J. Hyg., Camb. (1961), **59**, 49
Printed in Great Britain

A comparison of plague vaccines by the mouse protection test

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(*Received* 31 *August* 1960)

INTRODUCTION

The use of anti-plague vaccines is still regarded as an important measure in the control of the disease, and many health authorities in countries overseas require evidence of vaccination against plague before allowing travellers to enter their ports. There are several different types of plague vaccine in common use, and many detailed differences in their method of manufacture and the strains of *Pasteurella pestis* employed for the vaccine may be virulent or avirulent. No attempt is made by some manufacturers to estimate the potency of the vaccine by any biological method; the fact that opacity tests indicate that it contains the requisite number of bacterial cells per ml. is accepted as satisfactory. An active mouse protection test is used, however, by some laboratories for the comparison of the potency of different batches of the same vaccine. For instance one manufacturer used six mice at each of five dose-levels for the determination of the 50% protection dose (PD 50), by the method of Reed & Muench (1938), against a constant virulent challenge. While this application of the mouse protection test may have some value in a simple comparative test, the variability of the results of different tests limits its value as a method for the assay of different vaccines or for the provision of evidence to show whether the living vaccine, prepared from strains of reduced virulence, is more or less efficient than a killed vaccine, prepared from a highly virulent human strain, in protecting mice against a virulent challenge.

The enormous difficulties associated with a statistically controlled field trial make it virtually impossible to assess the comparative value of dead and living vaccines in the prevention of plague in man. It was felt that some sort of mouse protection offered the only means of comparing the potencies of various types of vaccine, and it was decided to re-examine the test with a view to reducing the effect of the variables which interfere with the reproducibility of the results. There can be considerable variation in the mouse virulence of a particular strain of *P. pestis* when the challenge suspensions are from different batches in which there may be slight differences of preparation. It is therefore most important that the preparation and storage procedures of the challenge suspension should be standardized as completely as possible so that the titration of the LD 50 of different batches of the virulent challenge would give results which were reasonably close. It seemed to be equally important to have a standard vaccine preparation which could be included in each set of tests so that the potencies of the vaccines under test could be expressed in terms directly related to the mouse-protective efficiency

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of the Standard. Although a complete Standard vaccine was not available in the same form as those under test it was considered that the stored acetone-dried whole-culture of the Tjiwidej strain would be most suitable for the purpose, since, as a freeze-dried preparation it could be reconstituted in accurately controlled dosage as a vaccine of which the different reconstituted samples would have identical properties. Any differences in the assessment of samples of this preparation by the mouse protection test would be more likely to be related to the variability of the method than the variability of the vaccine preparation itself. In addition, it was considered that a suitable dose-range of vaccines for the tests in mice should be worked out and that a minimum of ten mice should be used for every group of animals employed.

The object of the experiments described here, which were done some 4 years ago, was to determine whether it was possible to assay a plague vaccine either in terms of its power to protect mice against a standard challenge of virulent *P. pestis* or in terms of its protective power when compared with a Standard vaccine. And if so whether such methods could be regarded as practical.

MATERIALS AND METHODS

Preparation of challenge suspension

In the first series of experiments *P. pestis* L. 37 was used, but for all subsequent experiments strain MP. 6 (Burrows & Bacon, 1954) was employed. Both strains were stored at 4° C. in the form of gelatin-ascorbic acid disk-cultures. As required, a disk-culture was reconstituted and grown on a plate of tryptic-meat agar, containing also peptic sheep blood, for 48 hr. at 37° C. Selected colonies were reseeded on to tryptic-meat agar (T.M.A.) slopes, and after 48 hr. incubation at 37° C. the growth from each tube was washed off in 1 ml. phosphate-buffer (pH 7·4). Two drops of this dense suspension were inoculated into a flask containing 25 ml. of a Casamino-galactose medium (Herbert, personal communication) and incubated for 36 hr. at 28° C. under conditions of continuous agitation. This constituted the 'stock culture-suspension'. It was dispensed in half-ounce McCartney screw-cap bottles with due precautions against contamination and stored at 4° C. Under these conditions the culture-suspension was suitable for use up to 1 month from the date of preparation; after this interval the virulence of the organism began to decrease.

On the day before a test one of the bottles containing the stock culture-suspension was removed from the refrigerator and after thorough agitation two drops of the suspension were seeded on to each of two T.M.A. slopes which were then incubated at 37° C. from 4 p.m. until 9 a.m. the following day. The growth from the two slopes was suspended in 1 ml. phosphate-buffer and diluted 1/5; this formed the challenge suspension. The number of bacterial cells per ml. was determined in a nepholometer (Evans Electroselenium Ltd.) which had been calibrated against Brown's Standard Opacity Tube no. 1 (Burroughs Wellcome and Co.) which is equivalent to 10^9 *P. pestis* cells/ml. This estimate was checked by plate viable counts (Miles & Misra, 1938) of the actual suspension being used for the challenge.

The medium used for the plate counts was either T.M.A. with added peptic sheep blood or T.M.A. containing a freshly prepared 1% solution of sodium bisulphite which was added to the melted agar after cooling to 55° C. With either medium the colonies of *P. pestis* were not sufficiently developed for counting until 48 hr. or more incubation whether the temperature of incubation was 28° or 37° C.

Vaccines

Details of the strains used and the essential methods of preparation are summarized in Table 1. The standard vaccine preparation used was made from an acetone-dried whole culture of the avirulent strain Tjiwidej.

Table 1. *Vaccines used for assay*

Vaccine	Recommended human dose	Strains	Growth conditions: Temperature (° C.)	Growth conditions: Time of cultivation	Growth conditions: Culture medium	Preservative	Dry weight (mg./ml.): Before dialysis	Dry weight (mg./ml.): After dialysis
Standard acetone-dried whole culture	—	Tjiwidej	37	40 hr.	Tryptic-meat broth+1% galactose	Nil	9·2	0·2
A	½ and 1 ml.	Tjiwidej and Soemedang 50/50	37	48 hr.	Meyer's hormone agar	Formalin (0·5%) and phenol (0·5%)	9·2	0·15
B	1 and 1½ ml.	Virulent human strain	32	14 days	Casein hydrolysate liquid medium	Formalin (0·07%) and phenyl mercuric nitrate	24·6	1·63
C	½ and 1 ml.	Tjiwidej and Soemedang 50/50	37	72 hr.	Beef heart peptone= Schutze hormone agar medium	Formalin (0·5%) and phenol (0·5%)	13·0	0·2
D (a replica of B using an avirulent strain)	—	Tjiwidej	32	14 days	Casein hydrolysate liquid medium	Formalin (0·07%) and phenyl mercuric nitrate	24·4	2·07

Mice

The Porton strain of laboratory white mice was used. The strain was derived from the Parkes's strain obtained from the National Institute for Medical Research, London, N.W. 7. At the time of first inoculation the animals weighed 18–22 g.

EXPERIMENTAL PLAN

For most experiments each of six groups of ten mice received 0·5 ml. of one of six successive twofold dilutions of vaccine injected intraperitoneally. Seven days later they received a second dose of the same amount. After an interval of 1 week all the animals included in the test were injected intraperitoneally with 0·2 ml. of the challenge suspension, i.e. about 2000 viable organisms.

The intraperitoneal route was adopted, but because some workers prefer the subcutaneous route a comparison of the two methods and the possible permutations of them was made. The route of inoculation did not influence the protective effect of the vaccine, but it is noteworthy that there were no toxic deaths in the groups inoculated subcutaneously.

In the first series of tests strain L. 37 was used for the challenge, but this was replaced by strain MP. 6 for the remaining six assays. Deaths included all mice dying within 21 days, but events showed that this holding time could have been reduced to 1 week; a few mice died during the second week, but the relative potencies of vaccines calculated at 7 and 21 days respectively proved to be the same.

From the representative experiment with strain L. 37, recorded in Table 2, it will be seen that the weaker vaccines did not protect 50% of the mice. In subsequent experiments the dilutions of the vaccine were accordingly adjusted to ensure that a 50% protective dose lay within the range of dilutions used. The 50% protective dose (PD 50) of each vaccine and the error of the estimate were calculated by the use of probits (Finney, 1947).

Table 2. *Example of assay of potency*

Tjiwidej acetone-dried	Dose (μg.)...	100	50	25	12·5	6·25	3·125
	Survivors...	5/6*	6/7*	6/10	5/10	4/10	3/10
Dilution of vaccine ...		1/50	100	200	400	800	1600
Vaccine A	Survivors	4/10	0/10	1/10	1/10	0/10	0/10
Vaccine B	Survivors	7/10	6/10	5/10	5/10	1/10	1/10
Vaccine C	Survivors	4/10	3/10	2/10	1/10	0/10	2/10

Challenge dose—2650 organisms of L 37 strain in 0·2 ml.

Titration of virulence of challenge inoculum

Viable organisms inoculated ...	2650	1100	662	330	264	130	66	13
Survivors	1/20	5/20	3/20	6/20	5/20	14/20	13/20	17/20

* 4 and 3 deaths due to 'toxicity'.

RESULTS

In the first seven assays (Table 3) vaccines were assessed in terms of the Standard: vaccine B was significantly better than vaccines A or C, between which the difference was not significant. However, to obtain this degree of accuracy some 2000 mice were needed for the seven replicate assays.

Though the challenge doses were prepared in exactly the same way for each test and regularly contained approximately 2000 viable organisms they varied substantially in killing power. Estimates of the LD 50 ranged from 3 to 152 viable organisms, with a mean of 80, for strain L. 37 and from 83 to 360, with a mean of 211, for strain MP. 6.

Even this wide variation in the LD 50 of what was virtually the same challenge suspension did not account for the variability of the test as a whole. When the LD 50 of organisms used in the experiment, as determined by the parallel virulence titration, was plotted against the PD 50 obtained in the same test there was no correlation between the two quantities. Neither was there any correlation between

PD 50 and viable count. Altering the route of injection of vaccine and challenge from intraperitoneal to subcutaneous did not affect the type of result.

With potent vaccines such as the Standard and vaccine B, the assay was disturbed by the occurrence of toxic deaths, mainly after the first injection. The toxic activity remained in the supernatant after the bacterial cells had been thrown down by centrifugation at 1300 *g.* for 30 min. (Table 4). But it was found that when the

Table 3. *Assay of pasteurella pestis vaccines by mouse protection test. Combined results of seven experiments such as that in Table 2*

Vaccine	Relative potency	95 % fiducial limits
Acetone-dried standard	100	—
A	0·68	0·33–1·39
B	15·0	8·2–27·6
C	1·66	0·84–3·28

Table 4. *Toxic effects of vaccines*

	Proportion of mice dying following intraperitoneal inoculation of 0·5 ml. vaccines			
Vaccine used	Undiluted	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Tjiwidej standard*	9/10	98/120	79/210	11/210
A	0/10	0/10	0/10	—
B	30/30	21/30	6/30	0/5
C	0/10	0/10	0/10	—
D	29/30	19/30	13/30	—
B supernatant	8/10	10/10	4/10	—
B deposit	0/10	0/10	0/10	—

* Made up to contain 200 μg. in 0·5 ml.

Table 5. *Protective effect of supernatant and deposit of centrifuged vaccine*

Vaccine used	50 % protection dilution	
	Supernatant	Deposit
A	12 (6–28)	No significant protection
B	170 (100–350)	70 (*)

* Finite limits could not be obtained.

sedimented bacteria were used in place of the whole vaccine the protective effect was much reduced so that the sediment contained only part of the protective antigens. As a rule, however, the protective dose of the vaccine was some 60 times lower than its 50 % lethal dose so that toxicity is unlikely to have affected the precision of the test to any significant extent.

Factors affecting the potency of the vaccine

Content of high molecular weight substances

In view of the very different methods used in preparing the vaccines it is possible that the differences in vaccine potency might be a reflexion of the amount of high molecular weight bacterial products which they contained. The content of non-dialysable solids (Table 1, cols. 8–9) was found to vary a great deal, but potency cannot be attributed wholly to the amount of these substances because when the assay results are expressed in terms of the contents of these solids it is clear that the Standard and vaccine B are still better antigens than either vaccines A or C (Table 6).

Table 6. *Protective potency in terms of content of antigen and of high molecular weight substances*

Vaccine	Numerical order of potency	Amount of non-dialysable in μg./PD 50
Tjiwidej Standard	2	5·8 (4·4–7·7)
A	4	23·0 (16·0–33·0)
B	1	4·6 (3·6–6·0)
C	3	12·0 (8·0–17·0)

Strain used for vaccine production

In the light of the observations recorded in the previous paragraph a fourth vaccine, vaccine D, was prepared from the Tjiwidej strain but by methods identical with those used for the preparation of vaccine B. The potency tests for vaccine D have not been included in the tables because they were not undertaken on the same scale, but the tests that were done showed that vaccine D was significantly less potent than vaccine B.

When correction factors, to account for the differences in dry weight, were applied it was found that, were vaccine D to have been included in Table 6, it would have been placed between vaccines A and C, close to the latter.

Vaccine B which was prepared from a human virulent strain is clearly superior to the vaccine prepared from the avirulent Tjiwidej strain, a fact which is in accordance with other observations on the greater potency of vaccines made from virulent cultures.

Content of protective antigens

Of the many antigens in *P. pestis* only one is known to have importance in protecting mice against infection. This is 'antigen 3' in the system described by Crumpton & Davies (1956) which is the main component of fraction I described by Baker, Sommer, Foster, Meyer & Meyer (1952).

Equal weights of dialysed vaccines A, B and C were set up in Ouchterlony plates against sera of known antibody content. Vaccines A and C each gave two faint lines of precipitation, one of which was identified as antigen 3 by tests with mono-specific sera. Vaccine B gave eight lines, one of the stronger ones being due to

antigen 3. This line was far more intense than the antigen 3 lines produced under identical conditions by vaccines A and C. The Tjiwidej Standard preparation gave at least 12 lines of which antigen 3 was one of the strongest and somewhat stronger than the antigen 3 line with vaccine B. It will be seen from Table 1 that vaccines A and C are prepared from equal parts of suspensions of the strains Soemedang and Tjiwidej. The Soemedang strain is known to produce less of antigen 3 than the Tjiwidej strain so that the faint antigen 3 lines noted with these two vaccines, A and C, would appear to be related to this fact. In addition both A and C vaccines have a higher formalin content (0·5 %) than vaccine B (0·07 %) and both of them also contain phenol (0·5 %) which is absent from vaccine B. The effect of phenol on the protective antigen is not known but formalin does reduce the potency to some extent (see below). The Standard preparation has no added preservative and since it was dried in acetone at $-30°$ C. it might be expected to have retained its antigens the least impaired.

Effect of formalin preservation on antigenicity

The addition of 0·1 % formalin was found to reduce the toxicity of the vaccine for mice fifty-fold; it also reduced the potency though to a less degree. The potency of the Tjiwidej strain was reduced by this means by a factor of 1/3·5 times (1·9–6·3) and that of acetone dried MP. 6 by a factor of 1/2·4 times (1·3–4·4).

The detoxifying and preserving effects of formalin are desirable. It is true that there is some loss of potency but this usually can be offset by using a highly virulent strain rich in antigen 3.

DISCUSSION

It is clear that in spite of observing the greatest care in growing the challenge culture from the same batch of dried seed under strictly comparable conditions the relation of the viable count to the LD 50 varied greatly. The 50 % protective dose (PD 50) of the acetone-dried Standard vaccine was in consequence even more variable. When, however, the potency of the vaccines was expressed in terms of the Standard vaccine and a sufficient number of replicate assays were made it was possible to place the vaccines in a dependable order of immunogenic potency. The vaccines differed greatly in the content of material remaining after dialysis and when equal weights of this material were compared the vaccines prepared from virulent strains were appreciably more protective than those prepared from avirulent cultures.

The addition of formalin, while valuable as a preservative and a detoxifying agent, was found to reduce the protective potency of the vaccine. When the non-dialysable material was compared in Ouchterlony plates, lines corresponding to known antigenic components were obtained; the one component known to protect mice was more in evidence in the vaccine prepared from a virulent human strain.

SUMMARY

1. In our hands the assay of a plague vaccine, in terms of its power to protect mice against what was hoped to be a standard challenge, was found to be impracticable.

2. Assay by mouse protection tests in terms of a Standard vaccine was found to be possible, but it proved to be a very time-consuming procedure and demanded the use of an almost prohibitively large number of mice for a sufficient number of replicate tests which are required to offset the variability in the method. Theoretically a single test might be adequate if the numbers of immunized mice were very large, but there are obvious limitations to the numbers of mice that can be included in a single test of this nature.

3. The Ouchterlony plate technique proved to be useful in indicating the antigens present in a particular vaccine, but not on a strictly quantitative basis.

4. The vaccine prepared from the virulent human strain of *P. pestis* was found to protect mice more efficiently than those prepared from avirulent strains and when the protective potency was expressed in terms of content of antigen and high molecular weight substances the vaccine prepared from the virulent culture was found to be even more potent than the Standard acetone-dried vaccine.

5. The use of formalin as a preservative results in some loss of potency of a vaccine but, provided the strain of *P. pestis* used for the preparation of the vaccine is highly virulent, this need not be serious and is offset by a number of advantages.

We wish to acknowledge our indebtedness to the Director of the Haffkine Institute, Bombay, for supplying details of their methods for the preparation of plague vaccine, to Dr D. A. L. Davies for the acetone-dried material, the dry weight measurements and the diffusion results, to Mr S. Peto for the great trouble taken over the statistical analysis and finally to Mr G. R. Moody for technical assistance.

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