THE JOURNAL OF INFECTIOUS DISEASES • VOL. 136, NO. 6 • DECEMBER 1977

MAJOR ARTICLES

Evaluation of a UDP-Glucose-4-Epimeraseless Mutant of *Salmonella typhi* as a Live Oral Vaccine

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A mutant (Ty21a) of *Salmonella typhi*, which lacks the enzyme uridine 5'-diphosphate-glucose-4-epimerase, was evaluated in volunteers for use as a live attenuated oral typhoid vaccine. Five to eight doses of vaccine (containing 3–10^{10} viable organisms per dose) were given to 155 men without significant side effects. The rate of excretion of the vaccine strain in stools was low, and the majority of isolations occurred on day 1 after vaccination. Revertants able to ferment galactose were not found in any of 958 stool isolates tested. The mutant, strain Ty21a, grown in brain-heart infusion broth (BHIB) with 0.1% galactose, produces more O side chain than the same vaccine strain cultivated without galactose. Volunteers vaccinated with strain Ty21a grown in galactose and then challenged with 10^5 virulent *S. typhi* were significantly protected from disease and also had decreased stool carriage of *S. typhi* as compared with controls. Strain Ty21a grown without galactose did not provide vaccinees significant protection nor decrease fecal excretion of *S. typhi* as compared with controls. Strain Ty21a, when grown in BHIB with 0.1% galactose, results in a safe, stable, and protective oral vaccine that warrants further study in field trials.

Although injectable killed typhoid vaccine is safe and relatively effective, it does cause a significant number of unpleasant side effects such as fever, headache, malaise, and localized pain and swelling [1, 2]. Attenuated bacterial vaccines administered orally provide protection equal to or greater than that produced by killed vaccine given parenterally; moreover, oral vaccines have minimal side effects and reduce enteropathogenic fecal carriage [3–5].

Two candidates for oral typhoid vaccine have been evaluated in the volunteer model; one consisted of killed typhoid bacilli, and the other was a streptomycin-dependent mutant. The killed preparation was ineffective [3], and the streptomycin-dependent vaccine lost efficacy after lyophilization and also required the addition of streptomycin [5].

A third type of vaccine strain, not previously tested in humans, the *gal* E mutant of *Salmonella typhi*, designated Ty21a, has been found to be an effective, safe, and stable vaccine in the mouse typhoid model [6]. Ty21a mutants are rough strains that lack the enzyme uridine 5'-diphosphate (UDP)-glucose-4-epimerase. These mutant strains have incompletely developed polysaccharide chains without O antigens [7].

When mutants are grown in glucose-free medium in the presence of galactose, two opposing effects are noted. The mutant can synthesize normal lipopolysaccharide, but in so doing it lyses since toxic amounts of galactose-1-phosphate and

Received for publication January 13, 1976, and in revised form July 7, 1977.

This study was supported by Army grant no. 49-193-MD-2867 from the U.S. Department of Defense.

We acknowledge the willing participation of the volunteers at the Maryland House of Correction (Jessup, Maryland). Also, we thank the staff of the Maryland House of Correction and the State of Maryland Department of Correctional Services for their cooperation; nurses Ronald Grochowski, Alfred Murphy, Donald Haines, Steve Castor, and George Kuhta for their assistance; V. Daya and J. Klaff for technical help; R. Cash for editorial assistance; and R. Keadle and J. Barnard for help with the organization. We also thank Dr. R. Germanier (Swiss Serum Institute, Berne, Switzerland) for providing the Ty21a strain of *Salmonella typhi* and Dr. R. Edelman (National Institutes of Health, Bethesda, Maryland) for drawing our attention to H antibody and disease protection.

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UDP-galactose accumulate. Ty21a mutants therefore are strongly antigenic but have limited viability in vivo.

The purpose of this study was to test in humans, the only natural host of *S. typhi,* the safety and protective efficacy of the oral vaccine strain Ty21a.

Materials and Methods

Vaccine. The vaccine strain Ty21a was provided by Dr. R. Germanier of the Swiss Serum Institute (Berne, Switzerland). In trials 1 and 3, the vaccine strain was grown in brain-heart infusion broth (BHIB) to which galactose was added (final concentration, 0.1%) 14–16 hr prior to harvest. The presence of glucose in BHIB prevents lysis of the organisms. In contrast, inoculation of infusion broth or heart infusion broth (which lack sufficient glucose concentrations) with added galactose results in autolysis of the bacterial cells. In trial 2, vaccine was grown on brain-heart infusion agar without galactose, harvested in saline, and diluted to obtain the appropriate vaccine dose. In all trials the number of viable cells per ml was determined by pour-plate technique both before and after vaccination. When grown as described above, strain Ty21a does not possess Vi polysaccharide antigen. It does synthesize sufficient lipopolysaccharide antigen to allow agglutination with antisera to group D *Salmonella.* Typical *S. typhi* H antigen is also present.

Studies in volunteers. Volunteers were healthy adult male inmates of the Maryland House of Correction, Jessup, Md. Studies were explained in detail, and informed consent was obtained. The study protocols and consent forms were reviewed and approved by the University of Maryland Committee on Human Experimentation. No coercion whatsoever was exerted in selection of volunteers, and men were free to withdraw from the study at any time.

Experimental design. Three vaccine trials were performed. In two trials strain Ty21a was cultivated in BHI media containing 0.1% galactose; in the other trial strain Ty21a was grown in galactose-free media. This latter trial was designed in the belief that sufficient galactose was present in vivo to support the bacterial synthesis of smooth cell wall lipopolysaccharide.

All vaccines were given orally in 45 ml of milk 5 min after a solution of 2 g of $NaHCO_3$ dissolved in 60 ml of distilled water was ingested. Vaccine contained 3–10 $\times$ 10^{10} viable bacteria per dose and was administered at three- to four-day intervals over a four-week period.

Vaccine trials. In vaccine trials 1 and 3, 35 and 65 men, respectively, were given five to eight doses of vaccine cultivated with galactose. In vaccine trial 2, 56 men were given five to eight doses of vaccine grown without exogenous galactose.

Intubation. Colonization of the small intestine was evaluated in nine men in vaccine trials 2 and 3 by passage of double-lumen, small-intestinal tubes with ports at 130 cm (jejunum) and 250 cm (ileum) 6–8 hr after ingestion of the first dose of vaccine. Passage of tubes prior to vaccination resulted in colonization of the tube by the vaccine strain. Tube location was ascertained by abdominal X ray, and succus entericus was aspirated daily for two to three days.

Observation of volunteers. After oral vaccination with strain Ty21a, volunteers were observed daily, and symptoms and temperatures were recorded. Any man with intestinal or systemic complaints was seen by a physician. Stool or rectal swabs inoculated in Selenite F broth (Baltimore Biological Laboratories, Cockeysville, Md.) were obtained daily if possible for a period extending seven days beyond the last dose of vaccine.

Bacteriology. Stool and small bowel aspirates were plated directly onto salmonella-shigella agar and MacConkey's agar, and swabs were taken of the stool sample and inoculated into Selenite F broth. All swabs were incubated for 24 hr in Selenite F at 37 C and then streaked onto salmonella-shigella and MacConkey's agar.

The vaccine strain tends to grow slowly and gives a typical *S. typhi* reaction on triple-sugar-iron (TSI) agar slants (acid butt/alkaline slant/no gas) except that there is no H_2S at 24 hr. Organisms picked from TSI are readily agglutinated with antisera to group D *Salmonella.* In trials 2 and 3, after incubation for 24 hr, five lactose-negative colonies were picked and presumptively identified on TSI agar as the vaccine strain. If the lactose-negative colonies did not include

the vaccine strain, five more lactose-negative colonies were picked and identified after incubation for 48 hr. In trial 1, three lactose-negative isolates were sampled at 48 hr.

Reversion. Identification of strain Ty21a was made by the failure of organisms, identified as *S. typhi* by a standard technique, to utilize galactose [8]. This marker (inability to ferment galactose) is lost if these strains revert to the virulent form. All Ty21a isolates were inoculated into a 0.5% solution of galactose containing a phenol red indicator. In contrast to Ty21a, all virulent *S. typhi* strains ferment galactose.

Challenge. Volunteers were accepted for challenge if medical history, physical examination, complete blood cell count, urinalysis, blood chemistries including liver function tests, electrocardiogram, chest X ray, and oral cholecystogram were normal. Five to nine weeks following completion of vaccination, all available vaccinees and a comparable number of unvaccinated men serving as controls were fed 10^5 virulent *S. typhi* (Quailes strain) in 45 ml of milk.

For purposes of therapy, typhoid fever was defined as the presence of fever and a blood or stool culture positive for *S. typhi*. The patient was treated with chloramphenicol when one of the following occurred: fever of >103 F (39.4 C) for one day, fever of >101 F (38.3 C) for three consecutive days, or fever of >100 F (37.8 C) for five consecutive days.

Blood was cultured in supplemented peptone broth. Stool and rectal swabs were processed by the methods described for the vaccine strain, and suspicious isolates were identified as *S. typhi* by standard methods [9].

Serology. Sera for determinations of antibody to O, H, and Vi antigens were obtained prior to vaccination and biweekly thereafter for eight weeks; sera were also collected before challenge and biweekly for eight weeks.

Results

Analysis. Results for men in trials 1 and 3 (who received vaccine with galactose) were similar. Therefore, all of the data for these two groups have been combined.

Adverse reactions. Altogether, 756 doses of Ty21a vaccine grown in galactose and 416 doses of galactose-free vaccine were ingested by volunteers. Among all of the vaccinees, adverse reactions occurred as follows: constipation (22%), abdominal cramps (17%), diarrhea (10%), nausea (7%), vomiting (3%), anorexia (2%), and fever (1%). No individual had diarrhea for more than two days, and only one of 16 actually had more than two loose or watery stools per day. Some symptoms, e.g., vomiting, occurred in association with an upper respiratory infection but were nonetheless included in the tabulation.

All symptoms were mild, and no volunteer required medical treatment. Symptoms almost invariably occurred only after the first one or two doses of vaccine. No placebo group is available for comparison.

Excretion of vaccine virus. The rate of recovery of a detectable number of vaccine organisms from the stools of recipients was low (table 1). Vaccinees in trials 1 and 3 had a significantly greater percentage of strain Ty21a isolations after the first two vaccine doses than did those in trial 2. This finding only applied to stool specimens obtained on the first day following vaccination. By day 2, the isolation rate never exceeded 8% in either group, and by day 3, there was only a single positive culture.

The vaccine strain could not be recovered from

Table 1. Fecal excretion of strain Ty21a *Salmonella typhi* in 155 volunteers after vaccination with this strain.

Vaccine trial*	No. of men	Percentage of volunteers with fecal isolates after indicated dose			
		1 and 2	3 and 4	5 and 6	7 and 8
1 and 3	99	29 (P = 0.0008)	13 (NS)†	14 (NS)	15 (NS)
2	56	11	6	13	11

*Volunteers in trials 1 and 3 were given vaccine strain grown with galactose, and those in trial 2 were given vaccine strain grown without galactose.

†NS = not significant.

Table 2. Humoral antibody response to vaccination with strain Ty21a *Salmonella typhi.*

Vaccine trial*	No. of men	Percentage with fourfold rise in antibody to indicated antigen		
		O	H	Vi
1 and 3	96	17 ($P = 0.04$)	51 (NS)†	2 (NS)
2	53	4	60	0

NOTE. Six men had no base-line sera available and are not included in this analysis.

*Volunteers in trials 1 and 3 were given vaccine strain grown with galactose, and those in trial 2 were given vaccine strain grown without galactose.

†NS = not significant.

Table 3. Effect of prior H antibody on seroconversion of volunteers vaccinated with strain Ty21a *Salmonella typhi.*

Vaccine trial*	Seroconversion in vaccinees with indicated base-line titer of H antibody	
	<1:20	≥1:20
1 and 3	73 ($P = 0.0001$)	31
2	74 ($P = 0.01$)	33

NOTE. Data are percentages of vaccinees.

*Volunteers in trials 1 and 3 were given vaccine strain grown with galactose, and those in trial 2 were given vaccine strain grown without galactose.

small bowel aspirates (six men in trial 2 and three men in trial 3) one or more days after vaccination. The vaccine strain was isolated from stool samples of two men, one from each trial, during the period of aspiration.

Reversion. Nine hundred fifty-eight stool isolates, 316 in trial 2 (galactose-negative) and 642 in trials 1 and 3, were tested for galactose fermentation, and all were negative.

Serologic response to vaccine. The percentage of men who developed fourfold or greater responses in titer of O antibody was low in both vaccine groups. However, it was significantly greater ($P = 0.04$) in trials 1 and 3 than in trial 2 (table 2). No appreciable differences were observed in the seroconversion rates of both groups with respect to H or Vi antibody. There was no correlation between fecal excretion of strain Ty21a organisms and seroconversion with respect to titer of any of the antibodies tested.

In both vaccine groups those vaccinees who had detectable base-line titers of H antibody (≥ 1:20) were less likely to seroconvert in response to vaccination than were those whose base-line titer was undetectable (table 3). The titer of antibody to O antigen was rarely elevated prior to vaccination.

Results of challenge. Recipients of vaccine grown in galactose were significantly protected after ingestion of Quailes strain *S. typhi* from developing clinical typhoid fever as compared with control volunteers (table 4). Vaccine efficacy amounted to 87% ($P = 0.0002$). Furthermore, the same vaccinees were significantly less likely to excrete virulent *S. typhi* for four or more days after challenge than were their respective controls.

Men given Ty21a vaccine grown without galactose were half as likely to develop illness or to excrete *S. typhi* for prolonged periods than were their controls, but these differences were not statistically significant. Four of the five vaccinees in trial 2 (without galactose) who developed typhoid fever had excreted the vaccine strain on at least one occasion. Neither of the two men who

Table 4. Development of clinical disease and fecal excretion of virulent *Salmonella typhi* in controls and in recipients of *S. typhi* Ty21a vaccine.

Vaccine trial, group*	No. of men	Percentage with typhoid fever	Percentage excreting *S. typhi*	
			0–3 days	4–30 days
Trials 1 and 3				
Vaccinees	28	7 ($P = 0.0002$)	36 (NS)†	11 ($P = 0.00009$)
Controls	43	53	49	60
Trial 2				
Vaccinees	27	19 (NS)	19 (NS)	19 (NS)
Controls	21	38	24	38

*Volunteers in trials 1 and 3 were given vaccine strain grown with galactose, and those in trial 2 were given vaccine strain grown without galactose.

†NS = not significant.

developed typhoid fever and had been vaccinated with strain Ty21a with galactose had excreted the vaccine strain at any time.

Serologic response to challenge. Unvaccinated control volunteers who had preexisting detectable titers of antibody to H antigen had a significantly lower attack rate (24%) than did control volunteers without H antibody (61%) ($P = 0.02$) (table 5). However, in vaccinees the presence of H antibody was not associated with protection from typhoid fever, nor was the presence of O or Vi antibody correlated with protection of vaccinees or controls.

The development of clinical typhoid fever was paralleled by a fourfold rise in titer of O antibody in 70% of control volunteers. In contrast, none of the seven vaccinees who developed typhoid fever had a fourfold change in titer of antibody to O antigen ($P = 0.003$) (table 6). Seroconversion for H antibody was also more frequent in controls than in vaccinees. In controls, a fourfold rise in titer of O antibody was a relatively specific indicator of clinical disease; seroconversion for H antibody was less reliable since 34% of controls without illness had a fourfold rise in titer of this antibody. Seroconversion for H antibody was not, however, significantly associated with fecal excretion of virulent organisms ($P = 0.12$).

Discussion

For a live enteric vaccine to be both effective and practical, it must (*1*) be stable, (*2*) produce few adverse effects, (*3*) provide significant protection from disease, (*4*) decrease intestinal carriage of the pathogen, and (*5*) be easily produced, packaged, and distributed.

The present study evaluated *S. typhi* strain Ty21a vaccine, cultivated with and without galactose, for its ability to fulfill the first four of these five criteria. Strain Ty21a vaccine grown without galactose, although stable and safe, did not provide significant protection from disease nor decrease stool carriage of pathogens significantly. However, the same vaccine, when grown in media containing galactose, fulfilled the four criteria tested. That is, side effects were mild, biochemical and pathogenic reversion did not occur, and volunteers were not only protected from developing disease but also had decreased stool carriage of *S. typhi*. It must be pointed out that these studies were performed with up to eight doses of vaccine and challenge with *S. typhi* occurred within two months of the last vaccine dose.

The lack of protection provided by the Ty21a vaccine grown without galactose is indirect evidence supporting the importance of the O antigen in immunity to typhoid fever. Galactose added in vitro increases the amount of cell wall lipopolysaccharide produced by the bacteria [8]. This is reflected in our studies by the significantly greater number of men who had a fourfold rise in serum titer to O antigen in trials 1 and 3 than in trial 2.

The short duration of fecal excretion of the vaccine strain and the inability to culture it from the small bowel strongly suggest that strain Ty21a does not proliferate well in vivo. Fecal excretion of typhoid vaccine strains and vaccine efficacy do not appear to be directly correlated [5].

During the first three days after challenge, excretion of virulent *S. typhi* was similar in both control and vaccinated volunteers. Thereafter,

Table 5. Correlation between results of challenge with virulent *Salmonella typhi* (Quailes strain) and base-line titers of antibody to H antigen of *S. typhi* in unvaccinated control volunteers.

Clinical outcome	No. with base-line H antibody titer of	
	<1:20	≥1:20
Well	14	13
Ill	22 (61%)*	4 (24%)*

*The difference between these two values is significant at the level of $P = 0.02$.

Table 6. Seroconversion for O and H antibody titers after challenge of recipients of *Salmonella typhi* strain Ty21a vaccine and of controls with virulent *S. typhi* (Quailes strain).

Group	No. of men	Percentage with seroconversion	
		O	H
With clinical disease			
All vaccinees	7	0 ($P = 0.003$)	14 ($P = 0.05$)
All controls	30	70	63
Without clinical disease			
All vaccinees	48	0 (NS)*	8 ($P = 0.009$)
All controls	32	6	34

*NS = not significant.

effective oral vaccination decreased fecal excretion of *S. typhi* in vaccinees as compared with controls. It appears that this decrease was due to activation of a local immune process since parenteral killed vaccines, although effective in preventing disease, do not significantly decrease fecal excretion of virulent *S. typhi* in vaccinees as compared with that in control volunteers (authors' unpublished data).

Any protection from typhoid fever provided by humoral antibody is enigmatic. Prior antibody to H antigen in unvaccinated control volunteers was directly correlated with subsequent protection from challenge with virulent *S. typhi*. This effect, however, may not have been specifically due to H antibody since vaccinated volunteers with high H titers were not preferentially protected. Indeed, humoral antibodies may be quite irrelevant to immunity to typhoid fever, which could be due to local or cell-mediated phenomena.

H antibody seroconversion is inhibited in volunteers with detectable titers of antibody to H antigen prior to vaccination. This finding is not unique to typhoid but has also been observed following vaccination of children with a streptomycin-dependent shigella vaccine [10].

The inability of vaccinees who developed typhoid fever to produce a rise in titer of antibody to O antigen was an unexpected finding. In animals, oral feeding of antigen can produce tolerance to a subsequent systemic immunization [11, 12]. A similar mechanism may be operating in orally vaccinated volunteers.

The protection of 87% of vaccinees afforded by the present vaccine is the highest of any vaccines studied in our laboratory. Parenteral killed vaccines were ~70% effective against an infectious dose of virulent organisms that resulted in a 25% attack rate in controls [3].

In trials 1 and 3, 10^5 virulent typhoid bacilli constituted a 53% infectious dose in unvaccinated controls, a level that overcame any protective effect of parenteral vaccine in our earlier studies. We feel that this finding helps to demonstrate the superiority of the present oral vaccine over the available parenteral counterparts.

The clinical protection of 87% of vaccinees with the Ty21a vaccine compares favorably with the protection of 51%–94% observed in various foreign field trials utilizing parenteral vaccines [3]. The significant efficacy of the Ty21a vaccine may be further enhanced in the endemic field situation, in which it might be expected to boost natural immunity already present.

These preliminary studies with strain Ty21a vaccine are encouraging. Further studies are necessary to determine whether a decrease in the number of doses or the use of a lyophilized preparation will change vaccine efficacy.

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