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Oral Immunization Against Experimental Salmonellosis

I. Development of Temperature-Sensitive Mutant Vaccines

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Mutant strains of *Salmonella enteritidis* were selected for their inability to proliferate at 37 C; when exposed to this temperature, these organisms formed tangled masses of long filaments in liquid media, presumably as a result of their inability to form cross septa. The mutants were also incapable of synthesizing flagella protein. A study of the biological charateristics of the mutants indicated that in most respects they resembled the parent strain of *S. enteritidis*; however, they were avirulent for mice, presumably because of the restriction of growth imposed by the body temperature of the animal. Preliminary studies have suggested that these mutants are highly effective in inducing protection against severe challenge infections of *S. enteritidis*; of especial interest is the fact that, when given orally, the mutants conferred a substantial degree of protection against oral infection with the virulent strain.

Though the incidence of typhoid fever in many countries is now low, its importance as a public health problem has never really been understated. For this reason, and over a period of almost 70 years, many attempts have been made to derive immunoprophylactic agents which may provide a solid and lasting immunity against the disease; the potential usefulness of such an agent in countries where it occurs in epidemic proportions has been noted on many occasions (6). At the present time, however, the efficacy of typhoid vaccines is accepted with some reservations (9, 19). Though there may be many reasons for this, it stems in part from the difficulty of initiating in animals an experimental infection with *Salmonella typhi*, which is similar to the natural human disease (7, 17). As an alternative, most experimental studies on the pathogenesis of enteric infections caused by the *Salmonella* (and the immune mechanisms which might operate therein) have relied on the use of the "mouse typhoid model" caused by *S. typhimurium* or *S. enteritidis*.

Though considerable controversy concerning these mechanisms in mouse typhoid currently exists (2, 13), with very few exceptions, most agree that protection is best induced by exposing animals to nonvirulent variants (14), mutants (11, 16), or nonpathogenic *Salmonella* which may or may not possess O antigens related to those of the pathogen (4). If direct extrapolation to human typhoid is permissible, one possible approach to typhoid prophylaxis would be the use of a living vaccine which might similarly provide solid immunity to the organism under natural circumstances. Because the natural route of infection is via the intestine, oral immunization techniques have often been mooted; apart from the practical advantages, the possibility that local immune phenomena arising in the intestinal tissues might lead to greater overall protection must be considered in assessing the potential worth of oral vaccines. Clearly, if these vaccines are to be effective, they should possess sufficient invasive properties to be capable of penetrating the intestinal tissues, and yet be unable to produce overt infection which might have undesirable consequences so far as the individual or the community is concerned.

This paper describes mutant strains of *S. enteritidis* which, so far as experimental enteric fever is concerned, seemed to satisfy these criteria. These mutants are classed as temperature sensitive and have been selected for their capacity to multiply normally at 28 C yet, at 37 C, produce filamentous organisms which seem incapable of producing infection in mice. In addition to noting some of the biological characteristics of these mutants, the results of preliminary

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studies on their effectiveness in protecting mice from otherwise lethal intraperitoneal or oral infections of *S. enteritidis* are described.

MATERIALS AND METHODS

Bacterial strains. *S. enteritidis* (Australian Type Culture Catalogue designation, *dansyz*) was obtained from the School of Microbiology culture collection. The organism was passaged through mice 12 times; on each occasion it was isolated from the spleen of an animal given 10^3 organisms intraperitoneally (ip) 3 days earlier. After the last isolation, the organism was lyophilized and it served as the parent strain for preparation of mutants and also as the challenge organism for protection tests. For routine purposes, the organism was recovered from the dry state at regular intervals and maintained on Dorset egg medium at 4 C. This procedure was also adopted for the temperature-sensitive mutant strains (*vide infra*).

Mice. Male Swiss-Albino mice of an outbred laboratory stock were kindly supplied by the Commonwealth Serum Laboratories, Melbourne, Australia. At the beginning of each experiment, the animals weighed 16 to 20 g; they were fed with Barastoc poultry-growers pellets with a lucerne supplement and were given water ad lib.

Mouse infection techniques: ip route. Mice were injected in the peritoneal cavity with 0.5-ml volumes of organisms suspended in nutrient broth (NB). The inocula were prepared by diluting log-phase cultures of organisms prepared by incubation in NB for 90 min at 37 C under aerated conditions. The number of viable organisms in the inoculum was determined by the method of Miles, Misra, and Irwin (18). The animals were observed daily for 4 weeks before the survival rate was recorded; the mean time to death for animals in each group was also calculated.

Oral route. Cultures of the organisms, grown in 250-ml volumes of NB for 16 hr, were centrifuged at 2,300 × *g* for 15 min and then suspended in the required volume of sterile milk. This suspension was fed in 0.1-ml volumes to mice which had been deprived of water for 48 hr; it was given from a 0.2-ml graduated pipette with a rubber teat attached and was readily taken by the animals. The number of viable organisms in the inoculum and record of survival of mice was determined as described above.

Isolation of temperature-sensitive mutants. The method of van de Putte et al. (23) was used. *S. enteritidis dansyz* was grown for 16 hr at 37 C in minimal-growth medium (MGB). The culture was diluted 1 in 20 in MGB and incubated at 37 C on a shaker in a water bath for 4 hr. A 5 ml-amount of the culture was centrifuged at 2,300 × *g* for 10 min and the deposited cells were suspended in 5 ml of 0.1 M sodium citrate buffer (*p*H 5.0). They were again centrifuged and were suspended in 0.4 ml of the buffer. The suspension was treated with 0.1 ml of a solution (4 mg/ml) of *N*-methyl *N*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) for 1 hr at 37 C; the action of the mutagenic agent was arrested by dilution of the preparation with 4.5 ml of MGB. The cells were then centrifuged, suspended in 5 ml of MGB, diluted 1 in 100 in 10-ml volumes of MGB, and incubated with mechanical rocking in a water bath at 28 C for 24 hr.

This culture was diluted 1 in 40 in 10 ml of MGB and incubated at 37 C for 3 hr. A 1-ml amount of this culture was passed through a membrane filter (5 μm pore size, Gesellschaft Gottinger, Germany), and organisms which had deposited on the filter were recovered in 0.85% saline, washed three times in saline, and resuspended in 10 ml of MGB which was then incubated at 28 C for 18 hr under aerated conditions. The procedure of alternating growth at 28 C with 3 hr of incubation at 37 C, followed by membrane filtration, was repeated daily until, by microscopic examination, a high percentage of filaments was observed in the deposits on the membrane filter; usually six to eight repeats were necessary. When this was achieved, the deposit was spread on nutrient agar (NA) plates which were incubated at 28 C for 24 hr to obtain single colonies. Selected colonies were "picked" onto three NA plates which were incubated at 28, 37, or 42 C; those colonies which failed to grow at 42 C were retested to confirm that they formed filaments at 37 C. Those which satisfied these requirements were designated temperature-sensitive filament formers (TSF); they have been numbered in order of their selection.

Preparation of cultures for immunization. For ip immunization, the mutant strains were grown overnight at 28 C in NB; these cultures were diluted 1 in 25 in NB and incubated under aerated conditions at 28 C for 2 hr to obtain log-phase cultures. These were appropriately diluted in NB, and 0.5-ml volumes were given by the ip route. In the case of oral immunization, because large numbers were required, the mutants were grown in Roux bottles on NA at 28 C for 18 hr. The growth was harvested in saline centrifuged at 2,300 × *g* for 15 min and then suspended in fresh sterile milk to the required concentration of organisms; 0.1-ml volumes were given to each animal.

In both instances, the number of viable organisms given was determined by the technique of Miles, Misra, and Irwin (18), incubating the plates at 28 C for 24 hr before counting colonies.

RESULTS

Biological characteristics of TSF mutants: (i) Morphology. The parent strain of *S. enteritidis* from which the mutants were derived, when grown at 28, 37, or 42 C, is morphologically typical of the genus appearing as a gram-negative bacillus approximately 3 by 1 μm and motile by means of peritrichous flagella. These same morphological characteristics were shown by cultures of 10 different TSF mutants grown in MGB or NB at 28 C, with the exception that none was motile. Preparations from NB cultures of two selected mutants (TSF11 and TSF19) were examined for the presence of flagella by the silver impregnation method of Blenden and Goldberg (3) and by electron microscopy; in neither instance were flagella detected on these organisms when grown at 28 C.

TABLE 1. *Frequency of temperature-tolerant variants in cultures of temperature-sensitive mutants of S. enteritidis*

Mutant	Viable count at 28 C	Viable count at 37 C	Frequency of variants
TSF 11	8.2×10^8	8.9×10^4	0.0108%
TSF 19	7.1×10^8	8.0×10^4	0.0112%

Apart from the lack of motility, the most striking characteristic of the mutant strains was shown when they were incubated in MGB or NB under aerated or static conditions. Within 3 hr, the cultures contained bacilli ranging in length from 5 to 50 μm; by 6 hr, they contained masses of interwoven clumps of filaments which were so long that they exceeded, by many times, the diameter of the oil-immersion microscopic field. Electron microscopic examination of the filamentous forms indcated that, although a large amount of deoxyribonucleic acid material was present, there was no evidence of cross septum or flagella formation.

(ii) **Colonial formation.** When grown at 28 C on NA or MacConkey agar plates, all mutants formed colonies typical of *Salmonella* species. In contrast to the parent strain which formed normal colonies at 37 and 42 C, when plated on these media at 37 C, the mutants produced occasional minute colonies, whereas at 42 C no growth occurred. An estimate of the frequency of organisms capable of forming colonies at 37 C was obtained by preparing cultures of TSF11 and TSF19 in NB by incubation overnight at 28 C. The cultures were diluted in 10-fold steps, and samples were plated on NA plates which were incubated at 37 or 28 C for 24 hr. In both mutant cultures, organisms capable of forming colonies at 37 C occurred with a frequency of approximately 0.01% (Table 1). These were termed temperature-tolerant variants; the fact that they were not revertants to the wild type was shown by their inability to form colonies on agar or to multiply in liquid medium when subcultured and incubated at 42 C, though subcultures of these colonies were always capable of growing slowly at 37 C.

(iii) **Growth in liquid medium.** The growth of the parent strain and the mutants TSF11 and TSF19 in liquid medium was compared by turbidimetry and viable counting. Cultures of the three organisms were prepared by incubation overnight in MGB at 28 C; 0.1-ml volumes were then transferred to 10-ml volumes of this medium in T-tubes which had been equilibrated to the temperature required for incubation (28 or 37 C). The cultures were rocked mechanically in a water bath held at 28 or 37 C, and spectrophotometric measurement of the turbidity was made at timed intervals after inoculation. At hourly intervals, 0.1-ml samples of the cultures were removed for determination of viable organism numbers. (Fig. 1 and 2).

At 28 C, apart from the length of the lag period, the growth rates of the parent strain and the two mutants were similar when assessed by both methods; the mean generation times varied between 102 and 114 min. Significant differences were found in cultures incubated at 37 C. The parent strain multiplied rapidly from the time of inoculation, with a mean generation time of 54 min. In contrast, both TSF11 and TSF19 showed a lag period of at least 60 min, after which the turbidity of the cultures increased slowly for a period of only 3 hr. Over a period of 6 hr, the number of viable organisms present in the mutant cultures decreased slowly in spite of the increase in turbidity. This finding did not appear to be related to the lack of some essential growth

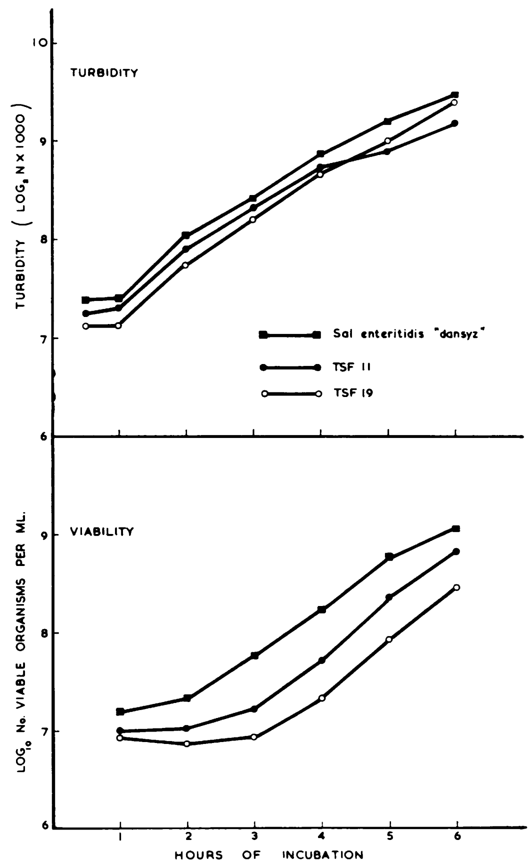

FIG. 1. *Growth of parent and TSF mutant strains of S. enteritidis in MGB at 28 C. Turbidity is expressed as log: N $\times$ 1,000, where N represents the galvanometer reading on the spectrophotometer.*

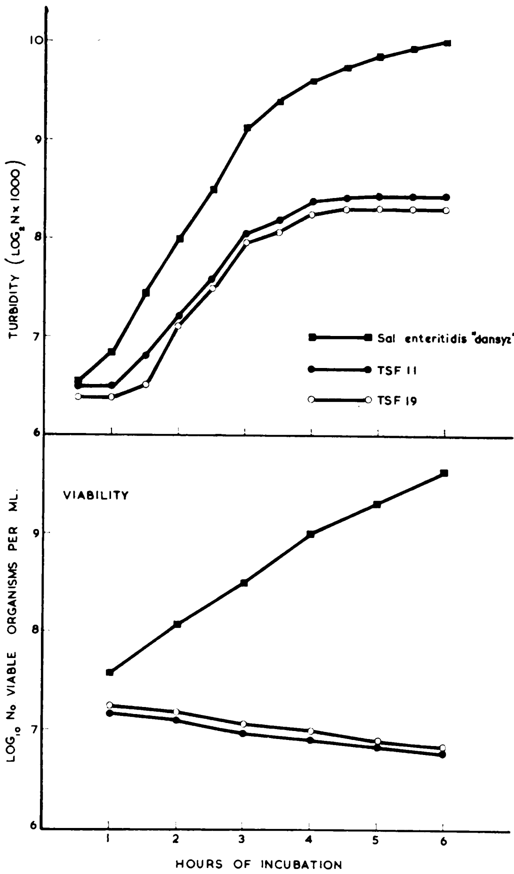

FIG. 2. *Growth of parent and TSF mutant strains of S. enteritidis in MGB at 37 C. Turbidity is expressed as explained in Fig. 1.*

factor(s) in MGB, for experiments repeated in NB yielded curves which were almost identical to those shown in Fig. 1 and 2.

(iv) Biochemical and antigenic activity. The biochemical activity of the mutants TSF11 and TSF19 were tested by using the methods described by Edwards and Ewing (8) for the identification and classification of *Salmonella*, except that the cultures were incubated at 28 C; in all respects, the mutant strains behaved in a manner identical to the parent strain (incubated either at 28 or 37 C), giving biochemical reactions characteristic of *S. enteritidis*. Antigenic analysis of the two mutants was also carried out by using single factor Salmonella O antisera and agglutinin-absorption tests with antisera prepared against the O antigens of the parent strain. These tests indicated that both TSF11 and TSF19 possessed O antigens identical to those of the parent strain. As might be expected from the above results, flagella antigens were not detected in the mutants grown at 28 C.

(v) Virulence. In these experiments, the virulence for mice of the parent strain and the mutants TSF11 and TSF19 was assessed by ip injection or by oral administration of graded doses of the organisms (Tables 2 and 3). When given by the ip route, the parent strain was highly virulent with an LD_{50} of 1.5×10^2 organisms for the strain of mice used. As might be anticipated, the organism was far less capable of causing a fatal infection when given orally, the

TABLE 3. *Virulence of TSF mutants for mice when given by the ip or oral routes*

Mutant	Route of injection	No. of viable organisms given	No. of deaths to no. of mice injected	Per cent mortality	Mean time to death (days)
TSF11	IP	5×10^8	19/20	95	1.0
		5×10^7	16/20	80	1.75
		5×10^6	0/20	0	
	Oral	2×10^{10}	0/20	0	
TSF19	IP	1×10^8	15/20	75	1.0
		1×10^7	6/29	21	1.5
		5×10^6	0/20	0	
	Oral	2×10^{10}	0/20	0	

TABLE 2. *Virulence of S. enteritidis dansyz for mice when given by the ip and oral routes*

Route of injection	No. of viable organisms given	No. deaths to no. mice injected	Per cent	Mean time to death (days)	LD_{50}[a]
IP	1.4×10^4	8/10	80	13.8	
	1.4×10^3	18/30	60	10.7	
	1.4×10^2	17/30	57	12.9	
	1.4×10^1	7/20	35	13.5	1.5×10^2
Oral	7×10^8	18/20	90	12.0	
	7×10^7	14/20	70	11.8	
	7×10^6	9/20	45	17.5	
	7×10^5	0/20	0		1.9×10^7

[a] Calculated by the method of Reed and Muench (22).

TABLE 4. *Resistance of mice immunized by IP injection of TSF mutants to IP challenge with S. enteritidis given 4 weeks later*[a]

Mutant	No. of mutant organisms injected	No. of mice surviving to no. of mice injected	Per cent survival	Mean time to death (days)
TSF11	1×10^7	53/63	84	10.8
	1×10^6	16/20	80	13.0
	1×10^5	11/20	55	10.0
	1×10^4	10/28	36	9.6
Controls		0/20	0	8.0
TSF19	1×10^7	21/23	91	10.0
	5×10^6	45/50	90	11.4
	1×10^6	24/28	86	17.7
	3×10^5	18/20	90	15.0
Controls		0/15	0	12.0

[a] Challenge dose of 8×10^4 organisms was equivalent to approximately 500 LD_{50}.

LD_{50} by this route being 100,000-fold greater than that given ip. Doses of the mutants greater than 10^7 organisms were lethal for many mice when given ip. However, in these animals the mean time to death of the animals was between 24 and 36 hr, a finding which did not seem consistent with the normal course of a *S. enteritidis* infection. All mice became severely ill within a few hours of injection, developed high temperatures, and produced watery diarrhea. At post mortem examination, extensive hemorrhage and congestion was found in the peritoneal cavity, suggesting that death was due to endotoxin activity rather than to multiplication of the organisms. Doses of the mutants containing less than 10^7 organisms were apparently inocuous for mice. Moreover, when given by the oral route as many as 2×10^{10} organisms failed to cause overt infection of the animals; because of the viscosity of the suspension, larger doses could not be given.

Immunity induced by mutant strains: (i) ip immunization and challenge. The mutants TSF11 and TSF19 were used. In the first experiments, the protection conferred by graded doses of the mutants, given to groups of mice by the ip route, was assessed by challenging the animals 4 weeks later with an ip infection of the virulent strain of *S. enteritidis* containing about 500 LD_{50} (Table 4). The immunity induced by injection of the mutants is substantial and is reflected not only by the high survival rate when doses of 10^5 organisms or greater were given but also by the prolonged time to death of the few animals which failed to survive the infection. For comparative purposes, groups of mice were injected by the ip route with nonviable vaccines prepared by killing suspensions of the virulent strain of *S. enteritidis* with a variety of physical or chemical reagents. These animals were challenged 4 weeks later with an ip injection of the virulent organism containing 200 LD_{50}; the results of this experiment (Table 5) illustrate the differences in protective activity of the living and dead vaccines.

The duration of the immunity after ip injection of the mutant TSF11 was tested by giving a group of 140 mice 10^6 viable organisms by this route. At various times, groups of 20 mice were challenged with approximately 500 LD_{50} of the virulent *S. enteritidis* given ip. Normal mice of the same age as the immunized animals were challenged at the same time. The results in Table 6 indicate that the immunity induced by a single injection of the mutant organism may be long lived; it showed no evidence of declining after 16 weeks.

(ii) Oral immunization and challenge. Although the size of the challenge inoculum was designed to contain approximately 100 LD_{50} (1.2×10^8 organisms), in these experiments some variation in numbers was unavoidable because of the method of preparation; moreover, in some instances, the challenge dose was not lethal for all control animals. For this reason, the results obtained in individual experiments are shown separately in Table 7 so that a direct comparison between the survival rates of immunized and

TABLE 5. *Resistance of mice immunized with nonviable S. enteritidis vaccines to ip challenge with virulent S. enteritidis given 4 weeks later*[a]

Vaccine no.	Method of prepn	No. of organisms given	No. of mice surviving to no. of mice injected	Per cent survival	Mean time to death (days)
1-HK	100 C for 60 min	10^8	1/15	7	9.2
2-HK	56 C for 60 min	10^8	7/24	29	10.0
3-UV	Ultraviolet light	10^8	2/19	11	7.4
4-ALC	70% Methyl alcohol	10^8	1/16	6	6.8
Controls	—	—	0/17	0	7.3

[a] Challenge dose of 2.8×10^4 organisms was equivalent to approximately 200 LD_{50}.

TABLE 6. *Duration of immunity to ip challenge in mice immunized IP with TSF11*[a]

Time between immunizing and challenge injections (weeks)	No. of mice surviving to no. of mice injected	Per cent survival	Mean time to death (days)
2	16/20	80	10.0
3	14/20	70	13.5
4	16/20	80	13.0
6	16/20	80	10.5
8	20/20	100	
12	19/20	95	22.0
16	19/20	95	11.0

[a] Immunizing dose of TSF11 was 10^6 viable organisms. Challenge dose of virulent strain was 9×10^4 organisms, equivalent to approximately 500 LD_{50}. Normal mice of the same age as immunized groups were challenged at the same time; no survivors were found amongst these animals. The mean time to death varied between 8.0 and 11.0 days.

control mice may be made. In these experiments, the protective capacity of graded doses of mutants TSF11 and TSF19 was assessed by oral challenge after 4 weeks.

In a second group of experiments, the effects of two large oral doses of the mutant vaccines given 4 weeks apart was assessed by oral challenge of the mice with 1.1×10^9 virulent organisms 4 weeks after the last vaccinating dose (Table 8). Though in both experiments assessment of the protection conferred by the oral immunizing procedure is made difficult because of the survival of small numbers of control animals, it is nonetheless evident that the mutant vaccines given by this route are capable of inducing a significant level of immunity to subsequent oral infection with the virulent strain.

DISCUSSION

After exposure to the mutagenic agent *N*-methyl *N*-nitro-*N*-nitrosoguanidine, several mutant strains of *S. enteritidis* were isolated on the basis of their capacity to grow as long filaments rather than as bacillary forms when incubated at 37 C. These mutants, termed temperature-sensitive filament formers, are capable of normal growth at 28 C; in contrast to the parent strain, they are unable to form colonies on solid media during incubation at 42 C. Two of these mutants (TSF11 and TSF19) were selected for more detailed examination.

At 28 C, the phenotypic characteristics of these mutants seemed similar to the wild-type strain; they multiplied at about the same rate in both minimal and nutrient growth media and produced similar colonies on solid media. A variety of biochemical tests suggested that the biosynthetic and metabolic properties of the mutants and wild-type strains were similar, as also was their antigenic composition judged by agglutination and agglutinin-absorption reactions. The only difference observed between the mutants and the parent strain at 28 C was the lack of ability of the former to synthesize flagella protein. At 37 C, striking differences were observed. In liquid medium, the turbidity of mutant cultures increased more slowly than did that of the wild-type cultures, and viable counting techniques revealed that during the period of incuba-

TABLE 7. *Survival of mice immunized by the oral route with TSF mutants after oral S. enteritidis infection given 4 weeks later*[a]

Mutant no.	No. of viable organisms in immunizing dose	No. of mice surviving to no. of mice infected	Per cent survival	Mean time to death (days)
TSF11	6×10^9	23/49	47	12.6
TSF19	5×10^9	29/50	58	10.9
Controls		0/20	0	12.0
TSF11	3×10^9	18/30	60	12.5
	3×10^8	14/30	47	11.2
Controls		2/20	10	10.2
TSF19	3×10^9	22/30	73	10.3
	3×10^8	19/29	66	13.1
Controls		3/20	15	12.3

[a] Challenge inocula for these experiments varied between 1.1×10^9 and 1.6×10^9 viable organisms.

TABLE 8. *Resistance to oral infection with S. enteritidis in mice given two oral doses of TSF mutants*[a]

Mutant no.	No. of organisms in first oral dose	No. of organisms in second oral dose	No. of mice surviving to no. of mice infected	Per cent survival	Mean time to death (days)
TSF11	2.8×10^{10}	1.8×10^{10}	26/30	87	13.3
TSF19	2.3×10^{10}	2.1×10^{10}	28/29	97	10.0
Control	None	None	2/20	10	12.6

[a] Challenge inoculum contained 1.6×10^9 organisms, equivalent to approximately 100 LD_{50}.

tion the number of viable colony-forming units in the mutant cultures declined. These observations reflected the failure of the mutants to divide at 37 C and, over 3 to 6 hr, the formation of tangled masses of extremely long filaments. Electron microscopic examination indicated that the mutants did not form cross septa in the filaments, and it is presumed that the genetic lesion is related to this rather than to their failure to utilize particular nutrients at 37 C.

Reversion to the temperature-insensitive form was not detected in the mutants. Though stable temperature-tolerant variants of the mutants were found, they were not revertants. More likely, these variants reflected the fact that within a population of mutant cells the genetic lesion was expressed over a narrow range of temperatures, e.g., 36 to 38 C rather than exactly at 37 C. Slight variations in the incubator temperature may also have contributed to their appearance.

Though large doses of the mutants were lethal for mice when given ip, the rapid onset of symptoms and the postmortem findings suggested this was the result of endotoxic activity of the cultures. Smaller doses (10^7 organisms or less) by the ip route did not cause lethal infection nor did doses of at least 2×10^{10} organisms given by the oral route; by either route, the mutants were at least 1,000-fold less virulent than the parent strain. It is logical to presume that this is the result of their inability to proliferate normally at the body temperature of the mice.

With this background of information, it seemed that the temperature-sensitive mutants might satisfy the criteria required for an effective, living, oral immunizing agent. When grown at 28 C, before inoculation of animals the mutants seemed to possess most of the phenotypic characteristics of the parent strain, and it was believed that they might be capable of invasion and sequestration in the tissues in the same manner as the virulent organism. Thereafter, because of the restriction imposed by the body temperature of the animal, proliferation of the mutant would be arrested, yet sufficient antigenic stimulation might be provided to induce specific resistance to a subsequent *Salmonella* infection. Though the results reported here can only be regarded as a preliminary assessment, it may be fairly claimed that the mutants are highly effective in this regard.

Although the methods of ip immunization and infection has been criticized in terms of its lack of relation to the normal route of *Salmonella* infection (1), it nonetheless provides some evidence of the protective capacity of immunizing agents (15, 20). Quite clearly, the injection of small numbers of either mutant by this route provides a solid and long-lasting immunity to severe challenge infection. The survival rates recorded (80 to 100%) are at least equal to, if not greater than, those recorded for other vaccines (living or dead) tested by the same method (14, 15, 20). Of the few mice that died, the increased time of survival compared with that of normal animals suggested they had acquired some, although insufficient, degree of resistance to the infection.

The method chosen to infect mice by the oral route has several disadvantages which are probably reflected by the failure, in most experiments, to obtain 100% mortality of normal mice infected with doses of the virulent organism containing 50 to 100 LD_{50} (about 10^9 organisms). Although the mice seemed to ingest the inoculum readily, there was no way to ascertain that all animals received the entire dose of organisms; although the inoculum was suspended in milk, the small volume may have been ineffective as a buffer against the acid *p*H of the stomach. Thus, considerable variation in the numbers of organisms which passed into the intestine and were then available to establish infection might be anticipated. Similar difficulties with the oral infection technique have been reported by Ocklitz et al. (21). More satisfactory methods of achieving intestinal infection have been described (5, 10); however, their use did not seem warranted in these experiments, particularly as oesophageal abrasion by stomach tubes may have provided a direct route for invasion of the blood stream by the infecting organisms. The above limitations would also apply to orally administered immunizing doses of the mutant organisms, and it is likely that a number of animals which failed to survive the subsequent challenge infection did so because they received an insufficient inoculum of the mutant to initiate an immune response. This is supported by the fact that, in contrast to ip immunized animals, these mice died at the same time as the control animals and, presumably, were no more resistant to the challenge infection than normal.

In spite of these difficulties, oral immunization with both mutant strains seemed to confer considerable protection against lethal oral infection with *S. enteritidis*; two doses of the vaccines given 4 weeks apart increased the survival rates, most probably because the second dose may have "taken" in a proportion of those animals which failed to receive a sufficient inoculum in the first place. It must be stressed that the dose of virulent organisms given in the challenge

infection probably represented an inoculum far in excess of that which might be received under natural circumstances.

Although direct extrapolation to typhoid infection is barely justified, it is of interest to note that the LD_{50} of *S. typhi* given orally to human volunteers [10^7 organisms (12)] was of the same order as the LD_{50} of the *S. enteritidis* for mice; these workers have demonstrated that, although parenteral immunization with typhoid vaccine may effectively protect against infection with an LD_{50} or less, it is unable to control infections in which greater numbers of organisms are given. Unpublished observations by one of us (G.N.C.) indicate that the same holds true for parenterally administered, killed vaccines in *S. enteritidis* infection of mice. Under these circumstances, the fact the oral immunization with the living mutant strains provides significant protection against doses containing at least 100 LD_{50} of *S. enteritidis* offers some hope that similar agents might eventually be considered for immunization against typhoid fever.

Apart from the potential use of the TSF mutants as immunizing agents, they may also be used to advantage in studies primarily concerned with the mechanisms of immunity which operate against *Salmonella* infections of animals and, possibly, of man. Although these mutants may be unable to multiply at body temperature, this does not deny the possibility that they may survive for limited periods in the host tissues; the fact that they are genotypically similar to the virulent strain of *S. enteritidis* makes them most suitable for use in studies designed to determine whether immunity is antibody dependent or mediated through the broader phenomena of cellular immunity and delayed hypersensitivity (2). Later papers of this series (Fahey and Cooper, *unpublished data*) will report the results of such studies in detail.

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