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Contribution to the study of live streptomycin-dependent *Salmonella* vaccines: the problem of reversion to a virulent form

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SUMMARY

The recovery of virulence by means of reversion of a live streptomycin-dependent (Sm D) *Salmonella typhimurium* vaccine was studied in CD-1 Swiss mice. Initially, a one-step Sm D mutant was obtained from a virulent streptomycin-sensitive (Sm S) *S. typhimurium* strain. Afterwards, two pools of streptomycin-independent (Sm I) revertants were prepared from the Sm D strain. The virulence of the Sm D strain and of the Sm I revertants was tested intraperitoneally. In the virulence testing the original suspension of the Sm I revertants, as well as their 1st and 10th passages on plain medium, medium + 50 μg. streptomycin/ml. and medium + 1000 μg. streptomycin/ml. were used. The results show that the Sm D mutant was avirulent, its avirulence being due to an intrinsic, genetic quality. The Sm I revertants, compared to the original Sm S strain, also displayed a lack of virulence. However, afterwards, the Sm I revertants behaved quite differently, according to their subsequent passages. Indeed, there was an increase in virulence after passages on plain medium, whereas after similar passages on medium containing the drug, the virulence not only failed to increase, but disappeared almost completely. Moreover, the passages on medium containing 1000 μg. streptomycin/ml. induced a return to the status of drug-dependence. The danger of recovery of virulence by means of revertants is evaluated.

INTRODUCTION

In spite of the early attempts of Besredka (1910, 1913) and Ciuca, Combiescu & Balteanu (1915), the use of live vaccines against typhoid fever has long been considered as a desideratum unlikely to be attained in current practice. The reason for this is because of the high risks implied by the use of such vaccines.

The success of live oral poliomyelitis vaccine has undoubtedly served as an important stimulus to the re-examination of this problem. Similarly, a leading role was played by the successful attempts to use such vaccines against bacillary dysentery (Formal *et al.* 1965*a*, 1965*b*, 1966; Istrati, 1961; Istrati *et al.* 1964;

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Istrati, Meitert & Ciufecu, 1965*a*, *b*, *c*, 1968; Mel *et al.* 1965*b*, 1968, 1971; Mel, Terzin & Vuksic 1965*a*, *c*).

Recent progress in microbial genetics has facilitated this task. Consequently, various techniques have been employed in order to obtain *Salmonella* strains both avirulent and effective as live vaccines (Archer & Rawley, 1969; Bacon, Burrows & Yates, 1951; Baron & Formal, 1960; Cooper & Fahey, 1970; Fahey & Cooper, 1970*a*, 1970*b*; Furness & Rowley, 1956; Herzberg, 1962; Kenny & Herzberg, 1967; Kishimoto, 1965; Reitman, 1967; Sergeev, Taratorina & Elkina, 1967). Among these strains the streptomycin-dependent (Sm D) mutants seem to offer, so far, the most promising solution. Particularly significant in this respect, are the favourable results obtained during the last years in volunteers (Du Pont *et al.* 1970; Hornick *et al.* 1970; Hornick & Du Pont, 1970) with the *S. typhi* 27 V strain (Reitman, 1967) and in chimpanzees (Cvjetanovic, Mel & Felsenfeld, 1970) with a variant of the same strain. These results constitute a real progress towards oral immunization with a live vaccine against typhoid fever. Considering the great advantages of the oral route in antityphoid immunization (Vladoianu *et al.* 1965; Vladoianu & Dimache, 1965; World Health Organization, 1972), and depending upon confirmation of these data in further trials, it may be hoped that the Sm D *Salmonella* strains will constitute a valid alternative in resolving the problem of vaccination against typhoid fever, as well as other *Salmonella* infections.

The use of live Sm D vaccines in the prophylaxis of salmonellosis and particularly of typhoid fever leads to the very important problem of the stability of the avirulence of these vaccines. Indeed, such preparations should be genetically stable, thus obviating the risks of reversion to a virulent form. This is an essential criterion which such preparations are required to meet (World Health Organization, 1972).

Trials performed so far seem to demonstrate that the Sm D *S. typhi* vaccine used is quite stable since no significant undesired reactions have occurred either in volunteers or in chimpanzees. However, considering the serious condition a return of this vaccine to a virulent drug-independent form would cause, we feel that particular attention has to be given to its possible reversion, and so complementary studies are necessary.

The present study is a result of such a consideration. Since no experimental model for reproducing human typhoid infection is available to date, and subhuman primates and volunteers are difficult to obtain, our experiments were carried out on mice, using *S. typhimurium*. Mice are naturally susceptible to *S. typhimurium* and develop an enteric-type illness bearing a resemblance to typhoid fever. For convenience, *S. typhimurium* infection of mice has been extensively used for many years as a model for typhoid fever and has yielded useful information which, as far as can be ascertained, correlates – within certain limits – with the pathogenesis of the human infection (Orskov, Jensen & Kobayashi, 1928; Hobson, 1957; Baron & Formal, 1960; Blanden, Mackaness & Collins, 1966; World Health Organization, 1972).

MATERIALS AND METHODS

Cultures

(1) *S. tym* 779 C–Sm S is a virulent (intraperitoneal LD50, 2×10^2), streptomycin-sensitive *S. typhimurium* strain, used by us in previous studies (Vladoianu *et al.* 1960; Vladoianu & Dimache, 1964).

(2) *S. tym* 779 C–Sm D is a one-step streptomycin-dependent mutant obtained from the former, according to the method of Paine & Finland (1948). This strain grows abundantly in the presence of streptomycin; it requires at least 50 μg. of streptomycin-base/ml. of medium (Difco tryptose-agar) for growth and tolerates at least 20,000 μg. of antibiotic/ml. of medium. It shows no dependence upon either neomycin or gentamicin, the only other aminoglycoside antibiotics with which it was tested.

(3) *S. tym* 779–Sm I rev. designates the streptomycin-independent revertants obtained from the *S. tym* 779 C–Sm D mutant, by plating cells of the latter on medium devoid of streptomycin. On plain Difco tryptose-agar the incidence of reversion was 2 to 8 colonies per 10^8 in a dependent cell population, after an incubation period of 5 days at 37° C.

The above-mentioned *S. tym* 779 C–Sm S, Sm D and Sm I rev., all belonged to the smooth form and displayed the morphological, biochemical and serological characters of the *S. typhimurium* species.

Experimental animals

Male, specific pathogen-free CD-1 Swiss mice (initially weighing 24–26 g.) were obtained from Charles River Breeding Laboratories. The mice were housed in stainless steel cages (up to 10 animals per cage) on sawdust bedding and fed with Charles River mouse pellets and tap water *ad libitum*. Cages, after preliminary sterilization or disinfection, were perfectly cleaned twice weekly.

Virulence testing

The object was to test the multiplication capacity of the Sm D and Sm I rev. mutants in the mouse host, by virtue of their growth to population levels resulting in death of the animal.

The behaviour of *S. tym* 779 C–Sm D strain was investigated both in the presence and absence of streptomycin. The Sm D cells were harvested from cultures on Difco tryptose-agar + 1000 μg. streptomycin/ml.

In the case of the *S. tym* 779 C–Sm I rev., a great number of revertant colonies was obtained. The drug-resistance level of each colony was examined by suspending it in 0·2 ml. saline and then depositing small drops of the suspension (by means of a fine platinum loop of 2 mm.) both on plain tryptose-agar and on that containing streptomycin in varying concentrations. Among these colonies, two groups of 30 each were selected. The first included only revertants displaying a high level of drug-resistance, while the revertants of the other group exhibited a wide range of phenotypes from drug-sensitivity to high-level resistance. The saline suspensions of the colonies corresponding to each group were pooled separately so that finally two pools of bacterial suspensions of revertants were

obtained. The original suspensions (of both the pools of Sm I revertants), as well as their 1st and 10th passages of plain medium (Difco tryptose-agar), medium + 50 μg. streptomycin/ml. and medium + 1000 μg. streptomycin/ml., were used in the virulence testing.

The virulence test consisted of the intraperitoneal inoculation of 0·5 ml. of the appropriate bacterial concentrations to groups of 10 mice each.

With the exception of the original suspensions, an 18-hour culture at 37° C., on Difco tryptose-agar (simple or, if necessary, containing the drug) was always used. From this a saline suspension corresponding to a turbidity of 10 international opacity units per ml. (World Health Organization, 1954) was made and using this suspension, a series of 10-fold saline dilutions was then prepared. Some of these dilutions served for the virulence testing whilst others were used to count the viable bacterial cells by a pour-plate technique.

The death of infected mice was registered daily for a period of 21 days. In preliminary experiments, mice that died after infection with *S. typhimurium* were autopsied, and samples of cardiac blood and spleen were cultured on appropriate media. Colonies that developed during incubation were identified serologically as *S. typhimurium*. Having demonstrated that all deaths were associated with recovery of the injected pathogen, autopsies were performed only if required by specific cases.

At the end of the control period, survivors were killed, their spleens removed and then ground separately in a tube containing 0·5 ml. sterile saline. Undiluted samples of 0·1 ml. were spread on Difco SS-agar and, if necessary, on the same medium supplemented with streptomycin, and the total bacterial count per organ was estimated. The identity of colonies was verified as *S. typhimurium* by slide agglutination, using specific antisera.

In parallel, live bacterial cell counts (on plain medium and, if necessary, on medium containing streptomycin), and the drug-resistances of the two Sm I rev. pools (original suspensions and their different successive passages), were also investigated.

RESULTS

The data from the virulence testing are summarized in Tables 1–4.

The results in Table 1 show that the *S. tym* 799 C–Sm D mutant is avirulent. In fact, animals inoculated intraperitoneally resisted very high doses of live organisms belonging to this strain. Under similar conditions, the parent strain *S. tym* 779 C–Sm S would have killed all the animals in 2–4 days. The *S. tym* 779 C–Sm D mutant remained avirulent even in the presence of streptomycin, since all the challenged animals receiving the drug in parallel survived up till the end of the control period. The presence of the antibiotic, which allowed abundant growth *in vitro*, did not favour the growth of these cells *in vivo* to population levels resulting in death, in spite of the high doses of Sm D cells inoculated.

The infected animals displayed only mild signs of infection within 12–24 hours after challenge.

Table 1. *Virulence testing of* S. tym 779 *C–Sm D mutant*

No. of infected animals	No. of inoculated organisms	Subcutaneous administration of streptomycin (0·5 ml.)	No. of dead animals	Survivors: With recovery of challenge organism from the spleen	Survivors: Volume of spleen
10	$4{\cdot}2 \times 10^7$	—	1*	2 (15)†	Very slightly increased
10	$4{\cdot}2 \times 10^6$	—	0	0	Normal
10	$4{\cdot}2 \times 10^7$	10 mg. daily‡ 5 days	0	9 (110)†	Slightly increased
10	$4{\cdot}2 \times 10^6$	10 mg. daily‡ 5 days	0	9 (50)†	Very slightly increased

* This mouse revealed negative hemoculture and spleen of normal volume.
† In parentheses: the mean number of recovered organisms/spleen.
‡ Given in two equal doses, at approximately 12 hours interval (first streptomycin dose was administered 30 minutes prior to challenge).

Table 2. *Virulence testing of* S. tym 779 *C–Sm I rev. mutants and their* 1*st and* 10*th passages on plain tryptose-agar*

Bacterial suspension	No. of infected animals	No. of inoculated organisms	No. of dead animals	Survivors: With recovery of challenge organism from the spleen*	Survivors: Volume of spleen
Original	10	$1{\cdot}6 \times 10^7$	8	2 (47·5)†	Much increased
	10	$1{\cdot}6 \times 10^6$	2	7 (42·5)†	Slightly increased
After 1 passage	10	$2{\cdot}9 \times 10^7$	10	—	—
	10	$2{\cdot}9 \times 10^6$	10	—	—
After 10 successive passages	10	$2{\cdot}0 \times 10^7$	10	—	—
	10	$2{\cdot}0 \times 10^6$	9	0	Much increased
	10	$2{\cdot}0 \times 10^5$	2	4 (30)†	Much increased

* Recovery on plain SS-agar.
† In parentheses: the mean number of recovered organisms/spleen.

The data in Table 1 concerning the recovery of the challenge organism from the spleens of the survivors indicate that a multiplication of Sm D cells was induced by the presence of the antibiotic. This process, however, was not able to overcome the mouse organism's defensive forces and lead to lethal bacterial levels.

The avirulence of *S. tym* 779 C–Sm D strain thus seems to be due to an intrinsic, genetic quality of this mutant, rather than to its dependence upon the drug.

It is also worth emphasizing that all the cultures isolated from survivors retained their streptomycin-dependency; not one independent colony was detected. Hence, one can conclude that Sm D cells are able to persist for a long time in the animal tissues, even in the absence of streptomycin.

Table 3. *Virulence testing of* S. tym 779 *C–Sm I rev. mutants after cultivation on tryptose-agar* + 50 μg./ml. *streptomycin*

				Survivors		
				With recovery of challenge organism from the spleen, on:		
Bacterial suspension	No. of infected animals	No. of inoculated organisms	No. of dead animals	Plain SS-agar	SS-agar + 50 μg./ml. streptomycin	Volume of spleen
After 1 passage	10	$5 \cdot 0 \times 10^7$	2	8 (21)*	3 (6)*	Much increased
	10	$5 \cdot 0 \times 10^6$	0	2 (85)*	2 (22·5)*	Normal
After 10 successive passages	10	$3 \cdot 3 \times 10^7$	0	1 (35)*	1 (45)*	Slightly increased
	10	$3 \cdot 3 \times 10^6$	0	0	0	Normal

* In parentheses: the mean number of recovered organisms/spleen.

Table 4. *Virulence testing of* S. tym 779 *C–Sm I rev. mutants after cultivation on tryptose-agar* + 1000 μg./ml. *streptomycin*

				Survivors		
				With recovery of challenge organism from the spleen, on:		
Bacterial suspension	No. of infected animals	No. of inoculated organisms	No. of dead animals	Plain SS-agar	SS-agar + 1000 μg./ml. streptomycin	Volume of spleen
After 1 passage	10	$3 \cdot 7 \times 10^7$	0	0	3 (11·5)*	Normal
	10	$3 \cdot 7 \times 10^6$	0	0	3 (5)*	Normal
After 10 successive passages	10	$4 \cdot 2 \times 10^7$	0	0	2 (7·5)*	Normal

* In parentheses: the mean number of recovered organisms/spleen.

Tables 2–4 present the results of the virulence tests carried out on *S. tym* 779 C–Sm I rev. mutants. Since the two pools of revertants behaved similarly, only data for the pool with initial high drug-resistance are presented.

As shown in Table 2, the virulence of Sm I revertants tested immediately after isolation (original bacterial suspension) was low. These revertants compared to the *S. tym* 779 C–Sm S strain, also displayed a lack of virulence. However, afterwards, the Sm I revertants behaved quite differently, according to their subsequent cultivation on plain tryptose-agar or tryptose-agar containing streptomycin. Indeed, there was an increase in virulence after passages on plain medium (Table 2) whereas after similar passages on medium containing the drug (Tables 3 and 4), the virulence not only failed to increase, but disappeared almost completely.

The differences in mortality rate observed are statistically significant (χ_c^2 test; $P < 0{\cdot}01$).

The increase in virulence was evidenced both by the increased mortality rate and by the severity of the signs of infection in the inoculated animals (prolonged apathy, lack of appetite, loss of weight). Moreover (see tables), the spleens of survivors were much increased in size. The challenge organisms were frequently recovered from these spleens.

The virulence of the organisms recovered from the spleens of the survivors, recorded in Table 2 after 10 successive passages on plain medium, was also tested and the LD50 was found to be 8×10^5. At the end of the control period, before being killed, these survivors proved to be intestinal carriers of the challenge organism; the virulence of the LD50 of the organism isolated from faeces was 8×10^4. It is also worth mentioning that the virulence of the organism isolated by hemoculture from the dead mice belonging to the same group was tested and the LD50 was found to be $5{\cdot}5 \times 10^5$.

The above data show that a recovery of virulence by means of revertants could be proved. This, however, could only be done in special circumstances, namely after cultivation of revertants on media devoid of streptomycin. This recovery was partial and stable enough. On the contrary, it follows that cultivation in the presence of the antibiotic impedes the process of virulence recovery in revertants.

Other interesting observations resulted from the investigations concerning the drug-resistance levels and from live bacterial cell-counts of the revertants. In fact, the behaviour of Sm I revertants towards the drug also varied considerably, according to their subsequent cultivation. Thus, after passages on plain medium, revertants gradually lost their initial drug-resistance, becoming similar to the original *S. tym* 779 C–Sm S strain. On the contrary, cultivation in the presence of streptomycin determined the maintenance of the initial high drug-resistance level. Moreover (see Table 4), the passages on medium containing 1000 μg. streptomycin/ml. induced the return to the state of drug-dependence.

DISCUSSION

The threshold doses used for virulence testing by the intraperitoneal route may be considered sufficiently high to demonstrate the avirulent character of a *S. typhimurium* strain. Adopting higher bacterial concentrations could have fostered errors, since the 'toxic' character of the inoculated microorganisms could have interfered at these concentrations. In fact, it has been demonstrated by Schewe (1958) that the total viable count of *S. typhimurium* in a mouse killed by infection was invariably between 10^9 and 10^{10} cells. This indicates that a population of this size yields sufficient toxicity to cause death. As the end-point which we adopted in the virulence testing was death of the animal, we used maximum inocula of 10^7–10^8 bacterial cells in order to avoid this problem of toxicity and deal only with the virulence of the organism. Consequently, absence of death with such high inocula practically indicated the total incapability of

the test strain to grow in the mouse organism and to overcome its defence forces, i.e. it indicated the avirulence of this strain.

Our findings concerning the benign behaviour of the Sm D *Salmonella* strains only partially agree with those of Reitman (1967). He, experimenting on mice, showed that the avirulence of the 27 V Sm D *S. typhi* strain was due only to the inability of this strain to multiply in the absence of streptomycin. When this essential nutrient was available, however, the virulence characteristics exhibited were similar to those of independent strains. This lack of correlation between our data, where the avirulence seems to be due to a genetic quality of the Sm D *Salmonella* strains rather than to their dependence upon the drug, and those of Reitman, might be explained by different experimental conditions. In fact, the reaction of the mouse to intraperitoneal challenge with *S. typhi* (in contradistinction to *S. typhimurium* infection) does not resemble typhoid fever in man. Besides, one has to bear in mind the fact that the *S. typhi* virulence testing technique in mice in the presence of mucin implies additional factors which operate both on the challenged organism and the infective germs (Felix, 1951). As a result, the defence mechanisms of the animal are blocked and completely artificial experimental conditions are created (Orskov, 1940). However, under natural conditions in volunteers (Du Pont *et al.* 1970), Reitman's *S. typhi* 27 V strain did not seem to display virulence characteristics similar to those of non-dependent *S. typhi* strains, in spite of its administration together with streptomycin.

Similarly, i.e. owing to the presence of an intrinsic, genetic quality, one could explain the behaviour of the *S. tym* 779 C–Sm I revertants grown in the presence of streptomycin. Here, a correlation is seen between the virulence of the Sm I revertants and the degree of drug-sensitivity. This is in agreement with the findings of Schnitzer & Grunberg (1957).

The observation concerning the return of Sm I rev. to Sm D is worth particular attention. This behaviour might be explained by the fact that Sm I rev. are double mutants in which the original Sm D mutation remains (Hashimoto, 1960; Goldschmidt, Matney & Bausum, 1962). This phenomenon of return to drug-dependence probably represents a major mechanism in the stability of the avirulence of live oral Sm D *Salmonella* vaccines. Return to drug-dependence is achieved only in the presence of high streptomycin doses. This constitutes a supplementary argument for a concomitant administration of sufficiently high antibiotic doses in oral antityphoid vaccination with live Sm D strains. In fact, the abundant multiplication of Sm D strains in the bowel and, implicitly, the efficiency of a local intestinal immunization, depends on the abiding presence of high antibiotic concentrations (Cvjetanovic *et al.* 1970).

It is worth mentioning that the two pools of Sm I revertants behaved similarly in all tests to which they were submitted, in spite of the difference in the initial drug-resistance of their components. In addition, the benign behaviour of Sm I rev. pools cultivated in the presence of streptomycin, seems to be in agreement with the absence of undesired reactions in vaccinated volunteers and chimpanzees. For these reasons, our system of testing pools prepared *in vitro* seems to agree with the situation observed *in vivo* during the experimentation of oral vaccination

in volunteers and chimpanzees. In fact, if the doses of vaccine used in these experiments and the frequency of reversion to drug-independence are taken into account, it may be concluded that the population of revertants in the vaccine doses was greater than 10^2. Consequently, the eventual state of the revertants was determined by their evolution as a 'pool'.

Extrapolating the observations made in mice to vaccinated volunteers, we would like to specify that the present findings must be considered only as guiding information. Indeed, conceding that our *S. typhimurium* model is a useful test model, particular investigations for each vaccinating *S. typhi* strain would eventually be necessary in volunteers.

Taking into account the results of this present work, and with the above attitude of reserve, the question arises whether recovery of virulence by means of revertants constitutes a danger during antityphoid vaccination with live Sm D strains. In order to obtain a correct answer, one has to consider separately the two principal methods of antityphoid vaccination, by subcutaneous or oral routes.

With subcutaneous vaccination, a concomitant administration of antibiotic would not normally be indicated. Consequently, the reversion to a virulent form would find favourable conditions, depending on three factors: the degree of virulence of the revertants, their number, and the degree of sensitivity of the parasitized host. In an experimental system similar to that used by us in the present study, a real danger would result only if the number of revertants reached 10^5-10^6, which implies the administration of a Sm D cell population much larger than the doses employed usually in subcutaneous immunization. However, this type of system does not seem to constitute a general rule. Thus, Baron & Formal (1960), experimenting on mice of the Bagg strain with a Sm D mutant derived from a virulent *S. typhimurium* strain, have noticed that some mice injected intraperitoneally with 10^7 washed cells of their mutant, died within 3 weeks after inoculation. Post-mortem cultures showed the presence of Sm I *S. typhimurium*. As the frequency of reversion of the Sm D mutant to streptomycin-independence was about 10^{-7}, the authors concluded that the presence of even one revertant cell was undesirable. Interesting observations to the same effect were made by Shuster *et al.* (1971), who studied the virulence properties of revertants obtained from Sm D mutants of *S. enteritidis* and *S. typhimurium*. A minor proportion of virulence was established amidst these revertants: about 85 % of them remained avirulent, and virulence properties were restored partially or completely in the rest. The frequency of revertants with complete recovery of virulence was very low: 1/82 for *S. enteritidis* and 0/107 for *S. typhimurium*. It could be concluded from the above data that in the case of a subcutaneous immunization (without streptomycin administration), a complete recovery of virulence is possible, but only at a relatively low rate.

Concerning oral vaccination, favourable conditions for the removal of the risk of complete reversion to virulence seem to exist. This is due to the fact that the concomitant administration of streptomycin finds its major indication here. The probable mechanisms by which the presence of streptomycin hinders the

formation of revertants in general, and particularly the selection of virulent revertants, result from the present study. One problem remains: if a revertant selection would have been hindered during oral immunization (that is by the presence of the antibiotic in the bowel), it should have become possible at the end of the immunization period, i.e. when the antibiotic administration ceases, and large numbers of Sm D cells find conditions similar to those which *in vitro* favour the process of recovery of virulence. In order to tackle this problem, an adequate experimental model of oral antityphoid vaccination is necessary. Unfortunately, the CD-1 mice proved highly resistant to experimental oral infection and consequently are not suitable for this purpose. In the hope of finding a favourable solution to this problem, we propose to test, in future studies, the behaviour of other strains of mice towards oral infection with *S. typhimurium*.

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