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A COMPARATIVE STUDY ON THE IMMUNOGENICITY OF LIVE AND INACTIVATED *SALMONELLA TYPHIMURIUM* VACCINES IN MICE

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ABSTRACT

CAMERON, C. M. & FULS, W. J. P., 1974. A comparative study on the immunogenicity of live and inactivated *Salmonella typhimurium* vaccines in mice. *Onderstepoort J. vet. Res.* 41(3), 81—92 (1974).

Mice were very successfully immunized against intraperitoneal infection with virulent *Salmonella typhimurium* by fluid and lyophilized formalin inactivated vaccines prepared from a smooth strain of the microorganism. A single subcutaneous injection of 0,2 ml containing 0,1% packed cells was sufficient to confer a solid immunity when tested 2 weeks after immunization. Fluid vaccine was fully potent after storage at 50 °C for 1 month.

A good immunity was also obtained with live vaccines prepared from rough mutants of *S. typhimurium*. The degree of protection varied with the vaccine strain used. Only those mutants which possessed some degree of residual virulence produced a solid immunity while absolutely avirulent mutants were ineffective unless excessively high doses were employed.

Neither inactivated nor live *S. typhimurium* vaccines conferred protection against *S. dublin* infection.

INTRODUCTION

In South Africa *Salmonella typhimurium* infection is responsible for up to 19,1% of all cases of calf paratyphoid and also occurs sporadically in other animals (Botes, 1965). Consequently, a satisfactory vaccine should not only afford protection against *S. dublin* but also against *S. typhimurium*.

Botes (1964) developed a vaccine consisting of avirulent rough mutants of both *S. dublin* and *S. typhimurium* which produced a good immunity in calves. However, in experiments aimed at elucidating the mechanism of immunity in mice, it was found that the degree of immunity was appreciably less than anticipated (Cameron, unpublished observations, 1973). In order to resolve the problem, a series of experiments was conducted in which the immunizing properties of live vaccines prepared from rough mutants and inactivated vaccines prepared from a virulent strain were evaluated quantitatively.

MATERIALS AND METHODS

Experimental animals

Conventionally reared male albino mice, obtained from the colony maintained at this institute, were housed in plastic cages on wood shavings and fed a balanced ration. All experiments on immunization were done in 6-week-old male mice whereas 8-week-old animals were used for the virulence assays.

Bacterial strains

A list of the smooth and rough strains of *S. typhimurium* used in this study is given in Table 1. All the cultures were lyophilized and kept at 4 °C.

TABLE 1 *Salmonella typhimurium* strains used in this study

Strain	Type	Origin	References
395M-RO–395M-R10	Rough.....	Dr T. Holm, Karolinska Serum Institute, Stockholm, Sweden	Holme, Lindberg, Garegg & Onn (1968), Edebo & Normann (1970)
M206.....	Rough.....	Mr B. Reynolds, University of Adelaide, Adelaide, S. Australia	Jenkin & Rowley (1965); Rowley, Auzins & Jenkin (1968)
C5.....	Smooth.....	Mr B. Reynolds, University of Adelaide, Adelaide, S. Australia	Jenkin & Rowley (1965); Rowley, Auzins & Jenkin (1968)
G30D.....	Rough.....	Dr R. Germanier, Institute Sérothérapique et Vaccinal Suisse, Berne, Switzerland	Germanier & Fürer (1971); Germanier (1972)
ILJ–15.....	Rough.....	Dr H. Jarolmen, American Cyanamid Co., Princeton, N. J., U.S.A.	Jarolmen *et al*, (1971)
T34–59.....	?	Dr L. le Minor, Institute Pasteur, Paris, France	Del Guercio, Tolone, Braga Andrade, Biozzi & Binaghi (1969)
SF1827.....	Rough.....	Mr N. L. van Jeney, Max Plank Institüt für Immunbiologie, Freiburg, Germany	Personal communication (1973)
1566.....	Smooth.....	Dr J. G. Collee, Dept. Bacteriology, University Medical School, Teviot Place, Edinburgh, Scotland	Cronly-Dillon (1972)
SL 1027.....	Smooth.....	Dr H. Nikaido, University of California, Berkeley, California, U.S.A.	Nakano & Saito (1969)
SR 11.....	Smooth.....	Dr L. J. Berry, University of Texas, Austin, Texas, U.S.A.	Venneman & Bigley (1969)
2656 34×O*1.....	Rough.....	Veterinary Research Institute, Onderstepoort	Botes (1964)
2656 34×S*2.....	Rough.....	Veterinary Research Institute, Onderstepoort	—
2656 34×DE*3....	Rough.....	Veterinary Research Institute, Onderstepoort	—
2656 51×.....	Rough.....	Veterinary Research Institute, Onderstepoort	Botes (1964)
2656 V*4.....	Smooth.....	Veterinary Research Institute, Onderstepoort	Botes (1964)
2659.....	Smooth.....	Veterinary Research Institute, Onderstepoort	—
1969.....	Smooth.....	Veterinary Research Institute, Onderstepoort	—

*1 Original rough strain
*2 Selected single colony from original rough strain
*3 Maintained on Dorsett egg medium for 1 year at 4 °C
*4 Virulent

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Assay of virulence

Smooth strains

The strains to be tested for virulence were cultured on nutrient agar overnight at 37 °C and the growth suspended in either tryptone water or saline. The density of the suspension was nephelometrically adjusted to contain approximately $4{,}0\times10^8$ or $2{,}5\times10^8$ live bateria/ml. Twofold, fivefold or tenfold dilutions of these suspensions were prepared and groups of 10 mice injected intraperitoneally with 0,2 ml of the various dilutions, as required for the particular experiments. Deaths were recorded for either 7, 14 or 21 days.

Rough strains

A similar procedure was followed. Fivefold serial dilutions were prepared from standardized suspensions containing $\pm2{,}5\times10^8$ bacteria/ml and groups of 10 mice injected with 0,2 ml subcutaneously. Deaths were recorded for 14 days.

Agglutination in acriflavine

In order to determine their degree of roughness, the ability of strains to agglutinate in acriflavine solutions was determined as described previously (Cameron, Fuls & Van Reenen, 1972).

Preparation of vaccines

Live fresh vaccines

The strains from which the vaccines were to be prepared were grown on nutrient agar at 37 °C for 18 h and the growth suspended in tryptone water. In the first immunity experiment, where high doses of bacteria were needed, the required density was obtained by measuring the packed cell volume (pcv) and preparing appropriate dilutions. In subsequent experiments the density was adjusted nephelometrically to contain approximately $2{,}5\times10^8$ bacteria/ml. Fivefold serial dilutions of this suspension were made in tryptone water in order to obtain graded doses of vaccine according to the requirements of the particular experiment.

A concentration of 0,02% packed cells was equivalent to $\pm3\times10^8$ bacteria/ml.

Live lyophilized vaccine

The bacteria were grown in D15 broth in shake cultures for 18 h at 37 °C (Schlecht & Westphal, 1966) and the growth harvested by centrifugation. The sedimented bacteria were resuspended in a buffer-lactosepeptone solution (BLP), lyophilized in 1,0 ml quantities *in vacuo* (Cameron & Fuls, 1970) and stored at 4 °C. The number of live organisms was determined by plate counts. For use the vaccine was reconstituted in a volume of tryptone water to give the desired number of organisms/ml and fivefold serial dilutions prepared as required for every particular experiment.

Inactivated fresh fluid vaccine

The bacteria were grown in D15 broth as described above and the pcv determined by means of Hopkin's tubes. The density was adjusted to 1,0% packed cells and 0,3% formalin was added. The flasks were kept at 37 °C for 48–72 h and sterility was checked by making plate and nutrient broth cultures. The formalinized bacterial suspension was diluted to contain either 0,5% or 0,1% packed cells and precipitated with alum and KOH as described previously (Cameron & Fuls, 1970). When sedimentation was complete, the supernatant fluid was decanted and replaced with an equal volume of saline containing 0,3% formalin. The sediment was resuspended in saline and again allowed to settle out. This washing process was repeated to remove as much endotoxin as possible. The final vaccine was suspended in the original volume of saline and stored at 4 °C.

For certain experiments vaccine was also prepared without alum.

Inactivated lyophilized vaccine

Bacterial cells were produced in D15 broth as described above and the density of the formalinized culture was concentrated to contain 2,0% packed cells. The cells were suspended in an equal volume of BLP lyophilized in 1,0 ml quantities *in vacuo* and stored at 4 °C. The dry material was reconstituted in either 2,0 ml or 10,0 ml volumes of distilled water for use to give vaccines containing either 0,5% or 0,1% packed cells.

Immunization of mice

Groups of 24 mice were injected with 0,2 ml of the different vaccines subcutaneously in all the experiments. The live as well as the inactivated vaccines were tested at various dosage levels depending on the requirements and object of the experiment. The details are given in the appropriate tables or figures.

Challenge of experimental mice

Mice were challenged 14 days after immunization with *S. typhimurium* Strain 2656 V.

In order to maintain a constant challenge dose in all the experiments a large stock of lyophilized bacteria was prepared as follows: The strain was grown on nutrient agar for 18 h at 37 °C, the growth washed off and a creamy suspension prepared in BLP. One ml volumes were lyophilized *in vacuo* and the number of live organisms per vial was determined by plate counts. The counts were repeated every 4–6 weeks and found to remain constant during the course of the study.

For assay of virulence and challenge purposes the dry material was reconstituted with sufficient tryptone water to give a suspension containing approximately $7{,}5\times10^7$ live organisms/ml.

At the time of challenge the number of mice was reduced to 20 per group. In all the experiments half of the 20 immunized and 20 non-immunized mice in each group were challenged by injection with $2\text{–}4\times10^6$ organisms and half with $4\text{–}8\times10^5$ organisms in 0,2 ml by the intraperitoneal route. For simplicity's sake the challenge doses in the figures are given as $\pm3\times10^6$/mouse and $\pm6\times10^5$/mouse respectively.

Deaths were recorded daily for 14 days and the percentage protection was calculated according to the following formula:

$$\frac{\text{Survivors in the immunized group} - \text{Survivors in the control group}}{10}\times100$$

Exactly the same procedure was followed for preparing the challenge material and infection of mice with *S. dublin* except that dosages of $\pm5\times10^7$ and $\pm1\times10^7$ were used.

Assay of virulence of smooth strains

Selection of diluent

The cumulative death rates of mice infected with different doses of *S. typhimurium* Strain 2656V suspended either in saline or in tryptone water are shown in Table 2. It is evident that the mice which received bacteria suspended in tryptone water died faster than those which received bacteria suspended in saline. Since tryptone water was also used routinely as diluent for viability counts and because it enhanced the infectivity of the organisms to some extent, it was used as suspending medium in all further experiments.

Selection of challenge strain

The LD_{50} of a number of virulent strains of *S. typhimurium* is shown in Table 3. A considerable variation is evident, Strain 2659 being the most virulent and Strain SL1027 the least. A strain with intermediate virulence (2656V) was selected as the challenge strain for further experiments.

Selection of challenge dose

In order to find a suitable challenge dose various dilutions were prepared from suspensions of Strain 2656V and their virulence was titrated in groups of mice. The results appear in Table 4.

TABLE 2 Effect of suspending medium on infectivity of a virulent smooth strain of *S. typhimurum* strain (S2656V) injected by the intraperitoneal route

Suspending medium	Dose/mouse	Cumulative deaths/10 mice over 7 days						
		1	2	3	4	5	6	7
Saline	8×10^7	2	5	6	8	9	9	9
Saline	4×10^7	1	3	5	7	7	8	8
Saline	2×10^7	0	0	0	4	4	6	7
Tryptone water	8×10^7	6	7	9	9	10	10	10
Tryptone water	4×10^7	3	5	7	8	8	9	9
Tryptone water	2×10^7	1	3	5	7	9	10	10

TABLE 3 Comparison of virulence of smooth strains of *S. typhimurium* for mice by the intraperitoneal route

Strain	No of bacteria/ml in original suspension	LD50 per 0,2 ml of the suspension	
		14 days	21 days
2656 V	$8{,}0\times10^7$	$10^{-3{,}38}$	$10^{-3{,}57}$
2659	$8{,}0\times10^7$	$10^{-5{,}50}$	$10^{-6{,}00}$
1969	$7{,}5\times10^7$	$10^{-4{,}00}$	$10^{-5{,}09}$
1566	$3{,}5\times10^7$	$10^{-2{,}57}$	$10^{-4{,}00}$
SR 11	$9{,}0\times10^7$	$10^{-4{,}50}$	$10^{-5{,}64}$
SL 1027	$6{,}4\times10^7$	$10^{-1{,}54}$	$10^{-1{,}80}$
C 5	$6{,}9\times10^7$	$10^{-3{,}15}$	$10^{-3{,}33}$

TABLE 4 Cumulative deaths of mice injected intraperitoneally with different doses of *S. typhimurium* strain 2656 V

Dose per mouse	Cumulative deaths/10 mice over 14 days													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$1–2\times10^7$	1	1	1	1	6	9	9	9	10	10	10	10	10	10
$1–2\times10^7$	2	4	7	9	10	10	10	10	10	10	10	10	10	10
$1–2\times10^7$	1	1	1	2	3	7	8	9	9	9	9	9	9	9
$2–4\times10^6$	1	1	1	4	7	8	9	9	9	9	9	9	9	9
$2–4\times10^6$	1	1	1	4	6	9	9	9	9	9	9	9	9	9
$2–4\times10^6$	1	2	6	8	9	9	10	10	10	10	10	10	10	10
$2–4\times10^6$	0	0	0	0	3	5	6	7	7	9	9	9	9	9
$1–2\times10^6$	0	0	1	5	8	8	8	8	8	8	8	8	8	8
$1–2\times10^6$	0	2	3	4	5	8	8	8	8	8	8	8	8	8
$1–2\times10^6$	0	0	1	6	9	9	9	10	10	10	10	10	10	10
$1–2\times10^6$	0	0	0	0	0	2	4	5	8	9	9	9	9	9
$0{,}5–1\times10^6$	0	0	0	2	2	5	5	6	6	8	8	8	8	8
$0{,}5–1\times10^6$	0	2	4	6	8	8	10	10	10	10	10	10	10	10
$4–8\times10^5$	0	0	0	0	3	6	6	7	7	7	7	7	7	7
$4–8\times10^5$	0	1	2	3	5	8	8	8	8	8	8	8	8	8
$4–8\times10^5$	0	0	0	0	3	6	6	7	7	7	7	7	7	7
$2–4\times10^5$	1	1	1	1	1	4	6	7	7	8	8	8	9	9

A considerable variation in the mortality rate was encountered in groups of mice given the same dose of bacteria. In order to compensate for this it was decided to challenge immunized mice at two levels, namely $2–4 \times 10^6$ and $4–8 \times 10^5$ bacteria per mouse.

Assay of virulence of rough strains

Agglutination in acriflavine

The agglutination of the rough strains in different concentrations of acriflavine is shown in Table 5. With the exception of Strains R4 and M206, there was no marked difference in the agglutinability of the strains.

Virulence for mice

Their virulence for mice, when injected subcutaneously, is shown in Table 6. In contrast to their agglutinability an appreciable variation in virulence was observed. Strains R4 and M206 were the most virulent, which corresponds to their low level of roughness, but a strain such as T34–59, which agglutinated well, was almost equally virulent.

However, the local strains as well as Strains R0, R10 and SF 1827, which all agglutinated well, were either totally or wellnigh innocuous at the dosages tested. The remaining strains were intermediate in position.

TABLE 5 Agglutination of local and foreign strains of *S. typhimurium* in acriflavine

Strain	Acriflavine concentration			
	1:1 000	1:2 000	1:5 000	1:10 000
R 0	****	****	**	—
R 1	****	****	****	*
R 2	****	***	*	—
R 3	****	****	**	—
R 4	*	—	—	—
R 5	****	****	**	—
R 6	****	****	*	—
R 7	****	****	***	*
R 8	****	****	***	—
R 9	****	****	***	*
R 10	****	****	**	—
M 206	—	—	—	—
930 D	****	****	***	**
ILJ–15	****	****	**	—
T 34–59	****	****	***	*
SF 1827	****	****	*	—
2556 34×O	****	***	*	—
2656 34×S	****	***	*	—
2656 34×DE	****	**	—	—
2656 51×	****	****	**	—

**** = Very rapid and complete agglutination
*** = Distinct agglutination
** = Distinct but incomplete agglutination
* = Slight agglutination
— = No detectable agglutination

TABLE 6 Pathogenicity of rough *S. typhimurium* mutants for mice by the subcutaneous route

Strain	Deaths/10 mice after 14 days				
	Dose/mouse				
	5×10^7	10^7	2×10^6	4×10^5	8×10^4
R 0	1	1	1	0	0
R 1	6	2	2	0	0
R 2	6	1	1	0	0
R 3	5	2	1	0	0
R 4	7	5	2	0	0
R 5	7	2	1	0	0
R 6	5	0	2	0	0
R 7	5	1	2	0	0
R 8	6	7	2	0	0
R 9	6	3	1	0	0
R 10	0	1	1	0	0
M 206	8	1	6	0	0
930 D	6	1	2	0	0
ILJ–15	6	4	1	0	0
T 34–59	9	2	2	0	0
SF 1827	0	0	0	0	0
2656 34×O	1	0	0	0	0
2656 34×S	0	0	0	0	0
2656 34×DE	0	0	0	0	0
2656 51×	0	0	0	0	0

Immunization with live vaccines

Experiments with local strains

In the first experiments the level of immunity induced by fresh live vaccine prepared from the rough strains [2656 34×O, 2656 34×S and 2656 34×DE (Table 1)] was compared with formalinized fluid vaccine prepared from Strain 2656V, a virulent smooth strain.

The challenge procedure is outlined above and the results obtained are illustrated in Fig. 1. An appreciable difference was found in the degree of immunity induced by the different live vaccines. Strain 2656 34×O elicited the best response while 2656 34×DE was extremely poor in this respect. It is also apparent that with these strains a large number of organisms ($\pm 1 \times 10^9$/mouse) is required in order to induce a satisfactory immunity irrespective of whether live or dead vaccine is used or whether 1 or 2 doses are given. The most striking feature of this experiment is, however, the finding that the formalinized vaccine prepared from a smooth strain gave by far the best protection. Even a single injection gave very good results.

Experiments with imported and local strains

In view of the poor results obtained with the local rough mutants, the immunizing value of a series of rough mutants obtained from abroad was investigated.

The immunogenicity of all the rough strains is compared in Fig. 2. Some of the strains were too virulent at dosages of $1–2 \times 10^7$ bacteria/mouse or higher to be tested at these levels while other strains which did not give a good immunity at $2–4 \times 10^6$ bacteria/mouse were not tested at lower concentrations.

There was no consistent correlation between virulence and immunogenicity. For example Strain RO, which is rather avirulent (Table 6), gave a good immunity while strains such as R2, R3, R5, R6, R7 and R8, which are more virulent (Table 6), were not as satisfactory. On the other hand, the more virulent strains such as R4 and R9 induced a good immunity. There were also strains such as G30D, which is moderately virulent and evokes a satisfactory immunity.

As in a previous experiment (Fig. 1), all the local strains proved to be very poor immunogens.

Further evaluation of inactivated vaccines

Since none of the strains which gave a good immunity were absolutely innocuous, experiments were undertaken to investigate the immunogenicity of formalinized vaccines more thoroughly.

Four different inactivated vaccines prepared from smooth strain 2656V were compared with live fresh vaccine prepared from strain 2656 34×O. The details of the composition of the inactivated vaccines are given in Fig. 3. Groups of mice were given either 1 or 2 injections 7 days apart. The results of the experiments are shown in Fig. 3.

FIG. 1 Comparison of immunity obtained with high doses of live fresh vaccines prepared from rough substrains of strain 2656 34× and formalin inactivated fresh vaccine prepared from the smooth virulent strain 2656V against challenge with strain 2656V by the intraperitoneal route

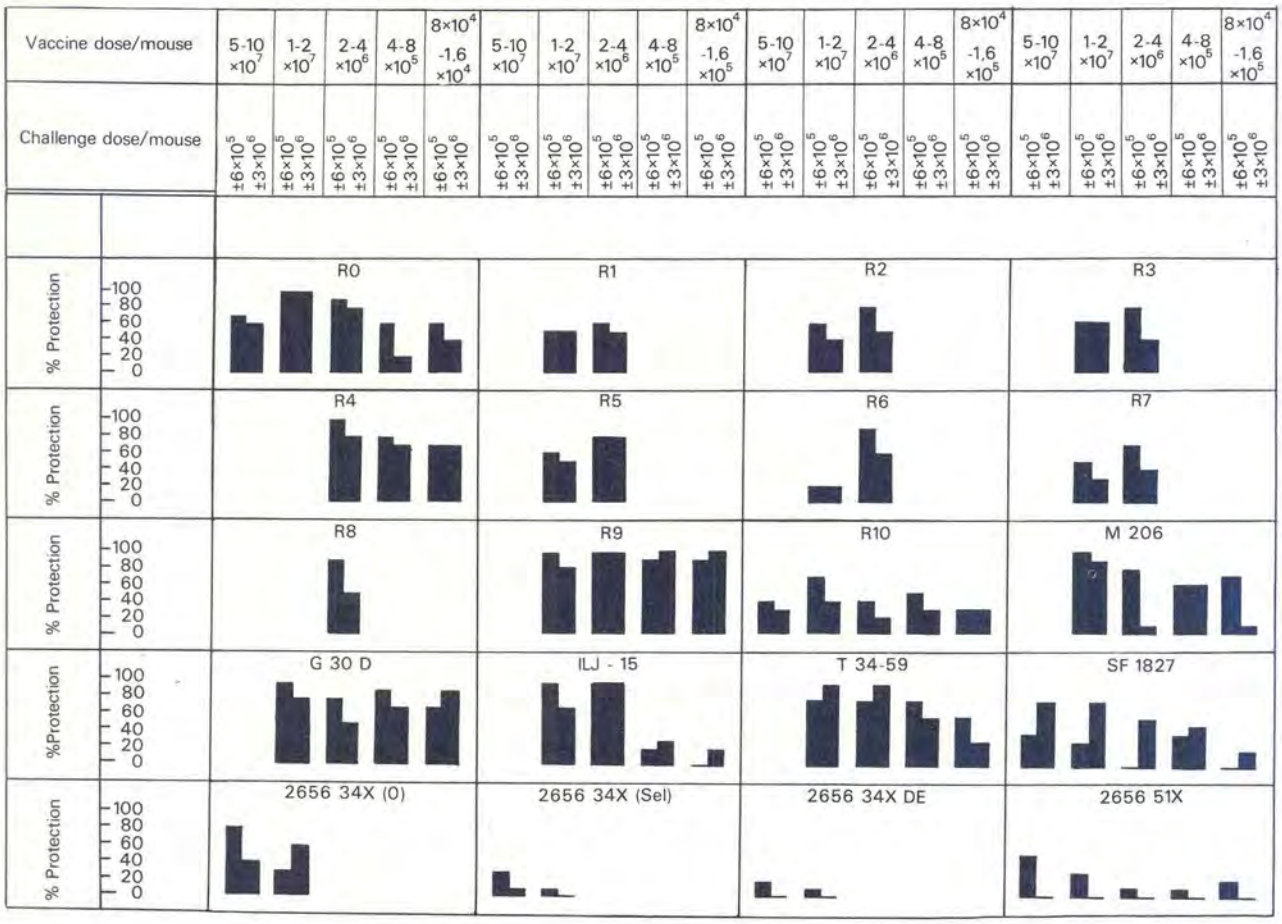

FIG. 2 Immunity obtained with live fresh vaccines prepared from different *S. typhimurium* rough mutants administered subcutaneously against challenge with *S. typhimurium* strain 2656V

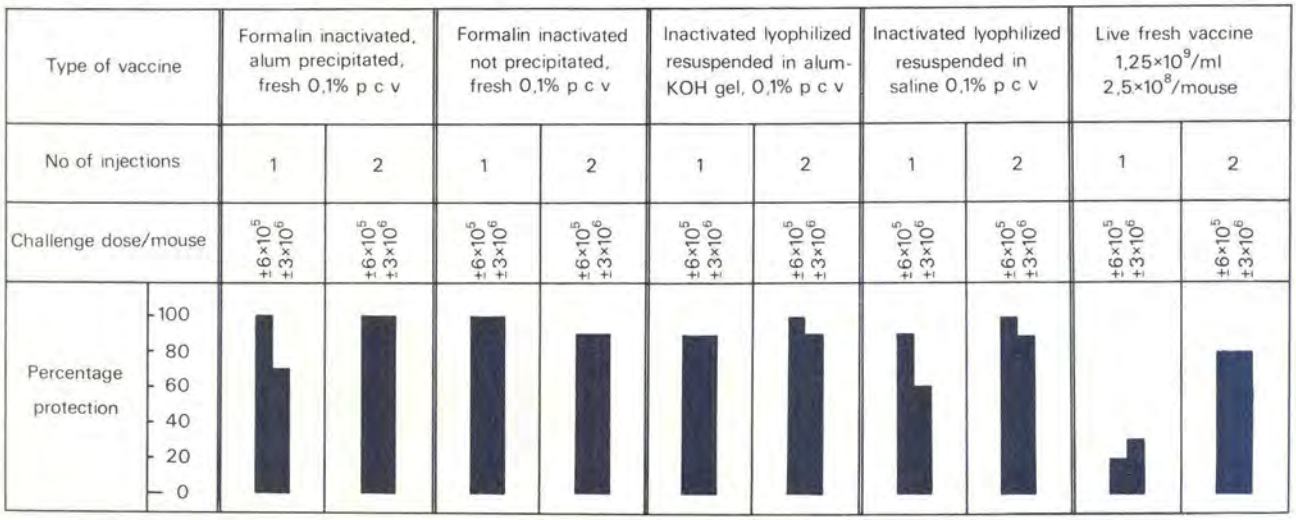

FIG. 3 Comparison of the immunogenicity of 4 different formalin inactivated vaccines prepared from the virulent strain 2656V and a live fresh vaccine prepared from the avirulent strain 2656 34×0

All the inactivated vaccines gave a high level of immunity and were superior to the live fresh vaccine prepared from strain 2656 34×O. Neither administration of 2 injections nor application of precipitated vaccine improved the degree of immunity of any of the vaccines.

Keeping quality of inactivated vaccine

According to Botes (1964, 1965) a major objection to formalinized *Salmonella* vaccines is their rapid loss of potency. Since the results obtained in this investigation indicate that only an inactivated vaccine would be acceptable for field application this aspect was investigated thoroughly.

A number of vials of both inactivated alum precipitated fluid vaccine and formalin inactivated lyophilized vaccines containing either 0,1% or 0,5% packed cells were stored at different temperatures. A number of vials were stored at 4°, 25°, 37° or 50 °C for 7 days or 1 month and their potency assayed in mice.

As shown in Fig. 4a and 4b both the fluid and the lyophilized inactivated vaccines withstood temperatures of up to 37 °C for 1 week. The fluid vaccine's potency was unaltered even after storage at 50 °C for 1 month (Fig. 5a), but there was a marked drop in potency of the lyophilized vaccine stored at 50 °C for 1 month (Fig. 5b). The deterioration of the latter vaccine was accompanied by an obvious change in the physical appearance of the lyophilized material.

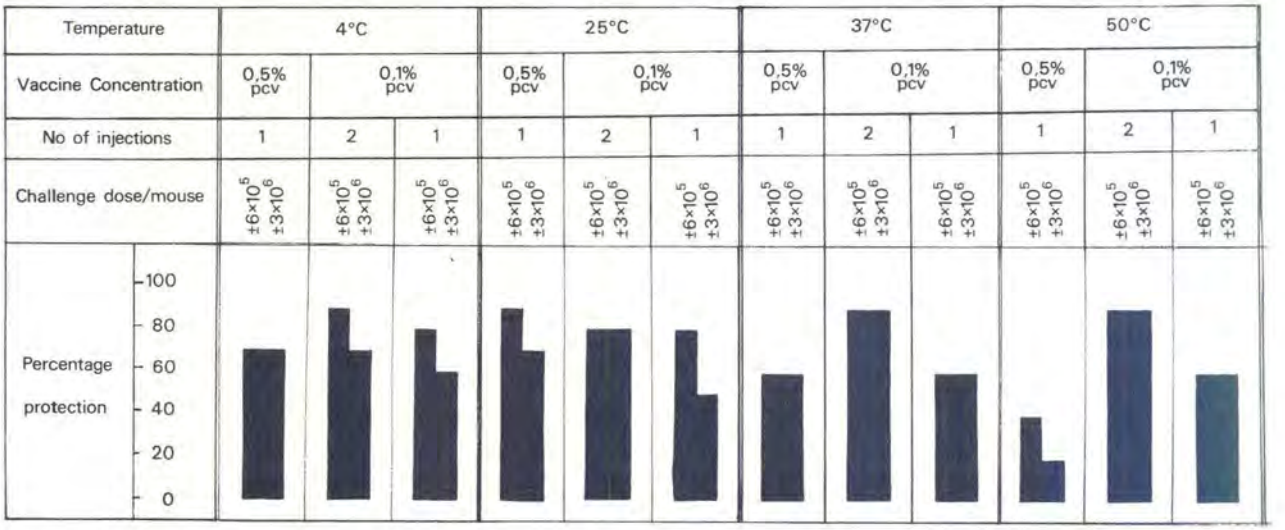

FIG. 4a Potency of formalin inactivated fresh *S. typhimurium* vaccine prepared from the virulent strain 2656V after storage for 1 week at different temperatures.

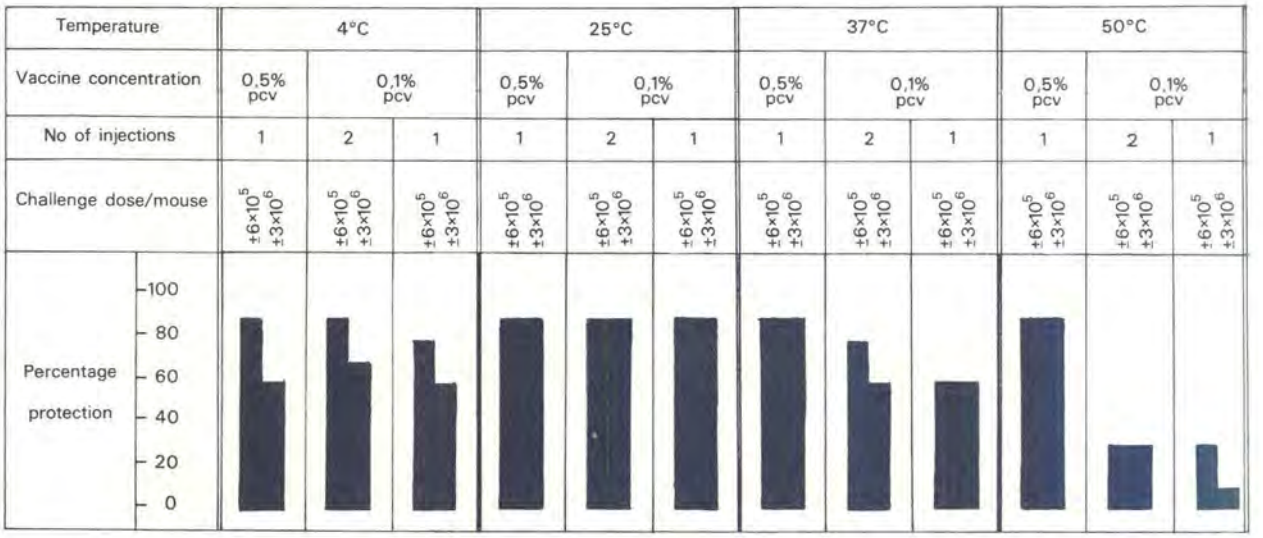

FIG. 4b Potency of formalinized lyophilized *S. typhimurium* vaccine prepared from the virulent strain 2656V after storage for 1 week at different temperatures

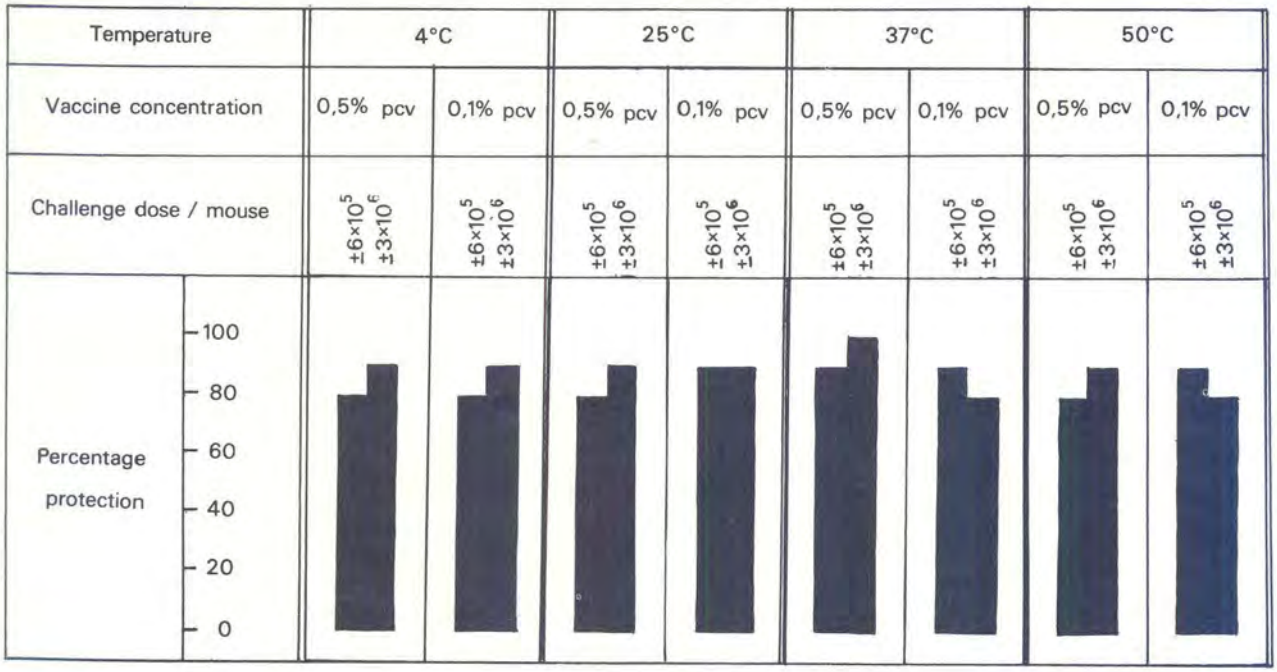

FIG. 5a Potency of a single injection of formalin inactivated fresh *S. typhimurium* vaccine prepared from the virulent strain 2656V after storage for 1 month at different temperatures

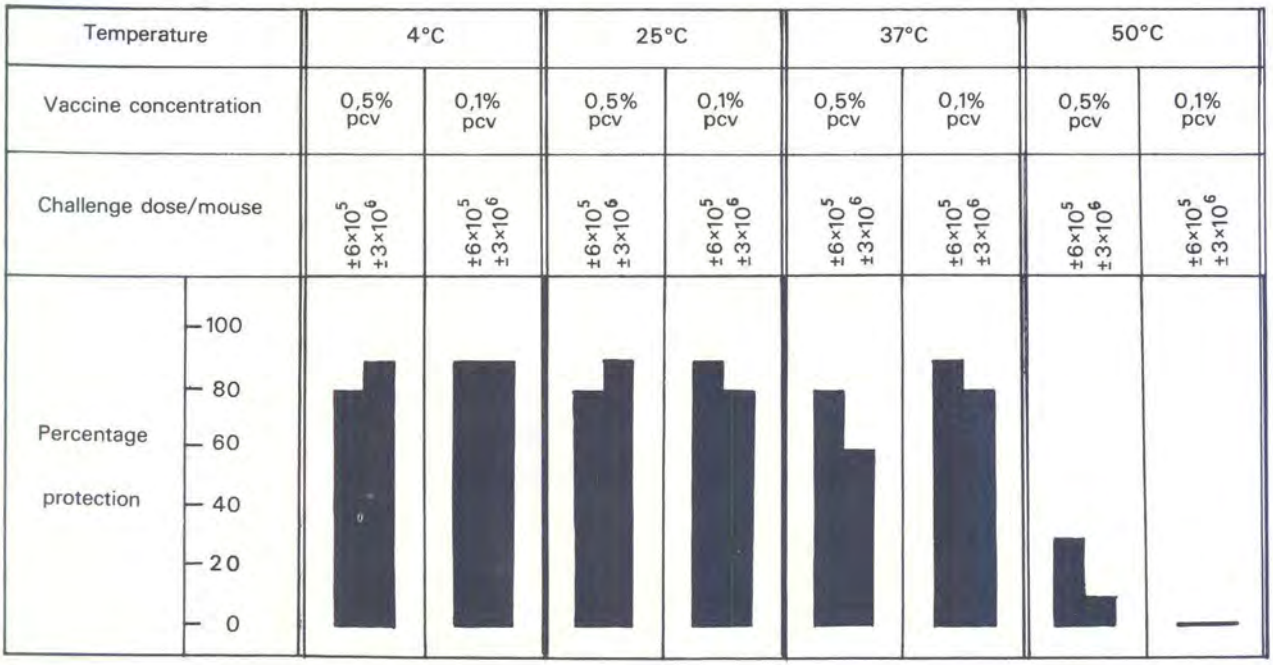

FIG. 5b Potency of a single injection of formalin inactivated lyophilized *S. typhimurium* vaccine prepared from the virulent strain 2656V after storage for 1 month at different temperatures

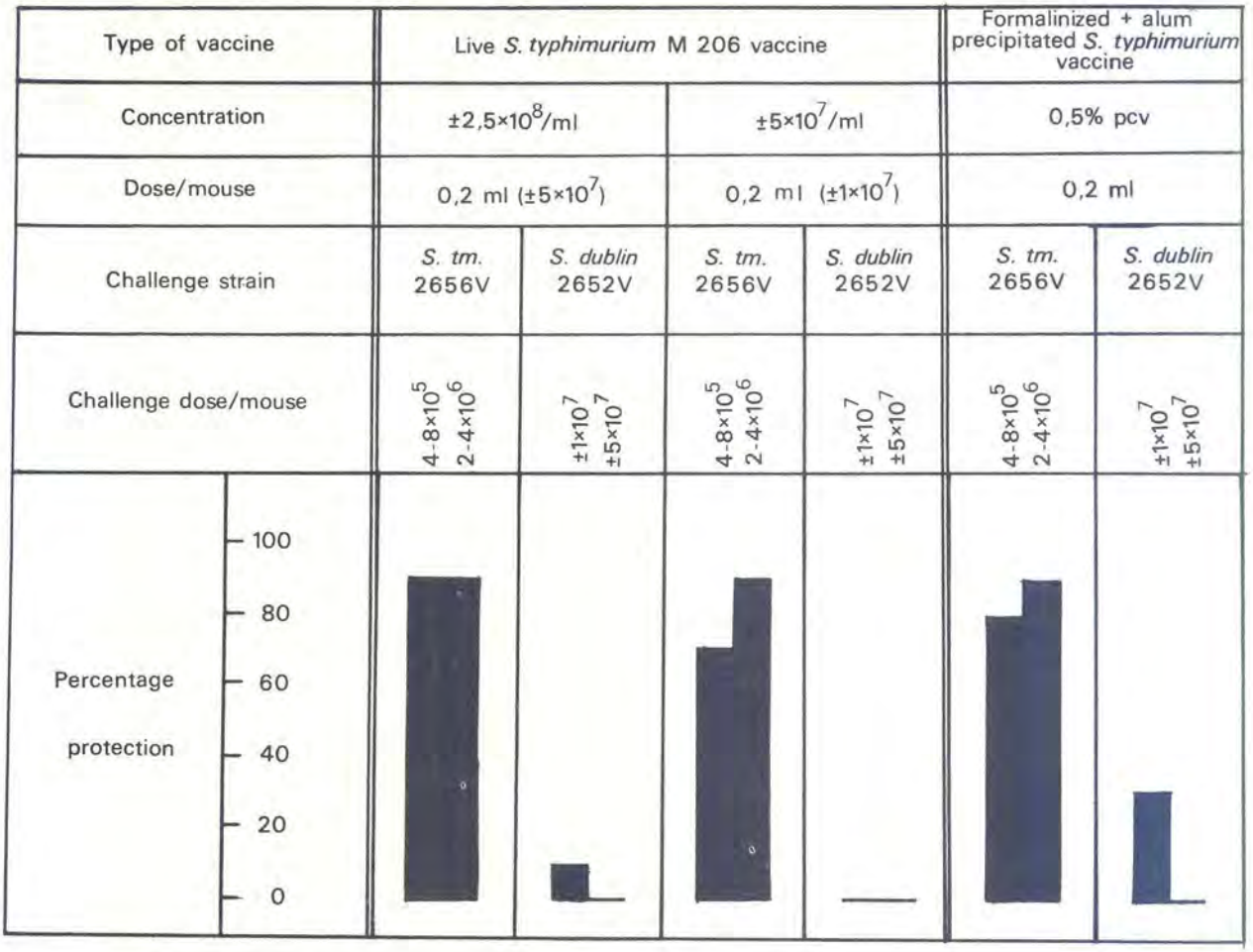

FIG. 6 A study on the level of protection induced by live and formalinized *S. typhimurium* vaccines to *S. dublin* infection
S. tm=S. typhimurium

Protection of S. typhimurium *immunized mice against challenge with* S. dublin

Finally it was of interest to determine whether either an inactivated alum precipitated fluid vaccine prepared from the virulent smooth strain 2656V or a fresh live vaccine prepared from the immunogenic rough strain M206 would protect mice against infection with *S. dublin*.

Groups of mice were immunized with the above vaccines in the usual way and challenged 14 days later by the intraperitoneal injection of a virulent strain of *S. dublin* (2652 V) (Cameron, unpublished data, 1974). Similarly immunized groups were challenged with *S. typhimurium* strain 2656V.

As shown in Fig. 6, neither the inactivated nor the live vaccine afforded any appreciable protection against challenge with *S. dublin* though they both provided good protection against homologous challenge.

Discussion

Avirulent rough mutants of *S. typhimurium* have been used successfully for immunizing mice by numerous authors (Jenkin & Rowley, 1965; Collins & Milne, 1966; Rowley, Auzins & Jenken, 1968; Nakano & Saito, 1969; Edebo & Norman, 1970; Germanier, 1970; Germanier & Fürer, 1971; Jarolmen, Hewel & Kain, 1971; Germanier, 1972, Houchens & Wright, 1973). It has been claimed that a good immunity to mouse typhoid can only be obtained when live bacteria are used for immunization (Jenkin & Rowley, 1963; Blanden, Mackaness & Collins, 1966), with the additional prerequisite that the immunizing strain should be able to multiply in the host (Jenkin, Rowley & Auzins, 1964; Collins, Mackaness & Blanden, 1966; Collins & Milne, 1966; Collins, 1968; Nakano & Saito, 1969; Germanier, 1970). On the other hand variable to good results have also been obtained with inactivated vaccines (Mitsuhashi, Kawakami, Yamaguchi & Nagai, 1958; Jonas, 1967; Kenny & Herzberg, 1967, 1968; Badakhsh & Herzberg, 1969; Collins, 1969; Jonas, 1970; Ornellas, Roantree & Steward, 1970; Venneman & Berry, 1971; Cronly-Dillon, 1972; Herzberg, Nash & Hino, 1972). In this respect the work by Herzberg *et al.* (1972) is particularly relevant. They conclude that: "Killed vaccines protected by their ability to induce an immune state which reduced the initial challenge population, prevented extensive multiplication, yet allowed 'cellular immunity' to develop due to response to the living challenge infection itself".

These observations are in agreement with our findings that a high level of protection can be induced with a formalin inactivated vaccine against intraperitoneal infection with *S. typhimurium*.

The poor results reported by some authors might be due to inadvertent destruction of labile antigens during the preparation of the vaccines, e.g. by heat (Blanden *et al.*, 1966; Collins & Milne, 1966; Collins, 1972; Germanier, 1972; Collins, 1973). In fact the possible significance of heat labile antigens has been raised by numerous workers in both *S. typhimurium*

(Jenkin & Rowley, 1965; Venneman & Bigley, 1969; Barber, Eylan & Heiber, 1972; Houchens & Wright, 1973) and *S. enteritidis* immunity (Kawakami, Osawa & Mitsuhashi, 1966; Milne & Collins, 1966). Our mild procedure of formalin inactivation would avoid such deterioration.

Although excessive heating during preparation may be deleterious, good results have nevertheless been obtained with heat-killed vaccines (Jonas, 1967; Kenny & Herzberg, 1967, 1968) and we found that storage of fluid vaccine for up to 1 month at 50 °C does not impair its efficacy. These conflicting results possibly depend on whether heat stabile 'O' antigens (Germanier, 1972) or other antigens (Nakano & Saito, 1969; Houchens & Wright, 1973) are the most important immunogens active in a particular experimental system.

One possible objection to the methods used in the present studies would be the fact that the death of challenged mice was used as the criterion for lack of immunity. However, since the natural disease in calves results in death (Stableforth & Galloway, 1959) and a challenge dose was used which produced a protracted disease, it is felt that our experimental design is acceptable in this respect.

A valid objection would be that the mice were challenged intraperitoneally instead of orally, because it has been found that immunity to intraperitoneal and oral infection is not necessarily synonymous (Blanden *et al.*, 1966; Collins, 1969; Cronly-Dillon, 1972). Similar studies designed to investigate immunity to oral challenge are therefor indicated.

The mechanism of immunity to mouse typhoid and related infections has been intensively studied and reviewed (Jenkin & Rowley, 1963; Turner, Jenkin & Rowley, 1964; Rowley, Turner & Jenkin, 1964; Blanden *et al.*, 1966; Collins *et al.*, 1966; Rowley *et al.*, 1968; Collins, 1971). It appears that both cellular and humoral immune mechanisms are involved, depending on the type of vaccine used and the particular phase of the immune response (Mitsuhashi *et al.*, 1958; Turner *et al.*, 1964; Jonas, 1967; Germanier, 1972). Furthermore, an appreciable level of non-specific protection towards heterologous serotypes, which has practical implications, has been recorded (Smith, 1966; Woolcock, 1973). We were, however, unable to demonstrate such cross protection with either live or dead *S. typhimurium* vaccine to *S. dublin* infection in mice.

In conclusion it can be stated that a good immunity to experimental *S. typhimurium* infection in mice can be established with either a formalin inactivated or a live vaccine prepared from rough mutants. Not all the mutants are, however, satisfactory and only those which retain some degree of virulence are effective, which unfortunately precludes their use in live vaccines destined for use in livestock. The only exception was strain SF1827 (Table 6) but subsequent investigations have shown that doses of 5×10^8 bacteria per mouse are often fatal (Cameron, unpublished data, 1974).

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