Recall of Immunity in Mice Vaccinated with
Salmonella enteritidis or Salmonella typhimurium

FRANK M. COLLINS
Trudeau Institute, Inc., Saranac Lake, New York 12983

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Mice were immunized with living Salmonella enteritidis or S. typhimurium and then were reinjected 8, 30, 60, and 150 days later with streptomycin-resistant strains of S. enteritidis, S. typhimurium, or a mixture of the two organisms. The level of resistance at the time of challenge and the rate at which resistance was recalled in late convalescence was determined by daily liver and spleen counts of the challenge population. An immediately effective specific antibacterial immunity was maintained only while the vaccinating strain could still be detected in the liver and spleen. Reinfection of vaccine-free mice with the homologous organism caused a rapid recall of antibacterial immunity (within 3 days), but the response to the heterologous organism was much slower (5 to 8 days). Simultaneous injection of both pathogens into the vaccinated mice resulted in liver and spleen growth curves which resembled those obtained when the two organisms were administered separately. The implications of these growth studies in the development of specific cellular immunity to Salmonella infections are discussed.

Antibacterial immunity to Salmonella infections in mice has been shown to be cellular in nature, with humoral factors playing a relatively minor role against an intravenous infection (9-11). Several reports have described cross-protection between antigenically unrelated salmonellae (1, 5, 6); this phenomenon has been attributed (7) to the sharing of a common "protective" antigen. However, the "protection" observed in the latter study was assessed only from survival data, which do not reveal the true state of resistance at the time of challenge (4, 5). The vaccinated animal can only slow the initial growth rate of the challenge organism without preventing a subsequent multiplication phase equal to that of unvaccinated animals. The slower emergence of the challenge infection merely provides the animal with an opportunity to develop acquired resistance in response to the challenge infection itself (5).

It was recently shown (3) that prior infection with either Salmonella enteritidis or S. typhimurium protected mice completely against the lethal effects of an intravenous challenge with the heterologous strain; but in neither case was the level of resistance sufficient to achieve complete elimination of the heterologous organism from the tissues, despite the sharing of a common somatic antigen. This finding suggested that immunity may be independent of the immune response to the dominant somatic antigens of the organism. The present investigation examines this possibility by studying (i) the decay of the antibacterial immunity induced by the two strains administered as living vaccines and (ii) the subsequent rate of recall of antibacterial resistance when challenged with the same strains alone or in combination.

MATERIALS AND METHODS

Organisms. S. enteritidis strains Se 795 and NCTC 5694 and S. typhimurium C5 have been described previously (3). The intravenous LD_{50} for strain Se 795 was approximately 10^8 organisms, for strain 5694 it was 10, and for strain C5 cells it was 2 \times 10^8. A streptomycin-resistant mutant (resistant to 10 \mu g/ml) of S. enteritidis 5694 SM^R had an LD_{50} of approximately 500 organisms, whereas the corresponding C5 SM^R mutant (resistant to 10 \mu g/ml) had an LD_{50} of 5 \times 10^8 organisms. Both mutants were produced by the gradient plate method (12).

Media. Media and cultural conditions were similar to those described earlier (5).

Vaccines. Living vaccines of S. enteritidis Se 795 and S. typhimurium C5 were prepared and administered intravenously as previously described (5). Intravenous challenge of vaccinated and unvaccinated control mice was made with logarithmic phase cultures of S. enteritidis NCTC 5694 SM^R or S. typhimurium C5 SM^R. The viability of the inoculum was checked immediately after the injections by spreading 0.1-ml samples of suitable serial dilutions on Blood Agar Base plates (Difco). Challenge was made 8, 30, 60, and 150 days after administration of the living vaccines.
Mice. Swiss white mice from a specific pathogen-free colony of COBS mice (Charles River Farms, Inc.) were used throughout this study. Only female animals which weighed 20 to 25 g at the beginning of the experiment were used. A group of mice were set aside in a clean animal holding room housing only noninfected stock until required as nonvaccinated controls of the same age as the vaccinated mice. The mice were housed in groups of 10 in plastic cages and were fed sterilized mouse cubes and sterilized water ad lib.

Bacterial enumeration in spleen and liver. Five mice were randomly selected at daily intervals from the vaccinated and control groups. The bacterial populations in each liver and spleen homogenate were determined separately by the double plating technique described previously (9). Results were expressed as average viable counts for the five determinations. The standard error in the counts was similar to that reported in earlier papers (5, 9).

RESULTS

A group of 500 COBS mice were vaccinated intravenously with $7 \times 10^4$ viable S. enteritidis Se 795 cells; a second group of 500 mice were vaccinated with $2.1 \times 10^5$ S. typhimurium C5 cells. The average liver and spleen populations found at intervals in mice from each group are recorded in Fig. 1. There were less than 100 (the limit of the counting method) viable S. enteritidis Se 795 cells present in the livers or spleens of randomly selected mice by day 25; the S. typhimurium population did not fall below this level until day 60. Detectable numbers of vaccine organisms may have been present in individual mice later than days 25 and 60, but periodic samples taken up to the time of completion of the experiment failed to disclose their presence.

Fifty-two of the mice vaccinated with S. enteritidis and 79 of those vaccinated with S. typhimurium died, mostly in the first 14 days of the vaccination period.

Rechallenge of mice vaccinated 8 days prior to challenge. Since both S. enteritidis- and S. typhimurium-vaccinated mice still had extensive liver and spleen populations on day 8, challenge was made with virulent streptomycin-resistant strains so that the challenge population could be readily identified.

S. enteritidis-vaccinated mice rapidly eliminated a 1,000 LD$_{50}$ challenge dose of S. enteritidis SM$^R$ (Fig. 2). Those mice challenged with 1,000 LD$_{50}$ doses of S. typhimurium SM$^R$ (heterologous challenge) were able to prevent a massive increase in the challenge population (compared with the nonvaccinated controls), but were unable to eliminate the organism within 7 days. More than 1,000 streptomycin-resistant organisms were still present in the livers of mice sacrificed on day 14. Two of the spleens contained less than 100 organisms, and the other three contained an average of 250 viable organisms at this time.

Essentially the same results were obtained with S. typhimurium-vaccinated mice challenged with the two SM$^R$ strains, except that the rate of inactivation of S. typhimurium by the liver was somewhat slower (Fig. 2). At first, S. enteritidis SM$^R$ was removed more slowly than the homologous organism, but it was virtually eliminated by day 10.

Rechallenge of mice vaccinated 30 days prior to challenge. In this experiment, mice from both vaccination groups were divided into three subgroups which were challenged as follows: (i) 1,000 LD$_{50}$ doses of S. enteritidis 5694 (SM resistant); (ii) 1,000 LD$_{50}$ doses of S. typhimurium C5 (SM resistant); (iii) 1,000 LD$_{50}$ doses of S. enteritidis 5694 (SM sensitive) plus 1,000 LD$_{50}$ doses of S. typhimurium C5 (SM resistant).

Normal nonvaccinated controls were also challenged with the first two inocula.

The growth curves of the two strains in mice vaccinated with S. enteritidis or S. typhimurium are shown in Fig. 3. It should be noted once again that the homologous strains were steadily eliminated from the tissues, whereas the heterologous organisms tended to persist in significant numbers in both the liver and spleen. Of particular interest is the finding that, when the two challenge organisms were mixed and injected simultaneously, the homologous strain gradually disappeared, whereas the heterologous population did not (Fig. 3).
The *S. typhimurium* (streptomycin sensitive)-vaccinated mice still contained small numbers of organisms (1,000 or less) when they were challenged with the mixture containing more than 10⁶ streptomycin-sensitive *S. enteritidis*. The relative proportions of the two sensitive strains were determined by slide agglutination tests, carried out on at least 20 randomly selected colonies from each plate count with specific absorbed “O” grouping sera (Difco). The absence of vaccinating organisms from the *S. enteritidis* mice at day 30 made this laborious checking procedure unnecessary for these animals.

Rechallenge of mice vaccinated 60 days prior to challenge. The in vivo growth of *S. enteritidis* 5694, *S. typhimurium* C5 (SM⁵), or a mixture of the two organisms in mice vaccinated with *S. enteritidis* or with *S. typhimurium* is shown in Fig. 4. The *S. enteritidis*-vaccinated mice (no detectable vaccine organisms present in the liver and spleen since day 25) no longer exhibited the early microbicidal activity which was expressed against the homologous strain by more recently vaccinated animals. The ability to inactivate the challenge organism was rapidly reestablished by the homologous but not the heterologous strain. In the *S. typhimurium*-vaccinated mice, the vaccine had only recently been eliminated from the tissues and the animals were, for this reason, somewhat more resistant to both challenge or-
RECALL OF IMMUNITY IN MICE

S. enteritidis &
S. typhimurium.

S. enteritidis &
S. typhimurium.

S. typhimurium.

S. typhimurium.

S. enteritidis.

S. enteritidis.

log of the number of viable bacteria per organ.

log of the number of viable bacteria per organ.

Time in days.

Time in days.

Fig. 3. Intravenous challenge of mice vaccinated 30 days previously with Salmonella enteritidis Se 795 (left) or Salmonella typhimurium C5 (right). The broken line represents growth of the challenge organisms in normal mice.

organisms than were mice of the other vaccinated group (Fig. 4).

Rechallenge of mice vaccinated 150 days prior to challenge. Mice in this group were shown to contain fewer than 100 vaccinating organisms of either type in their livers or spleens for at least 100 days. Challenge of these mice was followed by extensive in vivo growth of both the homol-
FIG. 4. Intravenous challenge of mice vaccinated 60 days previously with Salmonella enteritidis Se 795 (left) or Salmonella typhimurium C5 (right). The broken line represents growth of the challenge organisms in normal mice.

ogous and the heterologous organisms, regardless of which organism had been used for vaccination (Fig. 5). In every case, homologous organisms stopped increasing in number sooner than did the heterologous strain; but resistance always developed rapidly enough to control bacterial growth, so that none of the vaccinated mice succumbed to the 1,000 LD₅₀ challenge dose of either
organism. However, when subjected to the mixed challenge, resistance towards the homologous organism appeared 2 to 3 days earlier than resistance could be detected for the other strain.

**DISCUSSION**

*S. enteritidis* and *S. typhimurium* cause clinically similar infections in mice. Although the organisms occur in different Kauffmann-White groups,
they share the antigen 0–12, and vaccination with either one will protect mice against an enormous challenge dose of the other (3). The mere presence of a common somatic antigen in the two organisms does not explain the observed degree of cross-protection, since it has been demonstrated that specific immune serum does not significantly protect against either of these Salmonella species (2–5). Jenkin and Rowley (7) proposed that heat-labile antigens (such as antigen 0–5) can stimulate the production of "protective" antibodies against S. typhimurium infection. Criticism of this data has been made elsewhere (2, 3, 5). In addition, it has not been possible to demonstrate the presence of a similar protective antigen in S. enteritidis (4).

These living organisms have been shown to produce a persistent bacteremia in normal mice in the absence of opsonic antibody (5, 9). In the present study, vaccinated mice are presumed to have produced significant levels of this antibody active against both organisms, since neither of the organisms could be detected in the blood 1 hr, or any subsequent time, after massive challenge. The presence of opsonic antibody induced by killed vaccines has been shown to influence only the early part of the in vivo growth curve (4, 5, 9); it does not affect the ultimate size reached by the challenge population in vivo. Furthermore, it has been shown that S. typhimurium immune serum is not protective for mice challenged intravenously with S. enteritidis (3). Thus, it seems most unlikely that the present protection experiments can be explained in terms of conventional humoral antibody.

Examination of the growth curves of S. enteritidis in S. enteritidis-vaccinated mice shows that resistance, sufficient to eliminate a superinfecting inoculum within 3 to 5 days, is found only in recently vaccinated animals which are still heavily infected with the immunizing strain. When reinfeeted at the end of 2 months, a challenge population in S. enteritidis-vaccinated mice was observed to increase for a time before the onset of the inactivating mechanism produced a sharp decline in the microbial population. A similar situation was later observed in S. typhimurium-vaccinated mice (Fig. 4). A rapid decay in antibacterial immunity in the absence of a persisting bacterial population is characteristic of the cellular type of immunity (3, 8, 9).

The growth data obtained for the heterologous challenge was more difficult to interpret. Vaccination by one strain protects completely against challenge by the other in terms of ultimate survival (3); but the present enumeration studies revealed that this was not an antibacterial immunity capable of completely eliminating the entire population from the tissues. The heterologous organisms failed to increase in number or multiplied for only a short time and were then slowly eliminated. Even in mice able to eliminate a challenge dose of nearly $10^6$ virulent S. enteritidis cells (as on the eighth day of vaccination), a small proportion of the S. typhimurium challenge still managed to survive in the liver and spleen for at least 14 days. Such a residuum represents less than 1% of the original inoculum, but its continued survival poses a number of interesting questions which must be answered.

The foregoing observations indicate an element of specificity in the immunity expressed against the two salmonellae, which would have escaped detection by any other method of assay. It was anticipated that, when the two strains were presented simultaneously to the vaccinated animal, the resistance recalled by the homologous organism would have also extended to the heterologous strain. That this result was not realized implies that each of the bacterial populations exerts its influence on host resistance quite independently in the doubly infected animal.

Studies with other facultative intracellular parasites (8) have shown that activated macrophages, with an enhanced capacity to kill ingested bacteria, arise during the course of a delayed hypersensitivity reaction, set off by specific microbial antigens to which the host has become sensitized. In heavily infected animals, the macrophages in all parts of the body develop enhanced microbicidal activity. In more lightly infected animals, a systemic effect on the macrophages may be absent when the spleen and liver show an obviously increased resistance to challenge. This finding implies that higher levels of resistance develop in proximity to foci of infection and may provide the basis for explaining the present observations. Since the doubly infected animal appears to have dealt independently with the two bacterial strains and to have expressed resistance much more efficiently against the homologous organism, it would appear that the two strains are not identical with respect to the antigens involved in the activation of the cellular mechanism responsible for bacterial inactivation. After intravenous challenge, both S. typhimurium and S. enteritidis are rapidly sequestered in the liver and spleen (5, 9). There the organisms multiply rapidly to establish numerous small focal lesions which gradually increase in size. If there is a lack of cross-reactivity between the two strains at the cellular level, foci containing the heterologous organisms would be expected to develop for some time in the absence of specific antigenic stimulation to the macrophages surrounding them. In the animal reinfeeted with homologous organisms, on the other hand, each site of microbial implantation would be
expected to develop a high local level of resistance, which need not extend beyond the lesion to influence bacteria implanted elsewhere.

Recent observations have demonstrated that BCG-infected mice are highly resistant to challenge with *Listeria monocytogenes*, but that their lymphoid cells, unlike those from *Listeria*-infected mice, are incapable of passively protecting recipients against challenge with this organism (8). However, the recipients of BCG-sensitized cells become resistant to the heterologous organism immediately after a simultaneous injection of BCG. This means that the antimicrobial mechanism develops during a specific immune reaction which is mediated by living lymphoid cells. If the same is true of acquired resistance to *Salmonella* infections, the present results suggest that *S. typhimurium* and *S. enteritidis* differ from each other with respect to the antigens concerned in the cellular form of the hypersensitivity which develops in response to the infection. It has recently been found that delayed or cellular hypersensitivity does develop in *Salmonella*-infected mice (Collins, unpublished data). Cross-reactivity between strains and the relation of hypersensitivity to acquired resistance in these infections are presently being investigated.

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**LITERATURE CITED**


