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Oral Immunization of Mice with Killed *Salmonella typhimurium* Vaccine

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A study was undertaken to assess the efficacy of oral, parenteral, and intraperitoneal immunization methods of administering killed *Salmonella typhimurium* vaccine to mice and to evaluate the effectiveness of single and multiple doses of the vaccine containing varied numbers of the killed bacteria. A further objective of this study was to evaluate the effect of adding substances to the vaccine to which have been ascribed "adjuvant" properties. The protection was estimated by isolation of bacteria from the spleen and feces after oral challenge of the mice with live *S. typhimurium*. The results showed that one or more doses of 10^{10} organisms given orally led to significant protection. This rate of protection increased proportionately with the number of doses up to 10 doses, which offered 100% protection. Streptomycin, when added to multiple doses of 10^9 or more organisms given orally, increased the degree of protection, but beryllium sulfate and pertussis vaccine did not. Although multiple doses afforded similar systemic protection by all three routes of immunization, oral immunization yielded significantly greater local protection than that observed after subcutaneous or intraperitoneal immunization.

Besredka originally postulated the primary role of local immune mechanisms in protection against enteric bacterial infections over 40 years ago (2). Since then, there have been many studies pertaining to this subject (6–8, 12, 13), but, despite these, the mechanism of immunity in the intestine against oral bacterial challenge has not been elucidated.

Using the mouse as the experimental animal and oral challenge with *Salmonella typhimurium*, we undertook a series of laboratory studies to elucidate factors pertaining to the mechanism of local immunity against bacterial infection. This communication deals with a comparison of oral and parenteral immunization with the killed bacteria and with an evaluation of the effects of adding substances to which have been ascribed "adjuvant" or immune enhancement properties.

MATERIALS AND METHODS

Animals. Strain DC-1 female pathogen-free mice, weighing 18 to 22 g each (obtained from Charles River Laboratories, N. Wilmington, Mass.), were housed in air-conditioned quarters and given water and commercial pellets of complete mouse diet (Rockland Mouse Breeder Chow) ad libitum. Measures were taken to ensure that cross-infection from other mouse colonies could not occur.

Vaccines. *S. typhimurium* strain 7 (obtained from J. W. Shands, Jr., Department of Microbiology, University of Florida College of Medicine) was maintained by serial transfers on Trypticase Soy Agar (TSA) plates and was periodically inoculated and recovered from mice to maintain virulence. The bacteria were grown overnight in 10 ml of Trypticase Soy Broth (TSB) at 37 C; then 0.2 ml of this culture was streaked onto each of 10 TSA plates and incubated overnight. The surface of each plate was then washed with 10 ml of phosphate-buffered saline (PBS), *p*H 7.4, centrifuged, and washed twice; the sediment was resuspended in 10 ml of PBS. Bacterial counts were performed by the pour-plate method, and the counts were always 2×10^{10} to 8×10^{10} bacteria per ml. After this, cultures were heat-killed at 63 C for 3 hr, and 0.1 ml was streaked on TSA plates to insure the sterility of the vaccine. *Escherichia coli* strain B vaccine was prepared in an identical manner to the *S. typhimurium* vaccine. The vaccines were stored as long as 1 month at 4 C, at which time the unused part was discarded.

Immunization procedure. Mice were immunized into the stomach through a blunt needle on a syringe. Various vaccine schedules were used, as outlined below.

Challenge. Two weeks after the last dose of vaccine or placebo, the mice were challenged, at 24 hr after an oral dose of 25 mg of streptomycin, with approximately 10,000 streptomycin-resistant *S. typhimurium* organisms into the stomach (3). The organisms for challenge were grown overnight in TSB, washed once in PBS and diluted 10^{-4}. A 0.1-ml amount of the

diluted bacterial suspension was administered into the stomach of each mouse. One week later, the mice were killed and the spleens were removed and minced in 1.0 ml of PBS. In some experiments, feces were obtained from individual mice and handled similarly; 0.1 ml was streaked onto S S agar plates, the plates were incubated at 37 C for 48 hr, and the black colonies were counted. Animals were defined as "infected" if there were one or more colonies in the splenic culture. The livers were also cultured in early experiments, but culture of this organ was found not to be as sensitive an indicator of systemic infection.

RESULTS

Dose of vaccine. The number of doses of vaccine and the number of organisms per dose were varied to determine the effect these variables have on the protection rate. Administration of 10^8 organisms as many as 10 times gave no significant protection ($P > 0.1$); 10^9 organisms were protective only when given in three or more doses at weekly intervals ($P < 0.05$; Table 1). One dose of 10^{10} organisms gave significant protection, and the protection rate increased proportionately with the number of doses, up to 10 doses, which in all instances gave 100% protection. An *E. coli* vaccine, in a dose of 10^9 organisms given three times, gave no protection when compared with a water or saline control.

"Enhancement" effect. The killed *S. typhimurium* vaccine was used at various vaccine dosages with three substances to which have been ascribed "adjuvant" or "enhancing" effects: streptomycin (9), pertussis vaccine (10), and beryllium sulfate (15). The infection rate was compared with that in a group of mice receiving the same dose of vaccine without adjuvant (Table 2). Beryllium sulfate and pertussis vaccine had no adjuvant effect, as measured by increased protection against infection. Streptomycin did have a vaccine-enhancing effect, significantly increasing protection as compared to groups of animals receiving vaccine without streptomycin ($P < 0.05$). There was no significant increase in protection in the animals which received only one dose of vaccine. In the control groups, receiving no vaccine, streptomycin also decreased the infectivity rate, by 47%. With this taken into account, streptomycin significantly increased protection only in the groups receiving 10^9 organisms twice or three times, and in the group receiving 10^{10} organisms twice.

Local compared with systemic immunity. When various routes of immunization were compared, 10^{10} organisms given orally three times, 10^9 organisms given subcutaneously three times, and 10^9 organisms given intraperitoneally three times all gave equivalent protection rates, as measured by recovery of organisms from the spleens (Table 3). More than 10^9 organisms were lethal when given subcutaneously or intraperitoneally. When infection of the spleen was compared with isolation of organism from feces, it was observed that, whereas 13% of the animals immunized subcu-

TABLE 1. *Infection rates in mice immunized by various oral doses of killed S. typhimurium vaccine or placebo*

Vaccine	No. of doses of vaccine			
	1[a]	2[a]	3[a]	10[b]
10^9 Salmonellae	30/53[c] (57%)	26/52 (50%)	18/54 (33%)	—[d]
10^{10} Salmonellae	20/50 (40%)	12/57 (21%)	7/54 (13%)	0/50 (0%)
10^{10} *E. coli* cells	—	—	36/59 (61%)	—
Water	—	—	32/60 (53%)	—
Saline	—	—	41/52 (79%)	—

[a] Administered weekly.
[b] Administered daily.
[c] Number of mice infected/number challenged.
[d] Not done.

TABLE 2. *Effect of various "adjuvants" on protection afforded by oral killed S. typhimurium vaccine*

Immunization	Adjuvant			
	None	Streptomycin[a]	Pertussis[b]	$BeSO_4$[c]
10^9 Organisms				
Once	31/50[d] (62%)	23/51 (45%)	32/56 (57%)	34/50 (68%)
Twice	31/55 (56%)	6/50 (12%)	26/50 (52%)	27/53 (51%)
Three times	22/50 (44%)	8/57 (14%)	24/52 (46%)	16/53 (30%)
10^{10} Organisms				
Once	13/59 (22%)	12/55 (22%)	24/50 (48%)	19/58 (33%)
Twice	11/55 (20%)	2/58 (3%)	24/50 (48%)	13/52 (25%)
Three times	4/55 (7%)	0/51 (0%)	20/60 (33%)	8/56 (14%)
None	45/53 (85%)	23/51[e] (45%)	26/53[e] (49%)	38/53[e] (72%)

[a] The amount administered was 2.5 mg/dose of vaccine.
[b] Administered as 10^9 organisms/dose.
[c] Administered as 1.0 mg/dose.
[d] Number of mice infected/number challenged.
[e] Adjuvant alone given three times.

TABLE 3. *Comparison of route of immunization and site of infection*

Immunization	Infection	
	Intestine[a]	Spleen
10^9 Salmonellae subcutaneously, three times	31/31[b] (100%)	4/31 (13%)
10^{10} Salmonellae orally, three times	15/37 (41%)	8/37 (22%)
10^9 Salmonellae intraperitoneally, three times	—	7/39 (18%)
Nothing	31/31 (100%)	25/31 (81%)

[a] As determined by one or more positive fecal cultures.

[b] Number of mice infected/number challenged.

taneously had organisms in their spleens, 100% of the animals had organisms in their feces. By contrast, in the orally immunized animals, 22% of the spleens were infected and 41% had infected feces. Although there was no significant difference in the systemic protection rates, there was significantly greater local protection afforded by oral immunization.

DISCUSSION

The oral use of vaccines has a long history, and, despite the fact that they have not been accepted for general use, they have many practical and theoretical advantages. The practical advantage, of course, is that it is more pleasant to take a liquid or a capsule than it is to receive an injection. The theoretical advantages revolve around the concept of local mucosal immunity. IgA is the predominant immunoglobulin in external secretions, and the ability of secretory IgA antibody to protect against viral infections in the intestinal tract (11) and elsewhere (14) has been well established. The ability of secretory IgA antibody to protect against bacterial infections has been questioned. Since IgA does not fix complement in the classical manner, the question has been raised as to how secretory IgA can kill bacteria. One study indicated that secretory IgA plus complement and lysozyme can kill bacteria (1), but this observation has not been confirmed in the literature. Two recent observations have given impetus to renewed studies of oral immunization against bacterial infections: the findings that IgA can fix complement via the C'3 bypass system (5) and that IgA antibody has potent opsonic activity (9a, 17).

Oral immunization against bacterial infections in studies in which nonliving antigens were used has given mixed results. Oral immunization of human volunteers (7; Waldman et al., J. Infect. Dis., *in press*) and animals (6) with killed cholera vaccines has been shown to lead to the production of coproantibody, which in one study of humans was shown to be IgA antibody. In animal models, some studies have shown that oral immunization leads to good protection against challenge (8). Studies by Raettig et al. have shown that mice can be protected against challenge by oral immunization with *S. typhimurium* (12). Collins et al. were unable to demonstrate protection after oral immunization (4). Our studies support those of Raettig. Rauss was able to show protection in mice after oral immunization with killed *Shigella* vaccines (13).

The mechanism of the protection shown in this study is unknown, but is currently under investigation. There is evidence that the protection afforded by oral immunization is at least partly due to local (intestinal) immune mechanisms, since there was a significant reduction in intestinal infection (as measured by isolation of *S. typhimurium* from the feces) in the orally immunized, as compared to the parenterally immunized, mice. The nature of this local immunity needs further investigation, but could be a result of antibody (IgA or other class) or cell-mediated immunity. Local cell-mediated immunity, relatively independent of systemic cell-mediated immunity, has been demonstrated in the bronchopulmonary system of guinea pigs (16) and humans (Jurgensen et al., *unpublished data*).

S. typhimurium in mice was chosen as the model in our study because of its similarity to the human enteric fevers. The possibility that the analogy is a good one is attractive, since oral immunization for typhoid fever probably would be more convenient for public health officials, would be safer and more pleasant for the public, would be associated with fewer side effects, and, if the present study is any indication, would render excellent protection. Obviously, stretching analogies from mice to humans is fraught with pitfalls. The doses required were large but not unreasonable: 10^{10} organisms given three times gave excellent results and is not an impractical dose.

Three substances were given with the vaccine to determine whether they increased the vaccines effectiveness: the adjuvants beryllium sulfate and pertussis vaccine did not, whereas streptomycin did. Streptomycin was described as an "adjuvant" by Rauss in his studies on oral immunization of mice with *Shigella* (9). Those studies showed that, if the vaccines were given with streptomycin, the resulting protection rates were greater. The

present study confirms this. The mechanism is unknown, but it is conceivably caused by a decrease in certain bacterial flora, resulting in less antigenic competition. Studies are presently underway to investigate this phenomenon in two ways: by use of other antibiotics to see if they produce a similar effect, and by comparison of the coproantibody response in animals immunized with and without antibiotics.

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