A FILTERED NON-TOXIC PLAGUE VACCINE WHICH PROTECTS GUINEA-PIGS AND MICE

H. SMITH* AND L. P. PACKMAN

From the Microbiological Research Establishment, Porton, Salisbury, Wilts.

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THIS paper describes a filtered, non-toxic preparation from *Pasteurella pestis* which could be developed for vaccination of man against plague. The literature on plague prophylaxis will not be reviewed since this has been done elsewhere (Pollitzer, 1954 ; 1960). It is necessary however to consider, in relation to our previous work (Keppie, Cocking and Smith, 1958 ; Smith, Keppie, Cocking and Witt, 1960 ; Cocking, Keppie, Witt and Smith, 1960 ; Keppie, Cocking, Witt and Smith, 1960), the results of K. F. Meyer and his colleagues of the Hooper Foundation, San Francisco, regarding killed preparations from *P. pestis* which immunize guinea-pigs and mice, since it appears that a vaccine that immunizes both these animals will probably be effective in man (Girard, 1955). In the main, preparations without adjuvant will be discussed because the addition of oil adjuvant is not desirable for human vaccination, if adequate immunizing activity can be achieved in its absence.

Until recently, it was generally accepted that whereas live attenuated vaccines immunized guinea-pigs as well as mice, dead vaccines (without adjuvant) were relatively ineffective for guinea-pigs, although adequate for mice (Jawetz and Meyer, 1943 ; Pollitzer, 1954 ; Girard, 1955). Amongst the latter vaccines was a formolized preparation of virulent organisms used by the United States Army (Meyer, 1953 ; Spivack, Foster, Larson, Chen, Baker and Meyer, 1958). Baker Sommer, Foster, Meyer and Meyer, (1952) purified an envelope material—Fraction I from *P. pestis*. This material immunized mice but its activity did not account for the total immunizing activity of the whole organisms and other complex preparations (Baker *et al.*, 1952 ; Smith *et al.*, 1960 ; Keppie *et al.*, 1960). When injected without adjuvant, Fraction I did not immunize guinea-pigs (Baker *et al.*, 1952 ; Keppie *et al.*, 1960).

Working first with virulent *P. pestis* isolated directly from guinea-pigs dying of plague and then with virulent and avirulent immunogenic strains grown *in vitro* (Keppie *et al.*, 1958 ; Smith *et al.*, 1960 ; Cocking *et al.*, 1960) we obtained a non-toxic preparation which immunized both guinea-pigs and mice. This was the crude cell-wall material remaining after the treatment of *P. pestis* with ultrasonic waves and the removal of soluble material. It could be dissolved at pH 8·5–8·7 by treatment with ultrasonic waves (Keppie *et al.*, 1958) or directly at a higher pH by a short treatment with NaOH (Keppie *et al.*, 1960). The activity of this preparation accounted for all the immunizing activity of whole organisms for guinea-pigs but not for all of the activity for mice (much of the material immunogenic for mice was liberated and removed by treatment with ultrasonic waves, Keppie *et al.*, 1960). Using an early report of this work (Keppie *et al.*, 1958) as a

* Present address: Department of Microbiology, The University, Birmingham 15.

guide, Chen, Foster and Meyer (1961) prepared a similar cell wall vaccine and confirmed that it was non-toxic and immunized both guinea-pigs and mice. They were unable to dissolve their material at pH 8·7 but this did not indicate any difference from our materials since they neither used the ultrasonic treatment needed to effect solution at this pH (Keppie *et al.*, 1958) nor the higher pH used by us later (Keppie *et al.*, 1960). New information in this publication (Chen *et al.*, 1961) was that the formolized United States Army vaccine, which previously had been reported as being relatively ineffective in guinea-pigs (Meyer 1953 ; Spivack *et al.*, 1958) was now effective in these animals which were however of a different strain (Chen : private communication). A possible explanation for this difference in efficacy lies in the great variability of guinea-pigs in these tests which had been noted by Spivack *et al.* (1958) and has been confirmed in the work reported here.

This paper describes a filtered non-toxic vaccine prepared from an avirulent immunogenic strain (E.V. 76) of *P. pestis*. To obtain the best preparation for immunizing both guinea-pigs and mice, the cell-wall material of *P. pestis* (immunogenic for guinea-pigs and to some extent for mice) was supplemented by the material —immunogenic for mice—which was liberated when the organism was disrupted (Keppie *et al.*, 1960). However the latter material was very toxic for mice (Cocking *et al.*, 1960) and therefore could not be tested in this species unless detoxified in some way. Cocking *et al.* (1960) and Keppie *et al.* (1960) showed that a short treatment with N/10 NaOH not only dissolved cell-wall material but also largely destroyed mouse toxicity without reducing guinea-pig immunogenicity; the alkaline treatment reduced mouse immunogenicity but its rate of destruction was slower than that for mouse toxicity.

At first, ultrasonic waves were used to disrupt the cells prior to the alkaline treatment but later shaking with ballotini beads was substituted since it was equally effective and was more convenient for large scale production of the vaccine. This vaccine protects guinea-pigs and mice against subcutaneous and respiratory challenge with *P. pestis* and its efficacy has been compared with that of the formolized United States Army vaccine. This comparison was made on several occasions since a feature of the work was the great variability of batches of guinea-pigs in immunity tests either in their immune response or in their susceptibility to subsequent challenge.

Turning now to plague vaccines containing oil adjuvant (a mixture of light mineral oil and mannide oleate), K. F. Meyer and his colleagues (Spivack *et al.*, 1958 ; Chen *et al.*, 1961) have reported that such adjuvants may produce immunoparalysis with large amounts of vaccine but that they increase the efficacy of small amounts of vaccine for guinea-pigs and in particular render minute amounts of the envelope substance—Fraction I—immunogenic for these animals. In small scale experiments reported here we have confirmed these observations.

MATERIALS AND METHODS

Growth of Pasteurella pestis *(strain E.V. 76) for the vaccine*

For ease of production, an attenuated immunogenic strain of *P. pestis* was preferable to a virulent strain ; of the two strains (Tjiwidej and E.V. 76) which have been used to vaccinate man, E.V. 76 was chosen because it produced more of the antigens related to virulence than the Tjiwidej strain (Burrows, 1963).

Cultures of *P. pestis* (E.V. 76 obtained from Dr. T. W. Burrows) were maintained on slopes of tryptic meat agar stored at 4° ; they were subcultured at intervals of several months.

Subcultures on slopes of tryptic meat agar were incubated for 20 hr. at 28° and washed off with tryptic meat broth (1 ml. per tube). The suspension (0·4 ml.) was inoculated into tryptic meat broth containing 1 per cent w/v galactose (25 ml. in 250 ml. conical flasks) and the cultures were shaken (100 oscillations per min., 1·75 in. throw) at 37° for 22 hr. The cultures were checked for contamination (by microscopical examination) and added to more medium in Thomson bottles (each bottle received 10 ml. of culture and 250 ml. tryptic meat broth containing 1 per cent w/v of galactose). The Thomson bottles (20 in a normal batch, *i.e.* 5 l. of culture) were laid flat on a shaking machine aud shaken for 22 hr. at 37°. The purity of the cultures was checked and a total count (approx. 6×10^9 ml.) was obtained prior to collection of the organisms by centrifugation in the batch bowl of the Spinco Ultracentrifuge (16,000 rpm, 1 hr., 4°). The packed deposit of organisms was resuspended in sterile saline (200 ml.) by ball-milling in the batch bowl for 40 min. at 4°, and after removing the steel balls and diluting to 1,400 ml. the washed organisms were collected by recentrifugation at 16,000 rpm for 1 hr. The deposit was stored overnight at 4°.

Preparation of the vaccine

Preparation of the vaccine which is described in detail below, entails disrupting *P. pestis* by shaking with ballotini beads, dissolving the cell-wall material, detoxifying it by treatment with N/10 NaOH, sterile filtration after reducing the pH to 9·2 with glycine and finally adjusting the pH to 7·6. This treatment dissolved 90–95 per cent of the cell substance and at the standard strength equivalent to 1×10^{10} organisms per ml. the vaccine contained approximately 350 mg. of bacterial substance per 100 ml.

Preliminary experiments, the details of which are not reported here, showed that (1) initial disruption followed by treatment with NaOH dissolved more cell substance and produced better vaccines than simple treatment with NaOH (only about 85 per cent of the cell substance dissolved); (2) the optimum time for treatment with N/10 NaOH was 10 min. at room temperature, (3) shaking with ballotini beads was equally effective for the initial cell disruption as treatment with ultrasonic waves and much more convenient for the large scale production of the vaccine, and (4) the filtered vaccine was better for protecting both guinea-pigs and mice than a formolized preparation made from the same batch of organisms.

Method

The packed deposit of organisms described above was suspended in cold sterile distilled water (*c.* 150 ml.) by ball-milling in the ultracentrifuge bowl. The suspension was diluted to contain 1×10^{11} organisms/ml. (calculated from the original bacterial count of the culture) and 10 ml. aliquots were distributed into 1 oz. screw capped bottles containing ballotini beads (10 g., grade 16) with one drop of capryl alcohol to stop foaming. The bottles were shaken at 4° for 20 min. in a vertical shaking machine (500 oscillations per min.; 4 in. throw). The suspension of disintegrated cells and beads was bulked and diluted with sufficient distilled water to make the strength equivalent to $1{\cdot}1 \times 10^{10}$ organisms. After adding N NaOH to make the suspension N/10 with respect to NaOH the mixture was kept at room temperature for 10 min., then glycine (4·5 g./100 ml.) was added to reduce the pH to 9·2. When the glycine had dissolved and the ballotini beads had settled (10–15 min.), the supernatant was syphoned off and clarified by centrifugation in the batch bowl of the Spinco Ultracentrifuge (16,000 rpm, 1 hr.). The supernatant was sterilized by filtration through Oxoid cellulose acetate membranes which had been sterilized by treatment with 0·02 per cent merthiolate. After filtration, the pH of the vaccine was reduced to 7·6 by the addition of concentrated HCl (approx. 0·8 ml./100 ml. vaccine). The vaccine was checked for sterility and was stored in aliquots at −20° until required for use.

Adjuvant

A mixture of 9 parts Bayol 55 (light petroleum oil) and 1 part of Arlacel A (mannide oleate). The dose of vaccine was mixed with 0·5 ml. of adjuvant for guinea-pigs and 0·1 ml. for mice.

Formolized vaccine used by the United States Army

Several batches of this material (B6526, G7586 and G8216) were obtained from Cutter Laboratories, Berkeley, California, U.S.A. The material was made from the virulent strain 195/P (Chen *et al.*, 1961) and was equivalent to 2×10^9 organisms per ml.

Live vaccine (E.V. 76)

This strain was grown on a slope of tryptic meat agar at 37° for 20 hr. The organisms were washed off and diluted in gelatin-Locke solution. In immunity tests (see below), a single large dose (5×10^8 viable cells for guinea-pigs and $2{\cdot}5 \times 10^7$ cells for mice) was given subcutaneously at the same time as the second dose of a killed vaccine. The animals were challeneged 2 weeks after this dose in parallel with the animals receiving the dead vaccines.

Test for immunizing activity in guinea-pigs and mice

Two subcutaneous injections of the material under test were given to guinea-pigs (Porton strain unless stated otherwise, 400 ± 50 g.) or to mice (20 ± 2 g.) with an interval of two weeks between doses. Two weeks after the final dose, these animals and control groups were challenged with virulent *P. pestis* (strain L37).

(*a*) Subcutaneously—guinea-pigs received 1×10^4 organisms (approx. 100 LD_{50}) and mice 1×10^3 (approx. 100 LD_{50}).

(*b*) By the respiratory route—the apparatus designed by Henderson (1952) was used. Druett, Henderson, Packman and Peacock (1953) and Druett, Robinson, Henderson, Packman and Peacock (1956) should be consulted for details of the preparation of suspensions of organisms, for the method of cloud sampling and assessment, and for definitions of the terms used. Guinea-pigs were exposed for 1 min. to a cloud of concentration *c.* 6×10^4 organisms/l. the estimated LNt being 85. Mice were exposed for 5 min. to a cloud of concentration *c.* $0{\cdot}25 \times 10^6$ organisms/l. the estimated LNt being 75.

Tests for toxicity

Lethality for mice and guinea-pigs.—The materials were injected subcutaneously into mice (0·5 ml.) and guinea-pigs (5 ml.) and the animals were observed for 7 days. Since the maximum immunizing doses given to mice and guinea-pigs were 0·1 ml. and 1·0 ml. respectively, a preparation was considered non-toxic if no deaths occurred in 10 animals.

Tissue damage in the skin of mice, guinea-pigs, rabbits and monkeys.—Dilutions (0·2 ml.) of the test substances were injected into the shaven skin of these animals which were then observed every 2 hr. for the first 6 hr. and then each day for 7 days.

RESULTS

The results are a combination of those obtained in earlier experiments when the organisms were disrupted with ultrasonic waves and those of more recent experiments when the organisms were disrupted by shaking with ballotini beads. The latter experiments predominate and include all comparisons with the American Army (Cutter) Vaccine.

Protection of guinea-pigs against plague with the "filtered" vaccine

The variable results obtained from immunity tests with the "filtered" vaccine in guinea-pigs.—Table I shows the results of immunity tests (using a subcutaneous challenge) with numerous batches of vaccine prepared since 1960. The early results were similar to those already reported (Keppie *et al.*, 1960) but the results of subsequent tests showed an apparent decreased protection in guinea-pigs. However, the results of repeat tests on batches 8 and 9 (Table I) which were carried out 2–3 months after the original tests indicated that batches of guinea-pigs could vary either in their immune response or in their reaction to subsequent challenge (or both). This was confirmed by the results shown in Table II in which different batches of guinea-pigs (in one oxperiment, the same strain of guinea-pig on two different diets was used and in the other, two entirely different strains of guinea-pigs) were used in the same test on the same vaccine.

TABLE I.—*Immunity Tests in Guinea-pigs with Batches of "Filtered" Vaccine Prepared since* 1960

Batch	Guinea pigs given 2 doses of vaccine (1 ml.) No. survivors / No. in batch	(per cent)	Controls No. survivors / No. in batch
1	19/20	95	1/20
2	18/20	90	0/40
3	36/40	90	2/40
4	33/40	83	0/40
5	30/40	75	0/40
6	26/40	65	0/40
7	24/50	48	0/50
8 (1st test)	9/30	30	0/30
8 (repeat test)	24/40	60	0/40
9 (1st test)	17/30	57	0/30
9 (repeat test)	28/40	70	0/40

Details of immunity tests—see Methods; the animals were challenged subcutaneously.

The results of the first experiment shown in Table II demonstrate the lack of dose-response that occurs in these immunity tests which in itself indicates the large variability of individual guinea-pigs.

TABLE II.—*Parallel Immunity Tests with a Single Batch of Vaccine in Guinea-pigs Differing in Origin and Diet*

	Survivors (per cent) of guinea pigs (20) given two doses of vaccine (ml.)					
	4·8	2·4	1·2	0·6	0·4	Nil (Controls)
Vaccine 1						
(*a*) In Porton guinea-pigs without "green food" in their diet	35	65	35	60	45	0
(*b*) In Porton guinea-pigs with "green food" in their diet	75	80	65	80	80	0
Vaccine 2						
(*a*) In Porton guinea-pigs	—	—	80	—	—	0
(*b*) In guinea-pigs from the Chester Beatty Research Institute, London	—	—	45	—	—	0

Details of immunity tests—see Methods; the animals were challenged subcutaneously.

Comparison in guinea-pigs of the "filtered" vaccine with the American Army (Cutter) vaccine.—The results in Table III emphasize the variable results that are obtained in these tests with guinea-pigs but nevertheless clearly indicate the superiority of the "filtered" vaccine (3 batches) over the American Army vaccine (3 batches) in protecting guinea-pigs. The protection produced by the "filtered" vaccine in these tests was usually 60–80 per cent (Tables I, II and III) compared with 100 per cent produced by the live vaccine. The results in Table IV show that against respiratory challenge the superiority of the "filtered" vaccine over the Cutter vaccine and its inferiority to the live preparation also held true.

The effect of oil adjuvant on the response of guinea-pigs to the "filtered" vaccine.—The results in Table V show that the addition of oil adjuvant does not increase the

TABLE III.—*Comparison in Guinea-pigs of the "Filtered" Vaccine with the American Army (Cutter) Vaccine when the Animals were Challenged Subcutaneously*

	Survivors (per cent) of guinea-pigs given two doses of vaccine (ml.)		
	1·0	0·2	Nil (Controls)
A. Tests with "filtered" vaccine B33 (20 guinea-pigs per group)			
Test 1:			
B33	75	—	0
Cutter (B6526)	60	—	
Test 2:			
B33	85	—	0
Cutter (B6526)	65	—	
Test 3:			
B33	55	—	0
Cutter (G7586)	30	—	
Cutter (G8216)	32	—	
B. Tests with "filtered" vaccines B35 and B37			
Test 1* (30 guinea-pigs per group)			
B35	73	67	0
B37	77	80	
Cutter (G7586)	43	40	
Cutter (G8216)	57	60	
Test 2 (20 guinea-pigs per group)			
B35	50	—	0
Cutter (G7586)	30	—	
Cutter (G8216)	32	—	

Details of immunity tests—see Methods.

* In this test and on many other occasions, vaccination with live E.V. 76 organisms (5×10^8) resulted in 100 per cent protection.

TABLE IV.—*Comparison in Guinea-pigs of the "Filtered" Vaccine with the American Army (Cutter) Vaccine when the Animals were Challenged by the Respiratory Route*

	Survivors (per cent) of guinea-pigs (30) given two doses of vaccine (ml.)		
	1·0	0·2	Nil (Controls)
Filtered vaccine B35	53*	50	0
B37	66	54	
Cutter vaccine G7586	23	17	
G8216	30	30	
Live E.V. 76 (5×10^8 organisms)	100		

Details of immunity tests—see Methods.

* In similar tests with batches 1, 2, 3 and 4 of the "filtered" vaccine shown in Table I, the survival rates (per cent) after respiratory challenge were 55, 55, 44 and 50 in batches of 20–40 animals (dose of vaccine 1·0 ml.); no controls survived and the live vaccine produced 95–100 per cent protection.

immunizing effect of the doses of vaccine normally used. In fact, there are signs of immunological paralysis at the highest dose. However, the adjuvant produced good protection when it was combined with a very small amount of vaccine and also it rendered small amounts of envelope substance—Fraction I—immunogenic for guinea-pigs. These results are in accord with those of Spivack *et al.* (1958) and Chen *et al.* (1961).

TABLE V.—*The Effect of Oil Adjuvant on the Response to the "Filtered" Vaccine in Guinea-pigs*

	Survivors (per cent) of guinea-pigs (20) given two doses of the vaccine (ml.)			
	1·0	0·2	0·02	Nil (Controls)
Subcutaneous challenge				
B37 alone	80	65	—	0
B37 with adjuvant	40	70	85	
Respiratory challenge				
B37 alone	60	55	—	0
B37 with adjuvant	40	53	70	

Details of immunity tests—see Methods.

In a similar test, envelope substance or Fraction I (5 μg. and 0·5 μg.) protected guinea-pigs (10/20 and 13/20 respectively) when it was injected with adjuvant whereas it had no effect when it was injected alone (Keppie *et al.*, 1960).

Protection of mice against plague by the "filtered" vaccine

Tests with the vaccine in mice did not show the large variations that had been experienced in guinea-pigs. Since 1960 two doses of 0·1 ml. of numerous batches of vaccine have protected 70–90 per cent of mice against subcutaneous challenge.

Mice were less resistant to respiratory challenge than guinea-pigs. If challenged with a dose of organisms (animals exposed 5 min. to a cloud concentration $1{\cdot}3 \times 10^6$ organisms per l.) which killed 100 per cent of the control animals, animals receiving the "filtered" vaccine showed little protection. The challenge dose was therefore reduced (cloud concentration $0{\cdot}25 \times 10^6$ organisms per l.) so that only 60–90 per cent of the control animals died. Under these conditions significant protection by the "filtered" vaccine was demonstrated. Thus, in 8 similar immunity tests with different batches of the "filtered" vaccine two doses of 0·1 ml. protected a total of 304/405 (75 per cent) mice, whereas only 70/207 (34 per cent) of the control animals survived; in these tests the live EV. 76 vaccine ($2{\cdot}5 \times 10^7$ organisms) protected 155/205 (75 per cent) mice.

The results in Tables VI and VII show that there is no significant difference in the degree of protection conferred on mice by the "filtered" vaccine as compared with the American Army (Cutter) vaccine. The respiratory challenge administered in the experiment summarized in Table VII was slightly more severe than that used previously (see above).

Absence of toxicity shown by the "filtered" vaccine

Lethality.—The "filtered" vaccine did not kill mice (0·5 ml. given s.c.) or guinea-pigs (5 ml. given s.c.).

Tissue damaging activity.—Three different batches of the "filtered" vaccine (0·2 ml.) were examined (see Methods) in two or more animals.

In rabbits, the undiluted material produced raised pink areas (max. diam. 20 mm.) a few hr. after injection. At 24 hr. the area was decreasing (max. diam. 15 mm.) but there was slight oedema. At 48 hr. the area was much smaller and it disappeared in 3–6 days. Some rabbits were more reactive than others, and the vaccines produced similar (but smaller) reactions at 1/3 and 1/9 dilutions but not at 1/27.

TABLE VI.—*Comparison in Mice of the "Filtered" Vaccine with the American Army (Cutter) Vaccine when the Animals were Challenged Subcutaneously*

	Survivors (per cent) of mice (20) given two doses of the vaccine (ml.)		
	0·1	0·02	Nil (Controls)
Tests with "filtered" vaccine B33			
Test 1			
B33	70	50	0
Cutter (B6526)	75	65	
Test 2			
B33	90	65	0
Cutter (B6526)	85	60	
Test 3			
B33	80	40	0
Cutter (G7586)	90	75	
Cutter (G8216)	70	85	
Test with "filtered" vaccines B35 and B37			
Test 1			
B35	95	80	0
Cutter (G7586)	90	75	
Cutter (G8216)	70	85	
Test 2 (30 mice per group)			
B35	90	86	0
B37	87	83	
Cutter (G7586)	84	79	
Cutter (G8216)	80	89	
Live E.V. 76 ($2·5 \times 10^7$ organisms)	100		

Details of immunity tests—see Methods.

TABLE VII.—*Comparison in Mice of the "Filtered" Vaccine with the American Army (Cutter) Vaccine when the Animals were Challenged by the Respiratory Route*

	Survivors (per cent) of mice (30) given two doses of the vaccine (ml.)		
	0·1	0·02	Nil (Controls)
Vaccine			
"Filtered" vaccine B35	40	46	10
B37	74	47	
Cutter vaccine G7586	66	28	
G8216	32	43	
Live E.V. 76 ($2·5 \times 10^7$ organisms)	90		

Details of the immunity tests—see Methods.

In guinea-pigs, only the undiluted materials produced reactions. These were similar to those in rabbits but smaller; they reached their maxima in 24 hr., and faded more rapidly, *i.e.* 48–72 hr.

In monkeys, two batches of material had no noticeable effect. A third batch produced, when undiluted, a slightly raised inflamed reaction 10–15 mm. diam. which disappeared in 24 hr.

In mice, more severe reactions were evident with undiluted material and 1/3 and 1/9 dilutions. At 24 hr. the lesions were thick white opaque areas (10–20 mm. for undiluted material). At 48 hr. the centre of the area injected with undiluted and the 1/3 dilution, became dark red and remained so for 5–6 days when the surrounding opaque area disappeared.

The vaccine and the glycine buffer base were shown to be isotonic by the absence of haemolysis and crenation when mixed with human blood.

DISCUSSION AND SUMMARY

A filtered non-toxic vaccine suitable for trials in man has been produced from an avirulent immunogenic strain (E.V. 76) of *P. pestis*. It protects guinea-pigs and mice against challenge by virulent *P. pestis* given either by the subcutaneous or respiratory route. The extremely low dose-response (a 5–10-fold difference in dose made practically no difference in response at levels of 40–80 per cent protection) in tests on plague vaccines makes the comparison of potencies of vaccines difficult. However, at the doses tested (1·0 and 0·2 ml.) the filtered vaccine produced greater protection for guinea-pigs than the currently used American Army (Cutter) vaccine which is made by treating a virulent strain of *P. pestis* with formalin. In mice, at the doses tested (0·1 and 0·02 ml.) the two vaccines were indistinguishable in their protective effect. If the "filtered" vaccine is compared with the live E.V. 76 vaccine, the levels of protection (60–80 per cent in guinea-pigs and 70–90 per cent in mice against subcutaneous challenge and 50–60 per cent in guinea-pigs and 50–75 per cent in mice against a respiratory challenge) afforded by the dead filtered vaccine were less than those (95–100 per cent in guinea-pigs against either challenge and in mice 90–100 per cent against subcutaneous challenge and 70–90 per cent against respiratory challenge) produced by live E.V. 76 organisms. However, the latter were used in the large numbers recommended by Dr. G. Girard (private communication); and if these numbers of organisms (5×10^8 for guinea-pigs and $2{\cdot}5 \times 10^7$ for mice) were injected intraperitoneally rather than subcutaneously as in the immunity tests, a few animals had a fatal infection. Preliminary studies have indicated: (*a*) that the vaccine did not lose potency after 1 yr. at 0°, (*b*) that in 3 months the protection given to guinea-pigs waned more than that in mice, and (*c*) that oil adjuvant produced immuno-paralysis with large doses of vaccine but increased the protection from very small doses.

A feature of these studies on plague vaccines has been the variability of guinea-pigs either in their response to the vaccines or in their reaction to subsequent challenge. There is much to be said for using inbred strains of guinea-pigs or at least specific pathogen-free animals in future tests on plague vaccines.

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REFERENCES

BAKER, E. E., SOMMER, H., FOSTER, L. E., MEYER, E. AND MEYER, K. F.—(1952) *J. Immun.*, **68**, 131.

BURROWS, T. W.—(1963) *Ergebn. Mikrobiol. ImmunForsch. exp. Ther.*, **37**, 59.

CHEN, T. H., FOSTER, L. E. AND MEYER, K. F.—(1961) *J. Immun.*, **87**, 64.

COCKING, E. C., KEPPIE, J., WITT, K. AND SMITH, H.—(1960) *Br. J. exp. Path.*, **41**, 460.

DRUETT, H. A., HENDERSON, D. W., PACKMAN, L. AND PEACOCK, S.—(1953) *J. Hyg. Camb.*, **51**, 359.

DRUETT, H. A., ROBINSON, J. M., HENDERSON, D. W., PACKMAN, L. AND PEACOCK, S.—(1956) *J. Hyg. Camb.*, **54**, 37.

GIRARD, G.—(1955) *Annu. Rev. Microbiol.*, **9**, 253.

HENDERSON, D. W.—(1952) *J. Hyg., Camb.*, **50**, 53.

JAWETZ, E. AND MEYER, K. F.—(1943) *J. inf. Dis.*, **73**, 124.

KEPPIE, J., COCKING, E. C. AND SMITH, H.—(1958) *Lancet*, i, 246.

KEPPIE, J., COCKING, E. C., WITT, K. AND SMITH, H.—(1960) *Br. J. exp. Path.*, **41**, 577.

MEYER, K. F.—(1953) *Bull. Wld Hlth Org.*, **9**, 619.

POLLITZER, R.—(1954) ' Plague '. Geneva. (World Health Organisation).—(1960) *Bull. Wld Hlth Org.*, **23**, 313.

SMITH, H., KEPPIE, J., COCKING, E. C. AND WITT, K.—(1960) *Br. J. exp. Path.*, **41**, 452.

SPIVACK, M. L., FOSTER, P., LARSON, A. CHEN, T. H., BAKER, E. E. AND MEYER, K. F.—(1958) *J. Immun.*, **80**, 132.