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Oral Immunization in Experimental Salmonellosis

III. Behavior of Virulent and Temperature-Sensitive Mutant Strains in the Intestinal Tissues of Rats

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Infection of rats via a Peyer's patch has been used as a means of studying the behavior of *Salmonella enteritidis* in intestinal tissues. The course of infection in the Peyer's patch and draining mesenteric lymph node is characterized by multiplication of the organism over a period of 4 days followed by a gradual decline in numbers; the organism also passes to the liver and spleen and may be isolated from these organs as well as the intestinal tissues for at least 4 weeks. Temperature-sensitive mutants derived from the virulent strain are unable to multiply and do not pass to the liver and spleen; they remain viable for periods of less than 2 weeks. A quantitative technique based on the number of viable organisms remaining in the injected Peyer's patch 48 hr after infection has been used to assess the immune state of rats. The results have clearly demonstrated that *Salmonella* immunity can only be induced by living vaccines and that although viable organisms remain in the reticuloendothelial tissues, organisms given in a challenge infection are immediately subject to enhanced bactericidal activity within the intestinal tissues. Under the conditions used here, humoral antibody does not seem to offer any protective effect against *Salmonella* infection in the intestinal lymphoid tissues.

The choice of *Salmonella* infections in rodents as models for studying mechanisms of immunity which operate in human enteric fevers, particularly typhoid, is a necessary expedient. Unfortunately, it is obvious that quite different interpretations of the means by which immunity is mediated can be derived from these models, depending largely on the route by which infection is established. Most noteworthy in this respect are the results from experiments in which the intraperitoneal (ip) and intravenous (iv) routes have been used; the conflicting views on anti-*Salmonella* immunity that have arisen are vaguely reminiscent of the humoral versus cellular immunity arguments, heatedly debated at the turn of this century.

Quite appropriately, Akiyama et al. (2) and others (4, 16) have pointed out that the use of the ip challenge for studies of this kind is open to criticism on the grounds that the course of infection bears little resemblance to the pathogenesis of the natural disease. It cannot be denied, however, that this route of challenge demonstrates one type of immunity mechanism (i.e., humoral antibody) which is effective under certain circumstances; the important question which remains unsolved concerns the contribution of humoral mechanisms in defense against natural infections. The iv route has been recommended as an alternative, for it provides a means of directly establishing organisms in the liver and spleen, the major target organs of natural *Salmonella* infections (14). With this route, especial emphasis has been placed on the cellular immune mechanisms (4).

To be consistent, neither of these two routes properly accounts for the basic pathogenic feature of *Salmonella* infection, namely, that it is a disease acquired by the oral route; clearly the organism must gain access via the intestinal tissues and it is to be expected that the behavior of the intestinal lymphoid tissues may largely determine whether bacteremia and subsequent colonization of the liver and spleen occur. Theoretically, therefore, the oral route of infection should provide the most relevant information of all. Unfortunately, the practical difficulty of ensuring that all animals receive similar numbers of organisms in the intestine detracts considerably from its potential value as an experimental model; furthermore, it would be difficult to assess the course of this infection by any quantitative technique. As an alternative, we have used the Peyer's patch injection technique in rats, a method described earlier by Cooper and

Turner (7), to investigate the behavior of *Salmonella* organisms in the lymphoid tissues of the intestinal tract. The method has provided an opportunity to determine whether the course of infection in these tissues is similar to that which occurs in other reticuloendothelial (RE) organs (e.g., the liver and spleen).

MATERIALS AND METHODS

Bacterial strains. The virulent strain of *S. enteritidis* var. *dansyz* and temperature-sensitive mutants derived from it (TSF11 and TSF19) have been described in detail elsewhere (8). A strain of *Escherichia coli* 0111:B4 was obtained from the School of Microbiology, Melbourne; this organism was avirulent for rats. For injection, log phase cultures were preparted in nutrient broth (NB) by incubation on a reciprocal shaker in a water bath held at 37 or 28 C (for the temperature-sensitive mutants) for 2 to 3 hr; appropriate dilutions of these cultures were made in NB. The method of Miles et al. (13) was used to determine the numbers of organisms injected into animals.

Animals. Male rats of an outbred Albino stock weighing 200 to 250 g were used. These animals were bred in the School of Microbiology, Melbourne. Before each eoperiment, all rats were bled and their sera were tested for the presence of *S. enteritidis* O antibody to ensure that none had been inadvertently exposed to this organism or antigenically related salmonellae. In addition, occasional rats were selected at random and killed, and their livers, spleens. and intestinal contents were cultured to exclude the presence of the *Salmonella* carrier state. The animals derived from this particular colony satisfied these criteria.

Injection techniques. The technique for Peyer's patch injection (ipp) in anesthetized rats has been described fully elsewhere (7). The bacterial suspension was mixed with an equal volume of sterile carbon suspension (Pelikan India Ink, Hanover; diluted in 1% gelatin-saline to contain 8 mg of carbon per ml); this allowed demarcation of the injected Peyer's patch and its adjacent draining mesenteric nodes. The volumes of suspension deposited in a normal sized Peyer's patch were approximately 5 μliters (7).

Rats were also injected by the intraintestinal (ii) route in some experiments; laparotomy was performed under ether anesthesia, and a small section of the jejunal-ileal region was exposed. The suspension (0.5 ml) was delivered into the lumen by passing a 30-gauge needle, attached to a 2 ml Luer lock syringe, obliquely through the antimesenteric border of the intestine. After injection, the intestine was liberally flushed with Ringer's solution and returned to the peritoneal cavity. The incision was closed with sutures and a Michel-type clip.

Detection of organisms in rat tissues. The tissues (liver, spleen, Peyer's patch, or mesenteric lymph node) were removed after the animals had been killed by exsanguination. They were homogenized by passing through a stainless steel mesh into 2 ml of sterile distilled water. Quantitative estimates of the number of organisms in the suspensions were obtained by the method of Miles et al. (13). In other experiments, the suspensions were transferred to 10-ml volumes of Rappaport's medium (15) which were incubated at 37 or 28 C; after 24 hr, these were subcultured on MacConkey medium and incubated at 37 or 28 C, depending on whether the parent or TSF mutant strains were to be isolated. Confirmation of the isolation of the organism was obtained by biochemical tests and slide agglutination of selected colonies.

Serum antibody estimations. The hemagglutination technique described in a preceding paper was used (9). Serum samples from rats were collected by bleeding from the tail veins. The titers were entered on IBM punch cards and analyzed by computer by the method of Jaroslow and Nossal (12).

RESULTS

The strain of *S. enteritidis* used in these studies was highly virulent for mice; by the ip route, its LD_{50} was found to be 1.5×10^2 organisms and the minimal lethal dose, 3×10^3 organisms (8). Although only small groups were used, it is obvious from Table 1 that far larger doses of the organism are required to establish a lethal infection in rats. For present purposes, it was not considered necessary to determine the LD_{50} of the organism for rats given by the different routes. Tenfold lower doses of organisms than those shown in the table were not lethal. However, all animals which received these lower doses suffered a transient illness which was characterized by an incubation period of 7 to 14 days, development of a ruffled coat, pyrexia, and production of loose stools, which continued for 4 to 5 days. From these results, it is obvious that introduction of the organism by way of the Peyer's patch is more likely to establish a lethal infection than either the ii or ip routes.

Death is not the sole, nor necessarily the best, criterion of whether infection has been established in an animal; it may also be judged in terms of proliferation of the organism within the animal tissues. The fates of *S. enteritidis*, the temperature-sensitive mutant TSF11, and the nonvirulent *E. coli* strains after ipp injection were assessed by estimating the number of viable organisms present

TABLE 1. *Virulence of Salmonella enteritidis for rats*

Route of infection	No. of organisms injected	No. of deaths/no. of rats injected
Intraperitoneal	10^8	5/12
Intraintestinal	10^9	5/15
Intra-Peyer's patch	2×10^6	6/12

in the Peyer's patch and the draining mesenteric nodes over a period of 14 days; these tissues were selected because it has previously been shown that, after ipp injection with particulate material, almost all of it initially lodged there and was not rapidly disseminated to other tissues. Groups of four to six rats were killed at various times after injection; in all experiments, the first time chosen was approximately 60 sec after injection so that an estimate of the numbers deposited in the Peyer's patch could be obtained.

When sublethal doses of *S. enteritidis* were given, 50 to 90% of organisms were destroyed in the Peyer's patch within 4 hr of injection (Fig. 1); this reduction could not be accounted for by drainage of organisms to the mesenteric lymph nodes, for levels reached in these tissues at 4 hr were found to approximate to only 10% of the numbers injected into the Peyer's patch. Multiplication of the organisms then occurred over a period of 2 to 4 days, reaching a maximum level of approximately 5×10^5 organisms in the Peyer's patch. Thereafter, the numbers decreased and by the 14th day were barely countable. The infection was not confined to the intestinal tissues, for cultures prepared from the liver and spleen during the period of multiplication, in most instances, were found to contain *S. enteritidis* in numbers insufficient to be enumerated quantitatively (Table 2).

Irrespective of the initial challenge dose given in the Peyer's patch, multiplication occurred until the numbers reached a level of approximately 5×10^5 per patch. In a second experiment, 10^6 organisms were injected; after the initial decrease in numbers, they remained at this maximum level for 4 days before slowly decreasing (Fig. 2).

These results contrasted markedly with the behavior of *E. coli* and the mutant TSF11 in the Peyer's patch and mesenteric lymph node (Fig. 3). In neither instance did multiplication of the organism occur in the intestinal tissues. In the case of the avirulent *E. coli*, the organisms were no longer detectable 4 days after injection. On the other hand, the TSF mutant was able to survive for a

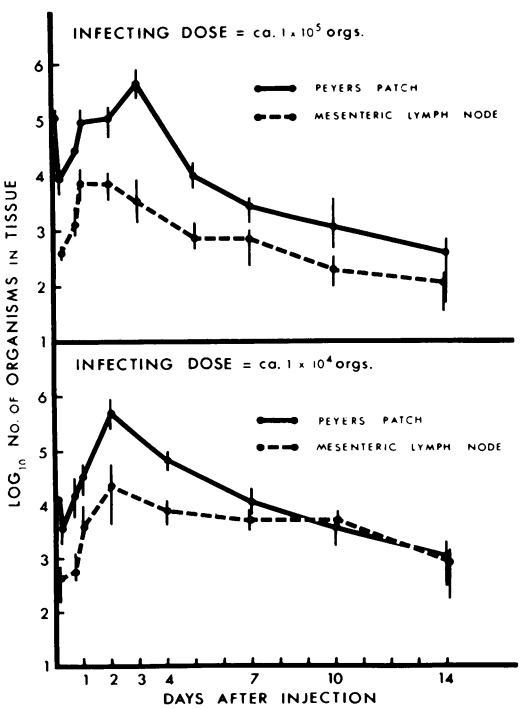

FIG. 1. *Growth of S. enteritidis in Peyer's patches and mesenteric lymph nodes of normal rats after ipp inoculation of 10^4 or 10^5 organisms.*

TABLE 2. *Detection of Salmonella enteritidis in the liver and spleen of rats after intra-Peyer's patch injection of 10^5 organisms*

Time after injection (days)	No. of rats with *S. enteritidis* in tissue/no. of rats injected	
	Liver	Spleen
4[a]	0/6	0/6
16[a]	0/6	0/6
1	4/6	5/6
2	2/6	3/6
3	4/6	4/6
7	6/6	4/6
10	3/6	4/6
14	4/6	1/6

[a] Hours.

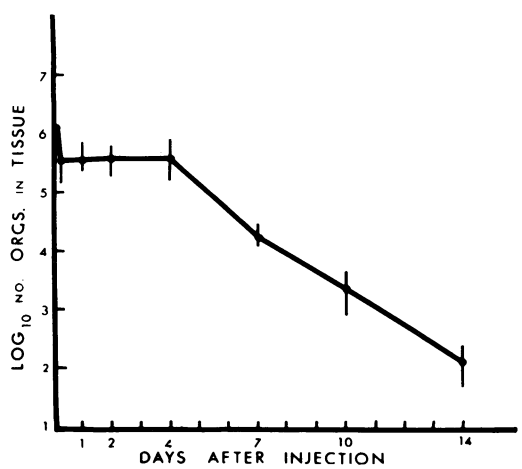

FIG. 2. *Growth of S. enteritidis in Peyer's patches of normal rats after ipp inoculation of 10^6 organisms.*

period of at least 7 days. Neither *E. coli* nor the TSF mutant strain could be isolated from the liver and spleen at any time after injection.

Course of infection in immunized rats. Many previous studies have conclusively demonstrated that ip injection of sublethal doses of salmonellae induce a long lasting immunity to lethal challenge infection; on the other hand, heat-killed vaccines given by the same route are generally ineffective. In the following experiment, two groups of rats were given ip injections of 10^6 organisms of a log phase culture of *S. enteritidis* or 10^9 organisms of the same strain which had been heated at 100 C for 60 min. Four weeks later, all rats were injected with 10^4 organisms of the virulent strain by the ipp route. At various times, groups of four rats were selected at random to determine the numbers of organisms present in the injected

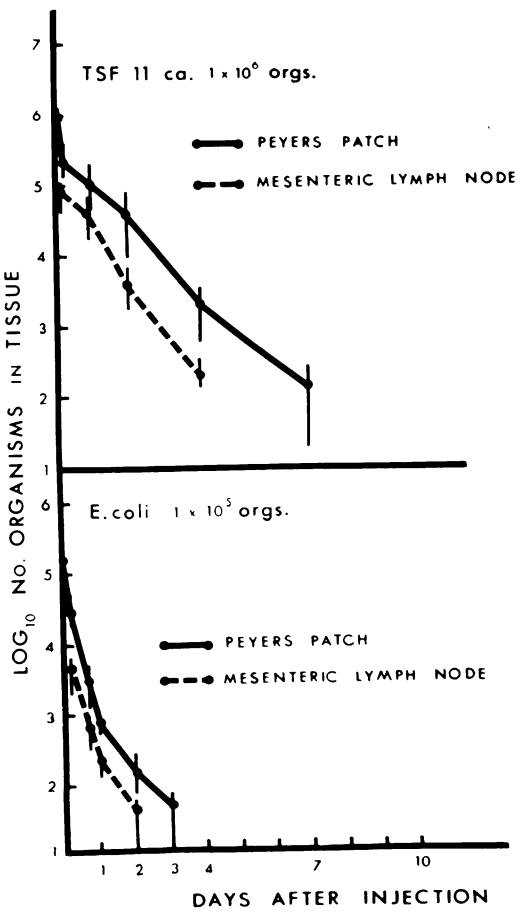

FIG. 3. *Survival of E. coli 0111:B and a temperature sensitive mutant of S. enteritidis in Peyer's patches and mesenteric lymph nodes of normal rats after ipp inoculation.*

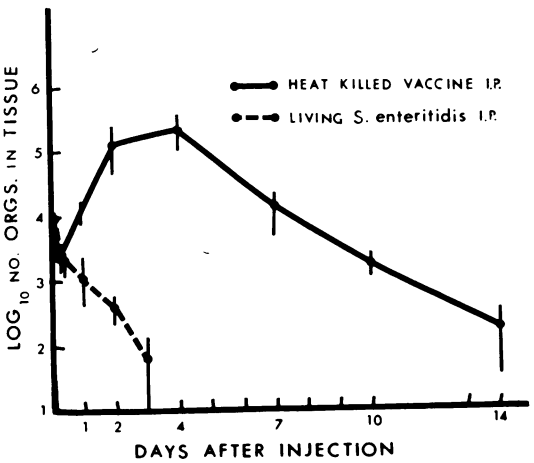

FIG. 4. *Growth of S. enteritidis in Peyer's patches of rats immunized 4 weeks earlier with heat-killed S. enteritidis vaccine or sublethal infection of S. enteritidis given by the ip route.*

Peyer's patch. The results (Fig. 4) clearly distinguish between the two groups of animals. In rats immunized with the living vaccine, the challenge organisms were treated in much the same manner as the nonvirulent *E. coli* (Fig. 3), whereas in those given the heat-killed vaccine, multiplication occurred in the Peyer's patch in exactly the same manner as described for normal animals (Fig. 1).

On the basis of these and similar results, it seemed that an assessment of the immune status of rats (at the time of challenge) could be made by comparing the numbers of viable organisms present in Peyer's patches of immunized and normal rats 48 hr after injection with *S. enteritidis*. Because of the number of animals involved in the experiments, it became clear that "normal" rats could not always be conveniently included in each experiment. A "standard value" for the Peyer's patch and draining mesenteric lymph nodes was therefore obtained; this represented the number of viable organisms present in these tissues of normal rats 48 hr after they receive a standard inoculum of a 2-hr log phase culture of *S. enteritidis*. By using a group of eight normal animals and an inoculum of 1.7×10^4 organisms, standard values of $2.4 \pm 0.8 \times 10^5$ organisms for the Peyer's patch and $3.3 \pm 1.3 \times 10^4$ organisms for the mesenteric lymph node were obtained. In the following experiments, to account for small variations in the size of the challenge inocula, the following formula was used to determine the "percentage of expected numbers" present at the 48th hr in treated groups of rats. Per cent of expected numbers = $(VT \times Is \times 100)/(Vs \times IT)$; VT = the number of viable organisms in tissues

of treated rats; VS = the number of viable organisms in tissues of normal rats (i.e., "standard value" for Peyer's patch or mesenteric node); IT = the number of organisms inoculated in treated rats; IS = the number of organisms inoculated in normal rats (i.e., 1.7×10^4 organisms).

To confirm the validity of this calculation, two further groups of eight rats were injected ipp with 1.1×10^4 and 5.7×10^3 organisms of the challenge strain; the number of viable organisms present in the Peyer's patches and mesenteric lymph nodes of these animals was then determined 48 hr later. By using the above formula, the percentage of the expected numbers for each tissue which those figures represented was calculated; the results are shown in Table 3. The figures were not significantly different from the expected percentage (i.e., 100%). Analysis of these results by the method of Bailey (3) indicated that statistically significant differences (at the $P \leqslant 0.05$ level) between treated and normal rats would be obtained if the "percentage of expected numbers" obtained in the former group was $\leqslant 33\%$ for the Peyer's patch and $\leqslant 17.5\%$ for the mesenteric lymph node.

TABLE 3. *Comparison of numbers of viable organisms in tissues of normal rats 48 hr after intra-Peyer's patch injection of Salmonella enteritidis*

No. of organisms in challenge inoculum	Percentage of expected numbers ± SE[a]	
	Peyer's patch	Mesenteric lymph node
5.7×10^3	99.2 ± 2.4	102.3 ± 4.7
1.1×10^4	101.5 ± 3.5	98.6 ± 7.3

[a] SE, 2× standard deviation.

The immune status of rats after injection of living cultures of *S. enteritidis*, the mutant TSF19, and the heat-killed vaccine was assessed by the above method. Groups of six to eight rats were used, the immunizing agent being given by the ip, ii, or the ipp routes. The challenge infection was given in the Peyer's patch 4 weeks later; in the case of the animals given the first injection ipp, the same patch was selected for the second injection. The number of viable organisms present in the injected patch and draining mesenteric lymph nodes was determined 48 hr later; using the above formula, the percentage of the expected numbers of these viable counts represented was determined. In all cases, graded doses of the immunizing agent were given; for the sake of brevity, however, the results presented in Table 4 have been restricted to recording figures for the smallest dose of the vaccine which had some, though not necessarily statistically significant, effect.

Irrespective of the route by which it was given, living *S. enteritidis* initiated an immune response which was reflected by destruction of the challenge organisms in the intestinal lymphoid tissues. A dose of only 40 organisms in the Peyer's patch was sufficient to induce a significant level of immunity; larger doses were required to produce the same effect when the ii or ip routes were used. In contrast, large doses of the killed vaccine were unable to restrict multiplication of the challenge organisms and must be presumed to have little immunizing capacity when given by any of the three routes. The TSF mutant possessed some immunizing activity when given via the intestine

TABLE 4. *Effects of living and killed vaccines on the survival of Salmonella enteritidis in Peyer's patches and mesenteric lymph nodes of rats after intra-Peyer's patch injection*

Route of immunization	Immunizing agent	No. of organisms in immunizing agent	Percentage of expected numbers ± SE[a] in		Statistical significance (P value)
			Peyer's patch	Mesenteric lymph node	
Intraperitoneal	*dansyz*	10^4	2.4 ± 1.1	1.4 ± 0.6	<0.05
	HK-100	10^9	64 ± 20.5	26.5 ± 7.3	>0.05
	TSF19	3.4×10^6	57 ± 14.6	29.3 ± 4.8	>0.05
Intraintestinal	*dansyz*	10^5	2.3 ± 0.9	1.3 ± 0.2	<0.05
	HK-100	10^{10}	51.0 ± 17.0	28.2 ± 13.5	>0.05
	TSF19	10^{10}	14.6 ± 1.6	6.7 ± 3.5	<0.05
Intra-Peyer's patch	*dansyz*	4×10^1	1.6 ± 0.7	1.3 ± 0.2	<0.05
	HK-100	1×10^8	39.7 ± 15.6	37.5 ± 5.7	>0.05
	TSF19	4×10^5	12.6 ± 2.5	6.9 ± 0.8	<0.05

[a] SE, 2× standard deviation.

or the Peyer's patch but apparently not after ip injection.

It was of interest to determine the time for development of immunity after injection of the living vaccines when given ip; in addition, attempts were made to demonstrate viable organisms in the liver and spleen at the time of challenge. Groups of rats were given 3×10^3 organisms of *S. enteritidis* or 3×10^6 organisms of the mutant TSF19 by the ip route. At selected times thereafter, groups of four rats were challenged ipp with ca. 10^4 organisms of the virulent strain; 48 hr later, the injected patch was removed for estimation of numbers of viable organisms. By using the above formula, these figures were converted to the percentage of expected numbers. At the time of challenge four rats were killed and their livers and spleens were removed for homogenization and culture in Rappaport's medium to demonstrate the presence of organisms of the immunizing strain. The results are shown in Table 5.

The capacity to restrict multiplication of organisms in the Peyer's patch develops approximately 7 days after the ip injection of both forms of living vaccine. In the case of animals given the parent strain *dansyz*, the immune state was retained for at least 4 weeks; although the TSF mutant was effective between days 7 and 14, the rats were no longer immune after 4 weeks. It is significant that multiplication of challenge organisms in the Peyer's patch was restricted only when the rats retained viable organisms of the immunizing strain in the liver and spleen.

Serum antibody after Peyer's patch immunization. Previous results have indicated that immunization via the Peyer's patch or the ii route may confer significant immunity against subsequent challenge infection initiated in the intestinal lymphoid tissues. In the following experiments, the serum antibody responses after immunization with *S. enteritidis*, the heat-killed vaccine prepared from it, or the living mutant TSF19 when given by the ipp or ii routes were examined over a period of 28 days. As in a previous experiment, graded doses were given, but, for the sake of brevity, results are only recorded for those doses referred to in Table 4. The results are shown in Fig. 5. Although the antibody titers were generally of a low order, this is to be expected in view of the relatively small size of the antigen doses given in most instances. The rat-to-rat variation was considerable; at the peak of the response, some animals were found to produce titers of 80 or greater, whereas others were as low as 10. Except in the case of the heat-killed vaccine, the mean peak titers were not significantly different between the various groups. The delay in response of animals which received *S. enteritidis* was, in all probability, due to the time required for multiplication of the organisms in the RE tissues until sufficient antigen was available to initiate antibody production.

It is evident from Fig. 5 and Table 4 that rats which showed immunity to *S. enteritidis* infection would normally possess significant levels of serum antibody at the time of challenge; the only exception to this was in the case of animals immunized with the TSF mutant by the ii route. It could, therefore, be argued that antibody was

TABLE 5. *Development of immunity in rats after intraperitoneal injection of living vaccines*

Organism injected by ip route	No. of organisms injected	Day of challenge	Percentage of expected numbers ± SE in Peyer's patch	No. of rats with positive cultures in liver and spleen at time of challenge[a]	
				28 C	37 C
Salmonella enteritidis	3.7×10^3	5	41.4 ± 7.6 NS[b]	4	4
		7	11.7 ± 2.5 S	4	4
		11	6.6 ± 1.3 S	4	4
		14	5.0 ± 2.0 S	4	4
		28	7.8 ± 2.8 S	4	4
TSF19	3.4×10^6	5	102.3 ± 14.3 NS	4	0
		7	25.7 ± 3.9 S	4	0
		11	19.6 ± 5.8 S	4	0
		14	25.3 ± 7.2 S	4	0
		28	115.7 ± 20.2 NS	0	0

[a] Four rats were examined at each time. Positive cultures at 28 and 37 C indicated *S. enteritidis*. Positive culture at 28 C but negative at 37 C indicated TSF19.

[b] Abbreviations are: NS, not significant reduction at 5% level; S, significant reduction at 5% level.

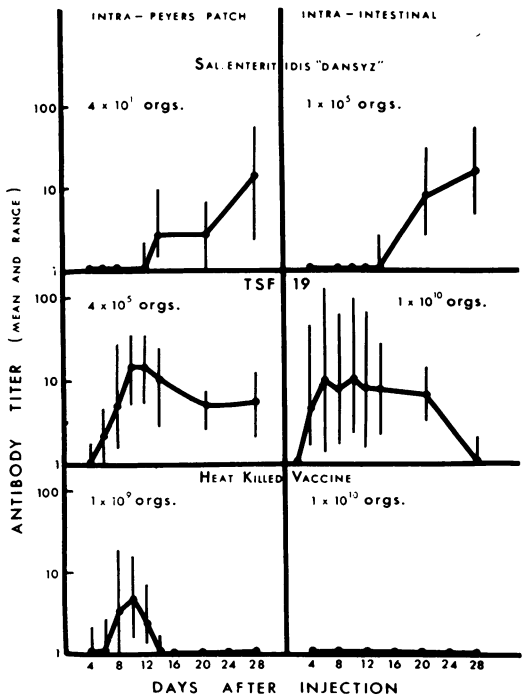

FIG. 5. *Serum antibody titers in rats inoculated by the ipp or ii routes with S. enteritidis, the temperature-sensitive mutant TSF19 or a heat-killed S. enteritidis vaccine. Vertical bars on the graphs indicate standard deviations.*

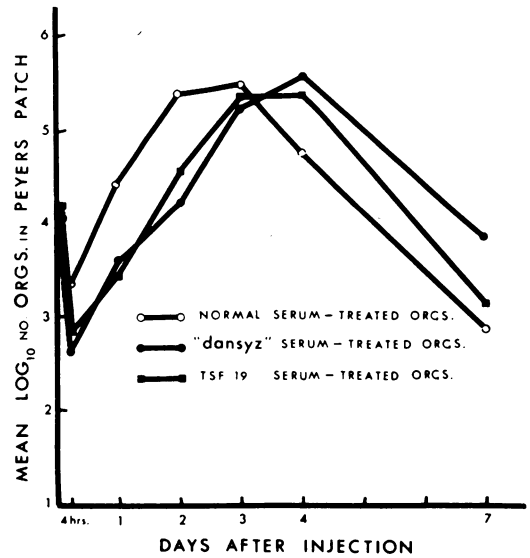

FIG. 6. *Growth of serum-treated S. enteritidis in Peyer's patches of normal rats after ipp inoculation.*

responsible for restriction of multiplication of the organism in these tissues. To test this possibility, the following experiments were undertaken. First, rats were injected in the Peyer's patch with 10^9 organisms of the heat-killed vaccine. They were challenged 10 days later with ca. 10^4 organisms of the virulent strain in the same patch; this particular time was selected because maximum serum antibody responses were expected (*see above*). The number of viable organisms in the Peyer's patch and draining mesenteric nodes was determined 48 hr later, and, as before, the percentage of expected numbers they represented was noted. In Peyer's patch, this value was 177 ± 56 (no significant difference from standard values); in mesenteric lymph node, 72 ± 38 (no significant difference from standard values). The vaccine had no protective effect in spite of the presence of serum antibody. (Serum antibody titer at the time of challenge was 10, determined in a group of four rats injected at the same time as above.)

In the second experiment, serum was obtained from rats 28 days after they had been immunized with 5×10^1 *S. enteritidis* or 6×10^5 TSF19 organisms by the Peyer's patch route. The hemagglutination titers of these sera were 20 and 10, respectively. A log-phase culture of the strain *dansyz* was diluted to contain 2×10^7 organisms per ml in NB; 0.1 ml of this suspension was added to 0.9-ml volumes of these sera. After 15 min at 4 C, the serum-treated organisms were injected in the Peyer's patches of two groups of normal rats; a third group of animals was inoculated by the same route with a suspension of organisms which had been incubated in normal rat serum. At selected times after injection, four rats were killed for estimation of the number of viable organisms present in the injected Peyer's patches. The course of infection is shown in Fig. 6. Organisms treated with normal rat serum behaved exactly the same as untreated organisms in normal rats, multiplying over 2 to 3 days (Fig. 1). Whereas the sera from rats immunized with the living vaccines caused greater initial reduction of the viable organisms in the Peyer's patch, it obviously had no lasting effect, for multiplication occurred thereafter for a period of 3 days; the numbers finally reached in the Peyer's patch were similar to those found in control animals. It is reasonable to assume that humoral antibody, by itself, was unable to prevent development of infection in this intestinal tissue.

DISCUSSION

The great bulk of facts presently known concerning the mechanisms of anti-*Salmonella* immunity have been derived from studies in mice (*see above*), an animal which is highly susceptible

to organisms such as *S. typhimurium* and *S. enteritidis*. In this respect, murine salmonellosis cannot be directly compared with typhoid infection in man; mortality figures for this disease, even during widespread epidemics, are never more than 10% among untreated cases (1). Neither, of course, do the commonly used routes of infection in experimental salmonellosis resemble those of human typhoid although some workers (10, 11) have stressed that involvement of the liver and spleen may be similar in both.

For these reasons, the present studies were undertaken in rats, animals which readily develop *Salmonella* infection yet are far less likely to succumb to its lethal effects; in addition, we have used the technique of Peyer's patch injection in an attempt to determine whether the same mechanisms of immunity are manifest in the intestinal lymphoid tissues as have been claimed for the liver and spleen of mice. This technique was used as the most reasonable alternative to oral infection which precludes any quantitative assessment of the course of infection or immune state of the animals.

The strain of *S. enteritidis* used in these experiments was lethal only when given in large numbers by the ip route (i.e., 10^8 or greater). It was also capable of killing rats when given ii, or more directly via the Peyer's patch. When sublethal inocula were given by this route, multiplication always occurred in the Peyer's patch and mesenteric nodes and was accompanied by migration of the organism to the liver and spleen. It was of some interest to note that irrespective of the size of the inoculum in the Peyer's patch, the organisms multiplied for sufficient periods to reach levels of approximately 5×10^5; within a short time of these numbers being attained, reduction occurred, presumably as a result of enhanced bactericidal activity of the RE cells within the tissue. When numbers in excess of this maximum were given, they were rapidly reduced to it and maintained at that level until the bactericidal mechanisms developed. Irrespective of the inoculum size, the reduction in numbers occurred at about the same time after infection. We may presume this to be evidence of the development of enhanced resistance to the infection. In contrast to the behavior of the virulent organism, the temperature-sensitive mutant derived from it and a nonvirulent strain of *E. coli* were unable to proliferate in the intestinal tissues, and in the latter case the numbers were rapidly reduced to undetectable levels. Although the TSF mutant survived longer, its numbers did not increase because of the temperature restriction placed on it; furthermore, it did not seem capable of invading beyond the intestinal tissues.

From these results, multiplication of organisms in the Peyer's patch and mesenteric lymph nodes might be considered as evidence of development of *S. enteritidis* infection; as a corollary, restriction of multiplication most probably indicates resistance to the organism. Based on this assumption, we have compared the numbers of viable organisms present in the Peyer's patches of treated and untreated rats 48 hr after injection of the virulent strain in an attempt to make some quantitative assessment of the immune state of the animals. The results we have obtained need not be discussed in any great detail as they agree almost entirely with those which have been reported by others who have used the iv infection route in mice (4, 5, 6). Although they, therefore, do not necessarily lead to any better understanding of the mechanisms involved in specific resistance to *Salmonella* infection, they undoubtedly are significant in that they demonstrate certain features which are common to different lymphoid tissues in another species of animal; in this respect, they might possibly strengthen the validity of analogies which are made between experimental *Salmonella* infections and human typhoid.

In short, it is obvious that living vaccines are generally effective in inducing a state of immunity to *Salmonella* infection. The immunity is of a general nature and may be induced whether the organisms are given parenterally or by the intestinal route. It is evident that the TSF mutants are capable of invading from the intestinal lumen in sufficient numbers to induce an immune response; this response is reflected not only by restriction of multiplication of virulent organisms in the Peyer's patch, but also by development of a significant antibody titer.

As recorded previously by Collins et al. (5, 6), immunity to infection is maintained as long as viable organisms are retained in the RE system; the greater protective effect of the *S. enteritidis* strain compared with the TSF mutant is readily explained in terms of the limited capacity of the latter organism to survive in the tissues. The immunity develops within 5 to 7 days of immunization and does not appear to be dependent upon development or presence of specific humoral antibody.

It should be pointed out that these results seem at variance with the views in the preceding publication in which we have stated that the presence of viable organisms is not essential for retention of immunity to *Salmonella* infection (9). We must stress that the two views are not mutually incompatible, for in the present experiments the technique allows only for demonstration of immunity which exists at the time of the challenge infection. This might be termed a "primary im-

mune state" which is dependent upon the continued survival of limited numbers of the immunizing organism in the RE tissues. This does not exclude the possibility that a "secondary immune state" might be induced in animals in the absence of viable organisms of the immunizing strain. The Peyer's patch injection technique in rats could not detect the secondary immune response which, as shown earlier, takes 5 to 7 days to develop; under the conditions of the present experiments, the challenge infection would already have been controlled by the bactericidal mechanisms of the rat intestinal lymphoid tissues.

Finally, it is obvious that, in terms of the dose of organisms needed to establish immunity in the Peyer's patch, the ipp and ii routes of immunization are more effective than the ip route. Similarly, far fewer organisms of the challenge inoculum remained viable at the 48th hr in the Peyer's patches of rats immunized with the virulent or TSF strains by the ipp or ii routes than in those immunized by ip injection. It may therefore be questioned whether local immune factors have enhanced the bactericidal efficiency of the intestinal lymphoid tissues. If this is the case, it could support the case for development of oral immunoprophylactic agents to be used against typhoid infections in man.

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