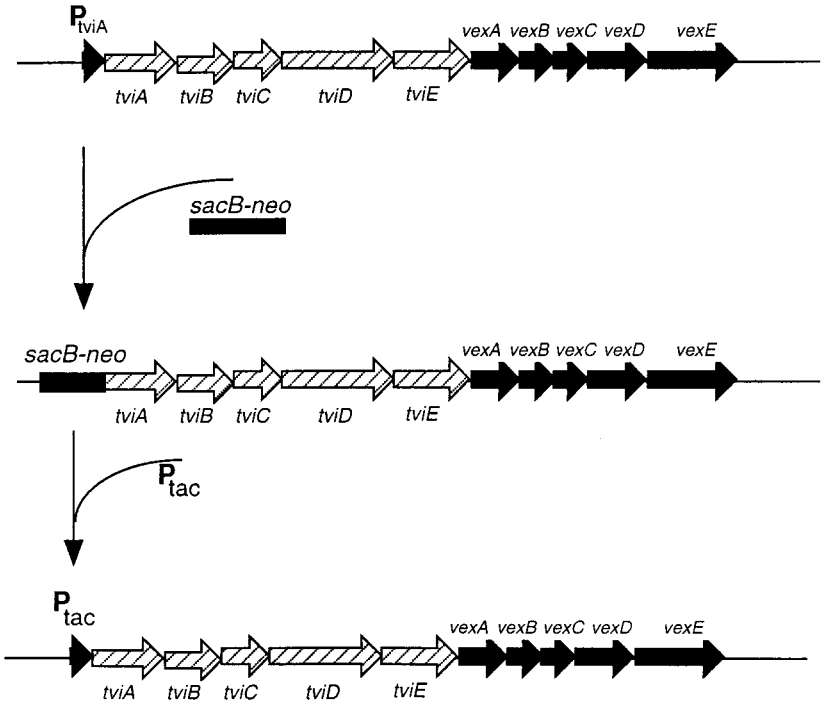

FIG. 1. Genetic map of the *viaB* locus, showing replacement of P_{tviA} by P_{tac}. The two-step strategy for promoter deletion and substitution is illustrated. In the first step, the *tviA* promoter is deleted and the *sacB-neo* cassette is inserted into the chromosome of CVD 908-*htrA*. In the second step, the replacement of the *sacB-neo* cassette allows for the selection of bacteria in which a second homologous recombination event results in replacement by the P_{tac} promoter.

oral dose have failed to stimulate serum Vi antibodies (21, 50, 51, 54, 55).

The likely explanation for the disparate observations cited above stems from the fact that the expression of Vi is highly regulated in relation to certain environmental signals, such as osmolarity, and that at least two separate two-component systems, *rcsB-rcsC* (2, 58) and *ompR-envZ* (45), are involved in the regulation of Vi expression. The supposition is that Vi expression ensues when the bacteria find themselves in certain extracellular environments, such as blood and bile (to protect them from the complement-mediated actions of O antibody) (9, 10, 46), but is turned off when the bacteria gain their intracellular niche within macrophages or intestinal epithelial cells. It follows that if Vi expression by a live oral vaccine strain is rendered constitutive, this may allow the stimulation of serum IgG Vi antibodies in orally vaccinated subjects, thereby enhancing overall protection against typhoid fever. Herein we describe the modification of attenuated Δ*aroC*, Δ*aroD*, Δ*htrA* strain CVD 908-*htrA* to derive CVD 909, which manifests the constitutive expression of Vi.

MATERIALS AND METHODS

Bacterial strains. Δ*aroC* Δ*aroD* Δ*htrA* serovar Typhi strain CVD 908-*htrA* (35) was derived from Δ*aroC* Δ*aroD* strain CVD 908 (22). In clinical trials CVD 908-*htrA* has proven to be highly immunogenic in stimulating serum IgG O and H antibodies, secretory IgA mucosal responses, and cell-mediated immune responses. In contrast, serum anti-Vi antibodies were not elicited (54, 55). Construction of Δ*aroC* Δ*aroD* Δ*htrA* P_{tac}-*tviA* strain CVD 909 is introduced in this study. All strains were grown on Aro agar as described previously (22).

Replacement of the *tviA* promoter. The construction of serovar Typhi CVD 909 was achieved in two phases. First, a deletion in the promoter region of *tviA* and insertion of the *sacB-neo* cassette was introduced into the chromosome of CVD 908-*htrA* by homologous recombination (42, 44). Second, the replacement of the *sacB-neo* cassette allowed counterselection for the replacement of the promoter region with the P_{tac} promoter in a second homologous recombination event (42, 44).

In the first step, a 167-bp deletion in the promoter region of *tviA* was created by an overlapping PCR. Specifically, a 601-bp region upstream from the promoter was amplified from serovar Typhi strain Ty2 genomic DNA with the primers 5′-GGGGGAGCTCAATTCTGCAAACCAGCCCTGTACCATCAAGTTCATA-3′ and 5′-**CCTCATCCCGGGCCCGGATCC**ACCTGCACAATTCATTGTTTGTACCTATC-3′ (relevant restriction sites used are underlined). In parallel, a 770-bp region downstream from the *tviA* promoter was amplified using the primers 5′-**GCAGGTGGATCCGGGCCCGGG**ATGAGGTTTCATCATTTCTGGCCTCCGAATGATATC-3′ and 5′-ATCCTTGAATTCGGGGGATCCTACTAAAATTTTATATTTACAAAGTTAATTCTAGGT-3′. Homologous sequences engineered into the tails of the above primers (shown as bold letters) allowed the two PCR products to be used in an overlapping PCR, resulting in a 1,371-bp product encompassing the upstream region of *tviA* but with a deletion of the promoter and containing a unique *Sma*I site. This PCR fragment was recovered in pGEM-T, yielding pGEM-T::ΔP_{tviA}. A *sacB-neo* cassette, obtained as a *Sma*I fragment from pIB279 (4, 44), was inserted into the unique *Sma*I site in pGEM-T::ΔP_{tviA} to result in pGEM-T::ΔP_{tviA}::*sacB-neo*. The ΔP_{tviA}::*sacB-neo* segment was excised as an *Sst*I fragment and was inserted into the suicide vector pKTN701, resulting in pKT::ΔP_{tviA}::*sacB-neo* (43, 44). Following the transfer by conjugation of pKT::ΔP_{tviA}::*sacB-neo* into CVD 908-*htrA*, homologous recombination events were selected for by screening kanamycin-resistant colonies for chloramphenicol sensitivity.

In the second step, the P_{tac} promoter was substituted for the *sacB-neo* insertion in the *tviA* locus of CVD 908-*htrA*-ΔP_{tviA}::*sacB-neo* (Fig. 1). Specifically, a 257-bp *Bam*HI-*Eco*RI segment that included P_{tac} from pKK223-3 (Pharmacia, Piscataway, N.J.) was cloned into pBluescript (Stratagene, La Jolla, Calif.), resulting in pBluescript::P_{tac}. Regions flanking the *tviA* promoter, amplified with the primers listed above, were cloned upstream (as an *Sst*I-*Bam*HI fragment) and downstream (as a *Sma*I-*Sal*I fragment) from the P_{tac} promoter in pBluescript::P_{tac}. The resulting *tviA* locus with the inserted P_{tac} promoter was cloned into the suicide vector pJG14, yielding pJG14::P_{tac}-*tviA*, which was used to exchange P_{tac} for *sacB-neo* in CVD 908-*htrA*-ΔP_{tviA}::*sacB-neo* by homologous recombination as described previously (43, 44). The isolation of double crossover mutants was enhanced by the counterselection provided by the toxicity of sucrose, which was conferred by *sacB*, and reversion to kanamycin sensitivity. The P_{tac} insertion was confirmed by PCR amplification of P_{tac}-*tviA* using the primers 5′-GGAATTGTGAGCGGATAACAATTTCACACAGG-3′ (based on the P_{tac} sequence) and 5′-ATCCTTGAATTCGGGGGATCCTACTAAAATTTTATATTTACAAAGTTAATTCTAGGT-3′ (based on the 3′ terminus of the *tviA* sequence). The expression of Vi was confirmed by slide agglutination of the bacterial clones with Vi antiserum (Difco, Chicago, Ill.).

Osmolarity regulation studies. The effect of NaCl on the expression of Vi antigen was assessed as described by Pickard et al. (45). Briefly, media with NaCl concentrations varying from 0.17 to 0.7 M were prepared by supplementation of Aro agar prepared without NaCl. Serovar Typhi colonies were grown overnight at 37°C and slide agglutination was performed with Vi antiserum.

Tissue culture invasiveness assay. Henle 407, a human embryonic intestinal cell line (ATCC CCL-6), was grown in stationary culture in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, N.Y.) supplemented with 15% fetal bovine serum (Gibco BRL) (15% FBS–DMEM) and penicillin-streptomycin (Gibco BRL) at 37°C with 5% CO_2. Gentamicin protection assays were performed using a modification of the method of Tartera and Metcalf (57). Briefly, 2×10^5 Henle 407 cells were introduced into each well of a 24-well tissue culture plate (Falcon 3847; Becton Dickinson, Lincoln Park, N.J.) and incubated overnight without antibiotics. Bacteria were grown overnight at 37°C on L agar, or Aro agar if required, harvested, and resuspended in 15% FBS–DMEM; 5×10^8 CFU/100 μl were then inoculated into each Henle 407 culture well for 90 min (multiplicity of infection, 1,000:1). Each well was washed three times with phosphate-buffered saline (PBS) supplemented with 100 μg of gentamicin (Vedco, St. Joseph, Mo.)/ml. The incubation was continued with 15% FBS–DMEM plus 100 μg of gentamicin/ml for another 60 min to kill all extracellular noninvasive bacteria.

Culture wells were then washed three times with PBS without gentamicin, and a subset was set aside for lysis (0 h); the remaining wells were incubated for an additional 4 h with 15% FBS–DMEM containing 50 μg of gentamicin/ml to allow for the intracellular growth of invasive bacteria. Infected Henle 407 cells (0 and 4 h) were lysed by incubation at 37°C with 1 ml of lysing buffer (1 mM NaH_2PO_4, 0.1% gelatin) per well for 30 min. Lysates from each well were then diluted for counting of viable cells in PBS, and 50-μl aliquots were plated on L or Aro agar as appropriate. The statistical significance of the difference between groups was determined by the Student *t* test evaluated at a *P* value of 0.025 for each of two experiments. An overall probability was obtained using Fisher's test of combined probabilities.

Serum antibody response. Three groups of 6-week-old male BALB/c mice ($n = 10$ per group) were immunized once intranasally (i.n.) with 10^{10} CFU of either serovar Typhi strain CVD 908-*htrA* or CVD 909 or PBS (control). Mice were bled on days 0 and 23 with respect to the first immunization. Serum IgG antibodies against the Vi and O antigens were determined by enzyme-linked immunosorbent assay (16). Titers in the different groups of mice were compared statistically by the Mann-Whitney test, and seroconversion rates were compared by Fisher's exact test.

Efficacy. Three new groups of 6-week-old male BALB/c mice ($n = 10$ per group) were immunized once i.n. with 10^{10} CFU of either serovar Typhi CVD 908-*htrA* or CVD 909 or PBS (control). Thirty days after primary immunization, mice were challenged with wild-type serovar Typhi strain Ty2, using the hog gastric mucin model (29, 63). Briefly, hog gastric mucin (type 1701W; Wilson Laboratories, Chicago, Ill.) was suspended in PBS at a 10% (wt/vol) concentration and autoclaved for 10 min. After autoclaving, 100 μl of this suspension was plated on L agar to check for lack of aerobic bacterial growth. Wild-type strain Ty2 was grown overnight on L agar, harvested, and suspended in 10% hog mucin at a concentration of 2.5×10^6 CFU/ml; 0.5 ml of this suspension was inoculated intraperitoneally. The survival of mice was monitored for 5 days. The statistical analysis of survival was performed by Fisher's exact test.

RESULTS

Strategy. The biogenesis and regulation of Vi polysaccharide in serovar Typhi are complex. Three widely separated chromosomal loci, *viaA* (which contains *rcsB-rcsC*) at centisome 43, *viaB* at centisome 92, and *ompB*, are responsible for the expression of the Vi antigen (2, 58, 59). The *viaB* locus, which is in all Vi-positive strains, contains the genes that encode the various enzymes necessary for the biogenesis of Vi (18, 20, 59). Intracellular synthesis of Vi is catalyzed by enzymes encoded by the serovar Typhi Vi genes *tviB*, *tviC*, *tviD*, and *tviE* (59, 61); these genes have also been referred to in the literature as Vi polysaccharide synthesis genes *vipA*, *vipB*, and *vipC* and open reading frame 4 (20). A set of Vi antigen export genes within the *viaB* locus, including *vexA*, *vexB*, *vexC*, *vexD*, and *vexE*, are responsible for the translocation of Vi and its anchoring to the bacterial surface (59). The first open reading frame of the *viaB* region, *tviA* (previously referred to as *vipR*) (19, 20), is a positive transcriptional regulator for its own expression (58, 59) as well as that of downstream genes. The TviA protein interacts in conjunction with the RcsB regulator protein at the promoter upstream of *tviA* to control the transcription of *tviB* (encoding an enzyme similar to GDP-mannose dehydrogenase involved in Vi monomer synthesis). The products of *tviC* (encoding an epimerase) and *tviD* are involved in polymerization of the Vi monomer. Two chromosomal regions, the *ompB* operon (comprising *ompR* and *envZ* [45]) and *rcsB-rcsC* (2), which are found in a number of *Enterobacteriaceae* (23), play a role in the expression of Vi in response to high osmolarity and other environmental signals.

TABLE 1. Agglutination with Vi-specific antiserum[a]

NaCl concn (M)	Agglutination of strain		
	Ty2	CVD 908-*htrA*	CVD 909
0.17	+++	+++	++++
0.3	+++	+++	+++
0.5	−	+/−	+++
0.6	−	−	+++
0.7	−	−	+++

[a] −, no agglutination; +/−, weak agglutination; +++, strong agglutination; ++++, very strong agglutination.

In order to change the expression of the Vi antigen from osmotically regulated to constitutive, we focused on the promoter of *tviA* (Fig. 1). It is thought that the products of *tviA*, *rcsB*, and perhaps *ompR-envZ* perform their regulatory action by binding the upstream region of *tviA* (58). We hypothesized that by replacing the promoter of *tviA* with the strong constitutive promoter P_{tac}, the down-regulation of *tviA* and therefore the regulation of expression of Vi in response to osmotic signals would be eliminated. P_{tac} is constitutive in *Salmonella*, since this genus lacks *lac*I.

Constitutive expression of the Vi antigen. After homologous recombination with pKT::ΔP_{tviA}*::sacB-neo*, the resulting ΔP_{tviA}*::sacB-neo* CVD 908-*htrA* derivative did not agglutinate with Vi antiserum (data not shown). This outcome resulted from the replacement of the *tviA* promoter with the *sacB-neo* cassette, thus confirming the essential role of this promoter and the role of *tviA* in the expression of the Vi antigen. Subsequently, homologous recombination of this strain with pJG14::P_{tac}-*tviA* yielded strain CVD 909, which strongly agglutinated with Vi antiserum. Whereas the expression of Vi by wild-type serovar Typhi strain Ty2 and the parent attenuated strain CVD 908-*htrA* was strongly regulated and disappeared in NaCl concentrations that were ≥0.5 M, the expression of Vi by strain CVD 909 did not diminish in the presence of increased salt concentrations (Table 1).

Tissue culture invasiveness assay. Results of the tissue culture invasion experiments are summarized in Table 2. A clear pattern was observed. Both at 0 h and after intracellular growth for 4 h, viable counts for CVD 908-*htrA* were diminished by 1 log below those of wild-type parent Ty2 and the viability of CVD 909 was diminished by 1 log below that of CVD 908-*htrA*.

Antibody responses against the Vi and O antigens. As shown in Table 3, CVD 908-*htrA* and CVD 909 elicited similar rates of seroconversion of Vi antibody (90 and 100%, respectively) and O antibody (50 and 44%, respectively). However, the geometric mean titer (GMT) of IgG anti-Vi was significantly higher in the mice that received CVD 909. In contrast, the GMTs of O antibody after immunization were quite similar in the two groups.

Effect of constitutive expression of Vi on protection. All 10 control mice succumbed to intraperitoneal challenge with wild-type serovar Typhi, as did 9 of 10 animals immunized with a single dose of CVD 908-*htrA* (Table 3). In contrast, the mor-

TABLE 2. Invasiveness of wild-type serovar Typhi strain Ty2 and vaccine strains CVD 908-*htrA* and CVD 909 for Henle 407 cells in tissue culture

Strain	Relevant phenotype	Expt	Geometric mean CFU[a] per well after Henle 407 cell lysis (SD)	
			0 h	4 h
Ty2	Wild type; regulated Vi expression	1	6.86×10^4 (1.42) b	5.03×10^5 (2.05) c
		2	4.68×10^5 (1.02) d	2.54×10^6 (1.66) e
CVD 908-*htrA*	Vaccine strain; regulated Vi expression	1	5.43×10^3 (1.04) f	1.30×10^4 (1.41) g
		2	9.03×10^3 (2.27) h	3.00×10^4 (1.04) i
CVD 909	Vaccine strain; constitutive Vi expression	1	7.52×10^2 (1.08) j	1.05×10^3 (1.39) k
		2	8.72×10^2 (1.70) l	1.08×10^3 (1.26) m

[a] Geometric mean of duplicate wells. Comparisons by Student's *t* test: b versus f, $P = 0.06$; f versus j, $P = 0.005$; d versus h, $P = 0.092$; h versus l, $P = 0.095$; c versus g, $P = 0.049$; g versus k, $P = 0.017$; e versus i, $P = 0.048$; and i versus m, $P = 0.026$. Overall probability using Fisher's test of combined probabilities: Ty2 versus CVD 908-*htrA*, $P = 0.034$ and $P = 0.017$ at 0 and 4 h, respectively; CVD 908-*htrA* versus CVD 909, $P = 0.004$ and $P = 0.004$ at 0 and 4 h, respectively.

tality among mice immunized with CVD 909 (3 of 8 [38%]) was significantly lower ($P = 0.0065$), yielding a protection rate of 62% (Table 4).

DISCUSSION

The Vi antigen was discovered in the 1930s by Felix and Pitt (8, 9), who went on to describe many of the relevant bacteriological, clinical, and epidemiological features. Using a mouse model of parenteral infection, they observed that this antigen appeared to protect the O antigen from the actions of O antibodies (8, 9, 13). The heat-inactivated, phenol-preserved whole-cell typhoid vaccine was the standard typhoid vaccine at the time. However, Felix contended that alternative chemical inactivators of serovar Typhi (e.g., alcohol) preserved the Vi capsule more efficiently and conferred greater protection in their mouse model (12). This led to alcohol-inactivated parenteral vaccine becoming the typhoid vaccine routinely used in the British military for several years. However, in a large-scale field trial in Yugoslavia initiated in the late 1950s, it was found that the protective efficacy conferred by alcohol-inactivated parenteral vaccine was lower than that provided by the classic heat-inactivated, phenolized parenteral vaccine (64). Felix (11) also first demonstrated that chronic typhoid carriers manifest high levels of serum Vi antibody.

In the 1970s, it was shown that a high yield of Vi polysaccharide largely free of lipopolysaccharide could be obtained from serovar Typhi using the detergent hexadecyltrimethylammonium bromide (46, 62), paving the way for large-scale production methods. In large-scale field trials, purified Vi polysaccharide parenteral vaccine prepared in this way was found to confer a moderate level of protection against typhoid fever (55% vaccine efficacy over 3 years of follow-up) by stimulating serum Vi antibodies (1, 25, 26). In contrast, Germanier and Furer (17), following a completely different approach, developed live oral typhoid vaccine strain Ty21a, which lacked activity of the epimerase encoded by *galE* and, in addition, was independently a Vi-negative mutant. In field trials, Ty21a was also found to confer a moderate level of protection, presumably by eliciting cell-mediated and additional immune responses other than the stimulation of Vi antibodies (31, 32, 34). Over 3 years of follow-up, 3 doses of the enteric-coated capsule formulation of Ty21a (given every other day) conferred 67% (32) and 33% (34) efficacy in two field trials, whereas the liquid formulation that is now licensed in a number of countries conferred 77% protection (34). Levine et al. (33) suggested that a much higher level of protection against typhoid fever might be achieved if the distinct immune responses stimulated by these two types of vaccines could be concomitantly elicited. A new generation of attenuated serovar Typhi vaccine strains has appeared, characterized by strains such as CVD 908, CVD 908-*htrA*, Ty800, and X4073 that are well tolerated but more immunogenic than Ty21a in eliciting serum O and H antibodies (21, 50–52, 54, 55). However, these new live oral vaccines only rarely stimulate serum Vi antibodies in subjects.

The expression of capsular polysaccharides by some *Enterobacteriaceae* is highly regulated in response to different environments, being activated by certain signals and suppressed by others. Typically, regulation is achieved by two-component sensory systems, with one gene encoding a histidine kinase sensor and another encoding a transcriptional activator protein. Vi expression by serovar Typhi is subject to regulation by at least two separate two-component systems, *rcsB-rcsC* (2) and *ompR-envZ* (45). In addition to the Vi antigen, the expression of serovar Typhi flagellin and the cell invasion-promoting Sip proteins is also modulated by the RcsB-RcsC regulatory system and osmolarity both during transcription and posttrans-

TABLE 3. Serum O and Vi antibody responses in mice immunized with a single i.n. dose of CVD 908-*htrA* or CVD 909[a]

Strain or control	Serum IgG anti-Vi			Serum IgG anti-O		
	Day 0 GMT	Day 23 GMT (range)	Frequency of seroconversion[b] (%)	Day 0 GMT	Day 23 GMT (range)	Frequency of seroconversion (%)[b,c]
CVD 908-*htrA*	10	49 a (20–160)	9/10 b (90)	31	87 (20–320)	3/6 (50)
CVD 909	10	160 d (80–640)	10/10 e (100)	32	80 (20–160)	4/9 (44)
PBS	10	10	0/10 f (0)	26	25 (20–40)	0/7 (0)

[a] a versus d, $P = 0.0007$, Mann-Whitney test; b versus f, $P < 0.001$, Fisher's exact test; e versus f, $P < 0.0001$, Fisher's exact test.
[b] Number of mice which seroconverted per total number tested.
[c] Some mice had insufficient quantities of serum remaining for testing O antibody.

TABLE 4. Efficacy of CVD 908-*htrA* and CVD 909 in protecting mice against intraperitoneal challenge with wild-type serovar Typhi suspended in hog gastric mucin, 4 weeks after a single i.n. immunization

Strain or control	Mortality[a] (%)	% Vaccine efficacy
CVD 908-*htrA*	9/10 a (90)	10
CVD 909[b]	3/8 b (38)	62
PBS	10/10 c (100)	0

[a] Number of mice that died per total number tested. a versus c, $P = 0.0043$, *Fisher's exact test*; *b versus c*, $P = 0.0065$, Fisher's exact test.
[b] Two mice allocated to this group died during handling prior to challenge.

lation (2). Under conditions of low osmolarity, the transcription of *iagA*, *invF*, and *sipB* (encoding proteins involved in cell invasion) is negatively controlled by the RcsB regulator, acting in concert with the TviA protein, which is encoded by *tviA* within the *viaB* locus. In contrast, Vi polysaccharide is preferentially expressed under the same low-osmolarity growth conditions. If the osmolarity of the medium is increased, the transcription of *iagA*, *invF*, and *sipB* is markedly increased, whereas the transcription of genes involved in Vi biosynthesis is markedly reduced (2). It was thus of interest to investigate the invasiveness of a serovar Typhi strain that constitutively expresses Vi. The expectation would be that invasiveness might be diminished somewhat. Indeed, this is what was observed, as the invasiveness of CVD 909 was 1 log below that of its isogenic parent, CVD 908-*htrA* ($P < 0.01$) (Table 2).

Similarly, the *ompR-envZ* regulatory system regulates OmpC and OmpF porins and Vi in relation to osmolarity, with the expression of Vi being suppressed at high osmolarity (45). These observations suggest that when serovar Typhi attains its protected intracellular site within macrophages or intestinal epithelial cells, signals within this ecological niche may turn off the expression of Vi. Serovar Typhi bacteria residing extracellularly in the gallbladder in conjunction with gallstones presumably find themselves in an environment where Vi expression persists. This would explain why chronic carriers manifest high titers of serum IgG Vi antibody (27, 37). By extension, these observations lead us to hypothesize that by making Vi expression constitutive in live oral vaccine strains, it may be possible to elicit serum IgG Vi antibodies, as is seen in chronic carriers (in the gallbladder) of serovar Typhi.

CVD 909, a further derivative of the promising vaccine strain CVD 908-*htrA* (54, 55), was constructed by replacing the native promoter of *tviA* with the strong constitutive promoter P_{tac}. Constitutive expression of Vi that did not turn off in the presence of a high concentration of saline was thereby achieved. In mice immunized mucosally (i.n.) with a single dose of vaccine, CVD 909 stimulated a significantly higher GMT of serum Vi antibodies than did CVD 908-*htrA*.

Although the intraperitoneal challenge of mice with serovar Typhi does not closely mimic the pathogenesis of human typhoid infection, it is nevertheless particularly sensitive in measuring the protective effect of Vi antibodies (9, 62, 63). It is thus encouraging that a single mucosal dose of CVD 909 conferred significantly greater protection than CVD 908-*htrA* (Table 4). However, the definitive test of the hypothesis must come from a proof-of-principle clinical trial in which increasing oral doses of CVD 909 vaccine are administered to groups of consenting volunteers. Such a clinical trial will be of interest from the perspective of reactogenicity and pathogenesis as well as of immunogenicity. In theory, the constitutive expression of Vi by CVD 909 could lead to short-lived vaccinemias, as was seen with CVD 908 (51). On the other hand, as a consequence of the significantly diminished tissue culture cell invasiveness of CVD 909, there may be no notable alteration in the clinical tolerability of the derivative. If, in the preliminary clinical trials, CVD 909 proves to be as well tolerated as CVD 908-*htrA*, does not cause vaccinemias, and elicits serum IgG Vi antibody in addition to the various other serous, mucosal, and cell-mediated immune responses stimulated by CVD 908-*htrA* (48, 54, 55), it will generate enthusiasm for more extensive clinical trials.

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REFERENCES

1. **Acharya, V. I., C. U. Lowe, R. Thapa, V. L. Gurubacharya, M. B. Shrestha, M. Cadoz, D. Schulz, J. Armand, D. A. Bryla, B. Trollfors, T. Cramton, R. Schneerson, and J. B. Robbins.** 1987. Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of *Salmonella typhi*. A preliminary report. N. Engl. J. Med. **317:**1101–1104.
2. **Arricau, N., D. Hermant, H. Waxin, C. Ecobichon, P. S. Duffey, and M. Y. Popoff.** 1998. The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. Mol. Microbiol. **29:**835–850.
3. **Black, R. E., M. M. Levine, C. Ferreccio, M. L. Clements, C. Lanata, J. Rooney, and R. Germanier.** 1990. Efficacy of one or two doses of Ty21a *Salmonella typhi* vaccine in enteric-coated capsules in a controlled field trial. Chilean Typhoid Committee. Vaccine **8:**81–84.
4. **Blomfield, I. C., V. Vaughn, R. F. Rest, and B. I. Eisenstein.** 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. **5:**1447–1457.
5. **Cryz, S. J., Jr., E. Fürer, L. S. Baron, K. F. Noon, F. A. Rubin, and D. J. Kopecko.** 1989. Construction and characterization of a Vi-positive variant of the *Salmonella typhi* live oral vaccine strain Ty21a. Infect. Immun. **57:**3863–3868.
6. **D'Amelio, R., A. Tagliabue, L. Nencioni, A. Di Addario, L. Villa, M. Manganaro, D. Boraschi, S. Le Moli, R. Nisini, and P. M. Matricardi.** 1988. Comparative analysis of immunological responses to oral (Ty21a) and parenteral (TAB) typhoid vaccines. Infect. Immun. **56:**2731–2735.
7. **Edwards, P., and W. Ewing.** 1972. Identification of Enterobacteriaceae. Burgess Publishing Co., Minneapolis, Minn.
8. **Felix, A., and R. Pitt.** 1934. A new antigen of *B. typhosus*. Lancet **ii:**186–191.
9. **Felix, A., and R. Pitt.** 1934. Virulence of *B. typhosus* and resistance to O antibody. J. Pathol. Bacteriol. **38:**409–420.
10. **Felix, A., and R. Pitt.** 1951. The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. J. Hyg. Camb. **49:**92–109.
11. **Felix, A.** 1938. Detection of chronic typhoid carriers by agglutination tests. Lancet **ii:**738–741.
12. **Felix, A.** 1941. New type of typhoid and paratyphoid vaccine. BMJ **i:**391–395.
13. **Felix, A., and S. S. Bhatnagar.** 1935. Further observations on the properties of the Vi antigen of *B. typhosus* and its corresponding antibody. Br. J. Exp. Pathol. **16:**422–434.
14. **Ferreccio, C., M. M. Levine, V. Solari, A. Misraji, L. Astroza, G. Berrios, and C. Pefaur.** 1990. Detección de portadores cronicos de *S. typhi*: metodo practico aplicado a manipuladores de alimentos del centro de Santiago. Rev. Med. Chile **118:**33–37.
15. **Forrest, B. D., D. J. C. Shearman, and J. T. LaBrooy.** 1990. Specific immune response in humans following rectal delivery of live typhoid vaccine. Vaccine **8:**209–211.
16. **Galen, J. E., O. G. Gomez-Duarte, G. A. Losonsky, J. L. Halpern, C. S. Lauderbaugh, S. Kaintuck, M. K. Reymann, and M. M. Levine.** 1997. A murine model of intranasal immunization to assess the immunogenicity of attenuated *Salmonella typhi* live vector vaccines in stimulating serum antibody responses to expressed foreign antigens. Vaccine **15:**700–708.
17. **Germanier, R., and E. Furer.** 1975. Isolation and characterization of galE mutant Ty21a of *Salmonella typhi*: a candidate strain for a live oral typhoid vaccine. J. Infect. Dis. **141:**553–558.
18. **Hashimoto, Y., and A. Q. Khan.** 1997. Comparison of ViaB regions of Vi-positive organisms. FEMS Microbiol. Lett. **157:**55–57.
19. **Hashimoto, Y., A. Q. Khan, and T. Ezaki.** 1996. Positive autoregulation of *vipR* expression in ViaB region-encoded Vi antigen of *Salmonella typhi*. J. Bacteriol. **178:**1430–1436.
20. **Hashimoto, Y., N. Li, H. Yokoyama, and T. Ezaki.** 1993. Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. J. Bacteriol. **175:**4456–4465.

21. **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.
22. **Hone, D. M., A. M. Harris, S. Chatfield, G. Dougan, and M. M. Levine.** 1991. Construction of genetically defined double aro mutants of *Salmonella typhi*. Vaccine **9:**810–816.
23. **Houng, H.-S. H., K. F. Noon, J. T. Ou, and L. S. Baron.** 1992. Expression of Vi antigen in *Escherichia coli* K-12: characterization of ViaB from *Citrobacter freundii* and identity of ViaA with RcsB. J. Bacteriol. **174:**5910–5915.
24. **Kantele, A.** 1990. Antibody-secreting cells in the evaluation of the immunogenicity of an oral vaccine. Vaccine **8:**321–326.
25. **Klugman, K., I. T. Gilbertson, H. J. Kornhoff, J. B. Robbins, R. Schneerson, D. Schulz, M. Cadoz, and J. Armand.** 1987. Protective activity of Vi polysaccharide vaccine against typhoid fever. Lancet **ii:**1165–1169.
26. **Klugman, K. P., H. J. Koornhof, J. B. Robbins, and N. N. Le Cam.** 1996. Immunogenicity, efficacy and serological correlate of protection of *Salmonella typhi* Vi capsular polysaccharide vaccine three years after immunization. Vaccine **14:**435–438.
27. **Lanata, C. F., M. M. Levine, C. Ristori, R. E. Black, L. Jimenez, M. Salcedo, J. Garcia, and V. Sotomayor.** 1983. Vi serology in detection of chronic *Salmonella typhi* carriers in an endemic area. Lancet **ii:**441–443.
28. **Lanata, C. F., C. Tafur, L. Benavente, E. Gotuzzo, and C. Carrillo.** 1988. Detección de portadores de *Salmonella typhi* mediante la prueba de hemaglutinación indirecta con antigeno Vi muy purificado. Bol. Oficina Sanit. Panam. **105:**283–289.
29. **Landy, M.** 1957. Studies of Vi antigen. VII. Characteristics of the immune response in the mouse. Am. J. Hyg. **65:**81–93.
30. **Lesmana, M., R. C. Rockhill, and W. R. Sanborn.** 1980. A coagglutination method for presumptive identification of Salmonella typhi. Southeast Asian J. Trop. Med. Public Health **11:**302–307.
31. **Levine, M. M., C. Ferreccio, P. Abrego, O. S. Martin, E. Ortiz, and S. Cryz.** 1999. Duration of efficacy of Ty21a, attenuated Salmonella typhi live oral vaccine. Vaccine **17**(Suppl. 2)**:**S22–S27.
32. **Levine, M. M., C. Ferreccio, R. E. Black, R. Germanier, and Chilean Typhoid Committee.** 1987. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet **i:**1049–1052.
33. **Levine, M. M., C. Ferreccio, R. E. Black, C. O. Tacket, and R. Germanier.** 1989. Progress in vaccines against typhoid fever. Rev. Infect. Dis. **11**(Suppl. 3)**:**S552–S567.
34. **Levine, M. M., C. Ferreccio, S. Cryz, and E. Ortiz.** 1990. Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in randomised controlled field trial. Lancet **336:**891–894.
35. **Levine, M. M., J. Galen, E. Barry, F. Noriega, S. Chatfield, M. Sztein, G. Dougan, and C. Tacket.** 1995. Attenuated *Salmonella* as live oral vaccines against typhoid fever and as live vectors. J. Biotechnol. **44:**193–196.
36. **Lin, F.-Y. C., J. M. Becke, C. Groves, B. P. Lim, E. Israel, E. F. Becker, R. M. Helfrich, D. S. Swetter, T. Cramton, and J. B. Robbins.** 1988. Restaurant-associated outbreak of typhoid fever in Maryland: identification of carrier facilitated by measurement of serum Vi antibodies. J. Clin. Microbiol. **26:**1194–1197.
37. **Losonsky, G. A., C. Ferreccio, K. L. Kotloff, S. Kaintuck, J. B. Robbins, and M. M. Levine.** 1987. Development and evaluation of an enzyme-linked immunosorbent assay for serum Vi antibodies for detection of chronic *Salmonella typhi* carriers. J. Clin. Microbiol. **25:**2266–2269.
38. **Murphy, J. R., S. Baqar, C. Munoz, L. Schlesinger, C. Ferreccio, A. A. Lindberg, S. Svenson, G. Losonsky, F. Koster, and M. M. Levine.** 1987. Characteristics of humoral and cellular immunity to *Salmonella typhi* in residents of typhoid-endemic and typhoid-free regions. J. Infect. Dis. **156:**1005–1009.
39. **Murphy, J. R., S. S. Wasserman, S. Baqar, L. Schlesinger, C. Ferreccio, A. A. Lindberg, and M. M. Levine.** 1989. Immunity to *Salmonella typhi*: considerations relevant to measurement of cellular immunity in typhoid-endemic regions. Clin. Exp. Immunol. **75:**228–233.
40. **Nisini, R., R. Biselli, P. M. Matricardi, A. Fattorossi, and R. D'Amelio.** 1993. Clinical and immunological response to typhoid vaccination with parenteral or oral vaccines in two groups of 30 recruits. Vaccine **11:**582–586.
41. **Nolan, C. M., J. C. Feeley, P. C. White, Jr., E. A. Hambie, S. L. Brown, and K.-H. Wong.** 1980. Evaluation of a new assay for Vi antibody in chronic carriers of *Salmonella typhi*. J. Clin. Microbiol. **12:**22–26.
42. **Noriega, F. R., G. Losonsky, C. Lauderbaugh, F. M. Liao, J. Y. Wang, and M. M. Levine.** 1996. Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a strain CVD 1205: construction, safety, immunogenicity, and potential efficacy as a mucosal vaccine. Infect. Immun. **64:**3055–3061.
43. **Noriega, F. R., G. Losonsky, J. Y. Wang, S. B. Formal, and M. M. Levine.** 1996. Further characterization of Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203 as a mucosal *Shigella* vaccine and as a live-vector vaccine for delivering antigens of enterotoxigenic *Escherichia coli*. Infect. Immun. **64:**23–27.
44. **Noriega, F. R., J. Y. Wang, G. Losonsky, D. R. Maneval, D. M. Hone, and M. M. Levine.** 1994. Construction and characterization of attenuated Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. Infect. Immun. **62:**5168–5172.
45. **Pickard, D., J. Li, M. Roberts, D. Maskell, D. Hone, M. Levine, G. Dougan, and S. Chatfield.** 1994. Characterization of defined *ompR* mutants of *Salmonella typhi: ompR* is involved in the regulation of Vi polysaccharide expression. Infect. Immun. **62:**3984–3993.
46. **Robbins, J., and J. Robbins.** 1984. Reexamination of the protective role of the capsular polysaccharide Vi antigen of *Salmonella typhi*. J. Infect. Dis. **150:**436–449.
47. **Simanjuntak, C. H., F. P. Paleologo, N. H. Punjabi, R. Darmowigoto, Soeprawoto, H. Totosudirjo, P. Haryanto, E. Suprijanto, N. D. Witham, and S. L. Hoffman.** 1991. Oral immunisation against typhoid fever in Indonesia with Ty21a vaccine. Lancet **338:**1055–1059.
48. **Sztein, M. B., S. S. Wasserman, C. O. Tacket, R. Edelman, D. Hone, A. A. Lindberg, and M. M. Levine.** 1994. Cytokine production patterns and lymphoproliferative responses in volunteers orally immunized with attenuated vaccine strains of *Salmonella typhi*. J. Infect. Dis. **170:**1508–1517.
49. **Tacket, C. O., C. Ferreccio, J. B. Robbins, C. M. Tsai, D. Schulz, M. Cadoz, A. Goudeau, and M. M. Levine.** 1986. Safety and immunogenicity of two *Salmonella typhi* Vi capsular polysaccharide vaccines. J. Infect. Dis. **154:**342–345.
50. **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.
51. **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.
52. **Tacket, C. O., S. M. Kelly, F. Schödel, G. Losonsky, J. P. Nataro, R. Edelman, M. M. Levine, and R. Curtiss III.** 1997. 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 system. Infect. Immun. **65:**3381–3385.
53. **Tacket, C. O., G. Losonsky, D. N. Taylor, L. S. Baron, D. Kopecko, S. Cryz, and M. M. Levine.** 1991. Lack of immune response to the Vi component of a Vi-positive variant of the *Salmonella typhi* live oral vaccine strain Ty21a in human studies. J. Infect. Dis. **163:**901–904.
54. **Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65:**452–456.
55. **Tacket, C. O., M. B. Sztein, S. S. Wasserman, G. Losonsky, K. L. Kotloff, T. L. Wyant, J. P. Nataro, R. Edelman, J. Perry, P. Bedford, D. Brown, S. Chatfield, G. Dougan, and M. M. Levine.** 2000. Phase 2 clinical trial of attenuated *Salmonella enterica* serovar Typhi oral live vector vaccine CVD 908-*htrA* in U.S. volunteers. Infect. Immun. **68:**1196–1201.
56. **Tagliabue, A., L. Villa, M. T. De Magistiris, M. Romano, S. Silvestri, D. Boraschji, and L. Nencioni.** 1986. IgA-driven T-cell-mediated antibacterial immunity in man after live oral Ty21a vaccine. J. Immunol. **137:**1504–1510.
57. **Tartera, C., and E. S. Metcalf.** 1993. Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. Infect. Immun. **61:**3084–3089.
58. **Virlogeux, I., H. Waxin, C. Ecobichon, J. O. Lee, and M. Y. Popoff.** 1996. Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi: rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. J. Bacteriol. **178:**1691–1698.
59. **Virlogeux, I., H. Waxin, C. Ecobichon, and M. Y. Popoff.** 1995. Role of the *viaB* locus in synthesis, transport and expression of *Salmonella typhi* Vi antigen. Microbiology **141:**3039–3047.
60. **Wahdan, M. H., C. Serie, Y. Cerisier, S. Sallam, and R. Germanier.** 1982. A controlled field trial of live *Salmonella typhi* strain Ty21a oral vaccine against typhoid: three-year results. J. Infect. Dis. **145:**292–296.
61. **Waxin, H., I. Virlogeux, S. Kolyva, and M. Y. Popoff.** 1993. Identification of six open reading frames in the *Salmonella enterica* subsp. enterica ser. Typhi *viaB* locus involved in Vi antigen production. Res. Microbiol. **144:**363–371.
62. **Wong, K. H., J. C. Feeley, R. S. Northrup, and M. E. Forlines.** 1974. Vi antigen from *Salmonella typhosa* and immunity against typhoid fever. I. Isolation and immunologic properties in animals. Infect. Immun. **9:**348–353.
63. **Wong, K. H., J. C. Feeley, and M. Pittman.** 1972. Effect of Vi-degrading enzyme on potency of typhoid vaccines in mice. J. Infect. Dis. **125:**360–366.
64. **Yugoslav Typhoid Commission.** 1962. A controlled field trial of the effectiveness of phenol and alcohol typhoid vaccines. Bull. W.H.O. **26:**357–369.

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