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Cross-Protection Against *Salmonella enteritidis* Infection in Mice

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Mice were vaccinated with six strains of *Salmonella* and two strains of *Escherichia coli*, as well as with *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Serratia marcescens*. The amount of in vivo growth of each organism was followed by viable-counting techniques on organ homogenates. The vaccinated mice, along with unvaccinated controls, were challenged intravenously with 1,000 LD_{50} of a streptomycin-resistant strain of *Salmonella enteritidis*. The ability of the vaccine to protect the mice against virulent challenge correlated with the ability of the strain to establish a persisting population in the liver and spleen. Enumeration of the liver and spleen populations in the challenged mice revealed that extensive growth of *S. enteritidis* occurred in animals which showed "protection," as assessed by progressive mortality data. No evidence was obtained for a major role of humoral factors in the cross-protection against intravenous *S. enteritidis* challenge.

The greater effectiveness of the immunity developed by living vaccines compared to that afforded by killed bacteria has been clearly established for several infections caused by facultative intracellular parasites (5, 11, 13). Earlier studies have shown that this is also true for *Salmonella enteritidis* infections in mice immunized with living and dead *S. gallinarum* vaccines (2).

In the past, claims have been made that antigenically unrelated organisms can produce cross-protection against different infections (4, 6). Jenkin and Rowley (7) reported the isolation of a "protective" antigen from *S. typhimurium*; they asserted that a number of other unrelated bacterial species, which could protect mice against virulent challenge by *S. typhimurium*, also contained this antigen. However, attempts to detect the presence of a similar type of protective antigen in *S. enteritidis* were unsuccessful (3).

In a series of earlier studies, immunity to *Salmonella* infections was shown to be cellular in nature, with antibody playing a relatively minor role (1, 2, 10). The degree of immunity to *S. enteritidis* challenge in mice vaccinated with living suspensions of the antigenically unrelated *S. montevideo* varied with the size of the residual vaccinating population (2). At the same time, an antigenically related organism, *S. pullorum*, was unable to establish a stable liver and spleen population in mice and to generate an effective antibacterial immunity against *S. enteritidis*.

The present study was designed to measure, with some precision, the degree of cross-protection against *S. enteritidis* after immunization with living vaccines of a number of unrelated gram-negative bacilli. The ability of any given organism to protect mice against a subsequent infection by the facultative intracellular parasite depended on the ability of the vaccine strain to persist in the liver and spleen in sufficient numbers to generate an adequate level of cellular immunity. Such nonspecific stimulation was sufficient to modify the growth pattern of the pathogen and thus to protect some of the mice from a lethal infection.

MATERIALS AND METHODS

Organisms. S. enteritidis NCTC 5694 was obtained from the National Collection of Type Cultures, Colindale, U.K. *S. enteritidis* strain Se 795 was described in an earlier paper (3). *S. adelaide*, *S. inverness*, and *S. seftenberg* were obtained from N. Atkinson, Microbiology Department, University of Adelaide, S. Australia. *S. typhimurium* strains C5 and M 206 were described by Jenkin et al. (8). *S. paratyphi* C (strain 159-64) was obtained from W. H. Ewing, National Communicable Disease Center, Atlanta, Ga.

Escherichia coli strains O14 and O55 were obtained from R. Mushin, Bacteriology Department, University of Melbourne, Melbourne, Australia. *Pseudomonas aeruginosa* NCTC 6750 and *Proteus vulgaris* NCTC 4175 were obtained from Colindale, U.K. *Serratia marcescens* ATCC 13880 was obtained from the American Type Culture Collection, Rockville, Md.

Media and cultural conditions were similar to those described in a previous paper (2), except that the *S. marcescens* cultures were always incubated at 30 C for 4 days to allow maximal pigmentation of the colonies.

Living vaccines were prepared and administered as described previously (2). The viability of all living vaccines and challenge bacterial suspensions was checked immediately after the injections had been completed by plating suitable 10-fold dilutions of each preparation on Blood Agar Base plates (Difco). *P. aeruginosa*, *P. vulgaris*, and *S. marcescens* could be readily distinguished from *S. enteritidis* on cultural grounds. Vaccinated and normal control mice were challenged with 1,000 LD_{50} of a streptomycin-resistant strain of *S. enteritidis* 5694 (LD_{50} equals 500 organisms by the intravenous route) 10 days after vaccination, except where indicated otherwise in the text. Mice were always challenged intravenously.

Mice. White Swiss female mice (20 to 25 g) were used throughout this study.

Bacterial enumeration in spleen, liver, and blood. For daily counts, five randomly selected mice from vaccinated and normal groups were used. Combined liver and spleen homogenates were counted by the double-plating technique described previously (10). Results are expressed as the average of the five mice. The standard deviation was similar to that reported in earlier growth studies (2). Groups of mice were always set aside to establish the progressive mortality rate in vaccinated and normal animals.

Antisera were prepared by injecting mice intravenously with three doses of 3×10^9 ethyl alcohol-killed organisms at weekly intervals. The mice were bled by heart puncture 1 week after the final injection, and the serum was pooled. The serum was sterilized by Seitz filtration and stored at −20 C. Bacteria (10^7) were mixed with a 1:5 dilution of serum, and they remained at 4 C for 30 min. The suspension was exposed to sonic vibration (Bronwill Biosonic II oscillator) for 5 sec to break up any clumps of agglutinated bacteria. After suitable dilution in sterile saline, the viability of the opsonized bacteria was determined before injection into mice by the intravenous, intraperitoneal, or subcutaneous routes. The LD_{50} was established by the method of Reed and Muench (12).

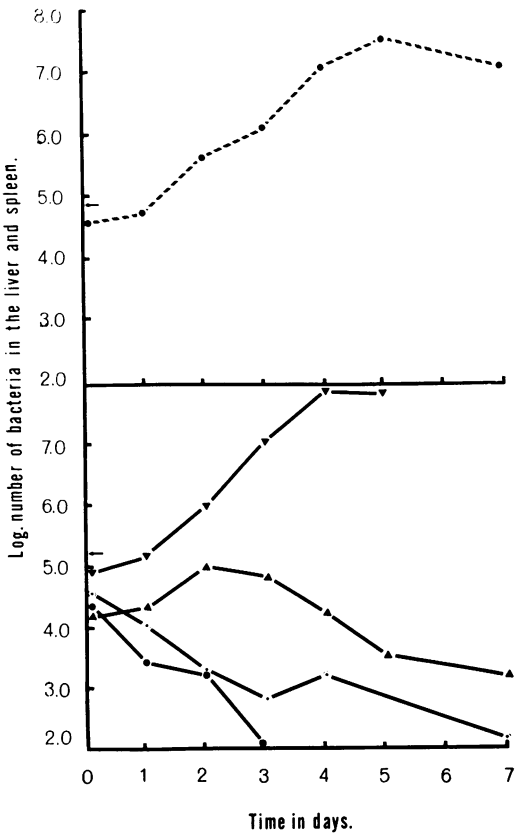

FIG. 1. *(Top) Growth curve for Salmonella enteritidis Se 795 inoculated intravenously in normal mice. Each point represents the average for five randomly selected mice. The arrow represents the size of the challenge population in each experiment. (Bottom) Intravenous challenge of S. enteritidis Se 795 vaccinated mice at increasing time periods. S. enteritidis 5694, streptomycin-resistant (10 μg/ml), was used for the challenge. Symbols: ●, 14-day vaccinated mice; •, 30 day; ▲, 90 day. Unvaccinated control mice, ▼. All mice in the latter group were dead by day 5.*

RESULTS

S. enteritidis-vaccinated mice. Intravenous injection of 9.0×10^4 *S. enteritidis* Se 795 cells into 400 mice produced the growth pattern shown in Fig. 1. Challenge of the 164 surviving mice with 1,000 LD_{50} of streptomycin-resistant *S. enteritidis* cells at 14 days, 30 days, and 90 days showed that the vaccine conferred complete protection on the mice (Table 1). The liver and spleen counts illustrated that the challenge organisms were not eliminated immediately or completely from the 30- and 90-day mice (Fig. 1); however, the overwhelming increase in numbers observed in normal controls was entirely averted. On the average, after 30 days, the vaccinated mice contained fewer than 1,000 viable bacteria in their livers and spleens; two of the five mice tested at this time contained fewer than 100 organisms (the lowest number detectable by the counting methods used in these tests). By 90 days, no residual vaccine organisms could be demonstrated in the livers and spleens of sacrificed mice.

This immunity was apparently caused by cellular factors because of the inability of immune sera to protect mice significantly against challenge by *S. enteritidis* (Table 2). When bacteria were opsonized with serum obtained from animals vaccinated with either living or killed *S. enteritidis*, the LD_{50} after intravenous or intraperitoneal challenge was not significantly altered. Injection of immune serum into intravenously challenged

mice with opsonized *S. typhimurium* had no effect on the growth of the virulent organisms in the livers and spleens of the recipient mice (10). Similarly, injection of 0.5 ml of serum obtained from mice immunized with living *S. enteritidis* failed to affect the in vivo growth of an intravenous challenge of virulent *S. enteritidis* (Collins, *unpublished data*). Blanden et al. (1) showed that immune serum had protective value against *S. typhimurium* infections only when the intraperitoneal route of challenge was employed, presumably because the opsonic antibody increased phagocytosis so that the organisms were removed from the extracellular environment before extensive multiplication in the peritoneal cavity could occur. *S. enteritidis* seemed almost as virulent when given intravenously as when injected intraperitoneally; thus, the inability of serum to protect against intraperitoneal challenge is hardly surprising.

S. typhimurium-vaccinated mice. Intravenous injection of 1,000 *S. typhimurium* C5 cells (approximately 0.5 LD_{50}) into 200 mice produced the growth pattern shown in Fig. 2. The 110 survivors were challenged with 1,000 LD_{50} of streptomycin-resistant *S. enteritidis* cells 30 days later. Protection against the virulent challenge was very high (Table 1). The liver and spleen populations of the challenge organisms (Fig. 2) revealed that, although *S. enteritidis* was almost completely prevented from multiplying in the tissues, it was only gradually eliminated from the liver and spleen. The avirulent strain of *S. typhimurium* (M 206) gave virtually no cross-protection in animals challenged after 30 days (Table 1).

TABLE 1. *Progressive mortality figures for vaccinated and control mice challenged with 1,000 LD_{50} of Salmonella enteritidis 5694*

Vaccine strain	Time (days)									
	5	6	7	8	9	10	11	12	14	28
S. enteritidis Se 795[a]	0	0	0	0	0	0	0	0	0	0/20[b]
S. typhimurium C5	0	1	1	1	1	1	1	1	1	1/25
S. typhimurium M206	3	8	17	18	19	19	19	19	19	19/20
S. paratyphi C	0	0	1	3	3	3	6	7	7	7/20
S. inverness	2	6	7	8	9	9	9	9	9	9/20
S. adelaide	2	9	21	29	29	33	33	33	33	33/40
S. seftenberg	2	3	6	6	8	8	8	9	9	9/40
Escherichia coli O55	11	16	20	20	21	22	24	29	33	33/40
Pseudomonas aeruginosa	8	10	14	19	19	19	19	19	19	19/20
Serratia marcescens	1	6	8	12	14	18	18	18	18	20/20
Proteus vulgaris	2	5	8	13	13	15	19	19	19	19/20
Control	9	11	12	12	14	15	15	15	15	15/15

[a] When challenged 14 and 90 days after vaccination, identical results were observed.
[b] Dead/total.

TABLE 2. *Determinations of the LD_{50} for a streptomycin-sensitive strain of Salmonella enteritidis NCTC 5694 after intravenous, subcutaneous, or intraperitoneal challenge*

Prepn	LD_{50}		
	Intraperitoneal	Intravenous	Subcutaneous
Unopsonized bacteria	2	10	4
Unopsonized bacteria in ethyl alcohol-killed *S. enteritidis*-vaccinated mice	80	50	—
Bacteria opsonized with ethyl alcohol-killed *S. enteritidis* antiserum	65	20	3
Bacteria opsonized with convalescent mouse serum (living *S. enteritidis* vaccine)	20	20	—
Opsonized with *S. adelaide* antiserum	5	10	—
Opsonized with *S. typhimurium* antiserum	—	20	—
Opsonized with pig serum (1/5)	20	10	—

S. paratyphi C-vaccinated mice. Vaccination of mice with 9×10^5 *S. paratyphi* C cells resulted in the establishment of stable liver and spleen populations for a period of at least 7 days (Fig. 3). However, this strain was nonlethal for mice in doses of as high as 10^7 organisms injected intravenously. Challenge of the vaccinated mice on day 10 showed increased survival compared with the controls (Table 1), but the liver and spleen counts demonstrated extensive in vivo growth of the challange organism after an initial 3-day lag (Fig. 3).

S. seftenberg-vaccinated mice. After injection of

5×10^6 living *S. seftenberg* cells, the organism was almost completely eliminated within 2 days. A phase of limited regrowth then occurred (Fig. 4). Challenge of these mice on day 10 with 1,000 LD_{50} of *S. enteritidis* cells showed a 1- to 2-day lag, followed by extensive growth of the challenge organism (Fig. 4). However, a considerable number of the challenged mice survived despite extensive multiplication of the challenge organism (Table 1).

S. adelaide-vaccinated mice. The strain used in this study showed little ability to multiply in normal mice (Fig. 5). On an average, only about 10^3 organisms survived in livers and spleens after 10 days. Challenge of these mice with 1,000 LD_{50} of *S. enteritidis* cells revealed that little cross-protection was afforded by this vaccinating organism (Table 1); thus, rapid and extensive in vivo growth occurred (Fig. 5), and most of the vaccinated mice died.

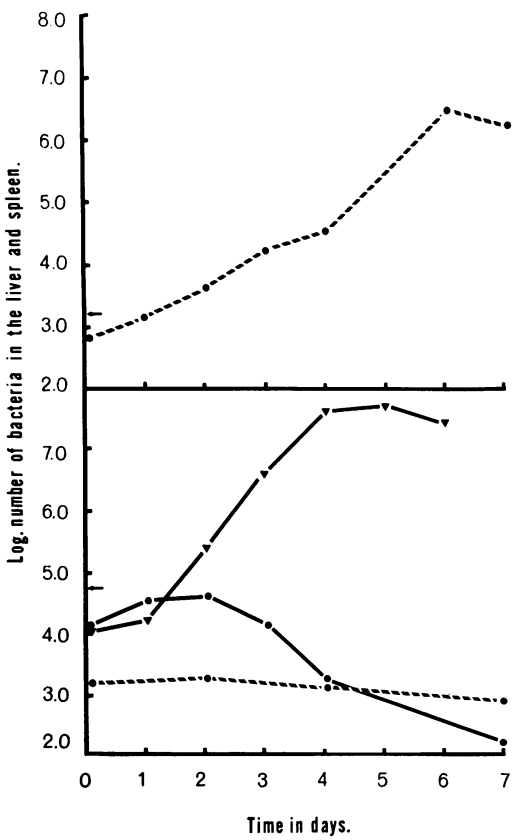

FIG. 2. *(Top) Growth curve for Salmonella typhimurium C5 inoculated intravenously in normal mice. (Bottom) Intravenous challenge of S. typhimurium C5 vaccinated mice with S. enteritidis 5694, streptomycin-resistant,* ●. *Residual S. typhimurium C5 are shown by the broken line. Unvaccinated controls,* ▼.

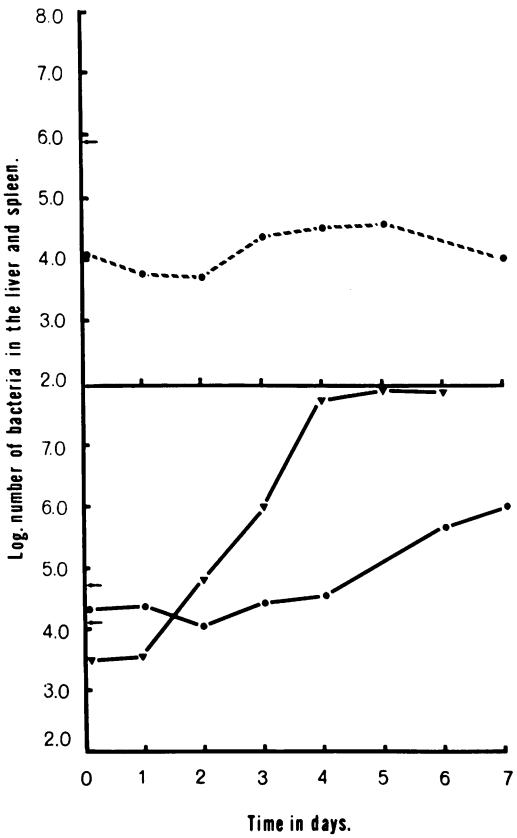

FIG. 3. *(Top) Growth curve for Salmonella paratyphi C inoculated intravenously in normal mice. (Bottom) Intravenous challenge of S. paratyphi C vaccinated mice by S. enteritidis 5694, streptomycin-resistant,* ●. *Unvaccinated controls,* ▼. *The residual S. paratyphi C population was too low for accurate estimation.*

S. inverness-vaccinated mice. This organism resembled *S. seftenberg* in its growth pattern (Fig. 6) and in the degree of protection afforded against virulent challenge (Fig. 6, Table 1).

Vaccination with E. coli O14 and O55, P. aeruginosa, P. vulgaris, and S. marcescens. Mice were inoculated intravenously with more than 10^6 living cells of each of these strains. All organisms were eliminated rapidly from the blood, liver, and spleen and could not be detected 48 hr later. The five vaccine strains behaved almost identically in vivo, and the growth curve for *P. aeruginosa* shown in Fig. 7 is representative of the group. When challenged with virulent *S. enteritidis*, the five groups of vaccinated mice seemed as susceptible to the infection as were the normal

controls (Table 1). Within the limits of experimental error, the in vivo growth curves for *S. enteritidis* in the five vaccinated groups resembled each other closely, and, therefore, only the typical data obtained with the *P. aeruginosa* vaccinated mice are presented (Fig. 7).

DISCUSSION

In a previous paper (2), in which the immunogenicity of living suspensions of three antigenically related *Salmonella* species were compared, it was shown that *S. gallinarum* grew well in vivo and established a persistent population. The vaccination resulted in complete protection against a subsequent challenge with *S. enteritidis* by promoting a fully effective antibacterial immunity. But, the antigenically related *S. pullorum* was unable to persist in the tissues of mice.

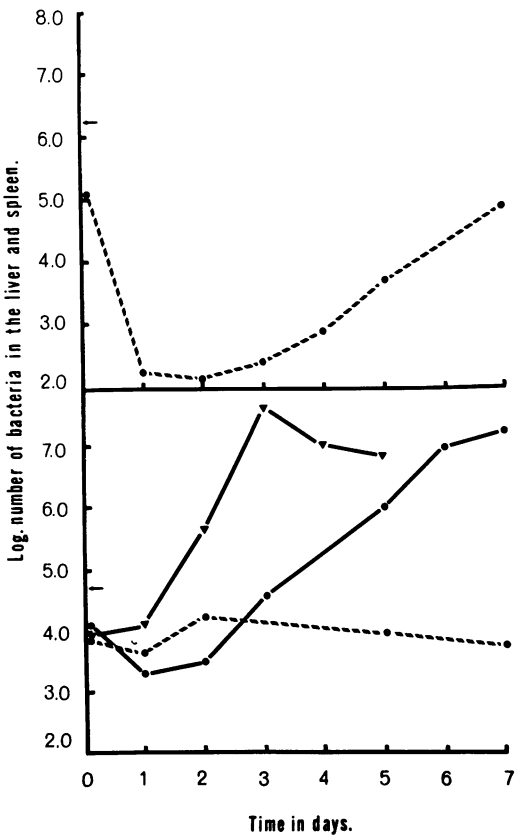

FIG. 4. *(Top) Growth curve for Salmonella seftenberg inoculated intravenously in normal mice. (Bottom) Intravenous challenge of S. seftenberg vaccinated mice by S. enteritidis 5694, streptomycin-resistant,* ●. *Residual vaccine strain is shown by the broken line. Unvaccinated controls,* ▼.

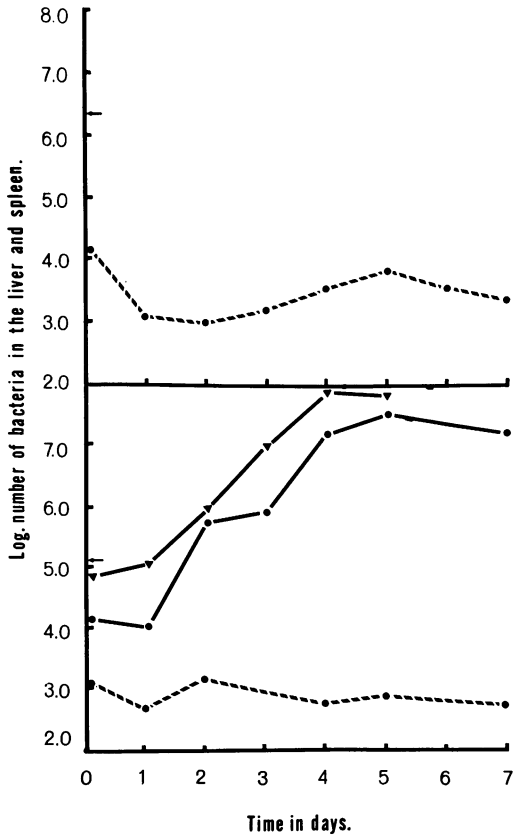

FIG. 5. *(Top) Growth curve for Salmonella adelaide inoculated intravenously in normal mice. (Bottom) Intravenous challenge of the S. adelaide vaccinated mice by S. enteritidis 5694, streptomycin-resistant,* ●. *Unvaccinated controls,* ▼. *Residual S. adelaide (vaccine strain) shown by the broken line.*

Animals vaccinated with either living or dead suspensions of this organism were equally susceptible to a virulent challenge of *S. enteritidis* that grew as rapidly in them as in the unvaccinated controls. From these studies it was predicted that any cross-protection against *S. enteritidis* infection, analogous to that reported by Jenkin and Rowley (7), would be produced only by those strains of organisms capable of sustaining themselves in the tissues. Thus, the demonstrated inability of *E. coli, P. aeruginosa, P. vulgaris,* and *S. marcescens* to grow in vivo was associated with a failure to protect these mice against subsequent challenge with *S. enteritidis* (Table 1, Fig. 7).

The antigenically unrelated *Salmonella* species used in this study to immunize mice could be claimed to have induced some protection against the *S. enteritidis* infection if mere death or survival

of the mice was used as the sole criterion of "protection" (Table 1). As discussed previously (2), such data gave no information on the relative effectiveness of the different vaccines in terms of host resistance at the time of challenge. The concurrent enumeration studies carried out in this investigation showed that only when the homologous strain was used as the vaccinating organism was an effective antibacterial immunity developed. Those unrelated *Salmonella* species effective in preventing the death of 50% or more of the mice merely generated a sufficient degree of resistance by virtue of their growth in vivo to retard the challenge organism in the early stages of infection. As a result, the liver and spleen populations failed to reach lethal proportions before an effective defense could be generated in response to the challenge organism itself. Even when the immunized mice were effectively protected against the lethal effects of the challenge

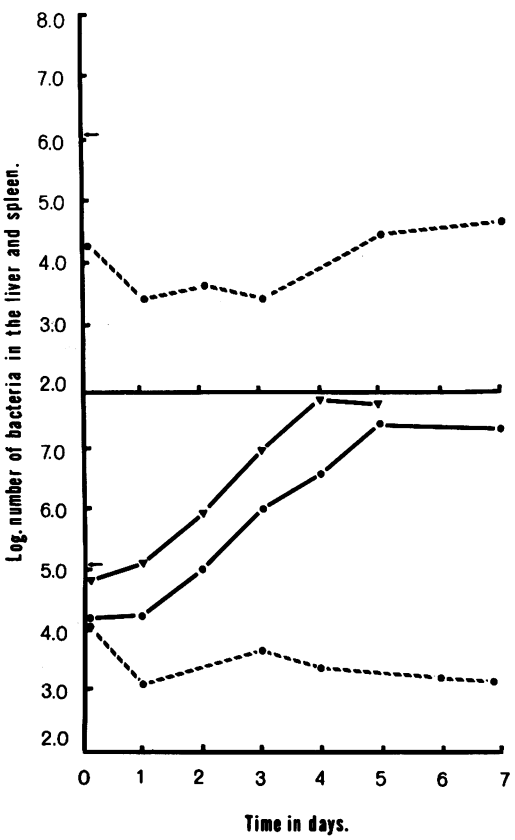

FIG. 6. *(Top) Growth curve for Salmonella inverness inoculated intravenously in normal mice. (Bottom) Intravenous challenge of the S. inverness vaccinated mice by S. enteritidis 5694, streptomycin-resistant, ●. Unvaccinated controls, ▼. Residual S. inverness shown by the broken line.*

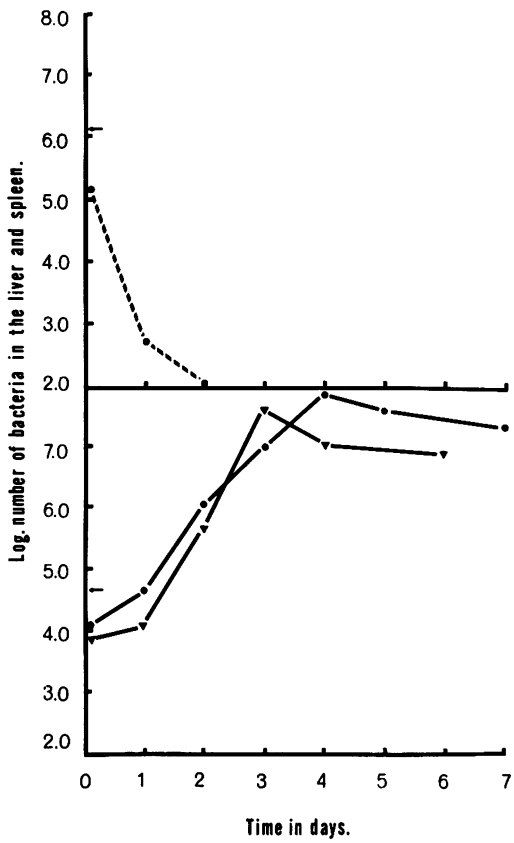

FIG. 7. *(Top) Growth curve of Pseudomonas aeruginosa inoculated intravenously in normal mice. Escherichia coli O14, E. coli O55, Proteus vulgaris, and Serratia marcescens inocula gave similar growth curves. (Bottom) Intravenous challenge of P. aeruginosa vaccinated mice by Salmonella enteritidis 5694, streptomycin-resistant, ●. Unvaccinated control, ▼. Challenge of mice vaccinated with E. coli O14, E. coli O55, P. vulgaris, or S. marcescens gave similar growth curves within the limits of experimental error.*

(e.g., in the *S. seftenberg*-vaccinated mice), extensive growth of the virulent organism occurred and caused severe signs of clinical disease despite ultimate recovery.

The only antigenically heterologous organism that caused a major increase in antibacterial resistance was a pathogenic strain of *S. typhimurium*. This organism resembles *S. enteritidis* in that it multiplies extensively in vivo even when small inocula are employed. Animals which survive the initial infection progress to a so-called "carrier state" (8) in which significant numbers of bacteria can be found in the liver and spleen for weeks or even months. In this study, *S. enteritidis* could be detected in vivo 2 months after the vaccinating infection, but not when it

was tested after 3 months. Challenge of these "carrier" mice with streptomycin-tagged *S. enteritidis* demonstrated that after 3 months the superinfecting organisms were eliminated more slowly than by 1- or 2-month old "carriers." Thus, persistence of the vaccine strain was apparently of prime importance for the continued maintenance of high degrees of antibacterial immunity (10).

The growth curves shown in Fig. 3–6 illustrate that all of the *Salmonella* species tested were capable of survival in vivo for at least 7 days. However, the subsequent growth of the challenge organism in all of these mice emphasizes that the mere survival of the *Salmonella* vaccine in vivo is not sufficient to generate and to maintain an effective level of nonspecific antibacterial immunity. Previous studies with *S. montevideo* vaccinated mice clearly demonstrated that the degree of antibacterial immunity to subsequent challenge with virulent *S. enteritidis* depended on the size of the vaccinating population still present at the time (2). Thus, the present growth curve data are entirely compatible with the thesis that the cross-protection produced by the antigenically unrelated *Salmonella* vaccines is caused by the low degree of cellular immunity produced and does not depend on the presence or absence of a specific "protective" factor, as postulated by Jenkin and Rowley (7).

Although conventional "protective" antibody does not appear to play any significant role in the present cross-protection experiments (Table 2), some degree of antigenic specificity is apparently involved between *S. enteritidis* and *S. typhimurium*. When mice vaccinated with *S. enteritidis* (Fig. 1) and *S. typhimurium* (Fig. 2) were rechallenged 30 days later with *S. enteritidis*, the *S. typhimurium*-vaccinated mice did not eliminate the antigenically heterologous challenge as rapidly as did the homologously vaccinated mice. Nonetheless, both groups of mice demonstrated complete protection from the highly lethal challenge in terms of death or survival. It appears inescapable that subtle antigenic differences must exist between the two pathogens and that the cells of the homologously stimulated animal can recognize these differences. Since opsonic antibody alone does not show any significant protective effect, the antigenic specificity which has been demonstrated in this study as an accelerated restoration in the level of antibacterial immunity by the homologous challenge population must be explained in terms of an immunological reaction other than that involving conventional antibodies. The possibility that delayed-type hypersensitivity is responsible for the specificity of the recall of host resistance (9) is currently under investigation.

ACKNOWLEDGMENTS

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