Comparative Immunogenicity of Heat-Killed and Living Oral Salmonella Vaccines

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CD-1 mice were vaccinated intragastrically or intramuscularly with one or two doses of 200 μg of heat-killed Salmonella enteritidis 5694. Control mice were vaccinated with sublethal doses of living S. enteritidis Se795. The mice were challenged intragastrically with approximately 10⁶ S. enteritidis 5694 SM® 7 to 14 days later, and the growth of the challenge population in the liver, spleen, mesenteric lymph nodes, lungs, and intestine was measured quantitatively. Mice receiving two doses of heat-killed vaccine by mouth were able to delay the systemic emergence of a gastrically introduced salmonella infection by 1 to 2 days. The corresponding liver and spleen populations were slightly lower than those seen in the normal controls. On the other hand, mice receiving the living, attenuated vaccine (either intravenously or intragastrically) developed an effective anti-salmonella immunity against subsequent reinfection.

Attempts to infect mice with Pasteurella multocida and Salmonella enteritidis intragastrically suggested that bacteria introduced into the stomach with a gavage needle did not result in pulmonary involvement nor was there systemic infection via minor abrasions of the esophagus or gastric mucosa. The significance of these findings is discussed with regard to their relevance for the oral mouse protection test used for the assessment of typhoid vaccines.

Vaccinating regimens currently used in the prophylaxis of typhoid fever largely stem from the pioneering studies of Wright about the turn of the century (19). In addition to the use of killed, parenterally injected Salmonella typhi suspensions, however, a number of attempts have been made (both accidentally and intentionally) to induce immunity with living, oral vaccines; the development of severe clinical typhoid fever in some of the vaccines forced the abandonment of these early human experiments (16, 39). Recently, protection studies have been carried out in chimpanzees (12, 20, 23), in human volunteers (17, 18), and in mice (7, 27, 34) which indicate that some protection may develop when inactivated vaccines are presented to the host by mouth, although the immunity developed in a recent human field trial appeared to be minimal, even with massive vaccine doses (3). However, anti-typhoid immunity is probably never absolute, even under ideal conditions, except perhaps in the case of the permanent typhoid carrier (8). The experimental finding that C57B1 mice vaccinated with a living attenuated vaccine can still be superinfected (9) is consistent with the well-known fact that second attacks of typhoid fever can occur naturally within months of the primary infection (17, 31). Thus, even during convalescence, resistance to reinfection may decline so rapidly that second (although usually milder) attacks of the disease can occur if the infectious dose is large enough (9) or the virulence of the reinfesting strain is very high (31).

Some mouse protection studies with orally vaccinated animals have suggested that killed salmonellae can increase the level of host resistance to enteric infection (33, 35). Oral inoculation of killed vaccines (26, 34) or the direct injection of antigenic material into Peyer’s patches (11) will induce specific immunoglobulin production, with rising immunoglobulin A titers in both the systemic circulation and in the intestinal secretions (40). Humoral responses to a wide variety of nonreplicating antigens are readily observed in orally vaccinated animals (15), although the precise nature of the immunoglobulin response may be quantitatively different from that seen after parenteral injection (28, 29). Interest in the role of secretory antibodies in the expression of resistance to enteric infection (40) has thus restimulated attempts to induce acquired resistance by means of oral vaccines. As a result, protection or local antibody responses within the intestinal contents, or both, have recently been reported both in man and animals after oral vaccination.
against cholera (37), dysentery (18), and enteric fever (17, 22).

Mice infected with sublethal doses of virulent *S. enteritidis* develop progressive systemic infections which can be monitored by means of serial viable counts carried out on saline homogenates of the intestine, draining lymph nodes, liver, spleen, and blood (7). Under these conditions, "protection" can be detected either by the absence of systemic infection or as a significant change in the bacterial growth pattern in vivo (7, 8). Most mouse protection tests have employed parenterally vaccinated mice, but the present study indicates that killed oral vaccines can also marginally increase host resistance against an oral infection by *S. enteritidis*.

**MATERIALS AND METHODS**

**Organisms.** *S. enteritidis* strains 5694 and Se795 were maintained under conditions described elsewhere (4, 7). *S. enteritidis* 5694 SMR is a virulent strain resistant to 20 μg of streptomycin per ml (5). The mean lethal dose (LD50) values for the three bacterial strains are shown in Table 1.

P. multocida strain SA was obtained from M. Solty, University of Guelph, Ontario, Canada. It was grown on heart infusion agar slants (Difco, Detroit, Mich.) and held at room temperature. All dilutions of the inoculum were made in Hanks balanced salt solution enriched with 1% fetal calf serum (Gibco, Grand Island, N.Y.). Plate counts were routinely carried out on heart infusion agar. The LD50 values for this organism are recorded in Table 1.

**Animals.** Specific pathogen-free CD-1 mice (Charles River Farms, Wilmington, Mass.) were maintained, 10 to a cage, under isocaps on sterile bedding (5). They were fed sterile, vitamin-enriched mouse cubes (Agway, Waverley, N.Y.) and sterile water ad lib. Female mice weighing 18 to 25 g were used throughout.

**Vaccines.** Mice were vaccinated intragastrically with approximately 10⁷, or intravenously with 10⁶, viable *S. enteritidis* Se795. The growth of the organism in the liver, spleen, mesenteric lymph nodes, and intestines was determined at 2- or 3-day intervals (7).

Heat-killed vaccine was prepared as described elsewhere (2). After heating at 56 C for 60 min, the suspension was lyophilized without further washing. Sterility tests carried out on more than 10⁸ bacteria were negative. The required weight of dried cells was suspended in sterile saline, homogenized with a Teflon grinder (Tri-R Instruments, Rockville, N.Y.), and then delivered intragastrically in a volume of 0.2 ml of saline by means of a gavage tube. Groups of 40 mice received 200 μg of heat-killed cells; a similar dose of antigen was given 1, 2, or 4 weeks later. Other groups of mice received 200 μg of heat-killed cells suspended in saline and injected intramuscularly; 2 weeks later the same dose was repeated. The vaccinated mice, together with normal controls of the same age, were challenged intragastrically or intravenously 7 to 10 days later.

**Intragastric challenge.** Mice were infected with approximately 10⁶ viable *S. enteritidis* 5694 SMR suspended in 0.2 ml of saline. The mice were not pretreated before challenge (9). The inoculum was introduced into the stomach with a bent, gauge 19, stainless-steel 2-inch feeding needle with a smooth, 3-mm ball on the end (Popper and Sons, New York, N.Y.). The mouse was held in a vertical position, and the needle was carefully introduced into the esophagus and down into the stomach. Accidental discharge of part of the inoculum into the mouth or esophagus resulted in the regurgitation of part of the inoculum; an excess of fluid appeared in the animal's mouth and, occasionally, bubbles were seen at the nose. All such animals were discarded from the experiment.

**Intravenous challenge.** Mice were injected with 10⁶ live *S. enteritidis* 5694 SMR suspended in 0.1 ml of saline via a tail vein. The viability of the challenge inocula was checked by plating suitable saline dilutions onto Tryptose soy agar (TSA) immediately after completion of the infection process.

**Bacterial enumeration technique.** The intestine, liver, spleen, lung, and mesenteric lymph nodes from five randomly selected mice were removed aseptically and homogenized separately in saline as described elsewhere (7). The gut counts were carried out on XL agar (BBL, Cockeysville, Md.) on which the *S. enteritidis* colonies produced a black center. Bacterial counts of the other organ homogenates were made on TSA. When in doubt, colonies were checked by slide imprint.

**TABLE 1. Median lethal dose determinations for S. enteritidis and P. multocida in normal CD-1 mice**

<table>
<thead>
<tr>
<th>Infection route</th>
<th>S. enteritidis</th>
<th>P. multocida</th>
<th>P. multocida</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5694</td>
<td>5694 SMR</td>
<td>Se795</td>
</tr>
<tr>
<td>Intravenous</td>
<td>1 × 10⁴</td>
<td>6 × 10⁴</td>
<td>5 × 10⁴</td>
</tr>
<tr>
<td>Subcutaneousb</td>
<td>5 × 10⁴</td>
<td>6 × 10⁴</td>
<td>7 × 10⁴</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>1 × 10⁶</td>
<td>1 × 10⁶</td>
<td>8 × 10⁴</td>
</tr>
<tr>
<td>Aerogenic</td>
<td>&gt; 10⁻³</td>
<td></td>
<td>&lt;10⁻³</td>
</tr>
<tr>
<td>Intragastric</td>
<td>2 × 10⁷</td>
<td>2 × 10⁷</td>
<td>1 × 10⁸</td>
</tr>
</tbody>
</table>

ᵃ Equal numbers of *P. multocida* and *S. enteritidis* were mixed and immediately injected. Death occurred within 36 hr from pasteurellosis. —, Not done.

ᵇ Injected into the right hind footpad in 0.04 ml of saline.

c Death occurred in 8 to 14 days from salmonellosis.
agglutination. The relative error for the counts was similar to that reported in earlier studies (7).

Aerogenic challenge. Mice were exposed to aerosols of *P. multocida* or *S. enteritidis* for 30 min in a Middlebrook chamber (Tri-R Instruments, Rockville Center, N.Y.). Overnight broth cultures were diluted immediately prior to aerosolization to contain 10³ to 10⁴ viable organisms per ml. Viable counts on lung homogenates were made 1 hr after infection to check the inoculum size.

Virulence tests. Virulence tests were carried out as described earlier (32), and the LD₅₀ was determined by the method of Reed and Muench (36).

RESULTS

Heat-killed intragastric vaccine. Mice vaccinated with a single dose of 200 μg of heat-killed *S. enteritidis* developed a systemic *S. enteritidis* infection very similar to that seen in the normal controls (Fig. 1). Mice receiving two doses of vaccine 1 or 2 weeks apart showed some protection, judging from the slower systemic emergence of the infection and a 10-fold reduction in the maximum bacterial population developing in the liver and spleen (Fig. 1). The difference between the liver and spleen counts in the doubly vaccinated mice and that seen in the corresponding control animals was small, although it was significant at the 5% level. Oral vaccination of CD-1 mice with killed suspensions of *S. enteritidis* was never able to prevent the development of clinical disease in the challenged mice.

When intragastrically vaccinated mice were challenged intravenously, the growth rate for the *S. enteritidis* 5694 in vivo was again reduced slightly compared with that for the controls (Fig. 2), but all of the vaccinated mice were obviously ill by day 5. It is doubtful if the observed differences in the growth rates in vivo had any practical significance so far as typhoid prophylaxis is concerned. All signs of acquired resistance to either intravenous or intragastric challenge had disappeared by 4 weeks.

Heat-killed vaccine injected intramuscularly. Mice receiving two doses of 200 μg of heat-killed *S. enteritidis* 5694 were challenged intragastrically or intravenously 1 week later. The growth curves for the intravenously infected animals resembled those published elsewhere (6, 10) and have been omitted; those for the intragastrically challenged mice are shown in Fig. 3. The host response to the killed systemic vaccine delayed the emergence of the challenge infection from the intestine for a day or so; but subsequently the in vivo growth curves for both vaccinated and control mice were very similar (Fig. 3).

Live *S. enteritidis* vaccine. The killed vaccine merely slowed the development of the salmonella infection but had marginal effects on the ultimate size of the in vivo population (both effects could have some protective advantage for the host under natural conditions, however). It was decided to compare this level of protection with that routinely seen in convalescent mice. Normal mice were vaccinated intragastrically or intravenously with a single, sublethal dose of *S. enteritidis* Se795 and then were challenged intragastrically with *S. enteritidis* 5694 SM₁₁ some 10 to 20 days later. The resulting growth curves for the intragastrically vaccinated mice are shown in Fig. 4 and for the intravenously vaccinated mice are shown in Fig. 5. The living vaccine effectively protected the mice against the lethal effects of the
challeng population of *S. enteritidis* 5694 SM\(^{\text{R}}\); the oral vaccine appeared to be a little less effective than the intravenous one in preventing the emergence of a transient infection in the liver and the mesenteric lymph nodes by the streptomycin-resistant organism. This may have been due to the smaller initial systemic vaccinating population which developed when the partially attenuated strain was given by mouth. Reduction of the oral vaccine dose to 10^7 viable Se795 organisms prevented the establishment of a systemic salmonella infection in most of the mice, so that their subsequent superinfection with virulent salmonellae resulted in the general development of severe clinical disease. To be effective, an oral vaccine must apparently establish a persisting systemic infection to engage the host's cellular defenses (8). The mere persistence of viable salmonellae in the gut contents may not immunize the host against a subsequent superinfection (7, 9).

**Aerogenic infection with *S. enteritidis***. Although *S. enteritidis* is an intestinal parasite, it can also invade the tissues via the lung (13). Mice infected aerogenically with as few as 10^3 or as many as 10^6 viable *S. enteritidis* develop a progressive pulmonary infection which usually continued for about 6 days and then slowly declined. Ultimately, this infection spread to the liver and spleen but bacterial growth in these organs normally ceased before reaching lethal proportions. The aerogenically induced infection always seemed to develop more slowly than was the case for the parenterally infected animal. As a result, it was not possible to accurately estimate the aerogenic LD\(_{50}\) for *S. enteritidis* (Table 1), largely because of technical difficulties associated with the introduction of very large numbers of salmonellae into the normal lung.

When mice were infected orally with *S. enteritidis* by placing 10^7 viable organisms in the mouth (in a volume of 0.05 ml of saline) and then allowing the animals to swallow the inoculum naturally, significant numbers of salmonellae could be isolated from the lungs of some of the animals. The variation in the lung counts after 1 hr was understandably high and, in fact, the lungs of three out of five test animals in one experiment did not contain detectable numbers of *S. enteritidis*; however, the other two animals had lung counts of 1,800 and 6,700 viable *S. enteritidis*, respectively. When the same inoculum (in 0.2 ml of saline) was carefully introduced into the stomach with a gavage needle, none of the 10 test mice contained detectable numbers of *S. enteritidis* in

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**Fig. 2.** Growth of an intravenous challenge dose of *S. enteritidis* 5694 in CD-1 mice vaccinated orally with two doses of heat-killed cells. *Lr*, Liver; *Sp*, spleen. The broken line represents the normal controls.

**Fig. 3.** Growth of an intragastric challenge with *S. enteritidis* 5694 in mice vaccinated intramuscularly with two doses of heat-killed cells. See legend to Fig. 1 for details.
the lung 1 hr after challenge. The lungs remained substantially free of bacteria until the terminal stages of a lethal infection. This experiment was repeated several times with the same result.

Further confirmation of the fact that when bacteria were carefully delivered into the stomach by means of a gavage tube there was no detectable lung infection was obtained when attempts were made to recover viable bacteria from the lungs of mice infected intragastrically with $10^7$ viable *P. multocida*. *P. multocida* has extraordinary virulence for CD-1 mice, whether introduced into the tissues parenterally or aerogenically (Table 1). They are, however, paradoxically resistant to intestinal infection. Mice were infected intragastrically with a mixture of $10^7$ viable *P. multocida* and $10^2$ *S. enteritidis*; the mice invariably developed salmonellosis some 5 to 10 days later, but none of the doubly infected animals showed any sign of pasteurellosis. Control mice infected subcutaneously with one viable *P. multocida* died 18 to 36 hr later with an overwhelming septicemia. Technical problems made it difficult to determine the precise fate of the pasteurellae within the gut, but it was assumed that the organisms were rapidly inactivated in the stomach or were eliminated.

**Fig. 4.** Growth curve for *S. enteritidis* Se795 intragastric vaccine in normal mice (bottom). Growth curve for an intragastric *S. enteritidis* 5694 SMr challenge in normal controls (top left). Challenge on day 14 of the vaccinating infection (top center). Challenge on day 22 of the vaccinating infection (top right).

**Fig. 5.** Growth curve for an intravenous *S. enteritidis* Se795 vaccine in normal mice (bottom). Growth curve for *S. enteritidis* 5694 SMr in normal controls (top left). Challenge on day 8 of the vaccinating infection (top center). Challenge on day 21 of the vaccinating infection (top right).
from the intestines before a systemic infection could be established.

In another experiment, small areas of the flank skin of normal CD-1 mice were shaved (30), and a drop of *P. multocida* or *S. enteritidis* 5694 culture was rubbed onto the bare skin with a throat swab. Five of the 10 pasteurella-treated mice died within 36 hr; however, none of the survivors had culturable pasteurella in the liver or spleen at 72 hr. All of the mice swabbed with the *S. enteritidis* 5694 developed salmonellosis, with counts of $6.3 \pm 1.2 \times 10^4$ viable organisms in the spleen and $6.0 \pm 1.4 \times 10^4$ viable organisms in the liver on day 8. When the experiment was repeated with the more attenuated Sc795 strain, no organisms could be detected systematically 5 or 8 days later. Infection through such minor skin abrasions apparently depends upon the virulence of the salmonella in question.

**DISCUSSION**

Although vaccination against enteric disease has been in use for more than 70 years, few immunologists have given serious consideration to the oral route of inoculation as a practical method for the prophylaxis of typhoid fever (16). Many qualitative (and frequently inadequately controlled) oral-protection studies were reported in the early literature but few definitive conclusions can be drawn about most of them. Even in recent years, there has been an ongoing controversy over the practical effectiveness of many of these inactivated vaccines (8, 25). It is only relatively recently that carefully and adequately controlled field trials have established statistical proof of the protective value of parenterally introduced killed typhoid vaccines for man (25). Very little information of an analogous nature exists for killed, oral salmonella vaccines (3, 16). The extensive studies of Raettig and his colleagues (27, 28, 33, 34) suggest that inactivated oral vaccines have some protective value for mice; however, experimental protection studies in chimpanzees (20, 23) and in human volunteers (17) indicate that the actual level of protection afforded against a controlled *S. typhi* challenge is, at best, marginal, and in practice many such immunogens appear to be valueless (3). The results from the present study indicate that mice receiving two doses of the killed, oral vaccine showed a slight, though statistically significant ($P < 0.05$), reduction in the size of the liver and spleen populations by day 6 to 8 compared with the normal controls (Fig. 1). Such “protection” could not be assessed in terms of increased survival rates because all of the animals received sublethal challenge doses of *S. enteritidis*. It seems reasonable to suppose that the oral vaccine had induced some type of local response on the part of the host and that this was responsible, in some manner, for the slower emergence of the systemic infection. However, such an immunizing regimen was never able to prevent the salmonellae from crossing the intestinal mucosa and ultimately infecting the liver and spleen. Furthermore, the rate of growth by the liver and spleen populations in the vaccinated and control mice was not apparently affected in mice pretreated with the killed vaccine, whether given intramuscularly (Fig. 3) or intragastrically (Fig. 1). In fact, the growth pattern for the salmonella challenge in both groups of vaccinated mice was characteristic of the humorally mediated response (2, 8) rather than of that seen in convalescent animals (cf. Fig. 1 and 4).

The intragastric infection route overcomes much of the criticism levied against the intraperitoneal mouse protection test (7, 8). In particular, the oral challenge method results in a better dose response with increasing numbers of virulent salmonellae than is the case for the intraperitoneal route. In the absence of specific opsonin, the extracellular bacteria in the peritoneal cavity multiply extensively, so that an initially small inoculum can increase many thousand-fold in a few hours, making it almost impossible to infect the animals with accurately graded challenge inocula. In the case of the orally infected mouse, the challenge infection is in an intracellular environment by the time it reaches the lamina propria (38), and there appears to be little tendency on the part of the salmonellae to multiply freely within the gut contents (7). In consequence, the number of living bacteria reaching the liver and spleen seems to be roughly proportional to the size of the initial infecting population (9). Nevertheless, the size of the oral challenge dose required to kill most of the normal control mice (1, 24) makes this model equally unrealistic in terms of human disease (8). Quantitation of the in vivo behavior of sublethal doses of *S. enteritidis* in vaccinated and control mice (9) permits a more realistic evaluation of the relative immunogenicity of killed salmonella vaccines (8). However, the test system is still subject to several potential artifacts. One of the most serious of these would be the systemic entry of viable salmonellae through the lungs (13), the tonsils (14), or by direct entry into the blood stream via minor mucosal abrasions caused by the introduction of the gavage needle into the esophagus (21). Such errors seem more remote in view of the inability to infect mice with *P. multocida* by the oral route. Normal CD-1 mice are highly susceptible to aerogenic challenge with *P. multocida*. The survival of the
mice infected intragastrically with *P. multocida* argues strongly against pulmonary involvement in these animals. This is consistent with the repeated inability to isolate *S. enteritidis* from the lungs of intragastrically infected mice. On the other hand, when the infectious dose was placed in the mouth and the animal was allowed to swallow the inoculum naturally (21), bacteria frequently reached the lungs, making infection by this method more difficult to control. In addition to possible aerogenic infection hazards, infection by the use of contaminated drinking water, milk, or bread (33, 41, 42) is further complicated by difficulties in controlling the viability and size of the challenge inoculum taken in by each animal.

Infection through mucosal abrasions is the second serious potential artifact. Both *P. multocida* and *S. enteritidis* entered the body through abrasions in freshly shaved (nonbleeding) skin. This confirms the earlier report by Liu et al. (30). The subcutaneous LD50 for *P. multocida* is one organism (Table 1), so that the absence of pasteurellosis in the intragastrically challenged CD-1 mice argued strongly against the entry of even small numbers of bacteria into the tissues through mucosal abrasions brought about by passage of the gavage needle down the esophagus.

It must, therefore, be concluded that intragastric inoculation of mice by using a 2-inch gavage needle is the most suitable means for delivering a standardized inoculum of salmonellae into the mouse gastrointestinal tract. Infection of vaccinated and control animals with carefully standardized, sublethal inocula delivered into the stomach in this way represents the most natural, reproducible, and reliable method presently available for measuring the level of acquired resistance developed against an experimental enteric infection.

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LITERATURE CITED


