Immunity in Experimental Salmonellosis

I. Protection Induced by Rough Mutants of *Salmonella typhimurium*

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Different rough mutants of *Salmonella typhimurium* were tested to determine their virulence and immunizing capacity when used as live vaccines for mice. All uridine diphosphate-galactose-4-epimeraseless mutants tested were much more potent immunizing agents than any other mutants. This capacity was not correlated with virulence or complexity of cell wall polysaccharide. For good protection, persistence of the rough strains in vivo was essential, but the protection lasted longer than the period during which bacteria were demonstrable in the liver and spleen of the mice. The outstanding immunizing capacity of the "epimeraseless" mutants is not dependent on the persistence of viable bacteria in the mouse.

Although mice which have recovered from infection with *Salmonella typhimurium* are highly resistant to reinfections, almost equally good protection is provided by immunization with live vaccines prepared from attenuated smooth or rough strains (3, 6, 8, 9, 17). There developed a consensus that neither active immunization with heat-killed virulent bacteria nor passive transfer of serum from resistant mice can produce an equivalent level of resistance (1, 11). The data developed in the present investigation show that not all rough strains are of equal effectiveness as live vaccines. These data were obtained by use of mutants with a known block in the biosynthesis of cell wall lipopolysaccharide.

MATERIALS AND METHODS

Organisms. *S. typhimurium* strains TV 119, TV 148, TV 161, and LT₂M₁ investigated by Nikaido (14) and strain G 30 isolated by Osborn (15) were obtained from W. Braun. Strains SL 1032, SL 1033, and SL 3657 were received from B. A. D. Stocker (18). These strains are rough mutants of the smooth strain LT₂. Strain 395 MS and the 395 MR mutants were received from T. Holme (7, 10). The characteristics of these strains are indicated in Fig. 1 and Table 1.

Cultivation. The bacteria were grown in Brain Heart Infusion at 37°C on a rotary shaker at 142 rev/min. In all experiments, cells obtained from 6-hr-old cultures were used. The cells used for immunization or challenge were suspended in sterile physiological saline, and their concentration was adjusted in a Hitachi spectrophotometer at 250 nm. At the time of use, the viability of the bacteria was checked by plating suitable dilutions on Endo-Agar.

Mice. Initial experiments were performed with ordinary JCR Swiss white mice. However, since the immunizing organisms were eliminated only very slowly from these mice, they were subsequently replaced by female SPF mice, F₁ Charles River × BALB C of the Breeding Farm Sisseln. These mice are significantly more sensitive to infection with *S. typhimurium*, and the surviving mice eliminate the bacteria much faster. Unless otherwise stated, the results in this paper refer to experiments with these latter mice. For virulence and protection tests, mice weighing 18 to 20 g were used, and for toxicity tests, mice weighing 15 to 17 g.

Virulence test. Groups of 10 mice were injected intraperitoneally (ip) with 0.2-ml amounts of decimal dilutions of 6-hr-old cultures. Deaths were recorded during 10 days, and the LD₅₀ was calculated by the method of Reed and Muench.

Toxicity test. Log-phase bacteria were harvested by centrifugation and, without washing, were suspended in saline and killed by heat (1 hr at 58°C). The sterility of the suspension was checked by incubation of a sample in Brain Heart Infusion. For testing of toxicity, 2.0-ml amounts of serial dilutions were injected by the ip route, and deaths were recorded 72 hr later.

Immunization. Immunization was performed by ip injection of 0.2 ml of a live bacterial suspension. The concentrations were approximately 2% of one LD₅₀. Some vaccinations were also performed by the intravenous (iv), subcutaneous (sc), or oral route. For sc application the concentration was doubled, and for oral application the concentration was 1,000-fold higher than for ip injection.

Protection test. At various intervals after immunization, mice were challenged by ip injection of 1,000 LD₅₀ of the virulent strain 395 MS. To distinguish
the immunizing bacteria from the challenge bacteria, a streptomycin-resistant (100 mg of streptomycin/liter) mutant of the latter was employed. This mutant, 395 MS SMR, possessed practically the same virulence as the parent strain. Deaths were recorded 1 week after the challenge.

Enumeration of bacteria in spleen and liver. The spleen and liver of five individual mice were removed aseptically and homogenized, with the aid of a Teflon pestle, in 10 ml of Brain Heart Infusion. After decimal dilutions, 0.1-ml amounts were plated in duplicate on Endo-Agar, Endo-Agar plus 100 mg of streptomycin/liter, and on Endo-Agar in which lactose had been replaced by galactose. All homogenates were counted separately and then averaged. The lowest number of bacteria per mouse which could be detected by this procedure was 10.

RESULTS

Relationship of cell wall composition, virulence, toxicity, and immunizing capacity of the mutants. As can be seen in Table 1, a decrease in the qualitative content of cell wall lipopolysaccharides (to which we shall refer as “degradation”) caused a sharp decrease in virulence. Loss of the O-specific side chains resulted in a 10,000-fold decrease in virulence. Further degradation of the polysaccharides reduced virulence only slightly. Various strains of the same type but derived from different parent strains sometimes showed appreciable differences in this respect, thus indicating that there are other minor factors apart from cell wall structure that play a role in virulence. The reason for the high virulence of strain 395 MR 9 in comparison with that of the two other epimerase-negative mutants may be that this mutant is “leaky,” which can be assumed from the cultural properties of this strain.

Table 2 shows the pattern of mortality produced by injection of cells of a smooth strain compared with that produced by cells of the “epimeraseless” mutant G 30. Mice injected ip with the smooth strain died as early as 3 to 6 days after injection. Both the time factor and the mortality rate were related to the number of injected cells. As few as 100 cells were sufficient to kill 4 of 20 mice, an observation which reflects the capacity of smooth strain cells to multiply in vivo. The epimerase-negative strain, on the other hand, required as many as 10⁴ cells to kill 19 of 20 mice. Moreover, the pattern of mortality was quite different; deaths occurred as early as the second day after injection. This undoubtedly represented the toxic effect of the injected cells. Only 1 mouse was killed in a group of 20 treated with 10⁷ cells. Thus, it can be assumed that cells of the “epimeraseless” mutant are essentially unable to multiply in the mouse. No wild-type S. typhimurium was reisolated, even from dead mice originally infected with the “epimeraseless” mutant G 30.

The loss in virulence did not involve a loss of toxicity. The decrease in toxicity with increasing degradation of cell wall polysaccharides in the series of mutants tested was very small compared with the decrease in virulence. These results agree with those of Nakano (13).
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TABLE 1. Principal characteristics of the different Salmonella typhimurium mutants

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Designation (chemotype)</th>
<th>Characterization</th>
<th>Virulence (LD₅₀)ᵃ</th>
<th>Toxicity (LD₅₀)ᵇ</th>
<th>Mouse protection (surviving/challenged mice)ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT 2 395 MS SMR</td>
<td>Smooth</td>
<td>Complete cell wall lipopolysaccharides</td>
<td>5 × 10⁴</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>TV 119 MR 0</td>
<td>R a</td>
<td>Lacks O-specific side chains</td>
<td>3 × 10⁴</td>
<td>12</td>
<td>7/10</td>
</tr>
<tr>
<td>TV 148 TV 161 395 MR 5</td>
<td>R b 1</td>
<td>Lacks glucosamine and O-specific side chains</td>
<td>6 × 10⁴</td>
<td>16</td>
<td>5/10</td>
</tr>
<tr>
<td>SL 1033 SL 3657</td>
<td>R b 3</td>
<td>Deficient in galactosyl transferase I</td>
<td>5 × 10⁴</td>
<td>16</td>
<td>3/10</td>
</tr>
<tr>
<td>G 30 LT 2 M1 395 MR 9</td>
<td>R c</td>
<td>Deficient in uridine diphosphate-galactose-4-epimerase</td>
<td>2 × 10⁴</td>
<td>20</td>
<td>10/10</td>
</tr>
<tr>
<td>SL 1032 395 MR 10</td>
<td>R d</td>
<td>Deficient in glucosyl transferase I</td>
<td>5 × 10⁴</td>
<td>20</td>
<td>3/10</td>
</tr>
<tr>
<td>G 30 C 21</td>
<td>R e</td>
<td>Lacks heptose KDOᵈ and lipid only</td>
<td>5 × 10⁴</td>
<td>18</td>
<td>4/10</td>
</tr>
</tbody>
</table>

ᵃ Deaths recorded 1 week after ip injection of living bacteria.
ᵇ Deaths recorded 72 hr after ip injection of heat-killed bacteria.
ᶜ Immunization with 2% of LD₅₀ of living bacteria; 4 weeks later, challenge with 1,000 LD₅₀ of the streptomycin-resistant, virulent strain 395 MS SMR. Deaths recorded 1 week later. Control: no survivors among 10 mice challenged.
ᵈ 2-Keto-3-deoxy-D-mannoctulosonic acid.

TABLE 2. Difference in mortality rate in mice given live cells of the smooth strain 395 MS and the "epimeraseless" mutant G 30 of Salmonella typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of bacteria injected</th>
<th>No. of mice killed / 20 mice injected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 10</th>
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</thead>
<tbody>
<tr>
<td>395 MS</td>
<td>10⁵</td>
<td>0 10 10 8 14 18 18 20</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>10⁴</td>
<td>0 10 10 2 10 12 14 16</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>0 0 0 2 6 6 9 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>0 0 0 0 0 2 4 4</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>G 30</td>
<td>10⁶</td>
<td>8 10 10 19 16 19 19 19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>0 0 0 0 1 1 1 1</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>10⁶</td>
<td>0 0 0 0 0 0 0 0</td>
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</tbody>
</table>

ᵃ Day after injection.

Although none of the mutants has an O antigen in common with the virulent challenge strain, they all produced a certain degree of immunity against this strain in mice. The degree of protection elicited was by no means equal for all mutants, and the protective capacity of the different mutant strains was not correlated with the degree of cell wall degradation and virulence. The most remarkable degree of protection was produced by immunization with any of the three "epimeraseless" mutants.

Route of immunization and challenge. For reasons of simplicity and accuracy, the ip route was chosen for the vaccination as well as for the challenge. It must be emphasized, however, that
the very same results were obtained when the vaccination was performed by the sc, iv, or oral route (Table 3).

**Development of immunity.** It is generally accepted that vaccination with live rough strains can elicit good protection only when the bacteria are able to produce a long-lasting infection in mice (17). It has also been suggested that the level of such resistance is related to the number of residual salmonellae remaining in the tissues (1, 3).

![Graph showing the growth curve of immunizing bacterium G 30 after ip inoculation of 5 × 10⁶ cells.](image1)

![Graph showing the growth curve of challenge bacterial strain 395 MS SMR after ip inoculation of 2 × 10⁶ cells at different times after immunization.](image2)

**Fig. 2.** (top) Growth curve of immunizing bacterium G 30 after ip inoculation of 5 × 10⁶ cells. (bottom) Growth curve of challenge bacterial strain 395 MS SMR after ip inoculation of 2 × 10⁶ cells at different times after immunization. The broken line represents growth of the challenge organism in normal mice.
Thus, it was conceivable that only mice still harboring the vaccinating bacteria were fully protected. To obtain information on this possibility, a group of 200 mice were vaccinated ip with 10⁶ living cells of strain G 30. At different intervals after the vaccination, groups of five mice were killed, and the number of bacteria in their liver and spleen was determined. At the same time, a group of 10 vaccinated mice were challenged ip with 2 × 10⁸ cells of the streptomycin-resistant virulent strain 395 MS SMR. At 7 and 14 days after the challenge, the liver and spleen of individual mice were homogenized and plated on Endo-Agar with and without streptomycin and on Endo-Agar in which lactose had been replaced by galactose. Thus, the vaccinating bacteria could easily be distinguished from the challenge bacteria by their difference in galactose fermentation. This method also permitted an assessment of whether any "epimeraseless" mutant had reverted to the wild type. The results of this experiment, given in Fig. 2, indicated that the vaccinating bacteria of strain G 30 can be demonstrated in the mice up to weeks after immunization; even after this period, no reversion to the wild type could be detected. Unfortunately, in this experiment ordinary JCR Swiss white mice were used. As will be seen later, the time required for elimination of the bacteria can be greatly shortened by using spf mice and by reducing the vaccinating dose.

In the control animals, the challenge was followed by a rapid increase of the bacteria up to approximately 5 × 10⁹. Five days after the challenge, all of the control mice had died whereas all vaccinated mice survived. The protective action can be measured as soon as 24 hr after the vaccination, but, as seen from the rate of inactivation of the bacteria, it is not until the ninth day that this protection is fully developed. Between the first and the sixth days after vaccination, the increase of the challenge bacterium was greatly inhibited as compared with the control mice, but even 14 days after challenge there were still some 100 living bacteria in the liver and spleen. Mice challenged 9 days after vaccination eliminated the challenge bacteria so quickly that after 7 days they could no longer be demonstrated. This good protection lasted as long as 2 months after vaccination and 4 weeks after the last demonstration of a vaccinating bacterium in the mice.

Elimination of the challenge bacteria after vaccination with different rough mutants. As stated above, vaccination with live bacteria of all rough strains provides some protection against challenge with a virulent smooth strain (Table 1), but among the rough strains the "epimeraseless" mutants have a special position. It is only after vaccination with such mutants that protection equal to that following a sublethal injection with the virulent strain can be achieved. The question which arises next is whether this unique capacity of the "epimeraseless" mutants might be due to a better infectivity with these mutants.

![Fig. 3. Growth of attenuated immunizing strains (left) and of virulent challenge strain (right). Immunizing dose: 5% of the corresponding LD₉₀ (LT, 10⁶; TV 119, 10⁸; all other strains, 2 × 10⁶). Challenge 30 days later with 1,000 LD₉₀ of virulent strain 395 MS. The survival rate after 13 days is shown at the right.](http://iai.asm.org/)
Groups of 20 mice were vaccinated with live bacteria of different strains in concentrations equal to approximately 5% of the respective LD50. As a consequence of this more adequate dosage and the use of SPF animals, more rapid elimination of the vaccinating bacteria occurred. One month after vaccination, the mice received an ip challenge with 1,000 LD50 of the virulent strain 395 MS. As in previous experiments, the growth of the vaccinating and challenge bacteria was measured by bacterial counts. The results (Fig. 3) show once again the outstanding immunizing capacity of the "epimeraseless" mutant G 30. The protection, as indicated by the number of surviving mice, was equally good after vaccination with the smooth strain LT2 as with the rough strains TV 119, TV 148, and G 30. When the rate of elimination of the virulent challenge bacteria is taken as a criterion for the degree of immunity, then it appears that only after vaccination with the mutant G 30 is the immunity as strong as after a sublethal injection with the smooth strain. Thus, it is only strain G 30 which produces an elimination of the challenge bacteria in as short a time as 10 days. This outstanding capacity is not linked with a longer persistence of the vaccinating bacteria in the mouse, because as early as 8 days after vaccination no bacteria could be demonstrated in the spleen and liver. The only other strains to be eliminated so quickly were the poorly protecting strains SL 1032, SL 1033, and G 30-C21. Normal nonimmunized mice died within 5 days, and at that time their liver and spleen harbored 10^9 to 10^10 salmonellae.

**DISCUSSION**

It has long been known that rough mutants derived from virulent smooth strains of *Salmonella* are far less virulent. This loss of virulence is not absolute but rather represents a 10,000- to 100,000-fold decrease, depending in part on the degree of roughness, i.e., on the composition of the cell wall lipopolysaccharides. Since the toxicity of the killed cells is relatively unaffected by alteration in lipopolysaccharides, the reason for the loss of virulence is presumed to be an increased sensitivity of these mutants to phagocytosis and intracellular destruction (2, 4, 5, 12).

It is generally accepted that vaccination with live attenuated strains of *Salmonella* provides protection superior to that achieved with killed virulent strains. This difference has been explained by the fact that the bactericidal capacity of macrophages is stimulated maximally by living bacteria. This "cellular immunity" does not exclude the possibility that some protection may also be obtained by specific humoral antibodies, nor is there anything to suggest that these two types of immunity are mutually exclusive. Other authors (16, 17) have discussed the question of whether in actual fact these two mechanisms might not be working independently. The conflicting literature on this issue may be explained by the fact that attenuated smooth strains as well as rough strains have been used as live vaccines (17). Our investigation concerning important differences in the protective value of various rough strains shows that it is not merely the presence of an endotoxin-containing bacterium in the liver and spleen cells of the mouse that provides protection against an infection with a virulent bacterium. It would seem that to produce good protection a bacterium must induce a process that resembles a real infection.

Measured by the rate of elimination of the virulent challenge bacteria, the protection is fully developed 7 to 10 days after vaccination. At that time, the mouse is still carrying the vaccinating bacterium. It must be emphasized, however, that as long as 1 month after the vaccinating bacteria could be demonstrated for the last time the protection persisted in essentially undiminished form. Despite limitations imposed by a method of detection not sensitive enough to detect fewer than 10 bacteria per liver and spleen, it is reasonable to assume that the protection lasts longer than the persistence of the vaccinating bacteria in the mouse. In that event, "infection immunity" is not the correct term for this late protection. On the other hand, it can also be assumed that early protection, measured as soon as 24 hr after vaccination, is due to nonspecific immunity involving effects by the endotoxin of the vaccinating organism.

The outstanding immunizing capacity of the "epimeraseless" mutants cannot be satisfactorily explained by data developed in this study. This capacity is certainly not due to a greater infectivity of these mutants, because rough mutants with more complete cell wall lipopolysaccharides causing a longer lasting infection in mice did not produce equivalent protection. Almost as improbable is the possibility that in this mutant some structures with peculiar antigenic properties, which could also be of some importance for the virulent smooth strain, are being uncovered. It may, however, be assumed that the excellent effectiveness of the "epimeraseless" mutants is related to the manner in which the block in lipopolysaccharide synthesis is achieved. The development of postvaccination immunity appears to be very similar to a normal antibody-formation curve. However, it must be emphasized that, even if this immunity is conditioned by antibodies against the virulent smooth strain, these antibodies are not formed through a reversion of the "epimeraseless" mu-
tant to the parental smooth strain. At no time was such reversion observed in any of the numerous experiments in mice.

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LITERATURE CITED