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Attenuated, Streptomycin-Dependent *Salmonella typhi* Oral Vaccine: Potential Deleterious Effects of Lyophilization

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Four studies were done with streptomycin-dependent *Salmonella typhi* as an oral, attenuated vaccine. Studies 1 and 3 employed freshly harvested vaccine, whereas studies 2 and 4 involved lyophilized vaccine. Five to eight doses (3×10^{10}–10^{11} organisms/dose) were given; oral streptomycin (1.0 g) was administered concomitantly in studies 2 and 3, with only two of the doses of vaccine in study 1, and was not given in study 4. No adverse reactions were encountered in 179 vaccinated men, and 94% of the men excreted the vaccine. In challenge studies (which included the control groups) with 10^5 virulent *S. typhi* organisms (Quailes strain), the fresh vaccine was highly protective (66%–78% efficacy), while lyophilized vaccine gave no clinical protection. Fresh vaccine also interfered significantly with intestinal proliferation of virulent *S. typhi*; only 17% of the vaccinees excreted organisms as compared with 75% of the controls. Studies of protection in mice showed no difference between immunogen content of the fresh and the lyophilized vaccines. Field trials with streptomycin-dependent, oral typhoid vaccine must await development of a lyophilized product that will retain the protective properties of the vaccine.

Parenterally administered, killed, whole-cell *Salmonella typhi* vaccines give considerable protection to adults and older children in areas where typhoid is endemic [1–4]. Nevertheless, these vaccines are unsatisfactory for several reasons. (*1*) They are associated with high rates of adverse reactions (fever, local inflammation) [1, 2, 5]. (*2*) Populations in areas where the disease is not endemic are not as well protected [6–8]. (*3*) Highly infective inocula overcome the protective effect, particularly in persons from areas where typhoid is not endemic [6–8]. (*4*) Local intestinal immunity is not induced.

A safe, immunogenic, oral typhoid vaccine might eliminate many drawbacks of the currently used parenteral vaccines. Because of the poor protection afforded by oral, killed typhoid vaccine in studies with volunteers [9] and in field trials [10, 11], live, attenuated strains of *S. typhi* are being evaluated as potential immunizing agents. This paper summarizes studies of streptomycin-dependent *S. typhi* as an oral typhoid vaccine.

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Materials and Methods

Volunteers. Volunteers were healthy adult male inmates of the Maryland House of Correction, Jessup, Maryland. Studies were explained in detail, and informed consent was obtained. The study protocols and consent forms were reviewed

by the University of Maryland Committee on Human Experimentation. No coercion whatsoever was exerted in solicitation of volunteers, and the men were free to withdraw from the study at any time.

Vaccination. Four trials of vaccine efficacy were done between 1970 and 1973 with the streptomycin-dependent 27v strain of *S. typhi* [12–14]. Trials 1 and 3 involved immunization with freshly harvested streptomycin-dependent *S. typhi*, while trials 2 and 4 tested reconstituted streptomycin-dependent *S. typhi* vaccine that had been lyophilized at the Walter Reed Army Institute of Research. Two grams of $NaHCO_3$ in 8 oz of water was given 5 min before administration of the vaccine, which was given in 45 ml of milk [14]. The number of organisms per dose was quantitated by viable-colony counts (replicate pour-plate method) in streptomycin-containing media at the time of vaccination.

For trial 1 (1970), weekly doses of vaccine (3×10^{10}–10^{11} organisms/dose) were administered to 45 men for five consecutive weeks. Oral streptomycin (1.0 g) was given concomitantly with the last two doses. For trial 2 (1971), 62 men received four weekly doses (5×10^{10} organisms/dose) of vaccine, each accompanied by streptomycin (1.0 g). In the third trial (1972), 44 volunteers received five to eight doses (3×10^{10} organisms/dose) of vaccine and 1.0 g of streptomycin twice a week for four weeks. In trial 4 (1973), seven or eight doses of vaccine (two per week) were given to 25 men. The dosage schedule included 3×10^{10} (1st dose), 4×10^{10} (2nd dose), and 5×10^{10} (3rd to 8th doses) organisms. Streptomycin was not administered.

Challenge. Six weeks after 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) organisms in 45 ml of milk. The base-line health status of volunteers prior to vaccination and challenge was ascertained from medical history, physical examination, electrocardiogram, chest radiograph, blood chemistry, urinalysis, complete blood count, and oral cholecystogram.

Typhoid fever was defined as acute illness with oral temperature of $\geq$ 101 F accompanied by isolation of *S. typhi* from blood or stool. Volunteers with temperatures of $\geq$ 103 F for 24 hr or $\geq$ 101 F for 48 hr received 1 g of chloramphenicol orally every 8 hr for seven days. One week later therapy was reinstituted for five additional days. Patients in relapse received 3 g of antibiotic daily for five days.

Bacteriology and serology. Stool or rectal swab specimens were obtained daily and plated directly onto salmonella-shigella (S-S) agar with and without streptomycin (200 μg/ml). Specimens were also inoculated into Selenite broth with (vaccine studies) or without (challenge studies) streptomycin (200 μg/ml). After incubation for 18 hr at 37 C, subcultures were made onto the above media. Blood was cultured in trypticase soy broth (trials 1 and 2) or supplemented peptone broth (trials 3 and 4). Colonies thought to be *S. typhi* were identified by standard techniques [15].

Sera for determinations of antibodies to O-, H-, and Vi-antigen were obtained before vaccination and weekly thereafter for eight weeks; sera were also collected before challenge and weekly for eight weeks afterward.

Tests of potency in mice. Tests of potency were conducted in mice according to the standard procedure for comparison of the antigenic content of fresh and lyophilized streptomycin-dependent vaccine with standard vaccine from the National Institutes of Health [16]. Fresh streptomycin-dependent vaccine was prepared and diluted to the same optical density as rehydrated lyophilized vaccine. Replicate plate counts showed viable colony-forming units to number 1.24×10^{10} cfu/ml for fresh vaccine and 2.58×10^{10} cfu/ml for the lyophilized vaccine.

Results

Safety. One hundred and seventy-six men received multiple doses of streptomycin-dependent vaccine. Neither vomiting nor fever was observed; an occasional patient had mild diarrhea.

Excretion of vaccine. These studies and those previously published [14] revealed that excretion of vaccine was increased by pretreatment with $NaHCO_3$, by concomitant administration of oral streptomycin, and by large initial dosages of vaccine organisms.

The proportion of vaccinees from whom vaccine was recovered, as well as the vaccine excretion rate by week, are presented in table 1. Strep-

Table 1. Excretion of vaccine organisms after immunization of volunteers with streptomycin-dependent *Salmonella typhi* oral vaccine.

Trial (year)	Vaccine preparation	No. who excreted vaccine/no. vaccinated (%)	Percentage with fourfold rise in antibody			Excretion by week of vaccination*				
			H	O	Vi	1	2	3	4	5
1 (1970)	Fresh	45/45 (100)	54	9	12	8/35 (23)	12/43 (28)	9/45 (20)	41/45 (91)	34/43 (79)
2 (1971)	Lyophilized	61/62 (98)	38	6	32	56 (92)	51 (84)	33 (54)	25 (41)	...
3 (1972)	Fresh	18/24 (75)†	65	8	12	17† (71)	3† (13)	3† (13)	2† (8)	...
4 (1973)	Lyophilized	22/25 (88)	18	0	0	10 (40)	18 (72)	13 (52)	15 (60)	...

* For trial 1, results for each week are expressed as no. who excreted/no. tested (%). For trials 2, 3, and 4, results are no. who excreted (%).

† Forty-four men were vaccinated, but bacteriologic data were available for 24 men only.

tomycin, given to all vaccinees with the last two doses of vaccine in trial 1, was considered responsible for increased recovery of vaccine organisms after these doses. The rate of excretion by week can be compared for trials 2, 3, and 4 since vaccination procedures were consistent for all doses within each trial, except for the vaccine dosage during the first week in trial 4. The recovery of vaccine organisms clearly decreased toward the end of the vaccination period in trials 2 and 3. The differences between the excretion rate in the first and fourth weeks of both trials 2 and 3 were highly significant, as was the difference between the excretion rate in the fourth weeks of trial 2 and trial 3. A low excretion rate (40%) occurred in week 1 of trial 4, when lower doses of vaccine (3×10^{10} and 4×10^{10}) were administered than during the subsequent weeks (5×10^{10}). Although the vaccine in trial 4 was administered without streptomycin, excretion rates during weeks 2, 3, and 4 were comparable to those observed in trial 2, in which the men received streptomycin along with lyophilized vaccine.

Vaccine efficacy. Comparisons of attack rates in vaccinated and control groups are presented in table 2. The two trials with freshly harvested

Table 2. Efficacy of freshly harvested and lyophilized streptomycin-dependent oral typhoid vaccine and excretion after challenge of volunteers with 10^5 virulent *Salmonella typhi* (Quailes strain).

Trial (year)	Vaccine preparation, group	No. with typhoid fever/total no. (%)	No. with positive cultures (%)			
			Blood	Stool		
				<72 hr*	>72 hr*	Anytime*
1 (1970)	Fresh					
	Vaccinees	5/30 (17)	3 (10)	9 (30)	6 (20)	10 (33)
	Controls	13/26 (50)	13 (50)	9 (35)	18 (69)	19 (73)
2 (1971)	Lyophilized					
	Vaccinees	16/49 (33)	7 (14)	16 (33)	22 (45)	28 (57)
	Controls	10/33 (30)	9 (27)	7 (21)	13 (39)	15 (46)
3 (1972)	Fresh					
	Vaccinees	3/26 (12)	3 (12)	7 (27)	5 (19)	7 (27)
	Controls	12/22 (55)	10 (45)	9 (41)	14 (64)	17 (77)
4 (1973)	Lyophilized					
	Vaccinees	3/17 (18)	3 (18)	2 (12)	5 (29)	7 (41)
	Controls	4/16 (25)	3 (19)	3 (19)	5 (31)	6 (38)

* Time after challenge.

streptomycin-dependent *S. typhi* vaccine demonstrated highly significant protective effects of the vaccine. In contrast, the lyophilized preparations conferred no clinical protection.

Compared with that in controls, fecal excretion of virulent *S. typhi* was significantly decreased in vaccinees who received fresh vaccine (table 2). Excretion of virulent *S. typhi* by recipients of lyophilized vaccine resembled that by controls. There was little difference between the recovery of *S. typhi* from vaccinees and controls in any trial during the first 72 hr after challenge. Excretion after 72 hr was clearly diminished in the men vaccinated with fresh vaccine, but not in the men who received lyophilized vaccine.

Serology. A greater percentage of vaccinees who received fresh vaccine (40/69; 58%) developed a fourfold rise in titer of circulating H-antibody than did the men vaccinated with lyophilized vaccine (30/90; 33%; $P = 0.003$). Rises in titer of O-antibody were seldom observed in recipients of either preparation of vaccine (table 1).

No correlation existed between presence of O- or Vi-antibody and protection against clinical disease. However, a curious association was found for H-antibody. Unvaccinated control volunteers who had preexistent titers of H-antibody of $\geqq 20$ had a lower attack rate than control volunteers without H-antibody (22% vs. 48%; $P = 0.005$). In contrast, this difference in attack rate was not seen in vaccinees with (21%) or without (26%) H-antibody at the time of challenge.

Tests of potency in mice. Both fresh and lyophilized vaccines were toxic for mice at a dilution of 1:5 and when undiluted. All mice inoculated with undiluted vaccine died within 24 hr. Deaths occurred within 48 hr in 17 of 20 mice inoculated with lyophilized vaccine and in 16 of 20 mice given fresh vaccine (1:5 dilution). In this assay no difference was detected between the protection conferred by fresh or by lyophilized streptomycin-dependent vaccine.

Discussion

Freshly harvested streptomycin-dependent *S. typhi* oral vaccine exhibited many attributes of an ideal immunizing agent against typhoid fever. The preparation did not cause adverse reactions, unlike the parenteral killed vaccines, which produce local tenderness and fever [1, 2, 5], and in contrast to oral, streptomycin-dependent shigella vaccines, which cause emesis in a small proportion of individuals after the first dose [17, 18]. The freshly harvested vaccine was significantly protective against clinical typhoid fever in the face of highly infective inocula (50% and 55% infectious doses). A high degree of local, intestinal antibacterial immunity was conferred on the vaccinees; in a vaccinated population in an area where typhoid is endemic, this immunity could be expected to alter the ecology of *S. typhi* by interfering with transmission by asymptomatic excretors.

Despite the advantages of the fresh vaccine, practical and economic considerations call for a streptomycin-dependent vaccine in lyophilized form that is effective without streptomycin and that can be reconstituted in the field and given to appropriate high-risk populations, such as schoolchildren, in areas where the disease is endemic. The failure of the lyophilized vaccine to confer clinical protection, even when administered with streptomycin, was disappointing and enigmatic.

Possible confounding variables other than vaccine preparation, including number of doses, size of doses, and administration of streptomycin, were examined in the four studies. Failure of protection could not be attributed to the number of doses or to the number of organisms per dose. Administration of streptomycin, we believe, can be discounted for three reasons. (*1*) In trial 1, which involved fresh vaccine that gave excellent protection, 1 g of streptomycin was given to all volunteers only with the last two doses. (*2*) In trial 2, which involved lyophilized vaccine and gave no evidence of protection, 1 g of streptomycin was given to all volunteers with every dose. Vaccine dosage was comparable to that in trial 1, and considerable excretion of vaccine was documented; 92% of the men excreted vaccine after the first dose and 84% did so after the second dose (table 1). (*3*) Although streptomycin was not administered with lyophilized vaccine in trial 4, the rates of vaccine excretion in weeks 2–4 were comparable with those of vaccinees in trial 2 who received streptomycin with each dose of lyophilized vaccine.

We are left with the firm conviction that the lyophilization process, in some as yet unexplained

way, alters the streptomycin-dependent organisms and renders them viable but nonprotective. Clinical protection is not provided by oral, killed vaccine [9–11]. Studies of potency in mice were not helpful in detecting a loss of immunogenicity.

Gilman et al. [19], in studies using a uridine diphosphoglucose 4-epimerase-less mutant of *S. typhi* as an oral vaccine, first noted that preexistant, natural H-antibody in control volunteers was associated with protection against clinical disease, whereas vaccine-induced H-antibody did not correlate with protection. Results with the streptomycin-dependent vaccine confirmed this observation. The explanation for this paradox is not clear at present. Edsall et al. [20] and Standfast [21] have pointed out that the ability to evoke H-antibody in rabbits was the only laboratory test that correlated with clinical protection in field trials of killed, parenteral vaccine in Yugoslavia. However, among participants in the field trial, the vaccines were comparable in elicitation of H-antibody response [22]. Furthermore, clinical relapse in typhoid usually occurs when levels of H-antibody are quite high [23], and Tully and Gaines showed that H-antibody is not protective in the mouse model [24]. The significance of the protective nature of H-antibody in control volunteers is unclear. There is no animal model or laboratory test that clearly predicts *S. typhi* immunity. Field trials of the efficacy of the streptomycin-dependent *S. typhi* oral vaccine must await development of a lyophilized product that retains its protective properties.

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