PROTECTION OF MICE BY LIVING Vi AND O VACCINES AGAINST DEATH CAUSED BY *SALMONELLA PARATYPHI C*

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INTRODUCTION

The development of vaccines against enteric fever has been directly influenced during the past 20 years by further elucidation of the nature and properties of the antigens of the causitive bacteria particularly those of *Salmonella typhi*. Thus Perry, Findlay & Bensted at first assumed that the ineffectiveness of the original 'Rawlings' strain of *Salm. typhi* in mouse protection, as shown by them (Perry, Findlay & Bensted, 1933*a*), was solely due to gross deficiency in O antigen but, following their successful mouse-passage experiments (Perry *et al.* 1933*b*) they found that new Rawlings was not only rich in O antigens but also possessed additional properties. Compared with some other smooth strains, equally rich in O antigen, the 'rejuvenated' Rawlings had far greater protective power in mice and Felix & Pitt (1934) were able to show that this additional property was due to an extra antigen which they designated the Vi antigen. The liability of this antigen to deterioration or modification (see Felix, 1951) led to the introduction of alcoholization and of acetone drying for vaccine preparation (Felix, Rainsford & Stokes, 1941; Rainsford, 1942; Landy, 1953). Typhoid vaccines killed and preserved with alcohol are better than vaccines preserved with phenol, after killing either by heat-phenol treatment or with alcohol, in producing Vi agglutinins and mouse-protecting antibodies in rabbits and in humans (see Felix, 1941; Felix & Anderson, (Landy, 1951; Young & Felsenfeld, 1947). Acetone-dried vaccine has been shown to (Landy, produce a better Vi antibody response in man than heat-phenol vaccine Gaines, Seal & Whiteside, 1954) and to afford superior mouse protection 1953).

On the basis of such findings alcoholized vaccine was adopted by the Army and its use became general in 1944, but opinion has remained divided as to the relative protective capabilities of the O and Vi components. Schütze (1930) had demonstrated the importance of the O antigens in the production of immunity to *Salm. typhimurium* and *Salm. enteriditis* infections in mice, but Landy (1952) showed that acetone-dried preparations of *Salm. ballerup* and of a coliform strain containing the Vi antigen were both more effective in protecting mice against *Salm. typhi* challenge than similar preparations of *Salm. typhi* itself, in spite of the absence of homologous O antigens. Furthermore, Landy and his collaborators have shown that purified preparations of Vi antigen alone have proved highly satisfactory in active protection tests against *Salm. typhi* for mice and caused a good antibody response in man, whose serum then had protecting power (Landy, 1954; Landy, Webster & Sagin, 1954). Nevertheless, the expectation that alcoholized vaccine

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would therefore be better than phenolized vaccine for the prevention of typhoid fever in man has not been borne out by experience. The analysis of the cases of typhoid fever occurring in persons inoculated with one or the other vaccine shows little evidence of difference in their relative protective effect.

On the one hand, the incidence of infection in the inoculated has led some workers to express doubt regarding the prophylactic value for man of any typhoid vaccine. On the other hand, the contrast between the clear cut superiority of Vi vaccines for mice and the failure of alcoholized vaccine, rich in Vi antigen, to show in man a similar superiority over phenolized vaccine has caused other investigators to mistrust mouse assay as a means of determining the relative value of different enteric vaccines for human use. The validity of the mouse-protection test for such an assessment has indeed long been questioned. Felix (1951) referred to the importance of remembering that the typhoid bacillus is not a natural pathogen of the mouse, while Landy (1953) pointed out that a vaccine superior in terms of mouse protection had not yet been shown to be superior in the protection of man against natural infection with typhoid. Thus, it may not be enough to determine the relative importance of the O and Vi antigens in protecting mice against *Salm. typhi*, for the 'infection' of this animal is only initiated by intraperitoneal inoculations of very large doses of this organism, and terminates in a rapid and fatal intoxication. Such experimental intoxication bears little relationship to the natural history of human enteric fever. Kauffmann (1936) and Griffitts (1944) make a similar differentiation between intoxication and infection.

It cannot be assumed that the demonstration of circulating antibodies is a satisfactory criterion of active immunity or that the production of these antibodies runs quantitatively parallel with that of other protective factors, (Williams Smith, 1956). The degree of cellular immunity must also be taken into consideration. Moreover, recent work has shown that the somatic antigens of gram-negative bacteria, or their endo-toxins, may considerably alter the functional activity of the reticulo-endothelial system (Biozzi, Benecerraf & Halpern, 1955; Rowley, Howard & Jenkin, 1956) and such changes may modify the susceptibility of animals to subsequent infection. Thus, it is most desirable that a more satisfactory method should be devised for the assessment of the protective power of a typhoid-paratyphoid vaccine than the mouse protection tests in current use.

It seemed therefore that an organism capable of setting up a true infection in mice, and which carried the Vi antigen in addition to its full complement of O antigen, might be more profitably employed to assess the relative values of the Vi and O antigens in prophylactic vaccines. *Salm. paratyphi C* appeared suitable for this purpose. Kauffmann (1936) showed that this organism killed mice when injected intraperitoneally in doses ranging from 1000 to 500,000,000 organisms. With the larger doses some mice died within 24 hr. At the lower dose level the mice died after an interval from 5 to 12 days. This was true for both Vi-positive and Vi-negative strains of *Salm. paratyphi C*. Kauffmann also found that this species can kill mice after administration by mouth.

In the work to be described we have employed *Salm. paratyphi C* var. East Africa, the Vi-positive strain used by Kauffmann whose findings outlined above,

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we have confirmed. The late J. B. Neal (unpublished) showed that the injection of small doses of 1000 or 10,000 organisms of this strain, resulted in delayed death and produced characteristic pathological changes in the gut and spleen which were not observed after a massive challenge with this or other salmonella organisms. Further, not only will *Salm. typhi* fail to kill mice in small doses, but in respect of large ones, the lethal dose is much less by intraperitoneal injection than by subcutaneous injection (Field, Howard & Whitby, 1955). By contrast a small dose (1000 to 10,000 organisms) challenge of *Salm. paratyphi C*, whether injected subcutaneously or into the peritoneum, will produce true fatal infection in the mouse after an incubation period of 5 days or more, but a massive dose into the peritoneum will produce a rapid fatal intoxication.

Intraperitoneal injections into mice of cultures of typhoid bacilli in doses too small to produce early toxic death do not appear to give rise to any ill effects. The animals quickly recover from the result of the inoculation, and although the organisms may persist in the spleen for varying periods they are eventually eliminated. In the case of similar injections with cultures of *Salm. paratyphi C* the picture is entirely different. Doses as small as forty-five organisms may set up an infection whereby the organisms are enabled to multiply in the tissues so that after a period of 5–10 days or perhaps longer, the total bacterial population has reached a fatal level. For the purpose of this communication death following the injection of these small doses is defined as *infective death* and the dose giving rise to this condition is called the *infective dose* (ID). The early death following the injection of massive doses of typhoid or paratyphoid organisms into the peritoneum is defined as *toxic death* and the dose itself the *toxic dose* (TD).

The term *toxic dose* is synonymous with the *lethal dose* introduced by Perry, Findlay & Bensted (1933*a*) as a measure of the mouse-virulence of typhoid bacilli. The variation in this lethal dose of different cultures of *Salm. typhi* was shown by Felix & Pitt (1936) and Bensted (1937) to depend on the amount of Vi antigen present in the culture, and the latter author found the average figure for recently isolated cultures was about 80×10^6 and seldom was the lethal dose lower than 50×10^6 (the classical strain Ty 2 was such an example). The above values were expressions of the LD_{100}; employing the method of Reed & Muench (1938) the LD_{50} for Ty 2 was about 30×10^6 and the TD_{50} for *Salm. paratyphi C* East Africa was between 65 and 90×10^6 which, bearing in mind the smaller amount of Vi antigen in the paratyphoid C cultures (see Discussion), is of the same order. With heat-killed organisms, where the Vi antigen is destroyed, one of us (J.L.W.) found the TD_{50} for both typhoid and paratyphoid bacilli to be about 8000×10^6, a figure in agreement with the findings of Felix (1938).

While the TD_{50} can be determined with a reasonable degree of accuracy and the results are reproducible there is at present no reliable method for the determination of the ID_{50} in mice for *Salm. paratyphi C*. Although very small doses, as indicated above, may set up a fatal infection the results of our experiments with graded doses were most irregular. For example, 20 % of the mice injected with 45 organisms into the peritoneum became fatally infected, 57 % after 530 organisms, 10 % after 13,000, 76 % after 16,000, 44 % after 18,000, and as Table 2 shows, less than

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50% after some 80,000; more than 100,000 were necessary for reliable results. Comparable doses of Vi-negative cultures of paratyphoid C were required, but experiments with graded doses gave equally irregular results.

There was a similar lack of correlation between dosage and infection rate with the intra-gastric route although 4/10 were fatally infected by an intra-gastric injection of 50,000 organisms. After Gaines, Landy, Edsall & Trapani (1956) had shown the enormous doses of *Salm. typhi* required to infect apes by the oral route we accordingly considerably increased our doses of *Salm. paratyphi C* and found that cultures containing 300×10^6 organisms introduced directly into the stomach regularly set up a fatal infection. The intra-gastric challenge route has given promising results in the preliminary tests, but obviously requires further investigation.

In our attempt to determine the relative importance of the O and Vi antigens in the active protection of mice against *infective* death and *toxic* death *Salm. cholerae-suis* var. Kunzendorf (O antigens 6 and 7) and *Salm. typhi* Ty6S (almost a pure Vi strain) were used as living vaccines, as described by Field *et al.* (1955). The same organisms and *Salm. paratyphi C* East Africa were used as killed (alcoholized) vaccines and *Salm. paratyphi C* East Africa was employed as the challenge either intraperitoneally or by the alimentary route. The latter form of challenge was used in our final experiment, after we had established an effective dose, in view of the findings of MacLeod (1954) that vaccines which gave significant protection against intraperitoneal challenge with *Salm. dublin* failed to do so against oral challenge with the same organism. No trace of Vi antigen in *Salm. cholerae-suis* var. Kunzendorf has ever been reported, but we thought we should confirm this with the particular culture we used by showing absence of agglutination on testing with high titre Vi sera, by the failure of four rabbits to produce Vi antibody after the third, fifth and eighth injection of freshly prepared alcoholized suspension of this organism and by the absence of Vi antibodies in the pooled serum of ten mice killed 18 days after, and a further ten killed 32 days after inoculation with living organisms of this strain.

MATERIALS AND METHODS

Strains of bacteria

The following were used: *Salm. paratyphi C* strain East Africa (Kauffmann), *Salm. typhi* strain Ty6S and *Salm. cholerae-suis* var. Kunzendorf.

Preparation of living bacterial suspensions for immunizing and challenge doses

Cultures were inoculated on to Difco nutrient-agar plates and incubated for 12 hr. at 37° C. Six smooth opaque colonies were selected, emulsified in physiological saline and used to inoculate Difco agar slopes. After 12 hr. at 37° C. the growth was washed off with saline and the bacterial concentration estimated by comparison with Brown's standard opacity tubes. Dilutions were made with saline to the required opacity, and checked by viable counts performed in accordance

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with the method of Miles & Misra (1938). Immunizing and challenge doses recorded in Tables 1, 2, 3, 4 and 6 were calculated from such living bacterial counts. In each of these experiments, however, the opacity of immunizing suspensions used was identical in comparable groups. In Table 5 the counts recorded are those estimated by opacity. The antigenic composition of bacterial suspensions was confirmed by routine agglutination tests.

Preparation of killed vaccines

Alcoholized vaccines were prepared essentially as described by Felix (1941). The dilutions for mouse immunization were made up by matching the concentrated vaccine against Brown's standard opacity tubes with subsequent dilution as required.

Animals

The *Strong A* strain of albino mice was used in these experiments. As a rule mice of one sex were used for each individual experiment, but where this was not possible equal numbers of each sex were used. The animals varied in weight from 18 to 25 g. although for each experiment mice of approximately the same weight and age were used. The mice were kept in jars with two mice to a jar. All animals were fed throughout on Pellet diet 41 (Bruce, 1950).

Inoculation methods

Volumes of 0·5 ml. were used for all inoculations, whether for immunization or challenge. Subcutaneous inoculations were made in the interscapular region. Intragastric inoculations were made with a metal tube 0·8 mm. in diameter attached to a syringe.

Procedure following challenge

Mice were observed for 7 days after a toxic challenge and for 4–5 weeks following an infective challenge. Postmortem examinations were made on mice dying after an infective challenge and their organs cultured to attempt to recover *Salm. paratyphi C*. In 75% of mice so examined the organisms were successfully recovered.

Agglutination tests

Agglutination tests were performed in round-bottomed tubes 12 mm. in diameter by the method described by Felix & Bensted (1954). Mouse serum was obtained from the heart blood and pooled samples from groups of ten mice examined for antibodies.

RESULTS

Earlier experiments had been carried out in these Laboratories by Lt.-Col. T. E. Field (unpublished) with killed vaccines of *Salm. typhi* Ty6S (Vi), *Salm. cholerae-suis* (O) or combinations of these two vaccines (Vi+O) and also with *Salm. paratyphi C* itself (Vi+O). Although significant protection was achieved on occasion with all three types of vaccine the results were not consistent. Field *et al.* (1955) had obtained very promising results in their mouse-protection experiments

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with living typhoid vaccines. We therefore decided in the present investigation to rely mainly on living vaccines of *Salm. typhi* Ty6S and *Salm. cholerae-suis* separately and in combination to assess the relative importance of Vi and O antigen as immunizing agents, but alcoholized cholerae-suis and paratyphoid C vaccines were included in one group of experiments.

Tables 1–4 show the results of two separate experiments in which duplicate groups of mice were immunized as set out in column 1 of each Table. All the mice

Table 1. *The effect of subcutaneous immunization with living organisms* 18 *days before intraperitoneal challenge with* 166×10^6 *living* Salm. paratyphi C (*East Africa strain*)

Immunizing agent and dose	Time after challenge (days) 1	2	3	4	5	6	7	Statistical significance
184×10^6 living *Salm. typhi* Ty 6S	17/20	17/20	13/20	4/20	0/20	0/20	0/20	Highly significant protection up to 3rd day
152×10^6 living *Salm. cholerae-suis*	6/20	6/20	6/20	4/20	2/20	2/20	2/20	Not significant
184×10^6 living *Salm. typhi* Ty 6S + 152×10^6 living *Salm. cholerae-suis*	18/20	15/20	14/20	12/20	9/20	7/20	7/20	Highly significant protection up to 7th day
Nil	7/20	6/20	2/20	0/20	0/20	0/20	0/20	

Results expressed as survivors/group total. Immunizing and challenge doses calculated from living bacterial counts performed by the method of Miles & Misra (1938). Mouse strain: Strong A females.

Table 2. *The effect of subcutaneous immunization with living organisms* 18 *days before intraperitoneal challenge with* 83,000 Salm. paratyphi C (*East Africa strain*)

Immunizing agent	Time after challenge (weeks) 1	2	3	4	5	Statistical significance
92,000 living *Salm. typhi* Ty 6S	20/20	5/20	5/20	5/20	5/20	No protection
184×10^6 living *Salm. typhi* Ty 6S	20/20	12/20	11/20	11/20	11/20	No protection
76,000 living *Salm. cholerae-suis*	20/20	18/20	18/20	18/20	18/20	Significant protection
152×10^6 living *Salm. cholerae-suis*	19/19	17/19	17/19	17/19	17/19	Significant protection
184×10^6 living *Salm. typhi* Ty 6S + 152×10^6 living *Salm. cholerae-suis*	20/20	20/20	20/20	20/20	20/20	Highly significant protection
Nil	20/20	12/20	11/20	11/20	11/20	

Results expressed as survivors/group total. Immunizing and challenge doses calculated from living bacterial counts performed by the method of Miles & Misra (1938). Mouse strain: Strong A females.

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Table 3. *The effect of subcutaneous immunization with living organisms* 18 *days before challenge with* 130×10^6 Salm. paratyphi C *and of subcutaneous immunization with alcoholized vaccines at weekly intervals the last immunizing dose being given* 7 *days before challenge with* 130×10^6 Salm. paratyphi C

Immunizing agent	Time after challenge (days) 1	2	3	4	5	6	7	Statistical significance
46×10^6 living *Salm. typhi* (Ty 6 S) + 62×10^6 living *Salm. cholerae-suis*	16/20	15/20	15/20	11/20	9/20	4/20	3/20	Highly significant protection up to the 5th day
46×10^6 living *Salm. typhi* (Ty 6 S)	15/20	14/20	12/20	4/20	0/20	0/20	0/20	Highly significant protection up to the 3rd day
62×10^6 living *Salm. cholerae-suis*	4/19	2/19	2/19	1/19	1/19	1/19	1/19	No protection
40, 80 and 160×10^6 alcoholized *Salm. cholerae-suis*	15/37	8/37	7/37	1/37	1/37	1/37	0/37	No protection
40, 80 and 160×10^6 alcoholized *Salm. paratyphi C*	31/38	31/38	30/38	24/38	9/38	5/38	4/38	Highly significant protection up to the 5th day
Nil	14/20	4/20	3/20	2/20	0/20	0/20	0/20	

Results expressed as survivors/group total. Immunizing and challenge doses calculated from living bacterial counts performed by the method of Miles & Misra (1938). Mouse strain: Strong A males and females. Equal numbers in each group.

Table 4. *The effect of subcutaneous immunization with living organisms* 18 *days before challenge with* 130,000 Salm. paratyphi C *and of subcutaneous immunization with alcoholized vaccines at weekly intervals, the last immunizing dose being given* 7 *days before challenge with* 130,000 Salm. paratyphi C

Immunizing agent	Time after challenge (weeks) 1	2	3	4	Statistical significance
46×10^6 living *Salm. typhi* (Ty 6 S) + 62×10^6 living *Salm. cholerae-suis*	20/20	17/20	17/20	16/20	Highly significant protection
46×10^6 living *Salm. typhi* (Ty 6 S)	20/20	12/20	9/20	9/20	No protection
62×10^6 living *Salm. cholerae-suis*	20/20	16/20	16/20	16/20	Highly significant protection
40, 80 and 160×10^6 alcoholized *Salm. cholerae-suis*	39/40	29/40	29/40	28/40	Highly significant protection
40, 80 and 160×10^6 alcoholized *Salm. paratyphi C.*	37/37	25/37	24/37	24/37	Significant protection
Nil	20/20	5/20	5/20	5/20	

Results expressed as suvivors/group total. Immunizing and challenge doses calculated from living bacterial counts performed by the method of Miles & Misra (1938). Mouse strain: Strong A males and females. Equal numbers in each group.

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receiving living vaccines were challenged 18 days after the immunizing dose, while those receiving killed vaccines were challenged 7 days after the last immunizing dose. One complete set of mice received a high level (toxic) challenge dose, determined by opacity as 200×16^6 organisms in each experiment. The viable counts, however, showed that the effective challenge was somewhat lower, becoming about 2 TD_{50}. The other set of mice received a low level (infective) challenge, determined by opacity as 100,000 organisms in the first experiment and 200,000 in the second. A lower challenge was not used in view of the variable mortality already described. In the first experiment high- and low-level doses were also used for immunization of the mice which were to receive an infective challenge. The lower immunizing dose (100,000 of each species by opacity) was chosen as being one presumed to require multiplication of the injected organisms to produce an effective immunogenic stimulus. Such a dose might favour one or the other antigen if there were species variation regarding rate and degree of multiplication after inoculation, an advantage more likely to favour the Vi vaccine (Young & Felsenfeld, 1947). The higher immunizing dose (200×10^6 by opacity) were considered an effective stimulus even if multiplication did not take place, or was minimal, for either species, but such massive doses might tend to reduce the delicacy of a test for differences in protective power. The failure of a large dose to protect and the success of a small one (either homologous or heterologous) seemed a possible continency of considerable interest. Only higher level doses were used in the second experiment. These were equal by opacity to those used in the first, but the viable count was considerably lower.

Table 5 shows the result of a third experiment carried out on larger groups of mice in a further attempt to obtain results which would show whether there is a significant difference between the protective power of a pure O vaccine and an O + Vi vaccine against infective death. Challenge doses were as in the first experiment. Table 6 records the results of intragastric challenge.

In the two experiments using *toxic* challenge (Tables 1 and 3) living Vi vaccine afforded significant temporary protection. This was shown to be specific by the negative results of control experiments in which mice were challenged with toxic doses of *Salm. cholerae-suis* or a Vi-negative strain of *Salm. paratyphi C*. Living and alcoholized Vi + O vaccine afforded temporary protection of slightly longer duration. Pure O vaccine, either living or dead, afforded no significant protection.

In all four experiments using *infective* challenge the living pure O vaccine afforded significant protection as did the alcoholized pure O vaccine in the single experiment in which it was used (Table 4). In the experiment recorded in Table 2 the death rate on the controls was rather low but protection, which was just significant ($0{\cdot}05 > P > 0{\cdot}01$), was evident after 3 weeks and the very small dose of living O vaccine given to one group seemed as effective as the large dose given to another. By contrast neither the large or the small dose of pure Vi vaccine appear to afford protection. Vi vaccine also failed to produce significant protection against infective intraperitoneal challenge in the second experiment (Table 4). Significant protection by pure Vi vaccine was, however, obtained against fatal intra-gastric infection.

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Table 5. *The effect of subcutaneous immunization with living organisms* 18 *days before intraperitoneal challenge with* 100,000 Salm. paratyphi C

Group	Immunizing agent	Time after challenge (weeks) 1	2	3	4	Statistical significance compared with control	Statistical significance compared with Group I
I	200×10^6 living *Salm. cholerae-suis*	59/59	30/59	28/59	26/59	Highly significant protection	—
II	200×10^6 living *Salm. cholerae-suis* + 200×10^6 living *Salm. typhi* (Ty 6S)	56/56	50/56	43/56	42/56	Highly significant protection	Highly significant protection
Control	Nil	29/30	7/30	3/30	3/30		

Results expressed as survivors/group total. Immunizing and challenge doses calculated by opacity. Mouse strain: Strong A females.

Table 6. *The effect of subcutaneous immunization with living organisms* 18 *days before challenge with* 320×10^6 Salm. paratyphi C *administered by stomach tube*

Group	Immunizing agent	Time after challenge (weeks) 1	2	3	4	Statistical significance compared with control	Statistical significance compared with Group I
I	112×10^6 *Salm. typhi* (Ty 6S)	37/37	15/37	12/37	10/37	Highly significant protection	—
II	82×10^6 *Salm. cholerae-suis*	40/40	31/40	28/40	28/40	Highly significant protection	Highly significant protection
III	112×10^6 *Salm. typhi* (Ty 6S) + 82×10^6 *Salm. cholerae-suis*	39/39	26/39	24/39	22/39	Highly significant protection	Highly significant protection
Control	Nil	37/40	4/40	1/40	1/40		

Results expressed as survivors/group total. Immunizing and challenge doses calculated from living bacterial counts performed by the method of Miles & Misra (1938). Mouse strain: Strong A females.

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Living Vi and O vaccine gave a significant protection against infective challenge in all four experiments; in two the effect of pure O vaccine was such as to leave no margin for demonstration of significant improvement by the addition of the Vi antigen. In the third (Table 5), Vi + O vaccine was very significantly better than pure O vaccine, yet, following intra-gastric challenge improvement was not demonstrable as a result of adding Vi antigen to the vaccine.

In view of the large size of most of the living inocula the possibility of the protection observed having some of the qualities of an interference phenomenon was considered. Such an effect has been suggested by Evans & Perkins (1955) with regard to the immediate effects of pertussis vaccine, and Williams Smith (1956) considered interference to be produced when the challenge was given up to 9 days after immunization or 2 days before immunization when using living *Salm. gallinarum* vaccines in chickens. Hence, though we did not challenge until 18 days after immunization, two experiments were made to determine whether any of the living inoculum was persisting in the mice at the time when they were challenged. *In the first, eighty mice were immunized with* 200×10^6 *living Salm. cholerae-suis.* Four mice were killed daily and cultures taken from the site of inoculation, the spleen and the heart blood. For the first 8 days after immunization *Salm. cholerae-suis* was recovered from almost all the mice examined. From 10 to 14 days after immunization positive cultures were obtained from 33 % of animals, but after that time less than 10 % of animals yielded positive cultures. In a second experiment forty mice were immunized with 200×10^6 *Salm. cholerae-suis* and a further forty with 200×10^6 *Salm. typhi* Ty6S. Fourteen days after immunization the mice were sacrificed and cultures taken from the liver, spleen and the site of inoculation. Positive cultures were obtained in eight mice, in two of these from the site of inoculation alone. Thus, it was concluded that at the time of challenge the living organisms injected were still persisting in only a very small percentage of animals.

DISCUSSION

The results presented here suggest that for the active protection of mice against death from infection with *Salm. paratyphi C* by bacterial vaccines a combination of the full O and Vi antigens is the most effective; the O antigen alone appears to have considerable value, but the Vi antigen alone is much less effective although it is the major factor in providing temporary protection against toxic death. However, before applying these findings to the prophylaxis of human typhoid there are a number of problems awaiting solution.

In the first place there is some divergence of opinion as to whether all Vi antigens are identical. Landy & Cepellini (1955) considered that they were indistinguishable in immunological specificity, although Landy (1954) had found some physico-chemical and immunological differences in these antigens—the latter, however, being mainly differences of antigenic potency. Craigie & Yen (1938) showed that there was modified sensitivity to the typhoid Vi phage 1 of the East Africa strain of *Salm. paratyphi C*, although Scholtens (1937) had reported that this and other Vi-positive strains of *Salm. paratyphi C* were insensitive to the typhoid Vi phages

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but could adsorb them. There is, however, general agreement that a 'fully Vi-positive' typhoid strain such as Ty2 contains much more Vi antigen than the Vi-positive strains of *Salm. paratyphi C*. Landy (1952) put the ratio at 50/1 and Felix (1952) comparing the Watson strain of *Salm. typhi* (less rich in Vi, than Ty2) with the Baghdad strain of *Salm. paratyphi C*, by absorption techniques, obtained a figure equivalent to 4/1. Bensted (personal communication) examined some freshly isolated strains of paratyphoid 'C' in India by the absorption technique and found that the Vi antigen in the cultures was less than 10% of that of high-virulence typhoid Vi cultures.

Relative Vi content is important in so far as there is correlation between the amount present and the functional effect. In respect of *Salm. typhi* this correlation appears to be close for mouse-virulence is directly proportional to the Vi content of the culture. The protection of the O antigen by the Vi antigen appears to reduce bacterial sensitivity to host resistance both natural and immune. An index of this shielding is abolition or reduction of O agglutinability; complete resistance to O agglutination is only found in organisms of the highest mouse virulence. *Salm. paratyphi C* has never been found in O-inagglutinable form, though reduced agglutinability of cultures of the East Africa strain is common. However, degrees of enhancement of virulence may be estimated by comparison of Vi-positive and Vi-negative strains. The ratio between the LD_{100} for the Vi-negative 901 strain of *Salm. typhi* and that for the Ty2 strain is 8/1 (Felix & Pitt, 1934). With regard to *Salm. paratyphi C*, tests on two Vi-negative strains suggest a TD_{50} for mice of 400×10^6; that for the East Africa (Vi) strain, as already stated, lies between 65×10^6 and 90×10^6; thus for this species the ratio between the TD_{50} for a Vi-negative strain and that of a Vi-positive one is about 5/1. On this basis the Vi antigen of *Salm. paratyphi C* has about half the functional effect in enhancing virulence of the Vi antigen of fully virulent strains of *Salm. typhi*. Our results show the significant effect of Vi vaccine against this enhancement, and had the experiments shown in Tables 1 and 3 been stopped after 48 hr. they would have resembled those of conventional mouse-protection tests against *Salm. typhi* challenge in indicating definite protection by Vi vaccine and none by O vaccine. However, *Salm. paratyphi C* East Africa owing to its O agglutinability would have been wholly unacceptable to Felix, who stressed (Felix, 1951) the importance of full resistance to the O antibody of the challenge dose, as a challenge in mouse protection tests. While such full resistance is clearly necessary if the value of the prophylactic under test against human infection with equally resistant strains is to be assessed, it is by no means certain that such strains are commonly involved in causing human typhoid fever. Bensted (1937, 1940) found only 16 out of 100 and in another series 4·7% of nearly 400 recently isolated cultures fully O resistant. In the second communication, however, he pointed out that this low figure was probably largely due to the loss of O resistance after subculture on ordinary laboratory media. Many more of these strains may thus have been O resistant while still in the human host. On the other hand, rapid loss of O resistance in the laboratory may be paralleled by rapid loss in other environments outside the human body, for example, in water or food which are the source of human

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infection, and evidence can rarely be produced to show whether the infecting strain has reached its host in a state of high O resistance or not. Nevertheless, it is of great importance that the Vi content and O resistance of a *Salm. paratyphi C* challenge should be raised to the highest possible level in any test to determine the value of Vi and O antigens for protection, and a similar enhancement of the Vi-antigen content in any para C vaccine proposed as an experimental parallel to a typhoid vaccine is necessary.

In the second place it is unfortunate that the infective intraperitoneal challenge with *Salm. paratyphi C* had to be so large, and that the lack of correlation of dose size with fatal effect made the use of graded doses unprofitable.

In the third place it is a pity that the work recorded here so largely dealt with the use of living vaccines, adopted because our previous experience of killed vaccines had given indefinite or conflicting results although those recorded here are very clear cut. With living inocula though the numbers of organisms injected may be varied (see Table 2), the effective dose cannot be computed and is not subject to controlled variation, which has been shown by Batson (1949) to be a better means of comparing typhoid vaccines than variation of the challenge. The latter, as stated above cannot, in any case, be effectively varied in the paratyphoid test.

Since the experiment recorded in Table 6 simulated human infection more closely than the others it is most desirable that this should be repeated in order to determine whether the observed failure of Vi+O vaccine to protect better than pure O vaccine against alimentary challenge can be established as a consistent finding or whether it can be dismissed as fortuitous. The latter would seem more likely since the recorded findings are anomalous in that pure Vi vaccine gave a significant measure of protection

The differences which appear in the values of the two antigens when assessed against fatal *infection* and against *intoxication* confirms the unsuitability of the conventional mouse test for evaluating different prophylactics against human enteric fever in which such antigens are quantitatively or qualitatively varied, and suggest the need for substituting (or adding) a test whereby identically made preparations, in which the paratyphoid C 'O' antigen replaces the typhoid 'O' antigen, are evaluated against paratyphoid C infection in mice. This cannot be done until an effective method of using dead inocula has been established. Dead inocula have not yet been tested against alimentary challenge. This step, with variation of the number of immunizing doses and of the route of inoculation (intravenous, subcutaneous intramuscular or intraperitoneal), is now proposed.

SUMMARY

In seeking a more satisfactory test than the conventional mouse-protection tests for the assessment of typhoid-paratyphoid vaccines in relation to their O and Vi antigens, it was decided to undertake a series of experiments with vaccines containing the somatic antigens of *Salmonella paratyphi C* and to use this organism to challenge mice thus immunized. *Salm. paratyphi C* contains the same Vi antigen

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as *Salm. typhi* and moreover it is capable of giving rise to a true infection in mice in contrast to the acute intoxication which follows a fatal dose of *Salm. typhi*.

Large doses, of the order of 100×10^6, of this paratyphoid organism injected intraperitoneally into mice resulted in acute intoxication with death in 24–72 hr., similar in every way to the picture with typhoid bacilli, but when the dose was reduced 100-fold or more an infective illness was set up which after an interval of 5–10 days or more, during which the organisms multiply freely in the tissues, terminated fatally. Typhoid bacilli do not produce a true infection in mice.

Living vaccines were prepared of *Salm. typhi* Ty6S (an almost pure Vi strain) and *Salm. cholerae-suis* (contains the same O-antigen complex as *Salm. paratyphi C*). These were used separately, and in combination, to immunize different batches of mice by the subcutaneous route against a subsequent intraperitoneal challenge with a Vi-positive culture of *Salm. paratyphi C* at both high- and low-dose levels. The different challenge doses were to assess the relative values of the O and Vi components in protecting against intoxication (high dose) and infection (low dose), respectively.

Against intoxication pure Vi vaccines were almost as effective as Vi + O vaccines, whereas pure O vaccines gave little or no protection.

Against fatal infection the combined Vi + O vaccine offered good protection and the pure O vaccine was but little inferior, whereas the pure Vi vaccine appeared to be without significant effect.

Quite small doses of *Salm. paratyphi C* administered by the intra-gastric route can give rise to a general infection in mice, but in order to obtain consistent results much larger doses were found necessary (about 300×10^6 organisms). Mice immunized with the same three vaccines and subsequently challenged by the intra-gastric route with this large dose showed significant protection throughout. The combined, O + Vi, vaccine did not appear to have any advantage over the pure O vaccine, but both were markedly superior to the pure Vi vaccine. Further experiments on the same lines are however necessary to see if the results obtained in this single experiment are reproducible.

The relationship of the above findings to the evaluation of agents employed in man for the prophylaxis of enteric fever and the need for further experimental work of a similar nature are discussed.

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