Published October 1, 1966

INFECTION-IMMUNITY IN EXPERIMENTAL SALMONELLOSIS*

By F. M. COLLINS, Ph.D., G. B. MACKANESS, M.B., and R. V. BLANDEN

(From the Trudeau Institute, Medical Research Laboratories, Saranac Lake, New York)

(Received for publication 18 May 1966)

Living *Salmonella* vaccines frequently protect against virulent challenge whereas vaccines prepared from organisms killed by a wide variety of methods do little more than prolong survival time (1–7). Questions have been raised as to whether the living vaccines are more effective because bacterial proliferation leads to greater antigenic stimulation in the host or because, in vivo, the organisms produce antigens which are not synthesized in vitro or which are destroyed during the preparation of the vaccine (8–10). Great ingenuity has been used to devise methods of inactivation which will preserve the antigenic constitution of the vaccines and so improve their immunogenicity (11, 12). Despite this effort, the simple fact remains that living organisms afford protection that has not yet been matched by any other immunizing regimen (13–15). It is interesting, however, that even living organisms vary in their immunizing potential in a manner unrelated to their known antigenic composition (5). Attempts have been made to explain these variations in immunogenicity in terms of the presence or absence of "protective" antigens not accounted for in the heat-stable antigens of the Kauffmann-White scheme of classification (10). Since host resistance to *Salmonella typhimurium* was found to be nonspecific in its antibacterial effects (16, 17), it seems necessary to look for biological properties other than "protective" antigens in those organisms with good immunizing ability. An obvious approach to this problem is to examine the relationship established between the host and the living vaccine in the hope of finding in the host-parasite relationship, features which could explain the peculiarities that enable some bacteria to protect against subsequent infection by pathogenic species of *Salmonella.*

Salmonella enteritidis was used throughout the present study. This organism is highly virulent for the mouse producing an infection which is indistinguishable from that caused by *S. typhimurium* (15). *S. enteritidis* was better suited to the present study because of its antigenic similarity to two other strains (*Salmonella gallinarum* and *Salmonella pullorum*) which are antigenically alike but differ greatly in their virulence and immunogenicity for the mouse. The contrasting

*This work was supported by Research Grant AI 07015 from the National Institute of Allergy and Infectious Disease, United States Public Health Service.

properties of the latter made them ideal as immunizing agents for the present study.

Materials and Methods

Organisms.—*S. enteritidis* (strain Se795), *S. pullorum* (strain 223), and *Salmonella montevideo* (strain 286) were obtained from Dr. Nancy Atkinson, University of Adelaide, South Australia. *S. gallinarum* (N.C.T.C. 9240) was obtained from Collindale, London. *S. enteritidis* has an LD_{50} of 500 to 1000, whereas *S. gallinarum* had an LD_{50} of 8×10^6. *S. pullorum* and *S. montevideo* were completely avirulent for the mouse in doses of 10^8 living cells. Whether living or dead, bacteria of all four strains were lethal at a dose of $1–5 \times 10^9$.

Media.—All bacteria were grown at 37°C in nutrient broth (Oxoid) enriched with 1% acid hydrolyzed casein and 0.1% yeast extract. Cultures were shaken at 120 CPM. and were harvested during the logarithmic growth phase.

Living Vaccines.—The strains used as living vaccines were made resistant to 25 μg of streptomycin per ml of medium. The bacteria were suspended in sterile physiological saline and standardized turbidimetrically to 10^8 bacteria per ml. The vaccines and suspensions used for challenge were diluted to the required density and used within 30 min. Viable counts were performed by the Miles and Misra method (18). In all experiments, white mice of the Swiss-Webster strain (15 to 20 g) were immunized by intravenous inoculation. Challenge was carried out 8 or 16 days later by intravenous injection of 1000 LD_{50}'s of *S. enteritidis*. Unvaccinated controls of similar age were challenged at the same time.

Killed Vaccines.—A washed saline suspension of logarithmic phase organisms was standardized optically to contain 2 mg dry weight of cells per ml and enough cold ethanol was added to give a final concentration of 70% (v/v). The suspension was shaken thoroughly and stood at 4°C overnight. The cells were deposited at 5000 *g* for 20 min in the cold, and the cells resuspended in sterile saline at a density of approximately 10^{10} per ml. Five 0.5 ml aliquots were inoculated into sterile digest broth, incubated at 37°C for 18 hr, and plated on digest agar. If sterile, they were dispensed in small volumes and stored at −20°C. Once thawed, any residual vaccine was discarded.

Bacterial Enumeration in Spleen, Liver, and Blood.—A sample of 0.02 ml of blood was obtained from the retroorbital plexus using a sterile capillary pipette. The blood was spread on a MacConkey agar plate and colonies counted after 24 hr of incubation. Contaminating bacteria were easily identified macroscopically, but typical colonies were always checked by slide agglutination. Combined liver and spleen counts, using a double plating technique, were made as previously described (15). Five randomly selected animals were used for each daily count. Obviously moribund animals were excluded from the experiments. Mice from each group were set aside to compare the overall mortality rates.

RESULTS

Growth Pattern of S. enteritidis in Normal and Infected Mice.—It was first necessary to study the behavior of *S. enteritidis* in normal mice in order to determine the degree of resistance that can be developed by mice against reinfection with the homologous organism. This was needed to provide a standard for evaluating the influence of vaccination on host resistance to *S. enteritidis*.

A group of 100 mice were infected with 1.9×10^5 *S. enteritidis* (streptomycin-resistant). The results of viable counts performed on blood, spleens and livers of 5 individual mice at intervals were plotted against time. After 16 days the infected mice and a group of normal con-

Published October 1, 1966

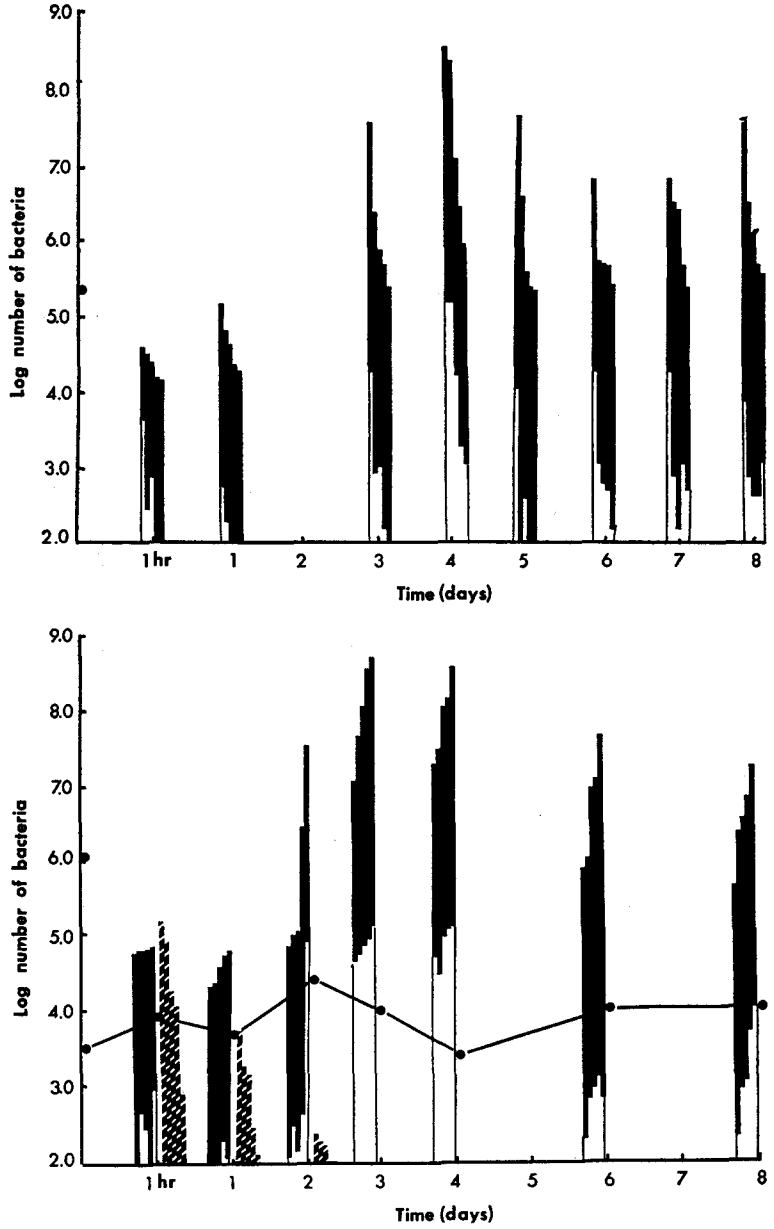

FIG. 1. *Top:* Numbers of *S. enteritidis* S^R in blood, liver, and spleens of normal mice injected intravenously with 1.9×10^5 organisms. Solid, *S. enteritidis* S^R in liver and spleen; open *S. enteritidis* in blood. *Bottom:* Numbers of *S. enteritidis* in normal mice and mice vaccinated with *S. enteritidis* S^R 16 days previously. The challenge of 1.1×10^6 virulent organisms was injected intravenously. Solid, *S. enteritidis* S^S in liver and spleens of control mice; hatched, *S. enteritidis* S^S in liver and spleens of vaccinated mice; and open, *S. enteritidis* in the blood. ●——●, *S. enteritidis* S^R in liver and spleen of vaccinated mice.

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trols were super-infected with 1.1×10^6 *S. enteritidis* (streptomycin-sensitive) and bacterial counts performed on liver, spleen, and blood.

Fig. 1 shows the growth pattern of the streptomycin-resistant mutant of *S. enteritidis* in the organs of normal mice. The streptomycin-sensitive strain

TABLE I

Progressive Mortality following Intravenous Challenge of Mice Immunized with Living Vaccines of S. enteritidis, S. pullorum, S. gallinarum, or S. montevideo

Vaccinating strain	Challenge organism	No. of deaths									
		Time in days									
		4	5	6	7	8	9	10	12	14	28
Nil	10^5 Se795-S^R	—	—	1	1	3	5	5	6	7	7/20*
Nil	10^6 S223	—	—	—	—	—	—	—	—	—	0/20
Nil	10^6 S9240-S^R	—	1	1	1	2	2	2	2	2	2/20
Nil	10^5 S286-S^R	—	—	—	—	—	—	—	—	—	0/20
10^5 Se795-S^R	10^6 Se795	—	—	—	—	—	—	—	—	—	0/20
10^6 S9240-S^R	10^6 Se795	—	—	—	—	—	—	—	1	1	1/20
10^6 S223	10^6 Se795	1	4	6	8	8	8	10	10	10	12/20
10^7 S223 (16)‡	10^6 Se795	—	—	—	—	—	—	—	1	1	1/20
10^6 S286-S^R	10^6 Se795	—	1	2	3	3	4	4	4	4	4/20
10^5 S286-S^R	10^6 Se795	—	—	—	—	—	—	—	—	1	1/20
Nil	10^5 Se795	1	2	5	7	8	10	12	15	16	18/20

S^R denotes that the organisms were resistant to 25 μg streptomycin per ml of medium.
* Total deaths/mice challenged.
‡ 10^7 viable *S. pullorum* injected daily for 16 days. Challenged on day 8.
Se795, *S. enteritidis*; S223, *S. pullorum*; S9240, *S. gallinarum*; and S286, *S. montevideo*.

had an identical growth pattern, but the ultimate total mortality was always higher (Table I). Both organisms behaved essentially as did *S. typhimurium*, and showed the same tendency to produce a secondary bacteremia (15). Fig. 1 also shows that the survivors of the primary infection were capable of eliminating a highly lethal inoculum of the streptomycin-sensitive strain of *S. enteritidis*. The graph also records the mean viable counts of the residual streptomycin-

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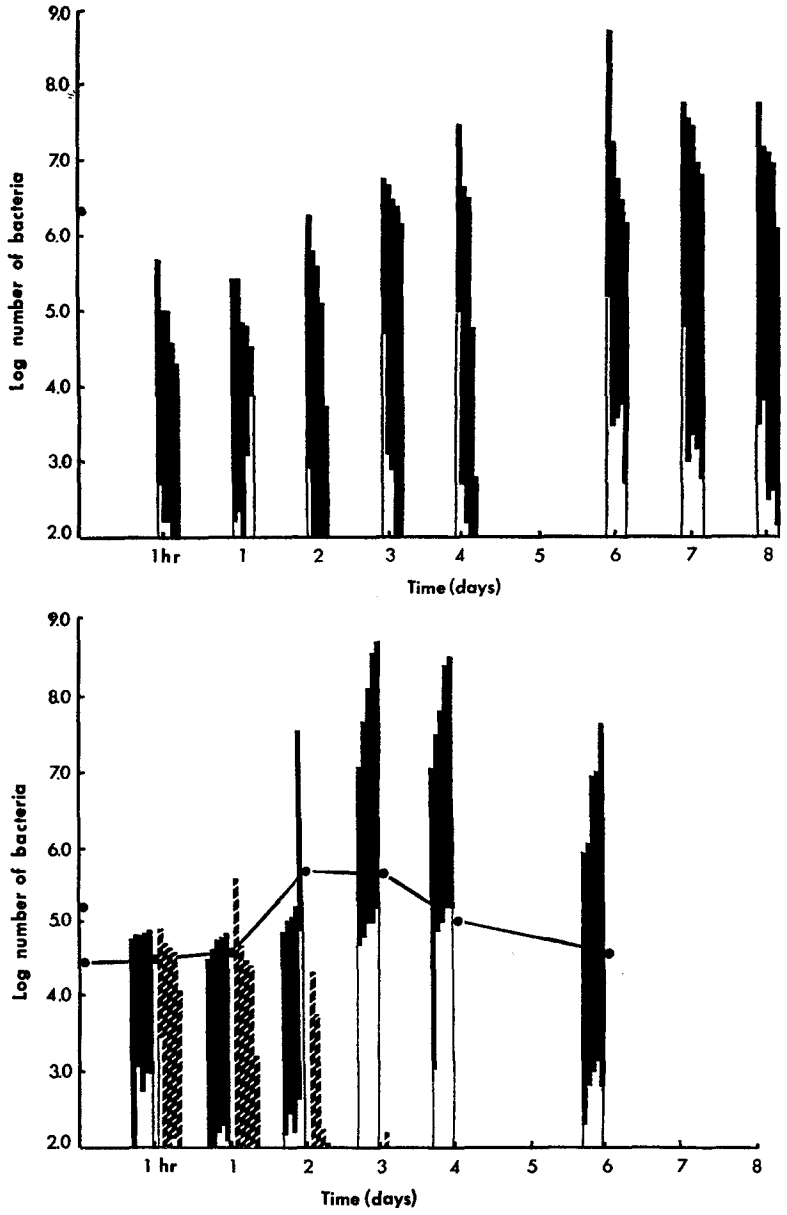

FIG. 2. *Top:* Numbers of *S. gallinarum* S^R in blood, livers, and spleens of normal mice injected intravenously with 2.0×10^6 organisms. Solid, *S. gallinarum* in liver and spleen; open, *S. gallinarum* in blood. *Bottom:* Numbers of *S. enteritidis* in normal mice and in mice vaccinated with *S. gallinarum* 16 days previously. All mice were challenged intravenously with 1.0×10^6 virulent organisms. Solid, *S. enteritidis* in liver and spleen of normal mice; hatched, *S. enteritidis* in liver and spleen of vaccinated mice; and open, organisms in the blood. ●——●, *S. gallinarum* in liver and spleen of vaccinated mice.

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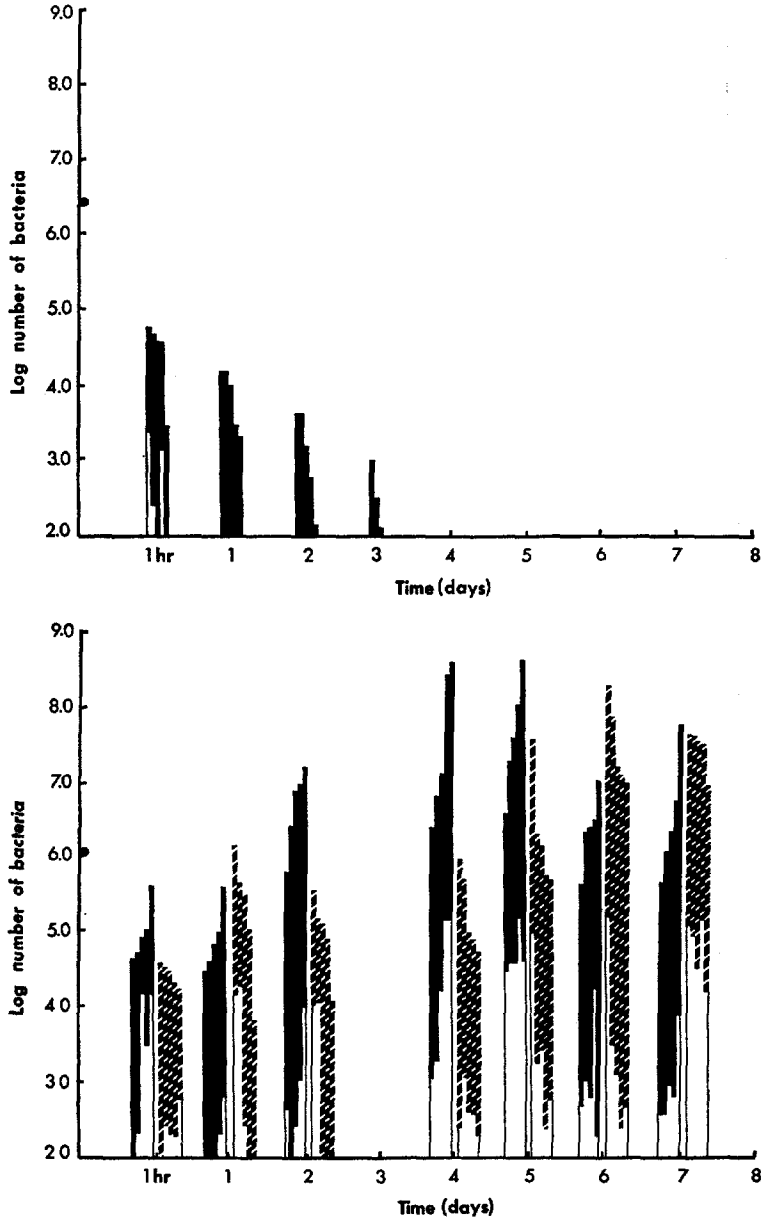

FIG. 3. *Top:* Numbers of *S. pullorum* in the livers and spleens of normal mice injected intravenously with 2.2×10^6 organisms. Solid, *S. pullorum* in liver and spleen; open, *S. pullorum* in blood. *Bottom:* Numbers of *S. enteritidis* in normal mice and in mice vaccinated with *S. pullorum* 16 days previously. All mice were challenged intravenously with 1.0×10^6 virulent organisms. Solid, *S. enteritidis* in liver and spleen of control mice; hatched, *S. enteritidis* in liver and spleen of vaccinated mice; and open, *S. enteritidis* in blood.

resistant organisms in the spleen and liver, and depicts the contrasting behavior of the challenge organisms in normal mice. It is evident that the highly effective resistance developed against this organism is comparable with that previously described for *S. typhimurium* (15, 16).

Having established that the mouse can develop an absolute resistance to reinfection with *S. enteritidis* [1, 9, 12; gm, −], it was possible to compare the

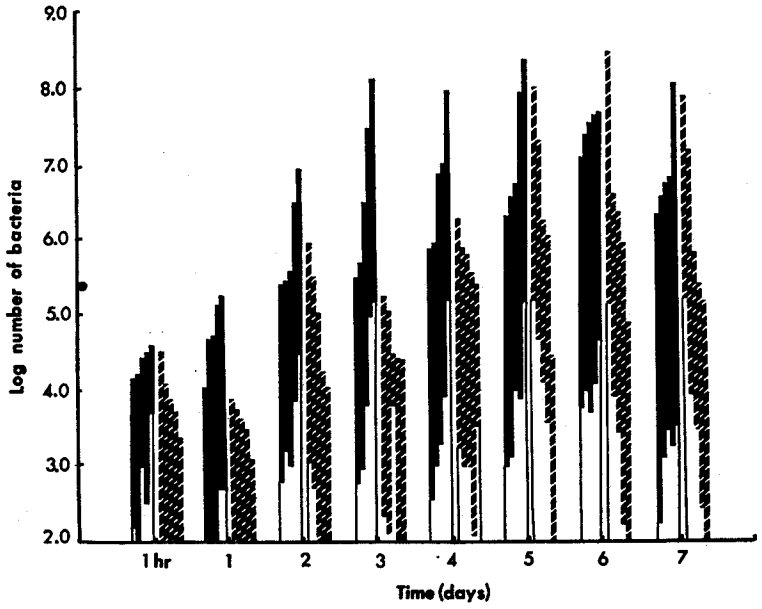

FIG. 4. Numbers of *S. enteritidis* in the blood, livers, and spleens of normal mice and mice injected intraperitoneally nine times with 10^6 alcohol-killed *S. enteritidis* prior to intravenous challenge with 2.5×10^5 virulent organisms. Solid, *S. enteritidis* in liver and spleen of control mice; hatched, *S. enteritidis* in liver and spleen of vaccinated mice; and open, *S. enteritidis* in the blood.

relative immunizing ability of this organism with that of two related *Salmonella* spp. of almost identical antigenic structure (*S. gallinarum* [(1), 9, 12; −] and *S. pullorum* [9,12; −]). It was known that living vaccines of these two organisms differed conspicuously in their ability to protect against challenge by virulent *S. enteritidis* despite the presence of common somatic antigens in all three organisms. On the basis of previous studies (16, 19), it was predicted that *S. gallinarum*, which is highly protective, would persist in the tissues, whereas *S. pullorum* would not.

Behavior of S. pullorum and S. gallinarum in Vivo—Figs. 2 and 3 record the growth patterns of *S. gallinarum* and *S. pullorum* in normal mice. The growth

curve of *S. gallinarum* S^R closely resembles that of *S. enteritidis*. However, less than 10% of the animals died in 16 days (Table I). Following injection, the organisms were rapidly cleared from the blood, but the bacteremia subsequently returned and persisted for at least 8 days. On the other hand, *S. pullorum* failed to establish a persisting population; it was virtually eliminated from the body by day 4. This result was reproduced on several occasions.

16 days after primary infection with *S. gallinarum* S^R or *S. pullorum*, groups

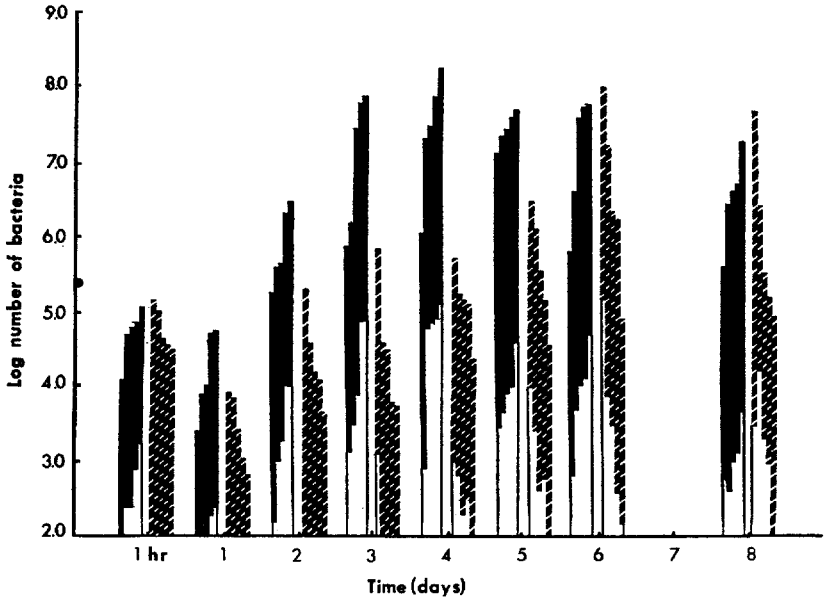

FIG. 5. Numbers of *S. enteritidis* in the blood, liver, and spleen of mice injected intraperitoneally nine times with 10^6 alcohol-killed *S. gallinarum* prior to intravenous challenge with 2.3×10^5 virulent organisms. Solid, *S. enteritidis* in liver and spleen of control mice hatched, *S. enteritidis* in liver and spleen of vaccinated mice; and open, *S. enteritidis* in the blood.

of mice (together with normal controls) were challenged with 1000 LD_{50}'s of *S. enteritidis*. The numbers of viable organisms of the primary and secondary infections found at intervals after challenge are also recorded in Figs. 2 and 3. *S. pullorum* was easily distinguished from *S. enteritidis* on the basis of colony size. *S. enteritidis* was as rapidly and completely removed from animals infected with *S. gallinarum* as it had been from mice infected with the homologous organism. On the other hand, the mice vaccinated with a larger dose (2.2×10^6) of living *S. pullorum* were not absolutely protected against the challenge infection. A steady increase in the number of challenge organisms occurred after a 2 to 3 day delay. Thereafter a normal growth pattern was observed (Fig. 3). However,

only 60% of the vaccinated animals died within 28 days. This is compared with a mortality of 90% observed with the unvaccinated controls (Table I).

Since *S. pullorum* and *S. gallinarum* are virtually indistinguishable antigenically but differ so strikingly in their ability to protect when used as living vaccines, it was of interest to compare the immunizing properties of alcohol-killed vaccines prepared from them. This method of inactivation was chosen because of a recent report (10) that protective antigens present in some *Salmonellae* are best preserved in vaccines inactivated by alcohol or acetone treatment.

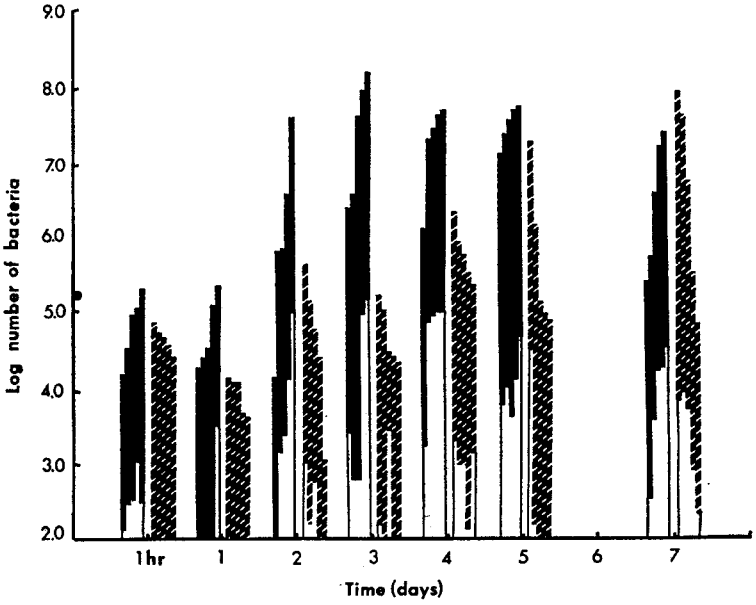

FIG. 6. Numbers of *S. enteritidis* in the blood, livers, and spleens of mice injected intraperitoneally nine times with 10^6 alcohol-killed *S. pullorum* prior to intravenous challenge with 1.8×10^5 virulent organisms. Solid, *S. enteritidis* in liver and spleen of control mice; hatched, *S. enteritidis* in liver and spleen of vaccinated mice; and open, *S. enteritidis* in the blood.

Growth of S. enteritidis in Mice Vaccinated with Alcohol-Killed vaccines.—

Groups of mice were given three intraperitoneal injections of 10^6 killed bacteria a week for 3 wk. The mice were rested for 7 days before testing 5 randomly chosen animals for the presence of living organisms in liver and spleen homogenates. The uniformly negative results of these sterility tests indicated that the immunized mice were free of viable *Salmonellae*. The vaccinated mice, together with a group of normal controls, were then challenged intravenously with 1000 LD_{50}'s of *S. enteritidis*. The bacterial populations of liver, spleen, and blood were determined on groups of 5 randomly selected mice 1 hr after challenge and thereafter at daily intervals.

The growth patterns shown in Figs. 4 to 6 record the behavior of *S. enteritidis* in mice immunized with alcohol-killed vaccines of *S. enteritidis*, *S. gallinarum*, and *S. pullorum*, respectively. Rapid and complete blood clearance occurred in all three groups of vaccinated mice; it was never complete in normal mice. A 100- to 1000-fold increase in bacterial numbers occurred in the livers and spleens of mice in all groups. However, the onset of this increase was delayed in the three vaccinated groups, and a corresponding delay occurred before the onset of a secondary bacteremia. As a consequence, the bacterial populations in a proportion of animals failed to reach lethal proportions before the onset of the acquired resistance generated by the challenge infection itself. This is reflected in

TABLE II

Progressive Mortality following Intravenous Challenge of Mice Vaccinated with Alcohol-Killed S. enteritidis, S. pullorum, or S. gallinarum

Vaccinating strain*	Challenge strain	No. of deaths									
		Time in days									
		4	5	6	7	8	9	10	12	14	28
10^7 Se795	10^5 Se795	—	—	1	2	8	9	12	12	12	12/20‡
10^9 Se795	10^5 Se795	—	1	4	5	7	8	9	11	14	14/20
10^7 S223	10^5 Se795	—	—	1	1	7	7	9	9	12	16/20
10^7 S9240	10^5 Se795	—	1	1	3	6	10	11	13	14	14/20
Nil	10^5 Se795	2	4	10	12	15	15	15	16	19	19/20

* See Table I for key to strain numbers.

‡ Total deaths/mice challenged.

the mortality rates recorded in Table II. Thus, identical doses of the three vaccines were not significantly different in their immunogenicity. Additional experiments showed that mice vaccinated with 10 or 100 times the number of alcohol-killed organisms showed similar growth curves following challenge and no significant improvement in total mortality (Table II). It is inferred that no real difference exists between the three vaccines in so far as their effects on host resistance are concerned.

In the past, a number of workers have commented on the long term persistence of living vaccines in the tissues of immunized mice. Such "carrier" mice have been shown to be highly resistant to reinfection by the virulent, homologous organism (6, 20, 21). Rowley and his colleagues (22) ascribe the enhanced killing ability observed in such mice to the production of specific antibody

rather than to any change in the phagocytic cells. Thus, it could be argued that living *S. pullorum* and *S. gallinarum* differ in their capacity to protect against infection by the related strain (*S. enteritidis*) simply because *S. gallinarum* provides a greater antigenic stimulus through its capacity to multiply in vivo. If this were the only difference it might be expected that repeated injections of *living S. pullorum*, given frequently enough to simulate an active infection, should give protection comparable with that produced by infection with *S.*

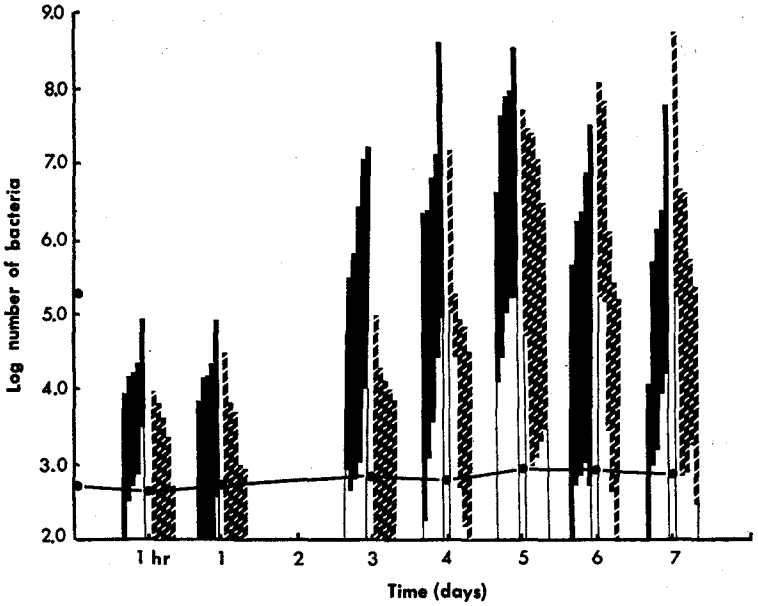

FIG. 7. Numbers of *S. enteritidis* and *S. pullorum* in the blood, livers, and spleens of mice injected daily with 10^7 viable *S. pullorum* and then challenged intravenously with 1.9×10^5 virulent *S. enteritidis* on day 8. Solid, *S. enteritidis* in livers and spleens of control mice; hatched, *S. enteritidis* in livers and spleens of vaccinated mice; and open, *S. enteritidis* in the blood. ●——●, *S. pullorum* in livers and spleens of vaccinated mice (average of 5).

gallinarum. In order to test this hypothesis the following experiment was performed.

The Effect of Repeated Injections of Living S. pullorum on Resistance to S. enteritidis Infection.—

Logarithmic phase cultures of *S. pullorum* were washed and resuspended in normal saline. They were standardized to 10^8 organisms per ml and the viable population was determined immediately before injection. A group of 80 normal mice were given daily intraperitoneal injections of 10^7 bacteria per mouse for 8 days. The mice were challenged on day 8 with 1000 LD_{50}'s of *S. enteritidis* along with a similar group of normal mice. The daily injections of living

S. pullorum were continued throughout the period of observation. Liver, spleen and blood counts were carried out on randomly selected mice prior to the daily injection of the vaccinating strain.

Preliminary experiments in mice infected with *S. gallinarum* had shown that resistance to *S. enteritidis* was fully established by the 8th day of the primary infection. The daily intraperitoneal injection of 10^7 *S. pullorum* produced an artificially sustained population of 10^8 to 10^4 living organisms in the livers and spleens of the vaccinated mice (Fig. 7). The fate of the intravenous challenge inoculum of *S. enteritidis* given on day 8 is also recorded in Fig. 7. In the vaccinated animals, the challenge organism was rapidly cleared from the blood, but

TABLE III

Toxicity of Killed Suspensions of S. enteritidis, S. pullorum, and Purified S. enteritidis Lipopolysaccharide in Normal and Immunized Mice

Vaccine	Toxic dose* (mg dry weight)		
	S. enteritidis (ethanol-killed)	*S. pullorum* (ethanol-killed)	LP‡
Nil	3.1	3.1	0.80
10^9 *S. enteritidis* (ethanol-killed)	5.0	3.5	1.25
16×10^7 *S. pullorum* (living)	5.2	4.1	1.25

* Estimated from number of deaths after 48 hr.

‡ Obtained from *S. enteritidis* by phenol-water extraction.

subsequently the numbers of *S. enteritidis* in the livers and spleens increased steadily until day 5. It then declined, presumably because acquired resistance had developed against the challenge organism itself.

It was concluded that no greater ability to kill or limit the growth of virulent organisms was generated by the repeated injection of living *S. pullorum* than could be obtained with similar numbers of alcohol-killed organisms (Fig. 6). However it was later demonstrated that immunized mice were more resistant to the toxic effects of heat-killed whole cells as well as to purified lipopolysaccharide (Table III). The survival of the mice immunized repeatedly with living *S. pullorum* was probably due to antitoxic immunity since the peak bacterial populations reached in these mice were frequently as high as those observed in unvaccinated controls (23), and comparable with the number present at death in animals infected with *S. typhimurium* (24). Thus, immunization with *S. pullorum* produced some protection without generating any marked degree of antibacterial immunity. On the other hand, the resistance produced during infection with *S. gallinarum* was a true antibacterial immunity, for only residual organisms of the immunizing strain could be detected 2 days after challenge (Fig. 2).

The foregoing experiments do not allow us to compare the relative magnitudes of the antigenic stimuli provided by the two living vaccines used. Nevertheless, the results suggest that only organisms which can persist in vivo give rise to a fully effective microbicidal mechanism not found in animals immunized with dead bacteria or even with a living vaccine of an organism incapable of sustaining itself in the tissues. Earlier studies with. *S typhimurium* (16) showed that acquired cellular resistance was not dependent on the presence of specific antibody but arose whenever animals were infected with a strain of bacteria

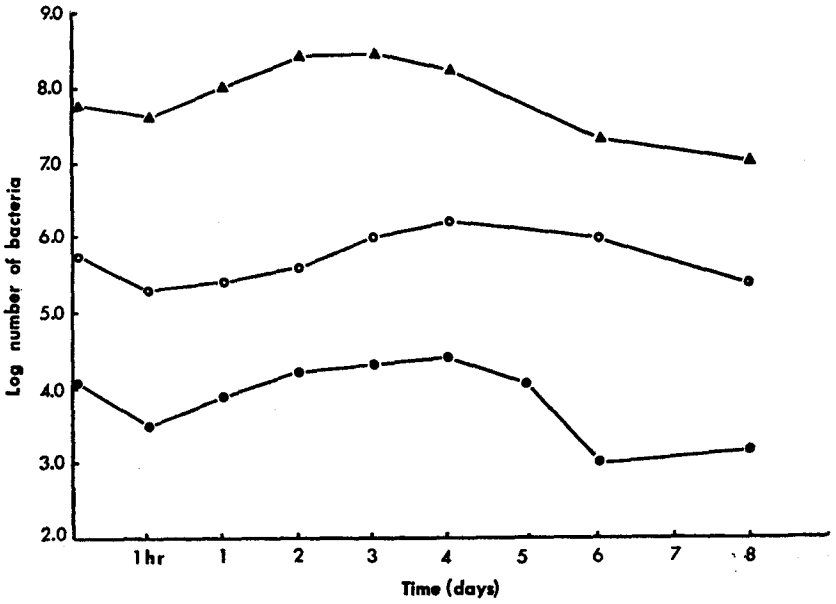

FIG. 8. Average numbers of *S. montevideo* S^R in the livers and spleens of 5 randomly selected mice injected intravenously with increasing doses of organisms. ▲——▲, 5.8×10^7 organisms; ○——○, 5.76×10^5 organisms; and ●——●, 1.0×10^4 organisms.

able to persist in the tissues for a prolonged period of time. The close antigenic relationship between the three organisms used in the present study made it impossible to exclude antibody as a factor in the development of immunity to *S. enteritidis*. For this reason it was decided to immunize mice with *S. montevideo* which does not share any known somatic antigens with the test organism. Its use as the immunizing strain would thus permit a study of the effect of antigenic dose on the level of resistance produced. *S. montevideo* was particularly appropriate for this purpose as it was shown to establish a chronic infection in mice in which the size of the bacterial population varied in proportion to the vaccinating dose (Fig. 8).

Published October 1, 1966

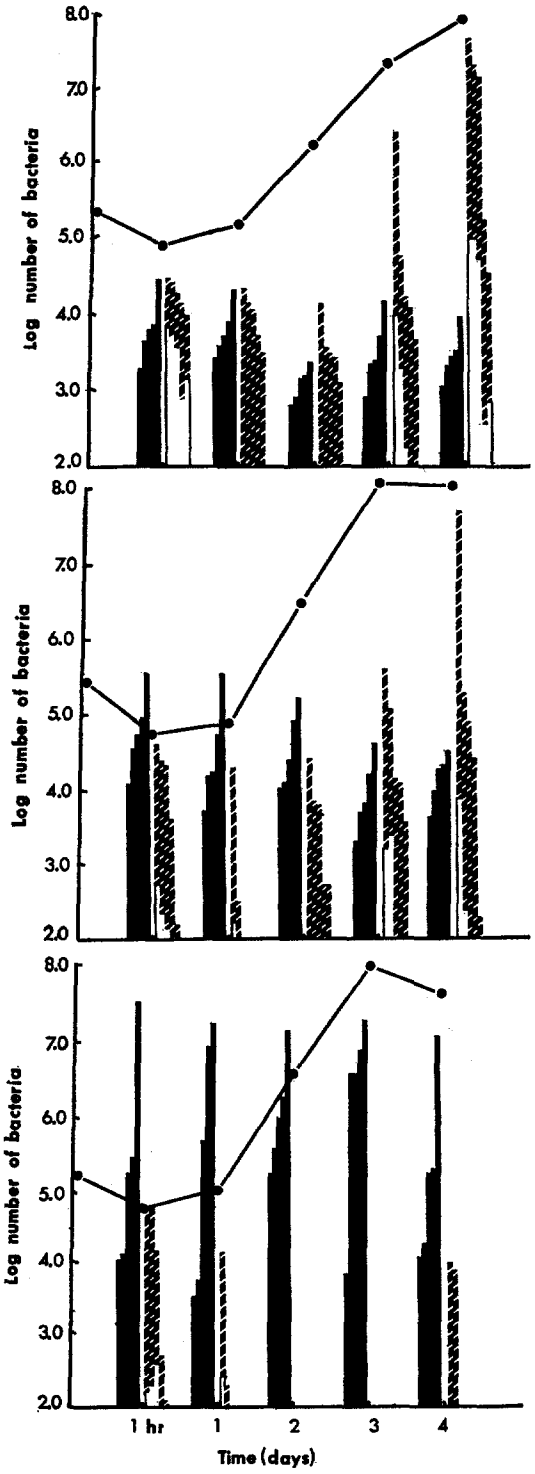

FIG. 9. *Top:* Numbers of *S. montevideo* S^R and *S. enteritidis* in the blood, livers, and spleens of mice vaccinated intravenously with 1.0×10^4 *S. montevideo* S^R 16 days prior to intravenous challenge with 2.6×10^5 virulent *S. enteritidis*. *Middle:* Numbers of *S. montevideo* S^R and *S. enteritidis* in the blood, livers, and spleens of mice injected intravenously with 5.76×10^5 *S. montevideo* S^R 16 days prior to intravenous challenge with 2.5×10^5 virulent *S. enteritidis*. *Bottom:* Numbers of *S. montevideo* S^R and *S. enteritidis* in the blood, livers, and spleens of mice injected intravenously with 5.76×10^7 *S. montevideo* S^R 17 days prior to intravenous challenge with 1.6×10^5 virulent *S. enteritidis*. Solid, *S. montevideo* in liver and spleen; hatched, *S. enteritidis* in liver and spleen; and open, *S. enteritidis* in blood. ●——●, *S. enteritidis* in control (average of 5 mice).

The Behavior of S. enteritidis [1, 9, 12; gm, −] in Mice Infected with S. montevideo [6, 7; gms, −].—

Three groups of mice were injected intravenously with approximately 10^4, 10^6, or 10^8 viable *S. montevideo* S^R. The liver and spleen populations of 5 randomly selected mice were determined after 1 hr and thereafter at daily intervals. All vaccinated mice were challenged with 1000 LD_{50}'s *S. enteritidis* on the 16th or 17th day after vaccination. Liver, spleen, and blood counts were made by the duplicate plate method after 1 hr and thereafter at daily intervals.

The behavior of *S. enteritidis* in the three groups of immunized mice is shown in Fig. 9. Despite the absence of homologous somatic antigens in the vaccinating organism, all three groups of mice were considerably more resistant to challenge than were the controls (Table I). Thus *S. montevideo* afforded similar cross-protection to that described by Howard (17) between *S. typhimurium* [1, 4, 5, 12; c, 1, 2] and *S. paratyphi* C [6, 7, Vi; c, 1, 5). The relationship between the level of resistance and the number of primary organisms still present in the tissues was evident, not only between groups but also between individual mice within these groups. The fact that opsonic antibody was not involved in the increased resistance of these mice to challenge with *S. enteritidis* is indicated by the persistence of organisms in the blood 1 hr after injection. This contrasts with the observations made in animals vaccinated with dead organisms which share somatic antigens with *S. enteritidis* (Figs. 4 to 6), but fail to produce an effective antibacterial immunity.

DISCUSSION

In the past great stress has been placed on the antigenic deficiencies of heat-inactivated vaccines as a means of explaining their failure to produce complete resistance to infection by virulent *Salmonellae* (1, 2, 13). Efforts to preserve the full spectrum of antigenic activity in organisms killed in various ways has led to the conclusion that "protective" antigens are better preserved in an active form in alcohol-killed vaccines (10, 11). Thus, the inability of the living *S. pullorum* vaccine to protect mice against *S. enteritidis* infection would be explained in terms of the absence of a "protective" antigen from the cell walls of *S. pullorum*. However, the fact that the alcohol-killed vaccines of *S. pullorum*, *S. gallinarum*, and *S. enteritidis* produced comparable effects on the host-parasite relationship suggests that the differences observed in the protective abilities of the living vaccines was not due to the absence of any hypothetical "protective" antigen from *S. pullorum*. Collins and Milne (23) investigated the immunogenicity of a number of antigenic extracts obtained from *S. enteritidis*. The protective ability of each fraction was estimated both by bacterial enumeration and from progressive mortality rates. The most protective fractions could do no more than slow the rate of growth of the challenge organism in vivo. It is significant that extracts of avirulent organisms were as effective as those from fully virulent strains. This result agrees with the findings of Mackenzie, et al.,

(25), Hobson (26), and Mitsuhashi, et al., (27) and further emphasizes the futility of attempting to isolate a fully "protective" antigen, particularly when evidence for their presence relies exclusively on mortality data, a stricture of particular importance when the peritoneal route of challenge is used (16).

In the present study only slight protection against *intravenous* challenge was observed in mice vaccinated with alcohol-killed organisms (Table II); but examination of Figs. 4 to 6 shows that this protection is not attended by any marked ability of the vaccinated mice to kill the bacterial inoculum. An ability to do so is not achieved until late in the challenge infection and is attributable directly to changes induced by the challenge infection itself. The foregoing arguments are not limited to animals immunized with killed vaccines. Animals injected *once* with living *S. gallinarum*, or repeatedly with living *S. pullorum*, showed a high degree of protection (Table I). In the absence of bacterial enumeration, both immunizing regimens would have been judged equally effective. It is clear, however, from the curves shown in Figs. 2 and 7 that the level of resistance in the *S. pullorum* vaccinated mice was far lower at the time of challenge than in mice infected with *S. gallinarum*. The *S. pullorum* mice survived because here, too, resistance developed after challenge. In the *S. gallinarum* mice on the other hand, absolute resistance was already present at the time of challenge. This clearly shows the inadequacy of merely recording cumulative mortality rates as a measure of host resistance.

In the past the effectiveness of a vaccine has usually been assessed on the basis of survival rates, supplemented on occasion by cultural evidence of the challenge organism in the tissues at the time of death. Quantitative data, which depicts the progress of the infection, has seldom been presented. The difficulties involved in evaluating the usefulness of a particular vaccine is well illustrated in the case of human typhoid. Despite the mass of clinical and laboratory data on all cases and suspected contacts of typhoid fever, the value of typhoid vaccine has only recently been clearly established on statistical grounds (28, 29). The experimental models used to assess the effectiveness of vaccines against *S. typhi* infections have, of necessity, employed highly artificial conditions in order to produce fatal infections. Under such conditions, immunity can only be assessed on an all-or-nothing basis depending on the relative proportions of survivors in the various groups of mice. No account is taken of subclinical or even severe clinical infections from which the mice ultimately recover. Such animals should properly be included in any over all assessment of the value of a vaccine, since absolute protection should be the ultimate aim of any rational immunization program. Only by a combination of bacterial enumeration studies with over-all mortality figures can the relative value of different immunizing procedures be properly assessed.

In the previous paper evidence was presented that acquired resistance to *Salmonella* infections depends above all on potentiation of the microbicidal

activity of host phagocytes. The only effect attributable to circulating antibody in *S. typhimurium* infections was an antitoxic effect at the cellular level which tended to supress the rate of development of primary lesions and the secondary bacteremia (16). The present results are entirely consistent with this view. Vaccines capable of establishing an active infection were able to produce an antibacterial mechanism, the efficiency of which was proportional to the residual level of vaccinating organisms in the tissues. In the present studies this was conclusively shown in mice immunized with *S. montevideo* because of the ease with which the size of the vaccinating population could be varied at will. These experiments also confirmed the nonspecific nature of the antibacterial mechanisms developed during a *Salmonella* infection (16).

The most interesting enigma emerging from the present studies was the failure to reproduce the effects of an active infection by repeated injections of living *S. pullorum*, an organism normally incapable of sustaining itself in the tissues of mice. It is presumed that *S. pullorum* failed in this respect either because it does not produce the background of hypersensitivity necessary for the induction of acquired cellular resistance (19), or because the cell is so rapidly destroyed that antigen levels cannot be maintained at a concentration high enough to activate the host's cellular defenses (30). It is interesting that *S. pullorum* is far more sensitive to serum inactivation than either *S. enteritidis* or *S. gallinarum* (31). It cannot be said at present whether this difference has any relevance to the present problem, but these questions are currently under investigation.

SUMMARY

Salmonella enteritidis is highly virulent for the mouse causing an infection resembling mouse typhoid. Survivors of the infection are completely resistant to reinfection and eliminate a large challenge dose of virulent organisms within 72 hr. The antigenically related *Salmonella gallinarum* was almost avirulent for the mouse but animals vaccinated with this organism were equally capable of eliminating a lethal dose of virulent *S. enteritidis*. Living *Samonella pullorum*, on the other hand, was quickly eliminated from the tissues of normal mice. Vaccination with this organism failed to evoke an effective bactericidal mechanism. Alcohol-killed vaccines of these three Salmonellae all produced an increase in blood clearance rate, but gave only marginal protection against *S. enteritidis*. Liver and spleen counts on these mice revealed a 1 to 2 day delay before any net increase in the total bacterial population could be observed. Immunization of mice with increasing doses of living *Salmonella montevideo* resulted in progressively greater killing of a challenge dose of *S. enteritidis* despite the absence of common somatic antigens between the two strains. The degree of protection varied with the size of the residual population of *S. montevideo* in the vaccinated mice. The significance of these findings in assessing the importance of various

factors involved in the development of acquired resistance to *Salmonella* infections is discussed.

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