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THE EVALUATION OF PASTEURELLA PESTIS VACCINES IN PRAOMYS (MASTOMYS) NATALENSIS

by

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INTRODUCTION

Pasteurella pestis (Yersinia pestis) is the causal organism of plague in animals and man. Its use as a live or killed vaccine has for many years been the subject of much discussion and experimental work, especially in relation to protection against the disease and the reactions produced by live attenuated strains.

The history of plague in man dates back many centuries and even some biblical writings are thought to refer to plague. The earliest certain plague epidemic and outbreaks however date back to the time of Justinian (A.D. 542) (Pollitzer, 1954) and are known as the First Pandemic. The present outbreaks are part of the Third Pandemic which originated in the Far East during the late 19th Century (Simpson, 1905). In recent years the disease as a health hazard has decreased throughout the world with the exception of a few areas such as Brazil, Bolivia, Congo and the Republic of Vietnam. Sporadic outbreaks however, still occur in many other areas. This decline in the incidence of plague has been due partly to improved standards of living, more efficient rodent and insect control measures, advances in therapy and also to a natural decline of the disease throughout the world.

The value of vaccination against plague now lies primarily in protecting laboratory staff, field personnel working in enzootic areas and certain other high risk groups under endemic conditions, such as those of war existing in Vietnam today.

The present investigation was initiated as a result of the concern of

many workers that most experimental animals at present used for testing plague vaccines are not suitable. Subhuman primates have recently come into more extensive use for this purpose in the United States of America, but these animals are costly and difficult to obtain.

The multimammate mouse Praomys (Mastomys) natalensis has been adopted to the laboratory at the South African Institute for Medical Research and due to its very high susceptibility to infection was adopted as the standard test animal for routine surveillance of plague. We therefore decided to determine whether this rodent species could be of use for the evaluation of both killed and live attenuated plague vaccines. With this in mind, the principal aims of the study were to investigate

a) the pathogenicity of certain live attenuated plague vaccines in experimental animals, and

b) the immunogenic efficacy of killed and live plague vaccines in these animals.

CHAPTER 1.

First attempts to protect man against plague date back to 1755 when Weszprem1 proposed a method similar to variolation (Wu Lien-Teh et al., 1936).

In 1781 Samoilowski contracted a mild form of plague after being infected with pus from a bubo, and consequently he recommended the method of applying a pad, previously inoculated with bubo pus directly to the arm without incision or scarification. This method was thought to confer immunity against plague by producing a mild infection. It soon fell into disrepute however, as it not infrequently resulted in disasterous consequences (Pollitzer, 1954).

Killed vaccines

Yersin, Calmette and Borrel showed in 1895 that rabbits could be protected against virulent infection by means of a vaccine prepared from fully virulent agar cultures killed by heating at 58°C for one hour (Wu Lien-Teh et al., 1936). About a year later Haffkine obtained similar results with broth cultures of freshly isolated, highly virulent P. pestis kept in the dark for six to eight weeks at room temperature and sterilised by placing them for one hour in a water bath at an average temperature of 65°C. This so called "whole vaccine" was then used in dosages of 3 to 4 ml in the first vaccination of man against plague. A small-scale study was carried out on 319 prisoners of Byculla Jail in Bombay in 1897. Haffkine inoculated 147 persons at the beginning of a 7-day outbreak and left the others uninoculated. During this period there were two cases in the inoculated group, neither of whom died and 12 cases with 6 deaths

in the uninoculated group. In a second study of plague vaccine, made in Umerkhadi Jail in Bombay, he inoculated "one individual among two". Within a 30-day observation period 10 cases of plague with 6 deaths developed among 127 uninoculated prisoners and 3 cases with no deaths in 147 inoculated (Taylor, 1933). Many millions were inoculated during the following years with the vaccine produced at the laboratory which later became the Haffkine Institute. Figures from the Punjab for the season 1902-03 show a reduction in case incidence from 7.7% among uninoculated to 1.8% among the inoculated. The case mortality was reduced from 60.1% to 23.9% in the same groups (Parish, 1965).

At a World Health Organisation seminar on plague control in the U.S.S.R. in 1965 Dr. B. Cvjetanovic stated that "... except for a few studies by Haffkine (1906) and Otten (1936) no other studies on the effectiveness of plague vaccine in man have been undertaken which may be called 'controlled' trials (as defined by Hill, 1951 and Pollock et al., 1965). The information available from these studies, in spite of some reservations, indicates that dead plague vaccine is to some degree effective and that live vaccine is perhaps superior. However, its exact degree of protection and duration are unknown".

The development of a killed plague vaccine for human inoculation by Haffkine was followed by numerous investigations using this vaccine and other preparations in various animal species. Widely differing results were obtained depending on the animals used and the mode of preparation of the vaccine. Some of the earliest experiments were conducted by Kolle and Otto (1903 and 1904) who inoculated white rats

and guinea pigs with Haffkine's vaccine and killed agar-grown cultures. After challenge with a virulent culture only 9 per cent of the guinea pigs survived, while the white rats showed a slightly higher degree of immunity.

This early observation that only a low degree of immunity could be established in certain experimental animals stimulated investigators to produce killed vaccines with greater potency. Rowland (1911a) found that it was possible to increase the potency of an agar-grown plague vaccine by culturing the organisms in the presence of heated horse serum. These preparations protected 5.-8% per cent of white rats against a virulent challenge dose which killed 90 per cent of the controls. The importance of using highly virulent organisms to produce an effective vaccine had also been stressed by Haffkine. This was also shown by Naidu et al. (1926) who protected 45.5% of rats with a killed vaccine prepared from a virulent culture which caused 86.6% mortality in these animals. A culture which caused 20.0% mortality, when used as a killed vaccine conferred immunity on 18.4% of rats. These workers also found that the potency of a vaccine increases with the incubation period in liquid up to a maximum at the end of five weeks. This potency remains fairly constant until the end of 13 weeks and decreases when incubated for 16 weeks or longer. Apart from attempting to increase the potency of killed vaccines as immunising agents, experimental work was aimed at reducing the local and general reactions which they caused. The toxicity of a killed vaccine was demonstrated in wild rats at an early date by workers at the Haffkine Institute.

Attempts to reduce this toxicity were made by Morison et al. (1924)

and Naidu et al., (1926) who centrifuged Haffkine's vaccine and used the supernatant, sediment and whole vaccine to inoculate wild caught rats. The sediment was slightly toxic and had little or no immunising properties, whereas the supernatant was less toxic than the whole vaccine and the immunising properties were about the same. Naidu and Jung (1929) found that the toxicity of the Haffkine vaccine could be demonstrated in rats, but not in guinea pigs.

Other attempts at reducing the toxicity were made by using bacteriophage lysed suspensions of plague organisms (Compton, 1928 and 1930, Flu, 1929 and Pirie, 1929). These preparations were less toxic than liquid vaccines both in rats and the inoculation of one human. Single doses of these vaccines were ineffective in conferring protection against virulent challenge, but two or more inoculations resulted in protection comparable with that of other killed vaccines. Furthermore, it was established that protection by these phage lysed vaccines was not the result of inoculation of phage particles, but from the lysed bacteria and their products.

Smidt (1929) tabulated all the published tests performed on rats with agar-grown vaccines at the Haffkine Institute during 1924-25. Inoculation of 5.9 mg of these preparations resulted in 25.3% deaths and protected 38.6% against virulent challenge. A tabulation of experiments performed only with agar-grown vaccines sterilised by heat at 60°C however, showed that 9.2 mg of these vaccines caused 54.5% mortality and protected 66.4% of the rats. The doses of 0.1 to 1.5 mg agar-grown vaccine which gave milder reactions than 3-4 cc of Haffkine's vaccine in humans was probably ineffective in conferring immunity. Proportional doses which gave high protection rates in rats would have been unacceptable for human inoculation.

The widely differing results of protection and toxicity of killed vaccines in various animals, revealed the importance of the animal species in plague vaccine investigations. Schutze (1925) compared the immunising power of three types of plague vaccine - Haffkine's broth, heat-killed agar-grown and phenol-killed agar-serum vaccine - in guinea pigs and rats. Differences between these vaccines could be detected in guinea pigs, but the rats respond equally well to immunisation. It was clear then from this work that rats respond differently from guinea pigs to plague vaccines. Other animals used were gerbils (Tatera sp.) (Pirie, 1927), Cricetomys gambianus (Burgess, 1927 and 1930) and monkeys (Macaca cynomolgus) (Strong, 1907). In a review of this early plague vaccine work, Otten (1933) concluded that the experimental conditions varied so widely that comparisons of them were not possible. He therefore conducted further work on the susceptibility and response to immunisation of different animals. It was shown that the guinea pig was the most susceptible to virulent infection, followed by wild rats and then white rats. Guinea pigs were protected the least in vaccination experiments and then wild rats. The greatest protection was obtained in the least susceptible animals i.e. white rats. He then suggested there may be an inverse relationship between the response to immunisation and susceptibility to plague. This was not confirmed in white mice however, which are highly susceptible, but in which 60% protection was obtained with the Haffkine vaccine.

Of great importance in the evaluation of plague vaccines was the introduction by Sokhey (1939) of the white mouse as a test animal. This was a great step away from the use of wild house rats (Rattus rattus) which gave very wide variations in results. Furthermore, Sokhey established that as few as 10 bacilli per animal were enough

to produce 100% mortality in the white mouse (Haffkine-Institute inbred strain). White rats, wild house rats and guinea pigs were much less susceptible to plague infection.

Although it is known that fully virulent plague organisms possess at least 10 (*Crumpton and Davies, 1956*) and possibly as many as 16 antigens (Lawton, Fukui and Surgalla, 1960), their part played in immunity is not fully known. Attempts to identify the immunising antigens of the plague organism have been made since Rowland (1914b) originally described a readily soluble outer covering. This substance was distinguished from the somatic antigen by Schutze (1932a) who demonstrated the immunogenic properties of the envelope substance in white rats (Schutze, 1932b and 1939). The heat-labile envelope antigen was shown to develop best at 37°C while the heat stable somatic antigen forms as well at 20°C as at 37°C. Although the outer covering of the organism has been termed a capsule by various authors (Sokhey, 1940 and Amies, 1951) the general concensus of opinion is that it should be termed an envelope (Englesberg and Levy, 1954 and Burrows, 1963). This is now commonly referred to as Fraction 1 or F1 antigen (Baker et al., 1947 and 1952).

Emphasis has been placed on the importance of Fraction 1 in conferring immunity to plague by Meyer and his associates at the George Williams Hooper Foundation, San Francisco. Their work showed (Baker et al., 1947) that by extracting virulent plague bacilli killed by acetone at -70°C with neutral salt or sodium acetate solution, water-soluble and water-insoluble antigenic components were obtained. The water-soluble fraction contained at least three antigenic components: (1) a carbohydrate protein (Fraction 1A), soluble at 0.2% saturation of ammonium sulphate at pH 7.0 to 7.5; (2) a carbohydrate-free protein

(Fraction 1B) soluble at 0.3 saturation of ammonium sulphate and (3) a toxic fraction, soluble at 0.33 saturation of ammonium sulphate. Fraction 1B was highly immunogenic in mice, white rats and monkeys, but not in guinea pigs. They then concluded that " ... Fraction 1B antigen is probably essential in the maximal protection of man and since this antigen is equally indispensible in the protection of mice against plague, it is deemed advisable to measure the immunogenic potency of a plague vaccine with mice rather than with guinea pigs".

The immune response of the guinea pig to a variety of preparations was further examined by Spivack et al. (1958) who established that guinea pigs could be immunised with small quantities of plague antigens if these were incorporated into an oil emulsion. Immunisation of guinea pigs with a killed vaccine preparation without adjuvant was however, achieved by Keppie, Cocking and Smith (1958), Chen, Foster and Meyer (1961) and Smith and Packman (1966). The latter authors used the attenuated EV76 strain to produce a filtered nontoxic vaccine which protected guinea pigs better than the American Army vaccine, but no difference in the two vaccines could be detected in the protection of white mice.

The assumption that subhuman primates resemble man more closely than guinea pigs or white mice in the immune response, stimulated the use of these animals in immunity studies. Otten (1933) stated " ... the problem concerning the protective value of plague vaccines in man is not solved. To approach the question, human susceptibility to plague infection and response to immunisation should be known, and on this point reliable information a priori is not available. The monkey seems to be the most suitable animal to bring this problem nearer solution". The individual inherent resistance of rhesus and cyno-

molgus monkeys has made it impossible to achieve reproducible, clear cut serologic responses to challenge by various routes in these two primate species (Chen and Meyer, 1965). Evaluation of plague vaccines through tests on subhuman primates might be more reliable if species less variable in susceptibility than these two species could be found. Hanuman langurs (Presbytis entellus) were shown by Chen and Meyer (1965) to be highly susceptible to plague and found by Meyer (1970) to be of great value for the investigation of live and killed plague vaccines. Meyer (1953) showed that Macaca mulatta could be protected against large doses of virulent P. pestis by suspensions of formalin-killed organisms or by heat- or chemically-killed broth vaccines using the normal human dose. Smaller doses conferred little or no immunity. Immunization with Fraction 1, irrespective of the dose, within a range of 0.1 - 5.0 mg, protected approximately 60% of the monkeys. Antibody production as a whole was much less extensive in non-human primates (M. mulatta) than in other species of animals. A similar rate of protection was obtained by Meyer (1970) using two doses of USP vaccine inoculated intramuscularly into langurs.

Amies (1951) used the wild rodent Praomys (Mastomys) natalensis, bred in the laboratory, for his investigations on the envelope substance of P. pestis. More than 2,000 of these animals were used for this work, which led him to conclude that P. natalensis stands midway between the guinea pig and the rat in its response to plague antigens and is thus comparable to man. Amies then stated that antigens intended to be used for human prophylaxis can therefore be assayed by protection tests on this animal.

Live Vaccines

The use of killed plague vaccines in animal experiments and in man has

established that immunity can be conferred by inoculation of killed broth cultures, agar-grown suspensions and extracts of the organisms. However, the value of killed vaccines was seriously questioned at an early date. Thus Kolle and Otto (1903 and 1904) and Rowland (1915b) demonstrated that live attenuated organisms could immunise guinea pigs and rats better against virulent infection than killed cultures. The use of live vaccines was more extensively investigated by Strong (1906 and 1907) who demonstrated the greater efficacy of these vaccines in guinea pigs and monkeys, and also vaccinated many human beings with a live attenuated strain. He then expressed himself as a firm supporter of inoculation against plague with live vaccines. Pirie (1927) however, demonstrated that strains may be attenuated for guinea pigs, but virulent for the gerbil (Tatera sp.), which is highly susceptible to plague. He concluded that the ideal attenuated strain for use as a vaccine would be one which had a very wide margin between the toxic dose and the dose which was sufficiently antigenic to produce immunity.

Interest shifted to the use of live plague vaccines for human inoculation again in the 1930's. Otten (1936) in Java selected a strain isolated from a rat found dead at Tjiwidej and then stored in a deep serum-agar stab-culture after rat passage. Four months later it was found to have lost its virulence completely for rats and guinea pigs. The Tjiwidej strain was shown to be superior to killed vaccines in guinea pigs and rats, but produced sterile abcesses in guinea pigs at the site of inoculation. However, the vaccine proved completely harmless to man in doses of 100 million viable bacilli. In the first trial of this vaccine in humans, Otten used the alternating method of vaccination i.e. one half of a household was vaccinated, the other half being left as a control. The mortality from bubonic plague in the vaccinated group of this trial was reduced to 10% of

that in the unvaccinated group. This vaccine was then used for the mass vaccination of a population of millions.

At about the same time as Otten developed his live attenuated vaccine in Java, Girard and his associates carried out similar work in Madagascar. These authors realised the shortcomings of the killed preparations and worked mainly on live attenuated vaccines, the outcome of which was the development of the EV vaccine (Girard, 1936). The strain EV was isolated in 1926 from a human bubo and after five years of subculture on nutrient agar was found to be avirulent for guinea pigs when inoculated subcutaneously. The strain produced solid immunity in guinea pigs and was found to be innocuous for man when administered subcutaneously in doses of 500 - 1,000 million organisms.

The vaccine was first tried in a few lepers in 1932 to determine the effects on humans (Girard, 1963). After this, a trial of the vaccine was conducted in 1,600 volunteers in a village in an infected area. Only one of the vaccinated contracted plague during the time when there were numerous cases in the neighbouring villages. These encouraging results led to the use of the vaccine for the mass vaccination of a population of more than one million people. According to Girard and Robic (1942) these vaccinations resulted in a reduction of the plague morbidity by 80% in Madagascar.

In South Africa the superiority of live vaccines over killed preparations was demonstrated by Pirie and Grasset (1938) in Rattus rattus. Live vaccines of the strains EV and Tjividej were then used extensively in the field for human inoculation to control plague outbreaks (Gale, 1941, Grasset, 1941, 1942 and 1946). The strains were first tried in

human volunteers and confirmed to be non-pathogenic in doses of 4,000 - 20,000 million organisms in guinea pigs and white rats. The vaccine consisted of a live emulsion of 1,000 million organisms per ml obtained from a 24-hour growth on nutrient agar at 37°C suspended in saline. Immunization consisted of a single injection of 1 ml of the vaccine administered subcutaneously. Proportionally lower doses were used for children. Reactions to the vaccine were very mild with local reactions in 4.2 per cent and moderate general reactions in 3.7 per cent of the vaccinated. The vaccine resulted in the development of a high degree of immunity to plague as only 15 plague cases were observed in a total of over 24,000 persons immunized with the attenuated vaccine during 14 outbreaks between 1941 and 1944.

The use of live attenuated plague vaccines for human inoculation in other countries has been limited mainly to the EV strain. In the former Belgian Congo nearly 500,000 vaccinations were carried out with the EV strain between 1939 and 1946 in the Lake Albert plague focus (Devignat, 1949). Of 132 plague cases between 1940 and 1946, 16 were recorded in vaccinated persons between three and six months after vaccination and 116 in non-vaccinated persons. In Tunisia the live EV vaccine was administered to about 60,000 persons between 1944 and 1945, and to about 100,000 persons in Senegal at that time (Girard, 1963). The value of these inoculations in preventing plague in the vaccinated was however, difficult to assess.

More recently the use of live attenuated plague vaccines has been confined mainly to experimental animals. From this work much of the present knowledge on the immunogenicity and pathogenicity of plague has been obtained. Working with genetically related strains of P. pestis, Burrows and Bacon (1956a and 1958) established the importance

of virulence determinants in immunity to experimental plague in white mice, guinea pigs and rabbits. This work with live vaccines confirmed the importance of the F1 antigen in conferring immunity to mice against experimental infection with virulent F1+ strains, and the importance of the VW antigens in immunity was also established. Laboratory techniques for the detection of these antigens have been described by Surgalla et al. (1970). The vaccine strains which were most immunogenic in both mice and guinea pigs were found to have the virulence characters of the strains EV76 (F1+ VW+ P-) and Tjiwidej (F1+ W- P+).

Immediately after the inoculation of mice, rats, guinea pigs and monkeys with protective attenuated P. pestis strains, large numbers of organisms are always present at the site of infection (Jawetz and Meyer, 1943 and 1944, Meyer, 1950). Within a few hours the afferent lymphatic system carries them to the regional lymph nodes from where the bacilli are transferred to the thoracic duct and the blood stream. If a sufficient number of organisms have been injected, bacilli may be deposited in the first 6 - 12 hours in the spleen, liver and bone marrow. The filtering action of the liver and spleen then usually become effective and this initial bacteraemia lasts only a very short time. The bacilli may be destroyed or may multiply in these locations and persist until recovery or death. The ability to resist phagocytosis, and hence to multiply in the animal, is associated with the antigens F1 and VW (Burrows and Bacon, 1954b, 1956a and 1956b). Only after massive multiplication are the splenic and hepatic filters overcome; when they are, bacteria appear again in the blood. Attenuated P. pestis produce an active invasion of the host which differs only in extent, not in kind, from a virulent infection. Death from virulent infection is due to a toxic effect. The inability of the host to stop multiplication and subsequent bacterial disintegration allows liberation of large quantities of

endotoxin which injure the blood vessels, myocardium and adrenals (Meyer, 1950).

The vast amount of experimental work on the live attenuated EV plague strain has resulted in the recognition of certain characteristics for the vaccine to be highly efficacious. One of the important characteristics stressed by Girard is the fact that the EV strain retains a certain degree of toxicity and residual virulence and he believes that the strain probably owes its protective power to the persistence of these properties. The residual virulence of the EV strain was demonstrated by Sablet, Girard and Robic (1937) who inoculated guinea pigs intraperitoneally with one thousand million bacilli. Two animals were then sacrificed daily for a period of 30 days. After the seventh day the spleen became enlarged and the surface had a rough rugose appearance. Tiny granulations appeared about the tenth day, then began to fade on the 15th day and had disappeared by the 20th to 25th day. By the 30th day the spleen had returned to its normal size and appearance.

Histological examination showed the presence of small nodular formations in the spleen red pulp after the 5th day. These were made up of a dense mass of reticular and polymorphonuclear cells containing coccobacilli. After this there was a massive proliferation of the reticular and endothelial cells. Regression began after the tenth day and by the 30th day the nodules had disappeared.

Multiplication of the EV strain in mice and guinea pigs was demonstrated by Girard and Badaody-Zalarosy (1940) and Walker et al. (1953). The liver and spleen were the main foci where multiplication took place within the first four days and these organs then became sterile after 9 to 14 days.

Tests for the evaluation of the immunity produced by vaccines have been outlined by Meyer (1953). These are (1) intentional challenge (2) inientional exposure to a natural infection (3) natural exposure, permitting the prevailing epidemic chances to act freely, and (4) measurement of the reactions in the body fluids to indicate altered refractoriness of the inoculated or immunised. As far as experimental evaluation of vaccines in animals is concerned, the first and fourth methods have been the most useful. Work on serological techniques for the appraisal of immunity to plague has resulted in the development of four useful tests i.e. agglutination, complement-fixation, haemagglutination and passive mouse-protection. Of these the last two have been used most extensively. The procedure of the passive mouse-protection test is as follows: at least ten mice are given intravenous injections of 0.5 ml of the undiluted serum to be tested (or diluted 1:2 if the protection is strong) and then immediately 100 MLD of a highly virulent P. pestis culture are given subcutaneously. The mice are then observed for about 14 days. The percentage mortality is then divided by the mean time of death to obtain the mouse protection index (MPI). This test can only be used for the evaluation of sera from humans, large animals, guinea pigs or pools of mouse sera as at least 5 ml of serum is required. Passive mouse protection indices below 10 and particularly below 5 are usually found in convalescent plague patients (McCrumb et al., 1955) and susceptible non-human primates which resist challenge infection (Meyer, 1970). An animal which has a MPI below 10 is therefore considered to have a high level of immunity to plague.

Despite the amount of work done on vaccination against plague, there are still many large gaps in our knowledge. Although the killed

vaccines in use at present have been shown to immunise mice and guinea pigs (Smith and Packman, 1966) and also primates, their value in protecting man against plague has been strongly questioned. If booster doses are given however, the killed vaccine is highly effective. This was revealed in Vietnam where persons vaccinated with a killed plague vaccine enjoy almost complete freedom from the disease, although frequently exposed to infection (Meyer, 1970). Live vaccines, particularly the EV and Tjiwidej strains, have been shown to be better vaccines in experimental animals and have been used on large scales for human inoculation. However, these live vaccines are pathogenic in non-human primates and produce unpleasant side reactions in man (Meyer, 1970 and personal communication). On the basis of experience in Malagasy, Payne, Smadel and Courdurier (1956) concluded that a single inoculation of living attenuated P. pestis, strain EV, elicits demonstrable antibodies in relatively few persons. However, antibody production was found to be enhanced by repeated vaccination.

The value of Praomys (Mastomys) natalensis as a laboratory test animal for plague has been established by Davis and Murray (1968b). This work was done after it was realised that guinea pigs, which were used as test animals at that time, were not uniformly susceptible to infection. Similar results in guinea pigs were obtained by Brygoo and Rajenison (1969) who found that white mice were better test animals.

The present work was therefore initiated to investigate the immunogenic and pathological effects of various live and killed vaccines in Praomys, and animal species which has been proved to be superior to both guinea pigs and mice in its uniform susceptibility to plague. Comparative studies with white mice, guinea pigs and African green monkeys (Cercopithecus aethiops py[illegible]ythrus) were also done.

MATERIAL AND METHODS.

Experimental animals.

Praomys (Mastomys) natalensis: The Praomys colony was built up from animals caught in Johannesburg in 1946 and maintained at the South African Institute for Medical Research since that time. They are now known as the Baragwanath strain. Praomys (fig. 1) is about twice the size of the white mouse, the body weight at maturity being between 50 and 60 g. For experimental work they were used when 9-14 weeks old at a body weight of 25 - 30 g. The breeding stocks are housed in a temperature controlled room about 21°C and maintained in standard metal mouse breeding cages 30 x 15 x 13 cm. The food given is standard mouse cubes supplemented with carrots, potatoes and cabbage once a week. Water is supplied by a drop bottle. The monogamous system of mating is used and breeding pairs are kept together for life. On the average eight young per litter are born, of which six are weaned (Davis, 1963). Animals were inbred to the twenty first generation when the fecundity declined to such an extent that insufficient animals were obtained for experimental use. The fertility was restored to the colony by cross breeding litters of the inbred strain.

Animals under experiment were caged singly in cages 13 x 13 x 18 cm. The top and bottom of these were made of galvanised iron and the sides were wire mesh. The bottom had short legs at each corner so that the cage could stand in a disinfected tray. Care has to be taken in handling Praomys as they are agile and bite viciously. One hand was covered with a calico bag consisting of 2 - 3 layers and the animal was held in the palm of the hand, being secured by the scruff of the neck between the thumb and forefinger.

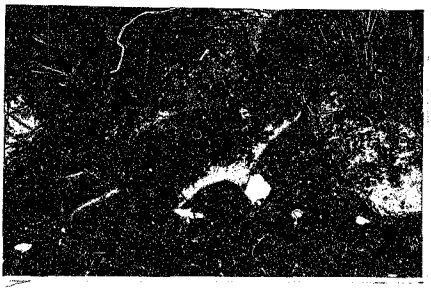

Fig. 1. Praomys (Mastomys) natalensis.

White mice and Guinea pigs:

The white mice and guinea pigs were non-inbred conventional animals from a colony maintained at the South African Institute for Medical Research for various purposes. The animals were fed on standard mouse biscuits daily and water was supplied by a drop bottle. The mice were 7 - 11 weeks old and the average weight was about 20 g. The guinea pigs weighed on an average about 200 g. During experiments 10 white mice were caged together in galvanised iron cages 60 x 30 x 13 cm and guinea pigs were kept together in wire-mesh cages 40 x 60 x 34 cm.

Cercopithecus aethiops pygerythrus:

African green monkeys (Cercopithecus aethiops pygerythrus) were wild caught in South Africa and then conditioned for approximately three months in large outdoor cages, which housed about 25 monkeys each. Healthy monkeys in good condition were selected from these and put in separate cages in the laboratory. The cages measured 75 x 77 x 105 cm with a false back which could be drawn forward to pinion the monkeys against the front, to facilitate handling the animals. The cages were suspended about 50 cm from the floor from a horizontal bar for ease of cleaning. The diet consisted of brown bread, sweet potatoes, bananas and oranges given twice daily. Water was always available in a trough.

Vaccines.

Four live attenuated and two killed vaccines were used in the experimental work. The live vaccine strain designated Tjiwidej Smooth or TS, was obtained in dried gelatin pellet form from Dr. T.W. Burrows, Porton, England. The other live vaccine strains designated LW76 (Paris), EV51f and M23 and the standard reference killed vaccine were obtained in lyophilised form, whereas the USP killed vaccine was in liquid suspension.

These vaccines were supplied by Dr. K.F. Meyer, San Francisco, U.S.A. The origin of these strains will be discussed under the experimental work.

The live attenuated strains were stored in the laboratory at 4°C in dried gelatin pellets according to the method of Stamp (1947). The method of preparation is as follows: Organisms from an 18 hour culture incubated at 28°C were seeded onto the surface of agar slopes (Oxoid blood agar base No. 2 pH 6.8) in MacCartney bottles. These were then incubated at 28°C for 18 hours. To each bottle the following gelatin ascorbic acid mixture was added:

A. Gelatin 100 g. Distilled water 500 ml.

B. Peptone 10 g. Sodium chloride 5 g, 'Lemco' beef extract 4 g. Distilled water 200 ml.

The pH was then adjusted to 9.3 and the solution boiled to precipitate the phosphates. It was then filtered through Green's 'Hydro' 904½ filter paper and added to solution A above. The pH was then adjusted to 7.6. Sterilisation was effected by steaming for 30 minutes on three successive days.

C. Ascorbic acid 0.125 g, sterile distilled water 4.5 ml. This was then sterilised by millipore filtration and 0.5 ml 20% lactose in distilled water added.

To each 4.5 ml of the 'Lemco' gelatin (A + B above), 0.5 ml of solution C was added.

The bacterial growth was emulsified in this mixture with the aid of glass beads and the suspension removed with a Pasteur pipette. Separate drops were placed on thin plastic sheets in petri dishes, previously sterilised in chloroform and dried under ultraviolet light

in a sterile chamber. The petri dish lids were propped open slightly and the dishes placed in a dessicator for three days at room temperature. The dried pellets were then removed aseptically and stored in a refrigerator at 4°C.

Agar-grown vaccines were prepared from dried gelatin pellets by incubation of the pellet on blood agar at 37°C for one hour to melt the gelatin. The inoculum was then spread over the agar surface and incubated at 26°C for 18 hours. This culture was then seeded onto trypticase soy agar in MacCartney bottles and incubated at 37°C for 48 hours. The growth was then harvested with sterile normal saline using glass beads to produce a uniform suspension.

Serial dilutions of all vaccines were made in normal saline using a separate pipette for each dilution. Total bacterial counts of inocula were done in a Thoma counting chamber after dilution in 1.5% formol saline. Viable counts were done by inoculation of 0.2 ml of serially diluted suspensions onto each of five blood agar plates and spread with metal spreaders. These were then incubated at 28°C for 72 hours before counting the colonies. All live vaccines and virulent challenge cultures were checked for purity and confirmed biochemically to be P.pestis after inoculation.

Vaccination techniques.

Three routes were used for vaccination of the animals i.e. intracutaneous, subcutaneous and oral.

Intracutaneous inoculation: These inoculations were made with bifurcated needles, which were solid stainless steel needles 1 mm thick and 65 mm long. One end was flat in which there was a bifurcation 2.5 mm long. The needles were charged with vaccine by dipping into the

vaccine dilution to a depth at which only the space between the forks was filled with fluid. The needle was then gently pushed into the skin and rapidly withdrawn without extracting blood.

Subcutaneous inoculation: Animals were inoculated subcutaneously in the right hind leg with the various dilutions of vaccine using a 2 ml syringe and a number 16 Record hypodermic needle.

Oral administration: Praomys (Mastomys) natalensis were vaccinated by the oral route using G18 x 2 inch Luer-lock hypodermic needles. The ends of the needles were rounded off to prevent injury. The animal was held with the abdomen facing upward and the needle was gently inserted into the back of the mouth. The vaccine was then introduced slowly as the animal swallowed.

Strains of virulent P. pestis used for challenge.

Two virulent plague strains were used in this work for the challenge of vaccinated and control animals. The strain used in the initial stages was designated P357 which was isolated in Lesotho on 11th July, 1968 from a flea, Xenopsylla sp., during a plague epidemic. After isolation, this strain was maintained in deep nutrient agar stab culture. For most of the challenge infections strain MP6 was employed. This strain was kindly sent by Dr. T.W. Burrows who isolated it from a mouse which has been inoculated with an X-irradiated culture of the Tjiwidej Smooth P. pestis strain (Burrows and Bacon, 1954a). The MP6 strain is highly virulent in mice and guinea pigs, the average lethal dose being less than 10 bacilli (Burrows and Bacon, 1958). It was maintained in dried gelatin pellets.

After vaccination or virulent challenge the test animals were kept under observation daily for at least 21 days. Autopsies on the animals

that died were carried out with strictest aseptic precautions. The macroscopic appearances were noted and smears were examined from the heart blood, liver and spleen. Cultures were made from the inguinal lymph node, liver, spleen, kidneys, lungs and heart blood. These were planted onto blood agar plates and incubated at 28°C for 48 hours. In some cases the tissues were enriched in nutrient broth at 28°C for 48 hours before subcultivation on blood agar. Pieces of tissue for culture were removed using sterile instruments for each organ.

Histology.

Tissues from animals which died were fixed in 10% neutral formol saline for histological section. These were then dehydrated in an Elliot tissue processor and wax blocks prepared. Sections of 4 microns thickness were cut and stained with haematoxylin and eosin or thionin.

Serology.

Blood for serology was collected aseptically by heart puncture from Pracnys as described by Hallett (1965) and from the hind leg of Cercopithecus. The sera were tested for haemagglutinating antibodies to the Fraction 1 antigen of P. pestis using the microtechnique as described by Chen and Meyer (1966). After inactivation the sera were absorbed with normal sheep red blood cells to remove the heterophile anti-species antibodies. Doubling dilutions of the sera were then made in a 1:100 dilution of inactivated and absorbed normal rabbit serum. A suspension of tanned sheep red blood cells sensitised to Fraction 1 was then added to each serum dilution and the titres were read after incubation at room temperature for 2 - 4 hours. This test has been used extensively throughout the world for the serological

detection of exposure to plague in man and animals (Levi et al., 1964, Cavanaugh et al., 1965, Davis et al., 1968a and Hallett et al., 1970). The Cercopithecus sera were tested for mouse protecting antibodies as previously described and the mouse protection indices were calculated.

Protection against virulent challenge.

Three to five weeks after vaccination the Praomys and white mice were challenged subcutaneously with a virulent agar-grown P. pestis culture. After a minimum observation period of three weeks the protection conferred by the vaccine was determined by calculation of the Active Protection Index (API) and the 50% protective dose (PD_{50}).

The active protection index as used by Burrows and Bacon (1958) takes both the percentage of deaths and the times of survival of challenged animals into account and was calculated as follows:-

$$A\ P\ I. = \frac{\text{mean time of death in days}}{\text{Percent deaths}} \times 100$$

This, as stated by Burrows and Bacon bears a direct relationship to the degree of protection, in contrast to the inverse relationship of the mouse protection index of Meyer and Foster (1948).

The 50% protective dose was calculated according to the method of moving averages described by Thompson (1947) and also outlined by Meynell and Meynell (1965). The other well known methods of obtaining the PD_{50} are those of Reed and Muench (1938) and Karber (described by Irwin and Cheeseman, 1939). These methods are however, unreliable if the doses happen to have been chosen so that there are many more on one side of the PD_{50} than on the other.

Safety Precautions.

The experimental work was carried out in the plague laboratory of the South African Institute for Medical Research. The laboratory consists of five rooms; (1) main bacteriological laboratory (2) autopsies (3) wash up and sterilisation (4) maintenance of routine test animals (5) maintenance of animals under experimentation.

Surgical gowns, rubber gloves and masks were worn at all times whilst working in the laboratory. Any work with cultures was done in the main laboratory in a safety chamber fitted with an extraction fan & filter. Goggles were worn when rodents were autopsied and gas masks and gum boots were worn when any work with monkeys was done. Animals under experimentation were maintained in a room fitted with an extraction fan and bacterial filter to maintain a negative pressure.

Cultures were disinfected with dilute lysol and then sterilised in an autoclave. Animal carcasses were incinerated after autopsy.

CHAPTER 2.

PATHOGENICITY AND IMMUNOGENIC EFFICACY OF THE LIVE ATTENUATED PLAGUE VACCINE STRAIN EV51f.

At a World Health Organisation Seminar on plague control in the U.S.S.R. in 1965, the view was expressed that although much is known about the virulence determinants and protective antigens of P. pestis, there are probably other antigenic components which have not been detected. Thus, although killed vaccines may possess all the known antigenic components, live vaccines possess the potential of producing antigenic components in vivo which are not formed in vitro. Live vaccines would therefore confer a higher degree of resistance to plague than killed vaccines.

Among the criteria adopted by the Soviet authors for the selection of attenuated vaccine strains were that these should genetically contain the F1 and V# antigens (F1+, V#+), must be independent of purines for growth (Pu+), and must not produce pigments on media containing haemin (P-). These are the determinants of the EV strain. The vaccine strains should also confer solid and long-lasting immunity on guinea pigs and mice.

It is well known that different attenuated plague strains have different immunising capacities, associated with the method of maintenance of the culture (Chen and Meyer, 1955 and Korobkova et al., 1964). A successful method which has been employed to stabilise attenuated vaccines antigenically and to increase the immunogenic capacity, has been to passage the strain through guinea pigs (Korobkova et al., 1964). By this method neither virulence of the strain nor adverse effects on the animal were increased.

To obtain a vaccine strain which possesses the full antigen complement, but which is P-, the EV strain was passaged through guinea pigs previously treated with ferrous sulphate. In animals not treated with iron salts, all the EV organisms are avirulent and are gradually eliminated from the animal. When iron is injected, the organisms which lack antigens for which the iron does not compensate (e.g. VW-) are still avirulent and are also eliminated. The cells which lack only the component for which iron compensates (P) however, multiply freely to provide a population of these cells which eventually results in death of the host (Jackson and Burrows, 1956b).

Details of the passage of an EV strain through guinea pigs to obtain the vaccine strain designated EV51f are as follows. In 1951 an EV76 strain was taken to the U.S.A. from Madagascar. A mass culture prepared from the original slant was grown on hormone agar at 37°C for 48 hours and lyophilised. In 1967 the lyophilised EV76 was reconstituted and grown on a blood agar slant at 37°C. A guinea pig given $FeSO_4$ in oil intramuscularly was inoculated with 0.5 ml of a 24 hour 37°C grown broth culture (approximately 100 million viable organisms) seeded with the growth from this slant. The animal was sacrificed six days later and the emulsified spleen was inoculated into a guinea pig pretreated with $FeSO_4$. Three additional spleen passages through treated guinea pigs were followed by the inoculation of an untreated guinea pig. From the enlarged spleen, studded with granulomas, a blood plate yielded numerous typical smooth plague colonies (Meyer, personal communication). The 50% protective dose (PD_{50}) by the subcutaneous route was 3,600 viable organisms for mice and 5 for guinea pigs (Meyer, 1970). The vaccine was lyophilised and sent by Dr. K.F. Meyer for experimental use in laboratory animals.

Subcutaneous inoculation of Cercopithecus aethiops pygerythrus and guinea pigs with a freeze dried EV51f culture.

Fifty Cercopithecus aethiops pygerythrus weighing 4 - 6 kg were cut into individual cages and divided into 5 groups of 10. Both sexes were fairly evenly distributed. Five to 6 animals were kept in a room to minimize cross infection with bacteria unrelated to P. pestis. To minimize rough handling and frightening of the animals, all handling was done after intramuscular injection of "Sernylan" (0.05 ml/kg body-weight) which fully anaesthetised them. In this manner all animals were weighed, their body temperatures taken rectally, and bled for white cell count and serological tests.

Each of 5 vials of the lyophilised EV51f vaccine were rehydrated with 10 ml sterile normal saline and pooled in a 100 ml Erlenmeyer flask. A total bacterial count was then done in a counting chamber and ten-fold dilutions done in normal saline. Five dilutions were selected for inoculation on the results of the total count and these were injected subcutaneously in 0.5 ml amounts into 10 monkeys for each dilution. At the same time five groups of ten guinea pigs each were inoculated subcutaneously with the identical doses. Plate counts were done as previously described. The groups of viable doses inoculated as determined from the plate counts and the vaccination fatalities of Cercopithecus which occurred are as follows:

Group	Vaccine Dose (viable bacilli)	Day of death	Total deaths
I	1.009×10^8	4,5 and 6 (2)	4
II	1.009×10^6	5 and 11	2
III	1.009×10^5	6,7,11 and 22	4
IV	1.009×10^3	8 and 28	2
V	101	9	1

The cages were inspected daily and examinations of monkeys were done initially on alternate days to determine reactions at the site of inoculation, size of inguinal and popliteal lymph nodes and rectal temperature. Where an animal had a temperature of 104°F or higher, blood was taken for white cell count, serology and quantitative blood cultures. After two weeks these examinations were reduced to twice weekly.

Local lesions at the inoculation sites were not observed, but the inguinal lymph nodes enlarged in 2 - 3 days and persisted as marked, firm lymphadenopathies for up to 25 days. A rise in temperature was recorded for every animal, and raised white cell counts were recorded in all those with temperatures of 104°F or higher (table 1). By the 28th day the white cell counts had returned to normal. Positive quantitative blood cultures were obtained from the fourth day (table 2) and these varied from 8 to innumerable bacilli per ml. Three monkeys had positive blood cultures of 8, 36 and 282 bacilli per ml and survived.

Raised serological HA titres were observed in monkeys within 5 - 7 days in those animals which had rectal temperatures of 104°F and over (table 2), but 3 of these 16 had negative titres. By the 28th day all but 2 of 38 surviving monkeys had serological titres between 8 and 4096.

The post-mortem appearances of the animals which died after vaccination were as follows:

Blood stained fluid in both pleural cavities in all cases; gross, nonpurulent congestion of the lungs in all cases, small amounts of blood stained peri-cardial fluid in most cases. The spleens were somewhat flabby and congested, while the livers appeared normal, except numbers 755 (vaccine dose : 1.009×10^6) and 746 (vaccine dose :

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TABLE 1. Temperatures and white blood cell counts of Cercopithecus aethiops vaccinated with attenuated P. pestis strain EV51f.

Group	No.	Sex	Pre-vacc. temperature	Highest temperature	on day	Temperature increase	Pre-vacc. white cell count	Highest white cell count	on day	White cell count on day 25
I	737	M	101.4	106.8	2	2.4	16,500			
	738	M	102.6	105.2	4	2.6	12,500	21,400	4	3,500
	739	M	101.4	104.1	4	2.7	12,000	23,600	4	
	740	M	101.8	103.7	2	1.9	6,500			
	741	F	102.6	105.1	7	2.5	14,500	17,400	7	
	742	F	101.6	104.0	2	2.4	7,400	60,500	5	
	743	M	100.4	103.7	2	3.3	7,500			
	744	F	100.0	103.0	2	3.0	12,700			
	745	M	102.4	104.5	11	2.1	8,300	15,000	11	
	746	M	100.4	103.1	2	2.7	6,400			
II	747	M	100.6	105.6	7	5.0	9,000	14,000	4	
	748	M	101.2	104.4	7	3.2	6,500	8,700	7	
	749	F	101.8	103.2	2	1.4	26,500			
	750	M	101.8	102.6	2	0.8	15,500			
	751	F	100.4	105.8	2	5.4	9,500			
	752	F	100.6	104.7	2	4.1	10,200			10,300
	753	F	100.0	102.5	2	2.5	11,500			9,200
	754	F	99.8	102.4	2	2.6	12,500			
	755	M	100.6	102.5	2	1.9	13,100			
	756	M	101.2	104.0	2	2.8	9,900			
III	757	M	102.2	102.6	11	0.4	15,300			16,700
	758	M	103.2	105.4	7	2.2	7,400	11,000	7	2,800
	759	M	102.6	104.7	7	2.1	9,800			4,800
	760	M	101.0	103.4	7	2.4	3,600			4,600
	761	M	102.0	103.7	2	1.7	7,600			6,600
	762	M	100.4	104.5	7	4.1	4,500	9,300	4	
	763	M	100.6	102.4	2	1.8	8,700			
	764	M	103.2	106.0	4	2.8	clotted			
	765	F	102.4	103.2	2	0.8	11,700	131,200	7	
	766	M	101.6	104.8	7	3.2	10,300	14,100	7	8,300
IV	767	M	101.8	104.4	4	2.6	8,400	13,400	4	7,900
	768	M	101.0	102.9	4	1.9	15,600			6,200
	769	M	99.6	104.8	7	5.2	14,200	22,300	7	5,500
	770	M	100.6	103.4	4	1.8	10,900			3,900
	771	M	101.1	104.2	7	3.2	6,900	15,[illegible]	7	4,600
	772	M	102.0	103.8	15	1.8	6,900			
	773	F	100.8	103.6	7	2.8	8,700			
	774	F	100.2	103.9	7	3.7	7,300			3,800
	775	M	102.8	105.[illegible]	7	2.[illegible]	12,800	34,[illegible]0	7	3,[illegible]
	776	F	2[illegible].8	10[illegible].[illegible]	4	2.[illegible]	7,500	19,[illegible]00	4	1,[illegible]
V	777	M	100.4	104.9	11	4.5	5,800			5,[illegible]
	778	M	102.2	103.[illegible]	2	1.[illegible]	7,700			12,[illegible]
	779	M	101.6	10[illegible].2	7	1.6	6,400			8,[illegible]
	780	F	102.2	103.6	18	1.4	9,700			
	781	M	100.4	103.8	7	3.4	6,900	27,[illegible]0	7	
	782	M	101.8	104.5	15	3.7	11,500			5,[illegible]
	783	M	102.0	105.0	7	3.0	10,100			
	784	M	100.6	103.4	18	2.8	3,900			
	785	F	100.8	103.5	22	2.7	4,500			11,600
	786	M	102.0	103.3	15	1.3	9,800			8,700

TABLE 2. Bacteriological, systemic and serological reactions and mortality recorded in 50 Cercopithecus aethiops, inoculated with attenuated P. pestis strain EV51f.

Group	No.	Quantitative Blood culture	Duration of Lymphadenopathy	Day of death	Intermediate HA titres (Reciprocal)	HA titres on day 28 (Reciprocal)	Geometric mean titre
I	737	negative (day 4	day 2 - 18			256	
	738	negative	day 4 - 25			0	
	739		day 4 - 25			4096	
	740		day 4 - 15			1024	
	741	negative (day 7)	day 4 - 25		1024 (day 7)	2048	
	742	innumerable/ml (day 5)	day 4 - 25	5	1024 (day 5)		
	743		absent	4			
	744		day 4 - 25	6			
	745	negative (day 11)	day 2 - 25		512 (day 11)	1024	
	746		day 2 - 25	6		512	512
II	747	56 orgs/ml (day 7)	day 4 - 25	11	16 (day 7)		
	748		day 4 - 25		512 (day 7)	1024	
	749		absent			0	
	750		day 2 - 25			256	
	751		day 4 - 15			4096	
	752		day 4 - 25			512	
	753		day 4 - 25			2048	
	754		day 4 - 25			2048	
	755		day 4 - 25	5			
	756		day 4 - 18			4096	948
III	757		day 2 - 25			512	
	758	negative (day 7)	day 4 - 15		64 (day 7)	512	
	759	282 orgs/ml (day 7)	day 4 - 11		128 (day 7)	1024	
	760		day 4 - 15			256	
	761		day 4 - 15			256	
	762	10 orgs/ml (day 4) 276 orgs/ml (day 7)	day 4 - 25	11	512 (day 7)		448
IV	763		day 4 - 25	6			
	764	269 orgs/ml (day 4) nil day 18)	day 12 - 15	22	256 (day 12)		
	765	innumerable/ml (day 7)	absent	7	512 (day 7)		
	766	negative (day 7)	day 7 - 15		0 (day 7)	256	
	767	innumerable/ml	day 4 - 11			256	
	768		day 4 - 11			4096	
	769	36 orgs/ml (day 7)	day 11 - 15		4 (day 7)	512	
	770		day 4 - 15			512	
	771	8 orgs/ml	absent			512	
	772		day 11 - 15			512	
	773		absent	6			
	774		absent			512	
	775	negative (day 7)			16 (day 7)	64	
	776	innumerable/ml on days 4 and 28	day 11 - 25	20		1024	448
V	777		day 11 - 25			512	
	778		absent			512	
	779		day 11 - 25			1024	
	780		day 11 - 25			64	
	781	scanty pos. (day 7)	absent	9	0 (days 7 & 8)		
	782	negative (days 4 and 15)	day 4 - 25		512 (day 15)	512	
	783	scanty pos. (day 7)	day 7 - 11		8 (day 7)	1024	
	784		day 12 - 25				
	785		day 4 - 25			512	
	786		day 11 - 15			512	362

1.009×10^8) which showed a marked mottled appearance. The spleen was grossly enlarged (about six times normal) in number 762 (vaccine dose : 1.009×10^5). Widespread adrenal haemorrhage was seen in the majority while the kidneys were universally pale. Glandular enlargement was restricted to the regional (popliteal and inguinal) glands except in number 742 (vaccine dose : 1.009×10^8) where a large right para-aortic gland was present. Number 762 (vaccine dose : 1.009×10^5) showed haemorrhage into a large right popliteal gland.

Cultures on blood agar of heart blood, lung, spleen, liver, kidney and inguinal lymph glands yielded abundant growths of P. pestis from all animals which died.

Tissues for histology were preserved in 10% formol saline and the sections were stained with haematoxylin and eosin.

Lymph nodes: Inflammatory reaction was only slight, but clusters of P. pestis were typical (fig. 2).

Spleen: Engorgement with red blood cells was distinctive, with a proliferation of monocytes. Some plasma was present in the sinuses with clumps of P. pestis common (fig. 3).

Liver: Marked congestion and foci of polymorphs and lymphocytes with clusters of P. pestis. Desquamation of the epithelium in bile ducts and portal spaces. P. pestis were seen in all liver sections but not in numbers as large as in the spleen and buboes.

Kidneys: Congestion of the glomeruli and degeneration of the convoluted tubular epithelium were constantly present. Monocytes containing P. pestis were seen in the capillaries and small numbers of organisms were present in the larger vessels (fig. 4).

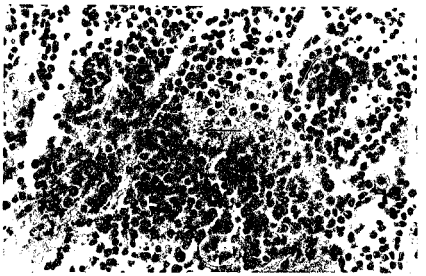

Fig. 2. Section of the inguinal gland of a Cercopithecus aethiops which died after subcutaneous inoculation of attenuated P. pestis strain EV51f. The arrow indicates the position of clusters of bacilli; x 375.

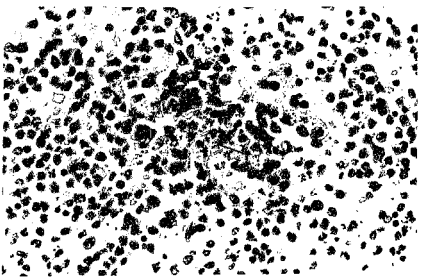

Fig. 3. Section of the spleen of a Cercopithecus aethiops which died after subcutaneous inoculation of attenuated P. pestis strain EV51f, showing a proliferation of monocytes. The arrow indicates clusters of bacilli; x 375.

Lungs: Congestion and desquamated cells were present in the alveolar spaces of all sections. The alveolar walls were thickened, containing masses of P. pestis and a dense infiltration of polymorphonuclear leucocytes (fig. 5).

All the guinea pigs survived vaccination without any abnormalities being observed.

The fifty guinea pigs were challenged with the virulent P. pestis 7537 strain on the 28th day and ten vaccinated Cercopithecus were challenged on the 170th day after vaccination.

The guinea pigs were challenged with 160,000 viable P. pestis (determined by plate counts) suspended in 0.5 ml normal saline, injected subcutaneously in the right hind leg.

Deaths of the control unvaccinated guinea pigs challenged in a similar manner, were as follows:

Challenge dose	Survivors/total	Days of death	Culture (heart blood)
800	0/3	6,7,12	Positive
80	0/3	10,11,12	Positive
8	2/3	8	Positive

Of the ten guinea pigs inoculated with [illegible] EV51f, three died on the 15th day after challenge (table 3). P. pestis (confirmed as virulent in white mice) was isolated from the heart blood and spleen of one of these animals. Three guinea pigs inoculated with 1009 EV51f died after 37 and 39(2) days, but only lactose fermenting gram negative bacilli were isolated on culture.

The high rate of protection conferred [illegible] EV51f vaccine on these

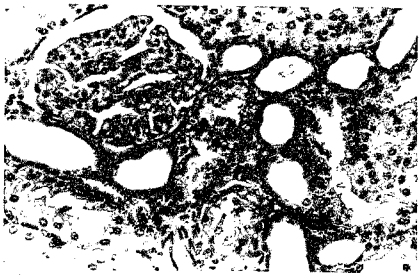

Fig. 4. Section of the kidneys of a Cercopithecus aethiops which died after subcutaneous inoculation of attenuated P. pestis strain EV51f, showing congestion of the glomerulus; x 240.

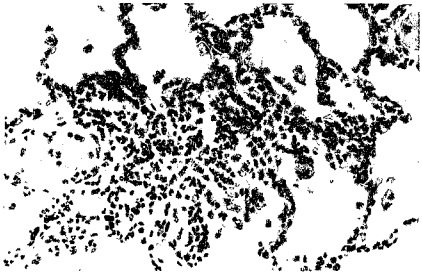

Fig. 5. Section of the lung of a Cercopithecus aethiops which died after subcutaneous inoculation of attenuated P. pestis strain EV51f, showing desquamated cells in the alveolar spaces, thickening of the alveolar walls and a dense infiltration of polymorphonuclear leucocytes; x 240.

TABLE 3. Resistance of guinea pigs to virulent P. pestis following subcutaneous inoculation with EV51f attenuated plague vaccine.

Viable EV51f dose	Virulent challenge dose	Results of challenge infection Survivors/total	Days of death after challenge	Culture (heart blood and spleen)
1.009×10^8	1.6×10^5	10/10	-	-
1.009×10^6	1.6×10^5	10/10	-	-
1.009×10^5	1.6×10^5	10/10	-	-
1.009×10^3	1.6×10^5	7/10	37,39,39	Lactose fermenting organisms
1.009×10^2	1.6×10^5	7/10	15	E. coli
			15	Proteus
			15	P. pestis

guinea pigs is in agreement with the results obtained by Meyer (1970).

As a result of the animal rooms being required for other studies, all but 10 monkeys were sacrificed on the 30th day after vaccination. The ten animals were housed together with 2 unvaccinated controls in one room in which an extraction fan had been installed to maintain a negative pressure. They were then challenged in the right thigh subcutaneously on the 170th day with 8,770 virulent P. pestis strain F357. Regular examinations were carried out, during which rectal temperatures were taken and blood collected for white cell counts and serology. Seven days before challenge the monkeys were bled for serological and mouse protection index (MPI) (Meyer and Foster, 1948) tests.

The results of the challenge are tabulated as follows:

Vaccine dose	Animal number	Reciprocal of HA titre day 163 after vaccination (7 days before challenge)	Rectal temp. (4 days after challenge	Day of death after challenge	White cell count	Blood culture
1.009×10^8	737	32	104.1	8	23,000 (day 4)	Negative (day 4)
	740	128	101.6	-	-	-
1.009×10^6	748	256	102.5	-	-	-
	750	128	105.6	12	21,900 (day 8)	(day 8) 150 cols/ml
	756	256	101.8	-	-	-
1.009×10^5	758	128	102.4	-	-	-
1.009×10^3	767	64	104.3	10	13,500 (day 4)	Negative (day 4)
	769	128	102.3	-	-	-
101	783	128	102.9	-	-	-
	784	Negative	104.2	-	-	-
Nil	Control 1	8	104.8	6	31,300 (day 4)	200 cols/ml (day 4)
Nil	Control 2	Negative	102.1	7	-	-

The post mortem appearances of the animals which died were typical of plague. The skin over the injection site was haemorrhagic with a local gelatinous oedema. The underlying tissues were haemorrhagic and indurated. Right inguinal glands were enlarged in both controls and in one of these the para-aortic and mesenteric glands were also enlarged. Livers were enlarged and had a mottled appearance. Spleens were normal in size but flabby and the cortex of the kidney was pale in colour. Adrenals were haemorrhagic. The lungs of one had a pulmonary oedema and the lung tissue of this animal was dark red in colour. In the other animal there was no pulmonary oedema and the lungs were very pale and collapsed. Straw coloured pleural fluid was present in both specimens, and the heart was dilated.

The post-mortem appearances of the vaccinated animals which died after virulent challenge were similar to the unvaccinated controls but the pathological changes were not as severe. There were no local lesions at the site of inoculation and the inguinal glands were slightly swollen. The livers were slightly enlarged and mottled, and the spleens were flabby but normal in size. The cortex of the kidney was very pale and the adrenals were haemorrhagic. There was a small amount of blood stained pleural fluid and the heart was dilated.

All the survivors of the virulent challenge were bled for MPI tests and sacrificed after 134 days. All the organs were normal at post-mortem, except number 748 which had an enlarged spleen about twice the normal size.

The results of the MPI tests are as follows:

Vaccine dose	Animal number	MPI 28 days after vaccination	MPI 163 days after vaccination	MPI 134 days after virulent challenge
1.009×10^8	737	3	17	-
1.009×10^8	740	-	17	9
1.009×10^6	748	6	5	-
1.009×10^6	750	3	17	-
1.009×10^6	756	1	5	6
1.009×10^5	758	1	13	5
1.009×10^3	767	3	15	-
1.009×10^3	769	2	14	1
101	783	3	10	00
101	784	11	14	2

Seven days before virulent challenge the HA titres of the three animals which died were 32, 64 and 128, in comparison with titres of less than 8, 128 and 256 of the 7 survivors, but all the titres had declined by the 163rd day after vaccination from values ranging between 1:256 to 1:4096, as determined on the 28th day after vaccination. The level of immunity as reflected by the mouse protection indices was high on the 28th day after vaccination, 8/10 of the values being below 10, but on the 163rd day after vaccination only 2/10 MPI values were below 10 and others varied from 10 to 17. This decline in the level of immunity as reflected by the mouse protecting and haemagglutinating antibodies was confirmed after challenge with virulent *F. pestis* when 3 of 10 monkeys succumbed. Two of these animals had MPI values of 17 and one a value of 15, seven days before challenge, while the others which survived had the same or slightly lower MPI values. There did not appear to be a relationship between the vaccine dose and the level of immunity,

as one animal in each of the groups vaccinated with 101 million, 1 million and 1009 viable EV51f died after challenge.

CHAPTER 3.

1. Subcutaneous inoculation of Praomys with a freeze dried EV5lf culture.

Each of 5 vials of the lyophilised EV5lf vaccine was rehydrated with 10 ml sterile normal saline and pooled in a 100 ml sterile Erlenmeyer flask (i.e. an initial 1:10 dilution of the vaccine). A total count was then done and the number of bacilli in the initial pool calculated to be 3.56×10^9 per ml. Tenfold dilutions of the vaccine suspension were prepared in sterile normal saline up to 10^{-7}.

Plate counts were done on dilutions 10^{-6} and 10^{-7} by adding 0.2 ml of the dilution to each of 5 blood agar plates. The number of colonies on each plate of the 10^{-6} dilution were as follows:

71, 65, 65, 68, 73 = 342.

$\therefore$ Number of viable organisms per ml in pool = 3.42×10^8

Total number of organisms in pool = 3.56×10^9

= 9.5% viability.

If certain technical details are attented to, the total count should not vary by more than $\pm$ 10 per cent from the real value (Wilson and Miles, 1955). It may however be as much as twice the viable count as a result of overfilling the counting chamber or the killing of some organisms when making the viable count (Meynell and Meynell, 1965). Despite these limitations of the techniques used for the total and viable counts, it was felt that there was a marked reduction in viability of the lyophilised culture.

Fifty Praomys were divided into 5 groups of 10 and each animal was injected subcutaneously on the inner side of the right hind leg with

0.5 ml of each of the following viable doses:

Group	Viable dose injected	Number of Praomys
I	1.7×10^8	10
II	1.7×10^6	10
III	1.7×10^5	10
IV	1.7×10^3	10
V	1.7×10^2	10

The animals were then observed daily.

a) Pathogenicity of the vaccine.

The Praomys in group I died on day three and one in group II died on day five. All the others survived. On post-mortem examination these four animals showed haemorrhages, gelatinous oedema and necrosis at the site of inoculation; hyperaemia, haemorrhages and gelatinous oedema of the abdominal and thoracic subcutaneous tissue and enlargement of the inguinal lymph nodes. The spleen, liver and adrenals of the group II animal were enlarged but not in the group I animals. None of the spleens or kidneys showed signs of necrosis but the liver of the group II animal had a mottled appearance. The lungs of the four animals were hyperaemic and oedematous and there was straw coloured fluid in the pleural cavities.

P. pestis was seen in direct smears and cultured from the heart blood and lungs of the four animals and also seen in direct smears and cultured from the inguinal lymph node, spleen, liver and kidneys of the Praomys in group II but not the group I animals.

Tissues fixed in 10% neutral formaline for histological exami-

nation were semi-autolysed but the following inflammatory reactions were seen:

Liver: Severe congestion of the sinusoids and venules and a marked increase in monocytes and polymorphonuclear cells in the venules. An increase of macrophages in the sinusoids was also seen. Clusters of P. pestis were distinct in the venules of the group II Praomys section.

Spleen: Engorgement of the red pulp with erythrocytes and a marked increase in mononuclear cells. Large numbers of P.pestis were seen throughout the section of the group II Praomys.

Lungs: Many alveolar spaces were filled with oedema fluid and some contained monocytes and erythrocytes. Alveolar walls were thickened and contained many mononuclear cells. No P. pestis could be seen.

b) Persistence of the vaccine strain.

Nine vaccinated Praomys from the following groups were sacrificed on day 27: group II - two animals, group III - two animals, group IV - three animals, group V - two animals.

The lymph nodes of one animal in each of groups III and IV were enlarged, and the liver of one in group IV was slightly mottled. The lungs in one animal in each of groups III and IV showed slight focal necrosis and hyperaemia. Cultures were done on blood agar of liver, spleen, kidney, lungs, heart blood and inguinal lymph nodes of these animals after maceration of the tissues but P. pestis could not be isolated.

c) Serological response after vaccination.

Forty four Praomys were bled by heart puncture on the 27th day after vaccination and the sera were tested for P. pestis F1 antibodies using the haemagglutination technique. Only two out of 19 Praomys inoculated with 1,700 (group IV) or 170 (group V) viable EV51f reacted serologically and these titres were very low (1:8 and 1:16). The Praomys inoculated with doses of 170,000 viable organisms reacted with titres ranging from 0 to 1:512, but more animals reacted with high titres when inoculated with doses of 1,700,000 and 170,000,000 (Table 4).

d) Protection conferred by a single EV51f vaccine dose against virulent challenge.

Six animals in each group were challenged on the 34th day with a recently isolated virulent strain of P. pestis (strain F357) from Lesotho. This strain had previously been shown to be highly virulent for Praomys by subcutaneous inoculation as follows:

P. pestis strain F357 viable dose	Praomys Survivors/Total	Days of death	Bacteriological culture
800	0/5	3	+
80	1/5	3 - 7	+
8	3/5	4 - 5	+

The vaccinated animals were challenged with a virulent culture passaged through a Praomys one week prior to challenge, the strain being isolated from the spleen of this animal after death. The challenge inoculum was then cultured on trypticase soy agar at 37°C for 48 hours. The culture was suspended in sterile normal saline and a total count done on a 1:10 dilution in formol saline.

TABLE 4. Resistance to virulent challenge and the serological response of Pracavs inoculated with various dilutions of lyophilised EV51f plague vaccine.

VACCINE DOSE	1.7×10^8			1.7×10^6			1.7×10^5			1.7×10^3			1.7×10^2		
Animal number	Reciprocal of HA titre		Day of death	Animal number	Reciprocal of HA titre		Animal number	Reciprocal of HA titre	Day of death	Animal number	Reciprocal of HA titre	Day of death	Animal number	Reciprocal of HA titre	Day of death
5	128		-	2	32	9	1	16	-	2	0	-	1	0	4
6	64		8	3	64	1*	2	32	-	3	0	7	2	0	5
7	1024		-	4	64	-	4	512	-	5	16	5	3	0	3
8	128		-	5	512	-	5	8	6	6	0	4	5	0	6
9	128		13	9	64	-	7	512	-	7	0	3	8	0	3
10	1024		-	10	16	7	8	128	5	9	8	3	10	0	4
Geometric mean titre (recipr.)	239				64			69			2			0	
Average day of death			11			8			6			4			4
% mortality		2/6 = 33%			2/5 = 40%			2/6 = 33%			5/6 = 83%			6/6 = 100%	
Active Protection Index(API)			33			20			18			4.8			4.0

* Intercurrent staphylococcal infection.

Tenfold dilutions were prepared to obtain a suspension of 2×10^4 organisms per ml and each animal received 0.5 ml of this dilution subcutaneously in the right hind leg. Ten unvaccinated control Praomys were challenged at the same time. Plate counts were done on lower dilutions by planting 0.2 ml of the dilution onto each of 5 blood agar plates and incubated at 28°C for 72 hours. The results of the plate counts showed that each animal received 6,000 viable virulent P. pestis strain F357.

All the control animals died, the average time of death being 4.6 days. With one exception, all the Praomys inoculated with 1,700 and 170 FV51f died of acute plague septicaemia after virulent challenge. P. pestis was cultured on blood agar and seen in smears of inguinal lymph node, spleen, liver, kidney, lungs and heart blood. Two animals inoculated with 1.7 million and two inoculated with 170,000 died of acute plague septicaemia; P. pestis was isolated from the tissues examined. One animal inoculated with 1.7 million died on the first day with a few pathological changes. Staphylococcus aureus was isolated from the spleen. Two animals inoculated with 170 million died on days 8 and 13 and showed severe lung involvement, but slight reaction in the other tissues. Cultures were positive for P. pestis.

The correlation between the degree of immunity and the antibody titre was not very striking although there did appear to be some relationship between the geometric mean titre of each vaccine dose and the rate of protection (table 4). The active protection indices of 1,700 vaccine bacilli and lower doses (table 4) are very low and these values correspond with those of vaccine strains which lack protective antigens (Burrows and Bacon, 1958). It is known

that strains which are Fl+ Wm+ P- and independent of purines for growth, such as the EV51f strain, are capable of limited growth in mice (Burrows and Bacon, 1958). The results of this experiment however show that multiplication of low doses of the EV51f strain is insufficient to stimulate detectable haemagglutinating antibodies in Praomys or confer immunity when inoculated subcutaneously.

The mortality and antibody production which followed the subcutaneous inoculation of the viable EV51f strain in doses of 170,000 or more organisms, revealed that this vaccine is invasive and multiplies in Praomys when inoculated in high doses. The impact on the immunity mechanisms however is not very great as 1.0 million organisms confer only 66% protection against virulent challenge. Although the mortality after virulent challenge in animals vaccinated with 1.0 million, 1.7 million and 17.0 million organisms was very similar, the protection as shown by the active protection indices was greater with the higher vaccine dose (table 4).

e) Pathogenesis of virulent P. pestis in immune Praomys.

The surviving Praomys which were challenged with virulent P. pestis on the 24th day after vaccination, were sacrificed on the 71st day after challenge. They were bled for serological tests, autopsies were done and the tissues were cultured on blood agar. The results (table 5) reveal that with three exceptions the survivors had gross lesions indicative of recovery from chronic plague. P. pestis, demonstrated as virulent in white mice, was recovered from one animal (group I, 5) immunised with 170,000,000 EV51f. There was no rise in the serological response of this group after challenge except in this one particular animal. The other groups showed distinct

TABLE 5. Serological response and pathological findings in 11 immune Pragnya which survived a severe virulent P. pestis challenge infection.

Animal number	Vaccine dose	HA titre 27 days after vaccination	Virulent challenge dose	HA titre 71 days after challenge	Post-mortem findings after sacrifice on day 71. Bacteriological findings
Group I	1.7×10^8		6,000		
5		128		32	All tissues normal except lungs which were haemorrhagic and had focal necroses. There was no oedema or pleural exudate. Culture of tissues: negative.
7		1024		1024	Lungs had focal necroses but no oedema or pleural exudate. Other tissues were normal. Culture of tissues: negative.
8		128		16,384	Inguinal lymph nodes enlarged, liver mottled, kidney necrotic lesions. Other tissues normal. Culture: lungs and heart blood P. pestis +++
10		1024		512	Inguinal lymph nodes enlarged, other tissues normal. Culture of tissues: negative.
Group II	1.7×10^6		6,000		
4		64		8,192	Oedema and necrosis at inoculation site, inguinal lymph nodes enlarged. Lungs had focal necroses. Culture of tissues: negative.
5		512		8,192	Inguinal lymph nodes enlarged, lungs had focal necroses. Culture of tissues: negative.
9		64		4,096	All tissues normal. Culture of tissues: negative.
Group III	1.7×10^5		6,000		
2		32		512	All tissues normal. Culture of tissues: negative.
4		512		1,096	Necroses at inoculation site, spleen enlarged, lungs had focal necroses. Culture of tissues: negative.
7		512		256	Lungs haemorrhagic and focal necroses, other tissues normal. Culture of tissues: negative.
Group IV	1.7×10^3		6,000		
2		0		8,192	Tissues normal. Culture of tissues: negative.

rises in titres, indicating multiplication of the virulent challenge organisms, as a result of a lower level of immunity.

f) Serological response after two vaccine doses and the protection conferred against virulent challenge.

Five vials of lyophilised EV51f vaccine were rehydrated as in the first vaccination. The total count of organisms in the initial pool was 2.6×10^9 per ml and the viable count was 3.4×10^8 per ml.

One Praomys of each of the previously unvaccinated groups (two in group V) was inoculated subcutaneously into the right hind leg with 0.5 ml of a 10^{-2} dilution of the vaccine i.e. 1,700,000 organisms. These Praomys had not been challenged with the virulent culture. They were then bled for serology on the 24th day after revaccination. Three died immediately after bleeding and the remaining three were challenged subcutaneously with 8,000 virulent P. pestis strain F357 on the 38th day after revaccination. After revaccination with a dose of 1,700,000 EV51f the six Praomys survived, whereas after primary immunisation this dosage resulted in 10% (1/10) mortality. Although the numbers were small there is an indication that the low primary vaccine doses protected the animals sufficiently against the invasiveness of a large second dose of the vaccine. The serological response to this second dose was inconsistent. One animal (group III, 6) responded with a dramatic rise in titre whereas others only showed a slight increase, or even a drop in titre 24 days after revaccination (table 6). One animal died after virulent challenge and the post-mortem was indicative of chronic plague with lung involvement, but the culture was negative (table 6).

TABLE 6. Serological response and protection of 6 Fresneys against virulent Y. pestis challenge following two doses of live attenuated EV51f plague vaccine.

Animal number	Vaccine dose	HA titre 27 days after vaccination	Revaccination dose day 70	HA titre 24 days after revaccination	Virulent challenge dose	Result of challenge
Group I						
4	1.7×10^8	128	3.4×10^6	32	6,000	Died day 17. Local oedema and necrosis. Lungs haemorrhagic and oedematous. Other tissues normal. Cultures negative.
Group II						
6	1.7×10^6	64	3.4×10^6	16,384	6,000	Survived.
Group III						
10	1.7×10^5	n.b.	3.4×10^6	negative	n.c.	Died after bleeding.
Group IV						
4	1.7×10^3	n.b.	3.4×10^6	256	n.c.	Died after bleeding.
Group V						
4	171	negative	3.4×10^6	512	6,000	Survived.
9	171	negative	3.4×10^6	32	n.c.	Died after bleeding.

n.b. = not bled

n.c. = not challenged

2. Subcutaneous inoculation of Praomys and white mice with an agar-grown EV51f culture.

The previous experiments with the EV51f vaccine inoculated in Praomys were done with a lyophilised culture. A fairly low viability (about 9%) was obtained after reconstitution of these cultures, and therefore very large numbers of dead bacilli were inoculated simultaneously. These organisms could have been toxic to the animals and therefore partly responsible for the deaths after vaccination. It was therefore decided to obtain a fresh culture grown on agar with a high viability, and determine whether a similar number of viable organisms with a lower total number was also pathogenic in Praomys. Comparative tests were also done simultaneously in white mice.

The lyophilised EV51f culture was seeded on to blood agar and grown at 37°C for 48 hours and then preserved according to the method of Stamp (1947). The dried cultures in gelatin pellets were grown initially on blood agar at 28°C for 48 hours and then subcultured on trypticase soy agar at 37°C for 48 hours. The growth from these agar slopes was suspended in sterile normal saline. Total and viable counts were done and tenfold dilutions in normal saline for inoculation in Praomys and white mice were then prepared. Six groups of 15 Praomys and six groups of 10 white mice were inoculated subcutaneously in the right hind leg with 0.5 ml of the dilutions containing from 1.1×10^7 to 1.1×10^2 viable EV51f bacilli.

Ten Praomys and ten white mice in each group were kept for virulent challenge and the other Praomys survivors were bled on the 29th day for haemagglutinating antibody tests. The animals were challenged subcutaneously on the 28th day with 21,650 virulent P. pestis strain MP6 as determined by plate counts. Autopsies were done on those

animals which died and tissues of fresh animals were preserved in 10% formol saline for histology. The animals which were bled on day 29 were sacrificed and autopsies done.

a) Pathogenicity of the vaccine strain.

Post vaccination deaths of 5/15 (33 1/3%) Praomys inoculated with 1.1×10^7 viable EV51f bacilli were recorded on day 2 (two animals) and day 3 (three animals). Of those vaccinated with 1.1×10^6 organisms 3/15 (20%) died on day 2 (two animals) and day 3 (one animal). One Praomys vaccinated with 1,100 EV 51f bacilli died on the 4th day but this was probably an intercurrent death as P. pestis was not isolated from the tissues and the post-mortem appearance did not resemble the other vaccination deaths.

All the white mice survived this vaccination.

The post-mortem appearances and cultures of Praomys which died after large EV51f vaccine doses were typical of death due to acute plague. Oedema was present at the inoculation site, the inguinal lymph nodes were enlarged, the spleens of animals which died on the 3rd day were enlarged, but not those which died on the 2nd day. The livers were slightly enlarged in 4 of 8 animals but the colour was normal. The kidneys were normal but the lungs in all cases were hyperaemic, haemorrhagic, oedematous and a pleural exudate was present. P. pestis was isolated from the inguinal lymph nodes of 6/8 animals and either from the lungs, spleen or heart blood of three of these. Cultures of two animals were negative.

Histologically, the liver venules were severely congested but there was not a marked increase in leucocytes in these spaces. Increased numbers of Kupffer cells and small granulomatous areas of macro-

phages and polymorphonuclear cells were present in the sinusoids. The red pulp of the spleen was engorged with erythrocytes and a marked increase in monocytes was evident. Numbers of giant cells were also present. The kidneys showed a marked congestion of the vascular spaces. The alveoli of the lungs were oedematous and the pulmonary vessels were congested.

These pathological changes are likely to be attributable to the action of toxin as P. pestis could not be identified in the sections and few organisms could be isolated from the tissues.

b) Serological response of Praomys after vaccination.

Only the Praomys inoculated with doses of 11,000 EV51f bacilli or higher reacted serologically 29 days after vaccination. Again there was no relationship between dose size and HA titre in the animals which reacted serologically:

Vaccine dose	Reciprocal of HA titre (day 29)	Reciprocal of geometric mean titre
1,100,000	64, 512	181
110,000	16, 64(2), 256	64
11,000	0, 16, 32, 128(2)	24
1,100	0(4)	0
110	0(5)	0

These results confirm those of the lyophilised vaccine trial in Praomys that doses of 1,000 EV51f bacilli or lower do not stimulate antibodies in this animal species.

c) Persistence of the vaccine strain in Praomys.

The twenty Praomys vaccinated with EV51f doses between 110 and

110,000 viable organisms which were bled 29 days after vaccination for serological tests, were sacrificed. Autopsies were done and the tissues were cultured on blood agar and examined histologically. All the cultures were negative and at autopsy the tissues appeared normal. Microscopically the tissues were singularly free of lesions except for some residual granulomatous areas of macrophage infiltration in the liver.

d) Protection against virulent challenge.

The deaths in white mice and Praomys challenged 28 days after vaccination are listed in the following table:

Vaccine dose	White Mice			Praomys		
	Average day of death	Mortality	Active Protection Index	Average day of death	Mortality	Active Protection Index
11,000,000	6.0	1/10=10%	60	> 21	0/10=0%	> 210
1,100,000	5.0	1/10=10%	50	> 21	0/10=0%	> 210
110,000	5.0	1/10=10%	50	8.0	1/10=10%	80
11,000	4.1	6/10=60%	6.8	5.3	4/10=40%	13.2
1,100	3.9	8/10=80%	4.9	3.3	10/10=100%	3.3
110	3.7	10/10=100%	3.7	3.7	9/10=90%	4.1
unvaccinated controls	2.9	10/10=100%	2.9	2.6	10/10=100%	2.6

The animals which died had post-mortem and bacteriological manifestations of bubonic-septicaemic plague. A local gelatinous oedema was present at the site of inoculation and in the subcutaneous tissue, which was also hyperaemic. The inguinal lymph nodes were typically enlarged and the liver and spleen were also enlarged in some cases. Severe pathological changes were present in the lungs of Praomys vaccinated with 110 EV51f organisms and those vaccinated with higher doses, which died after the third day. In these animals the lungs were hyperaemic, haemorrhagic in patches, oedematous and an exudate was present in the pleural cavity.

These manifestations were not present in the lungs of Praomys

110,000 viable organisms which were bled 29 days after vaccination for serological tests, were sacrificed. Autopsies were done and the tissues were cultured on blood agar and examined histologically. All the cultures were negative and at autopsy the tissues appeared normal. Microscopically the tissues were singularly free of lesions except for some residual granulomatous areas of macrophage infiltration in the liver.

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11,000,000	6.0	1/10=10%	60	> 21	0/10=0%	> 210
1,100,000	5.0	1/10=10%	50	> 21	0/10=0%	> 210
110,000	5.0	1/10=10%	50	8.0	1/10=10%	80
11,000	4.1	6/10=60%	6.8	5.3	4/10=40%	13.2
1,100	3.9	8/10=80%	4.9	3.3	10/10=100%	3.3
110	3.7	10/10=100%	3.7	3.7	9/10=90%	4.1
unvaccinated controls	2.9	10/10=100%	2.9	2.6	10/10=100%	2.6

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These manifestations were not present in the lungs of Praomys

vaccinated with 1,100 EV51f which died on the 3rd day. In partially immune animals the lung lesions take some time to develop, whereas in non-immune animals these lesions develop early.

Bacteriological cultures on blood agar of lymph nodes, liver, spleen, kidneys, lungs and heart blood were positive for P. pestis. Sections of the liver of these Praomys showed marked congestion of the venules and capillaries. Kupffer cells were loaded with ingested P. pestis and masses of free bacilli were present in all spaces (fig. 6). Severe multiplication of P. pestis was also present in the spleen, which was congested. Giant cells and increased numbers of monocytes were present in the splenic red pulp (fig. 7). Localised oedema and slight haemorrhages were present in the lungs with large bacterial masses in the alveolar walls (fig. 8). No myocardial lesions were seen.

The 50% protective dose (PD_{50}) calculated by Thompson's method was 11,000 viable EV51f bacilli for both Praomys and white mice inoculated via the subcutaneous route. Meyer (1970) obtained a PD_{50} value of 3,600 viable EV51f bacilli for white rice by subcutaneous inoculation. The simultaneous inoculation of Praomys and white mice with the vaccine indicates that the same number of EV51f organisms are required to immunise these two rodent species. The higher protection conferred in Praomys by each dilution of the vaccine, as shown by the protective indices, may be attributed to a greater invasiveness of the EV51f strain in Praomys than in white mice. This would result in a greater impact of the organisms on the immunity mechanism of Praomys.

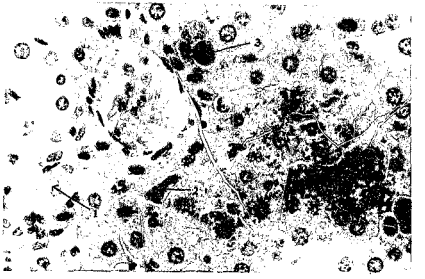


Fig. 6. Section of the liver of a Praomys (Mastomys) natalensis vaccinated with live attenuated P. pestis strain EV51F and which died after virulent challenge. Showing: (1) congestion of the vessels, (2) infected P. pestis in Kupffer cells and (3) masses of bacilli in the sinuses; × 375.

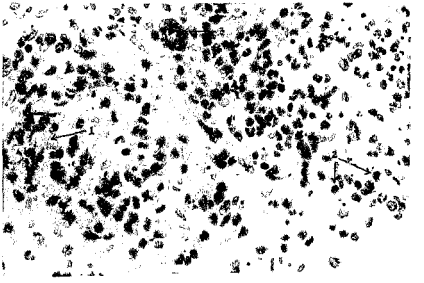


Fig. 7. Section of the spleen of a Praomys (Mastomys) natalensis vaccinated with live attenuated P. pestis strain EV51F and which died after virulent challenge. Showing (1) multiplication of P. pestis in the red pulp, (2) abundant monocytes and (3) a giant cell; × 375.

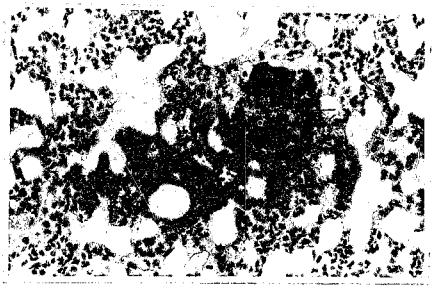


Fig. 8. Section of the lung of a Praomys (Mastomys) natalensis vaccinated with live attenuated P. pestis strain EV51f and which died after virulent challenge. Showing large bacterial masses in the alveolar walls; x 240.

The agar grown live EV51f vaccine appeared to have a slightly higher degree of pathogenicity than the lyophilised preparation in Praomys. The lyophilised vaccine in doses of 170,000,000 and 1,700,000 viable organisms caused 3/10 (30%) and 1/10 (10%) mortality respectively whereas 11,000,000 and 1,100,000 viable agar-grown organisms caused 5/15 (33%) and 3/15 (20%) mortality respectively. The numbers of animals were too small however to draw definite conclusions.

The protection conferred in Praomys by the agar-grown EV51f preparation against challenge with the virulent EP6 P. pestis strain was much greater than the protection conferred by the lyophilised vaccine against a challenge dose of the virulent P. pestis strain F357. The highest protection conferred by the lyophilised preparation was 60% with a dose of 1.7 million organisms and the protection indices after inoculation of 170 million, 1.7 million and 170,000 organisms were 33, 20 and 18 respectively. However, the inoculation of 11 million and 1.1 million agar-grown EV51f organisms resulted in solid protection of Praomys whereas 110,000 and 11,000 organisms conferred 90% and 60% protection respectively with active protection indices of 80 and 13.2. As both the EP6 and F357 P. pestis strains are highly virulent in Praomys it is concluded that the protection conferred by the agar-grown vaccine was greater than that of the lyophilised vaccine.

CHAPTER 4.

Invasiveness and persistence of attenuated and virulent P. pestis in normal and immune Praomys.

The fate of the EV plague strain in guinea pigs after subcutaneous inoculation in large doses was studied by Girard and Badaody-Balarosy (1940). These workers found that after 40 hours the organisms could not be detected anywhere in the body, but then appeared successively in the blood, spleen and liver and disappeared in the same order.

Investigating the invasiveness of virulent and attenuated plague strains in mice and guinea pigs, Jawetz and Meyer (1944a) showed that the route of infection followed the same pattern established for other bacterial infections, i.e. via the afferent lymphatics to the regional lymph glands and then via the efferent lymphatics to the thoracic duct and hence to blood stream, liver and spleen. In their investigations no correlation could be found between the immunogenic potency of a given attenuated strain for a certain animal species and the extent and duration of infection produced by such a strain in that species. This and other work led these authors to believe that the immunogenic activity of a virulent plague strain is a function of the antigenic make-up and not of the invasive or pathogenic properties.

Korobkova and Samoilova (1962) also emphasised the importance of the antigenic constitution of a live vaccine, but stated that those strains which could not multiply and remain in the body, do not confer immunity against plague. These workers found that doses of 50,000 - 100,000 different vaccine organisms inoculated subcutaneously in white mice multiply in the animal and can be isolated 10 - 18 days later from the site of inoculation, the regional lymph nodes, spleen and liver and also

from the blood and lungs. It was then postulated that immunity to plague develops in two phases, a "non-sterile" phase and a phase of "sterile" post-infectious immunity. The degree of immunity of the post-infectious phase was said to be dependent on the length and intensity of the "non-sterile" phase. A short retention of the organisms in the body of a vaccinated animal led to only weak immunity.

It was shown by the experiments described in chapters 2 and 3 that the live EV51f plague vaccine causes mortality in Cercopithecus aethiops and Praomys (Mastomys) natalensis. The invasiveness of the EV51f strain by subcutaneous inoculation was then investigated in greater detail in Praomys, and comparative experiments done with a strain in normal and immune animals of this species.

i) Invasiveness and persistence of attenuated P. pestis strain EV51f in normal Praomys.

An EV51f culture grown on trypticase soy agar at 37°C for 24 hours, was inoculated subcutaneously in the right hind l g of Praomys in two experiments. In the first, 130,000 bacilli were inoculated into 18 Praomys and in the second 150,000 bacilli were inoculated into the same number of animals. In previous experiments these numbers of bacilli caused no mortality and protected 90% of Praomys against virulent challenge. Two animals in each experiment were sacrificed by cervical dislocation after the following intervals: 6 hours, 12 hours, 1, 2, 3, 5, 8, 10 and 24 days.

Autopsies were done and the following tissues were cultured on blood agar and in nutrient broth after maceration using a pair of scissors: inguinal lymph nodes, spleen, liver, kidneys, lungs and heart blood.

The colonies on the agar were counted and the nutrient broth tubes examined after 72 hours. Growth was confirmed biochemically as P. pestis. Histological sections of the above tissues except the inguinal lymph nodes were also prepared.

The bacteriological results are tabulated as follows:

Time after inoculation	Animal number*	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
6 hours	1	0	0	0	0	0	0
	2	3+	0	0	0	0	0
	3	0	0	0	0	0	0
	4	3+	0	0	0	0	0
12 hours	1	4+	3+	0	3+	0	0
	2	0	0	0	0	0	0
	3	3+	0	3+	2+	0	0
	4	0	0	0	0	2+	2+
1 day	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	4+	0	3+	0	0	0
	4	4+	0	2+	0	3+	3+
2 days	1	4+	0	0	0	0	0
	2	4+	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
3 days	1	4+	0	0	0	0	0
	2	2+	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
5 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
8 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0

Time after inoculation	Animal number*	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
10 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
14 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0

Meaning of symbols: 0 Nutrient broth negative, plate negative
1+ Nutrient broth positive, plate negative
2+ Nutrient broth positive, 1-10 colonies on plate
3+ Nutrient broth positive, 11-50 colonies on plate
4+ Nutrient broth positive, >50 colonies on plate

* Animal numbers 1 and 2 were inoculated with 130,000 bacilli and numbers 3 and 4 were inoculated with 150,000 bacilli.

The organisms invaded rapidly and could be detected in the inguinal lymph nodes in 2 out of 4 Praomys after 6 hours. Spreading progressed throughout the body after 12 hours, when organisms could be recovered from the spleen, liver, kidney, lungs and heart blood. Plague bacilli remained generalised in the body for 24 hours when they could be isolated from the liver, lungs and heart blood. After this time they were cleared very rapidly, but persisted in the lymph nodes for 2 - 3 days. They were then cleared completely and could not be isolated from any tissue.

Autopsy findings.

The tissues of all animals sacrificed up to 24 hours after the subcutaneous inoculation of EV51f showed no abnormal appearances. After 2 days the subcutaneous tissue became hyperaemic but there was no oedema. The inguinal lymph nodes and spleens were enlarged and the livers were

pale in colour. The lungs were hyperaemic. No changes were observed in the kidneys. These pathological changes were observed for 5 days. By the eighth day the organs had returned to normal and no abnormalities were seen after this time.

Histology

Six hours after the inoculation of live EV51f organisms there was a proliferation of monocytes in the spleen red pulp and constriction of the sinuses (fig. 9). The follicles were distinct and few polymorphonuclear leucocytes were present. This proliferation of monocytes in the spleen became more extensive after 12 hours, and in the lungs the alveolar walls became thickened in places with a monocytic infiltration (fig. 10). Large numbers of polymorphonuclear and mononuclear leucocytes were present in the spleen red pulp after 24 hours and proliferation of the macrophages constricted the sinuses. At that stage there was also an increase in polymorphonuclears and mononuclears in the liver venules and small masses of these cells in the sinusoids (fig. 11). In the lungs there were large monocytic accumulations around the bronchioles and vessels and thickening of the alveolar walls in parts (fig. 12). In the kidneys there were monocyte aggregates around the glomeruli and amongst the convoluted tubules (fig. 13).

On the second and third day more polymorphonuclears and monocytes appeared in the liver capillaries and sinusoids (fig. 14), spleen sinuses and vessels (fig. 15) and in the lungs around the vessels, bronchioli and in the alveolar walls (fig. 16). The liver and spleen became acutely congested; the sinuses were dilated and filled with blood (fig. 14). Monocyte accumulations were also present in the glomeruli. By the fifth day there was no congestion of the liver or

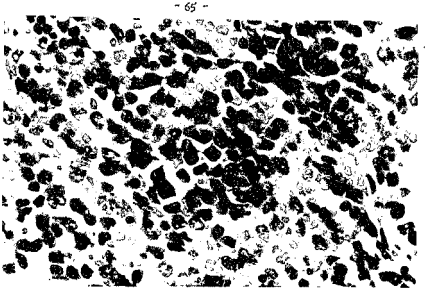


Fig. 9. Section of the spleen of a Praomys (Mastomys) natalensis killed six hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing proliferation of monocytes and constriction of the sinuses; x 600.

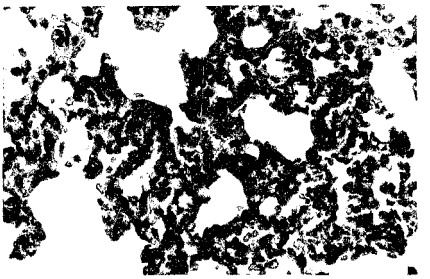


Fig. 10. Section of the lung of a Praomys (Mastomys) natalensis killed twelve hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing thickening of the alveolar walls and monocytic infiltration; x 375.

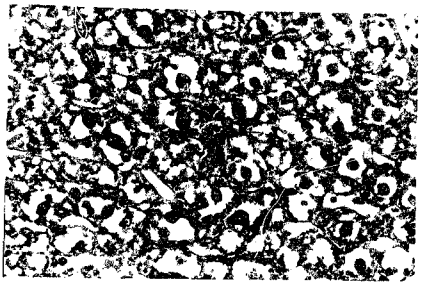


Fig. 11. Section of the liver of a Praomys (Mastomys) natalensis killed 24 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing accumulations of mononuclear and polymorphonuclear leucocytes in liver venules; x 375.

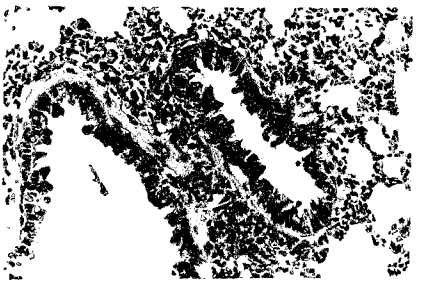


Fig. 12. Section of the lung of a Praomys (Mastomys) natalensis killed 24 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing monocytic accumulations around bronchioles and thickening of the alveolar walls; x 240.

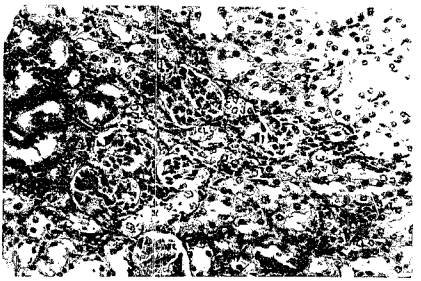


Fig. 13. Section of the kidney of a Praomys (Mastomys) natalensis killed 24 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing monocyte aggregates around the glomeruli and amongst the convoluted tubules; x 240.

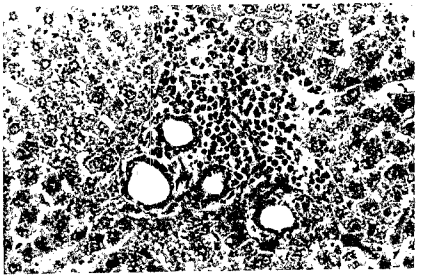


Fig. 14. Section of the liver of a Praomys (Mastomys) natalensis killed 48 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing mononuclear and polymorphonuclear leucocyte accumulations in the sinusoids; x 240.

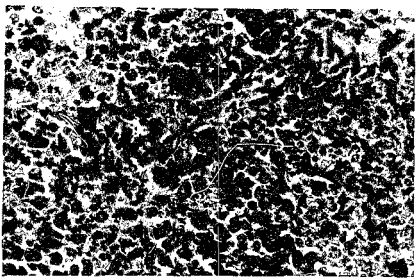


Fig. 15. Section of the spleen of a Praomys (Mastomys) natalensis killed 48 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing congestion of the sinuses and accumulations of mononuclear and polymorphonuclear leucocytes; x 375.

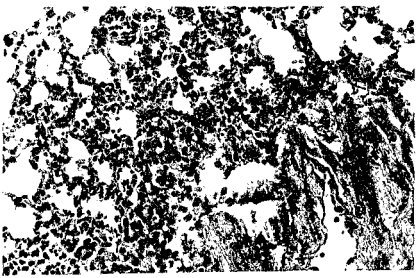


Fig. 16. Section of the lung of a Praomys (Mastomys) natalensis killed 48 hours after subcutaneous injection of live attenuated P. pestis strain EV51f. Showing monocyte accumulations in the alveolar walls; x 240.

spleen but a few polymorphonuclear and monocytic masses were present in the liver sinusoids. Numerous polymorphonucleare and monocytes were also still present in the spleen sinuses. Regression of the reactions then occurred rapidly; by the eighth day a few small monocytic masses were present in the liver sinusoids and other tissues were normal in appearance. The liver also returned to normal by the 14th day.

ii) Invasiveness and persistence of virulent P. pestis strain MP6 in normal Fraomys.

Virulent P. pestis strain MP6 was grown on trypticase soy agar at 37°C for 24 hours and three dilutions of a suspension were made, containing 12,000, 60,000 and 120,000 organisms. These were then inoculated subcutaneously in the right hind leg using 12 animals for each dilution. One animal inoculated with 120,000 organisms died on day 2, seven inoculated with 120,000, 60,000 or 12,000 organisms died on day 3, and one inoculated with 12,000 organisms died on day 4. The remaining animals were sacrificed by cervical dislocation at the following times after inoculation: 6, 12 hours, 1, 2, 3 days. Autopsies were done and the following tissues were inoculated onto blood agar and into nutrient broth after maceration: inguinal lymph node, liver, spleen, kidney, lungs and heart blood. These were then incubated at 28°C for 72 hours and the colonies counted. Growth in the nutrient broth was confirmed biochemically as P. pestis. The following tissues were preserved in 10% neutral formol saline for histological examination: liver, spleen, kidney, lungs.

Bacteriological results.

As shown in the tabulated results, bacilli could not be isolated from

any animals 6 hours after infection. P. pestis was first isolated after 12 hours from the inguinal lymph node of one of six animals inoculated with 120,000 organisms, but the other tissues of this animal and all others were negative at that time. Positive cultures were obtained from all tisues examined of one Praomys after 24 hours but the other animals were negative. Infection spread throughout the body after 48 hours and the first death was also then recorded. There were no survivors after 3 days.

The bacteriological results are tabulated as follows:

Time after inoculation	Animal number	Number of organisms inoculated	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
6 hours	1	12,000	0	0	0	0	0	0
	2	12,000	0	0	0	0	0	0
	3	60,000	0	0	0	0	0	0
	4	60,000	0	0	0	0	0	0
	5	120,000	0	0	0	0	0	0
	6	120,000	0	0	0	0	0	0
12 hours	1	12,000	0	0	0	0	0	0
	2	12,000	0	0	0	0	0	0
	3	60,000	0	0	0	0	0	0
	4	60,000	0	0	0	0	0	0
	5	120,000	2+	0	0	0	0	0
	6	120,000	0	0	0	0	0	0
1 day	1	12,000	0	0	0	0	0	0
	2	12,000	0	0	0	0	0	0
	3	60,000	0	0	0	0	0	0
	4	60,000	0	0	0	0	0	0
	5	120,000	4+	3+	3+	2+	2+	2+
	6	120,000	0	0	0	0	0	0
2 days	1	12,000	4+	0	1+	0	2+	0
	2	12,000	4+	2+	2+	0	1+	3+
	3	60,000	4+	4+	3+	4+	1+	3+
	4	60,000	4+	3+	0	2+	0	0
	5	120,000	4+	4+	4+	4+	4+	4+

Time after inoculation	Animal number	Number of organisms inoculated	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
3 days	1	12,000	4+	4+	4+	4+	4+	4+
	2	12,000	4+	4+	4+	4+	4+	4+
	3	60,000	4+	4+	4+	4+	4+	4+
	4	60,000	4+	4+	4+	4+	4+	4+

Meaning of symbols as in previous table.

Autopsy findings.

The inguinal lymph nodes were slightly enlarged six hours after inoculation but all other tissues were normal. After 12 hours the site of inoculation was oedematous and the inguinal lymph nodes were further enlarged. These reactions intensified after 24 hours, the lungs became hyperaemic and the liver was enlarged. The livers then developed a very dark red colour and the spleen also became enlarged. These changes were also noted three days after inoculation and were also characteristic of those animals which died of plague.

Histology.

No histological changes were observed until 24 hours after inoculation, when the liver sinuses and sinusoids became congested, and monocytes increased in these sinusoids. Very few polymorphonuclear leucocytes were seen in the liver sections at that time (fig. 17). The mononuclears also increased in number in the kidney glomeruli, the spleen sinuses and around the vessels in the lungs. After 48 hours the number of polymorphonuclears increased greatly in the liver sinusoids and spleen sinuses. The latter also became congested at that time (fig. 18). Oedema fluid was present in some alveolar spaces after 3 days and mononuclear and polymorphonuclear leucocytes infiltrated into the alveolar walls which were also thickened (fig. 19).

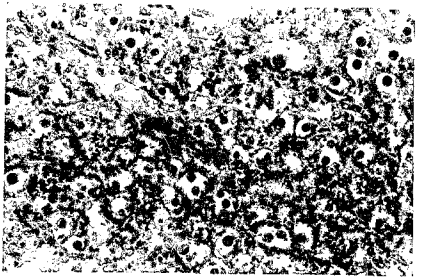


Fig. 17. Section of the liver of a Praomys (Mastomys) natalensis killed 24 hours after subcutaneous injection of virulent P. pestis strain 195. Shows a few polymorphonuclear leucocytes in the sinusoids; x 240.

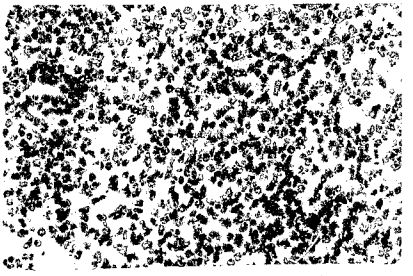


Fig. 18. Section of the spleen of a Praomys (Mastomys) natalensis killed 48 hours after subcutaneous injection of virulent P. pestis strain 195. Shows congestion and large numbers of polymorphonuclear leucocytes in the sinuses; x 375.

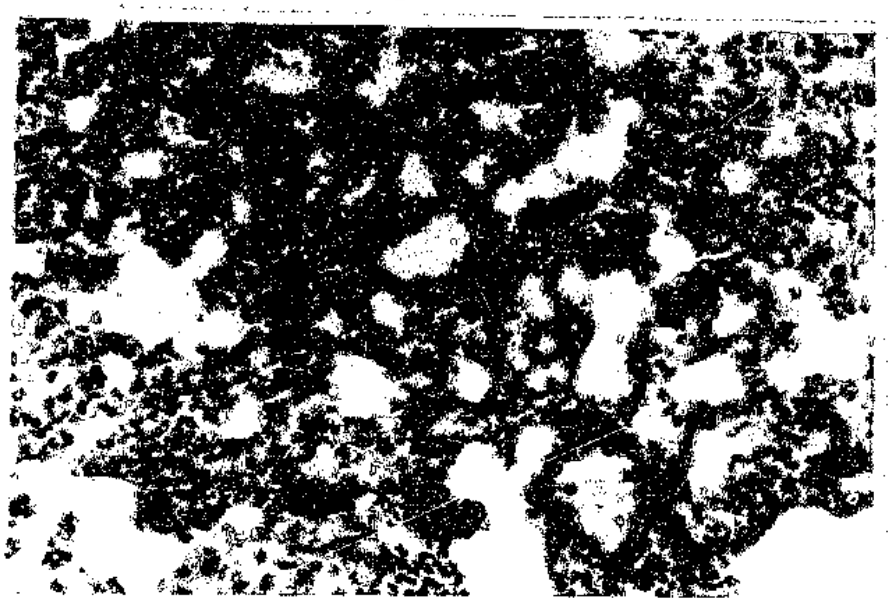


Fig. 19. Section of the lung of a Praomys (Mastomys) natalensis killed 72 hours after subcutaneous injection of virulent P. pestis strain MP6. Showing oedema, thickening of the alveolar walls and infiltration of polymorphonuclear and mononuclear leucocytes; x 240.

iii.) Invasiveness of virulent P. pestis in immune Praomys.

Praomys were immunised by subcutaneous inoculation in the right hind leg with 130,000 live agar-grown attenuated P. pestis strain EV51f. These animals were then inoculated subcutaneously 28 days later in the same site with 50,000 virulent P. pestis strain MP6. This culture was grown on trypticase soy agar at 37°C for 24 hours. Four animals were then sacrificed by cervical dislocation at each of the following times: 6 hours, 12 hours, 24 hours, 2, 3, 6, 9, 14 and 21 days.

The following tissues were cultured in nutrient broth and on blood agar: inguinal lymph nodes, spleen, liver, kidneys, lungs and heart blood. These were then incubated at 28°C for 48 hours and the colonies counted on the agar. Growth in the broth was confirmed biochemically as P. pestis. The following tissues were preserved in 10% neutral formol saline for histology: liver, spleen, kidneys and lungs.

Bacteriological results.

The results of the bacteriological cultures are given in the following table:

Time after inoculation	Animal number	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
6 hours	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	2+	0	0	0	0	0
12 hours	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0

Time after inoculation	Animal number	Inguinal lymph node	Spleen	Liver	Kidney	Lungs	Heart blood
1 day	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	2+	1+	0	0	0	0
2 days	1	2+	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	4+	2+	1+	1+	0	1+
3 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
6 days	1	4+	0	1+	0	0	0
	2	0	0	0	0	0	0
	3	0	1+	1+	2+	0	0
	4	2+	2+	0	0	0	0
9 days	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	4+	2+	1+	2+	0	0
	4	0	0	0	0	0	0
11 days	1	0	0	3+	0	0	0
	2	0	0	0	0	0	0
	3	2+	0	0	0	0	0
	4	2+	0	0	0	0	0
21 days	1	0	0	0	0	0	0
	2	0	4+	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0

Meaning of symbols as in previous table.

P. pestis was isolated from the inguinal lymph node of one animal 6 hours after inoculation. Organisms could not be detected in any other tissues until 24 hours after infection; at that time P. pestis

spread to the inguinal lymph node and n. After two days infection had spread throughout the body in one animal and the organisms could be isolated from the spleen, liver, kidneys, lungs and heart blood. This animal may not have been fully immune and may have eventually died from this infection. Bacilli could be isolated from the organs of other animals after 6 - 9 days and persisted in the tissues for at least three weeks. These organisms were confirmed to be virulent *P. pestis* by inoculation of white mice.

Autopsy findings.

All tissues were normal in appearance for 24 hours after inoculation. After 48 hours there was slight oedema at the site of inoculation and the inguinal lymph node was enlarged. Three days after inoculation oedema and a pus nodule were present at the site of inoculation. The lymph nodes, spleen and liver were slightly enlarged. These changes persisted for two weeks and after 21 days a creamy pus nodule persisted at the site of inoculation, the inguinal lymph node was slightly swollen and the spleen was slightly enlarged. All other organs appeared normal.

Histology.

Twelve hours after inoculation, small granulomatous areas of mononuclear and polymorphonuclear leucocytes appeared in the liver sinusoids (fig. 20). At that time there was also a proliferation of monocytes in the spleen red pulp, but few polymorphonuclears were present (fig. 21). In the lungs oedema fluid was present in a few air spaces and some alveolar walls were thickened, accompanied by macrophage infiltration (fig. 22). The liver and spleen then became congested and polymorphonuclears appeared in the spleen sinuses. By the 6th day these changes reached a maximum; at which stage there was severe lymphoid and mono-

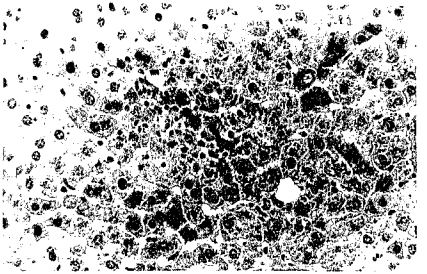


Fig. 20. Section of the liver of an immune *Praomys* (*Mastomys*) *natalensis* killed 12 hours after subcutaneous injection of virulent *P. pestis* strain EV6. Showing a small granulomatous area of mononuclear and polymorphonuclear leucocytes; x 240.

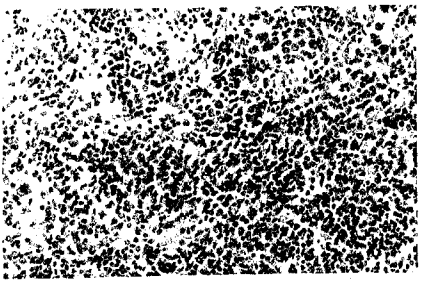


Fig. 21. Section of the spleen of an immune *Praomys* (*Mastomys*) *natalensis* killed 12 hours after subcutaneous injection of virulent *P. pestis* strain EV6. Showing proliferation of monocytes in the red pulp; x 240.

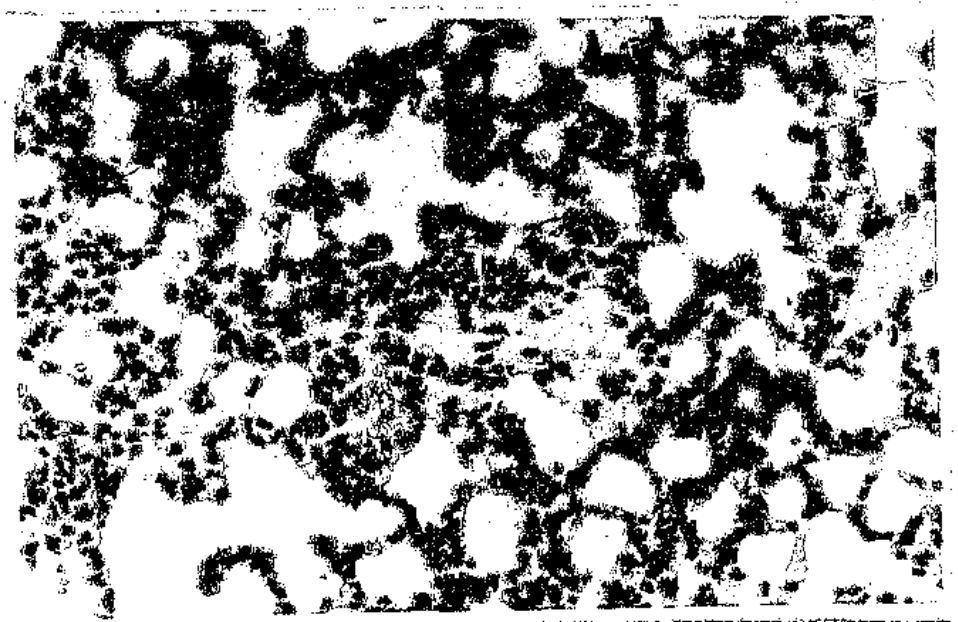


Fig. 22. Section of the lung of an immune Praomys (Mastomys) natalensis killed 12 hours after subcutaneous injection of virulent P. pestis strain NP6. Showing oedema in some air spaces and thickening of the alveolar walls, accompanied by macrophage infiltration; x 240.

cytic proliferation in the spleen. Regression of the inflammatory reactions then occurred, the lungs being the first to return to normal after 9 days. After two weeks the liver and spleen were no longer congested but small macrophage accumulations were present in the liver sinusoids. The monocytic and polymorphonuclear hyperplasia also persisted in the spleen.

Previous work on the fate of virulent and attenuated P. pestis in normal and immune experimental animals has shown that protective attenuated strains invade and spread rapidly throughout the body. After subcutaneous or intravenous inoculation, such strains persist in the tissues of mice and guinea pigs for 7 - 18 days (Walker et al., 1953; Jawetz and Meyer, 1944b and Korobkova and Samoilova, 1962). Korobkova and Samoilova (1962) maintained that the intensity of invasion and duration of persistence were of primary importance in the establishment of immunity, and Markenson and Ben-Efraim (1969) showed that protection by attenuated strains in mice and guinea pigs was in direct proportion to the duration of persistence.

In Praomys the invasion and spread of the attenuated EV51f strain after subcutaneous inoculation is as rapid and extensive as has been described for other attenuated strains in mice. The period of persistence of the EV51f strain is however much shorter in Praomys (3 days) than protective attenuated strains in mice and guinea pigs. The intensity of invasion therefore seems to be of great importance in establishing immunity in Praomys.

The route of infection of virulent P. pestis in mice after subcutaneous inoculation is via the lymphatics and lymph glands to the blood stream and then to the liver and spleen (Jawetz and Meyer, 1944b). In Praomys

the route of infection is similar to that in white mice, but the bacilli appear to remain localised at the site of inoculation longer, as virulent P. pestis could not be isolated from the regional lymph nodes or other tissues for some time after subcutaneous inoculation. The bacilli appeared in small numbers in the lymph node of one animal after 12 hours and then multiplied there to reach large numbers in this tissue after 2 days. At that time bacilli had also spread to other organs in small numbers. Multiplication then took place throughout the body and masses of bacilli were present in all tissues within 3 days.

In immune guinea pigs and mice the spread of virulent P. pestis differs only quantitatively from that observed in normal animals (Jawetz and Meyer, 1944b). Virulent bacilli persist in immune mice for 7 - 6 days. In immune Praomys the spread of virulent P. pestis is similar to that in normal animals and also differs quantitatively as was found in guinea pigs and mice. The persistence of the virulent strain is however much longer in immune Praomys (at least 21 days) than in immune mice or guinea pigs. This observation could be of epidemiological importance as the persistence of virulent bacilli in immune animals in the field may be one mechanism by which plague can persist in enzootic foci (Meyer et al., 1943). Under certain circumstances these animals may become bacteraemic and spread of infection by fleas could result. This may then be the ultimate source of a plague outbreak.

Goldenberg et al. (1964) reported the isolation of virulent P. pestis from apparently healthy Microtus californicus which were highly resistant to plague. Organisms were recovered from the blood and the blood filtering organs - the spleen, liver, kidneys and bone marrow. These voles were then implicated in the reservoiring mechanism of plague in the San Francisco area. A similar situation may exist in

South Africa if virulent P. pestis persists in Praomys or other wild rodents in enzootic areas.

Histological changes in the guinea pig spleen after subcutaneous inoculation of the attenuated EV strain were described by Bablet, Girard and Robic (1937). After the fifth day the red pulp contained small nodular masses of reticular cells, in the centre of which were groups of polymorphonuclear cells containing a few cocco-bacilli. This was followed by a proliferation of the reticular and endothelial elements, as a result of which the white pulp became indistinct. These changes reached a peak on the 10th day and then began to regress. By the 30th day the nodules had disappeared.

After intramuscular injection of white mice with cortisone, attenuated P. pestis strains produce bacteraemia and fatal infections similar to that caused by fully virulent strains (Payne et al., 1955). This effect of cortisone was attributed to an inhibition of local inflammatory reactions and infection-localising processes, particularly the phagocytes. The natural defence mechanisms against P. pestis infection were stated to be primarily cellular and depend on localization of the infection by derivatives of the reticulo-endothelial system in conjunction with the local mobilization of polymorphonuclear leucocytes.

Subcutaneous inoculation of Praomys with the attenuated EV51f strain resulted in an initial proliferation of monocytes within 12 hours in the spleen and alveolar walls of the lungs. This was then followed by the appearance of polymorphonuclear leucocytes in the liver, spleen and lungs, and resulted in a rapid clearing of bacilli from the tissues within 3 days. Regression of the inflammatory reaction then occurred rapidly and had cleared completely by the 8th to 14th day.

After subcutaneous inoculation of normal Praomys with virulent P. pestis there were no histological changes within the first 24 hours, but then accumulations of mononuclear leucocytes appeared in the liver sinusoids. Abundant polymorphonuclear leucccytes appeared after 48 hours in the liver and spleen sinuses and the alveolar walls of the lungs. Inoculation of immune Praomys with virulent P. pestis resulted in a reaction similar to that observed after inoculation of the attenuated strain in normal animals, but the inflammatory process lasted longer.

The reaction reached a maximum after 6 days and regression was slower as accumulations of polymorphonuclear and monocytic cells persisted in the liver and spleen after two weeks.

These histological reactions of Praomys to attenuated and virulent P. pestis are similar to those described by Jawetz and Meyer (1944b) for these strains in white mice, but the changes occurring in the latter animals persisted for longer periods.

Praomys therefore differs from white mice and guinea pigs in certain respects in its reaction to subcutaneous inoculation of virulent and attenuated P. pestis. Clearance of attenuated strains from normal Praomys is more rapid than in white mice or guinea pigs, although the rate and extent of invasion but not the duration of persistence in the three animal species are similar. Histological reactions to infection with attenuated P. pestis in normal Praomys and white mice were similar, but the shorter duration of the inflammatory reaction in Praomys agrees with a more rapid clearance of bacilli from the tissues.

Infection of normal Praomys with virulent P. pestis follows the same route as in white mice, but the rate of spread to the body tissues is slower. Localisation of the bacilli at the inoculation site is impli-

cated. Virulent P. pestis persists for longer periods in immune Praomys than in white mice or guinea pigs.

CHAPTER 5.

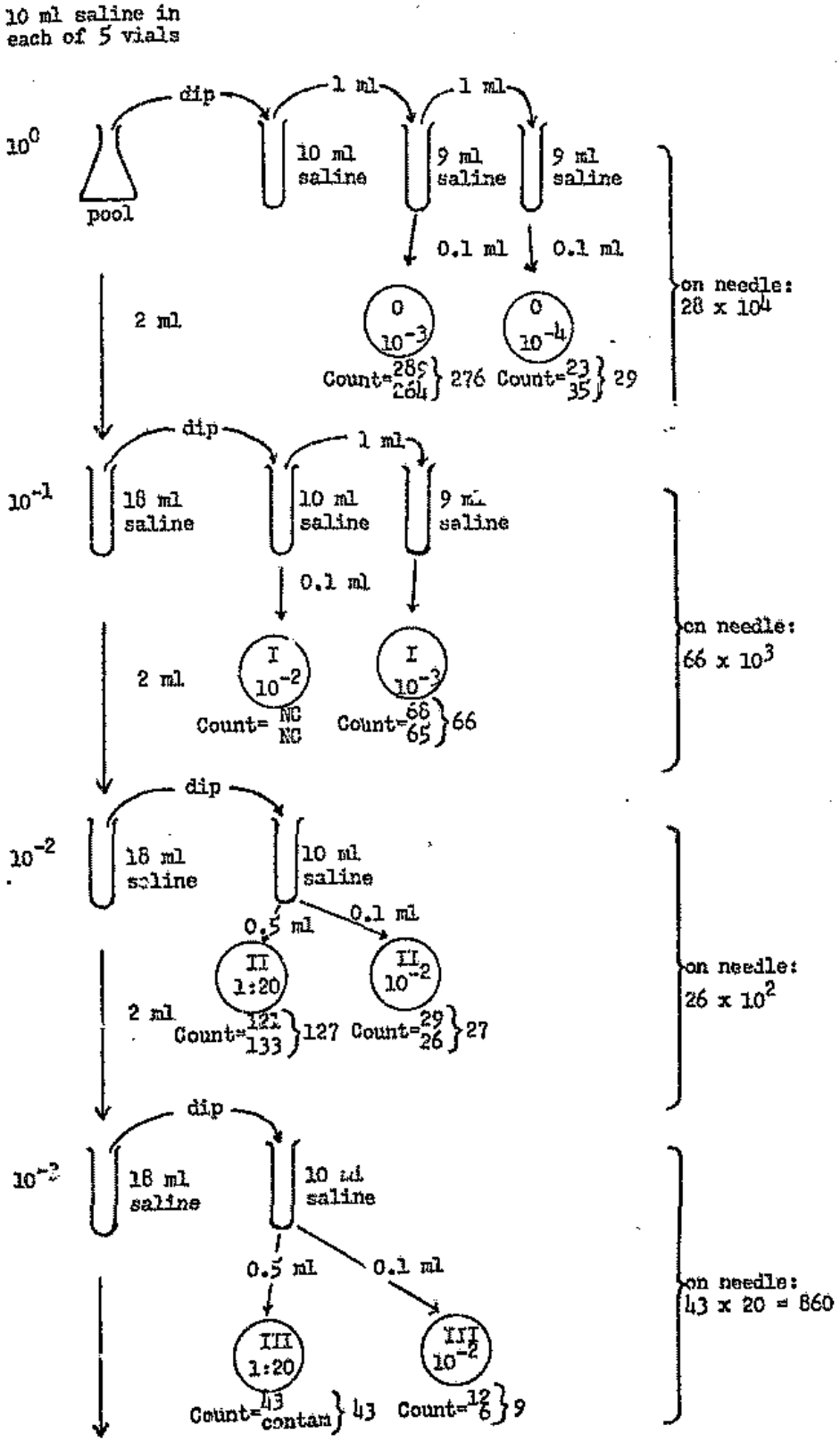
Intracutaneous inoculation of Praomys using the bifurcated needle technique

The unpleasant local and systemic reactions in man produced by live attenuated plague vaccines is reduced if the vaccine is administered by the intradermal or epidermal route (Novikova, 1956, Dermina, 1956 (both quoted in Pollitzer, 1960) and Meyer, 1970). To determine whether the residual virulence of an attenuated live vaccine is lower if administered intracutaneously, Praomys were inoculated with a lyophilised EV51f culture by this route.

Fifty bifurcated needles were sterilised separately in cotton wool plugged test tubes. The hair was then shaved off the right thighs of fifty Praomys to prevent small droplets of vaccine being picked from the needle before it was applied to the skin. The lyophilised EV51f vaccine was then reconstituted as for the subcutaneous inoculation of Praomys by adding 10 ml sterile normal saline to each vial. These suspensions were then pooled in a 100 ml Erlenmeyer flask. A total count was done on a tenfold dilution in 1.5% formol saline in a counting chamber. Sequential tenfold dilutions were then made on the pool by diluting 2 ml of the inoculum with 18 ml normal saline. A separate sterile needle was dipped into each dilution of the sequence and thus charged with inoculum. This was accomplished by holding the end of the needle with sterile forceps and the needle dipped to a point of about 1 mm above the origin of the bifurcation. If it was dipped further, droplets would adhere to the needle and run down onto the point of inoculation and thus substantially increase the dosage applied. The fully charged needle was then dropped into a tube of 10 ml sterile

normal saline. Sequential tenfold dilutions were then made of initial dilutions and 0.1 ml of each was plated on blood agar plates. The plates were then incubated at 28°C for 72 hours before counting the colonies. The number of viable organisms on the needles was calculated from these counts. Thus with the needle dipped into the pool, an average of 29 colonies was counted on 2 plates which received 0.1 ml of a 10^{-2} dilution of the tube into which the fully charged needle was dropped. This is equal to 29 x 1/0.1 (amount plated) x 10^2 (2 tenfold dilutions) x 10 (approximate dilution factor of the tube receiving the needle itself) or 29×10^4 viable organisms on the needle tip.

On those plates marked "direct" 0.1 ml saline was added to the petri dish and the fully charged needle was washed in the saline while tilting the plate. Dilutions and plate counts were made according to the following scheme:


10 ml saline in each of 5 vials
10^0
dip
pool
1 ml
10 ml saline
1 ml
9 ml saline
9 ml saline
0.1 ml
0.1 ml
2 ml
0 10^{-3}
Count= 285 264 } 276
0 10^{-4}
Count= 23 35 } 29
on needle: 28×10^4
10^{-1}
dip
18 ml saline
1 ml
10 ml saline
9 ml saline
0.1 ml
2 ml
I 10^{-2}
Count= NC NC
I 10^{-3}
Count= 68 65 } 66
on needle: 66×10^3
10^{-2}
dip
18 ml saline
10 ml saline
0.5 ml
0.1 ml
2 ml
II 1:20
Count= 121 133 } 127
II 10^{-2}
Count= 29 26 } 27
on needle: 26×10^2
10^{-2}
dip
18 ml saline
10 ml saline
0.5 ml
0.1 ml
III 1:20
Count= 43 contam } 43
III 10^{-2}
Count= 12 6 } 9
on needle: $43 \times 20 = 860$

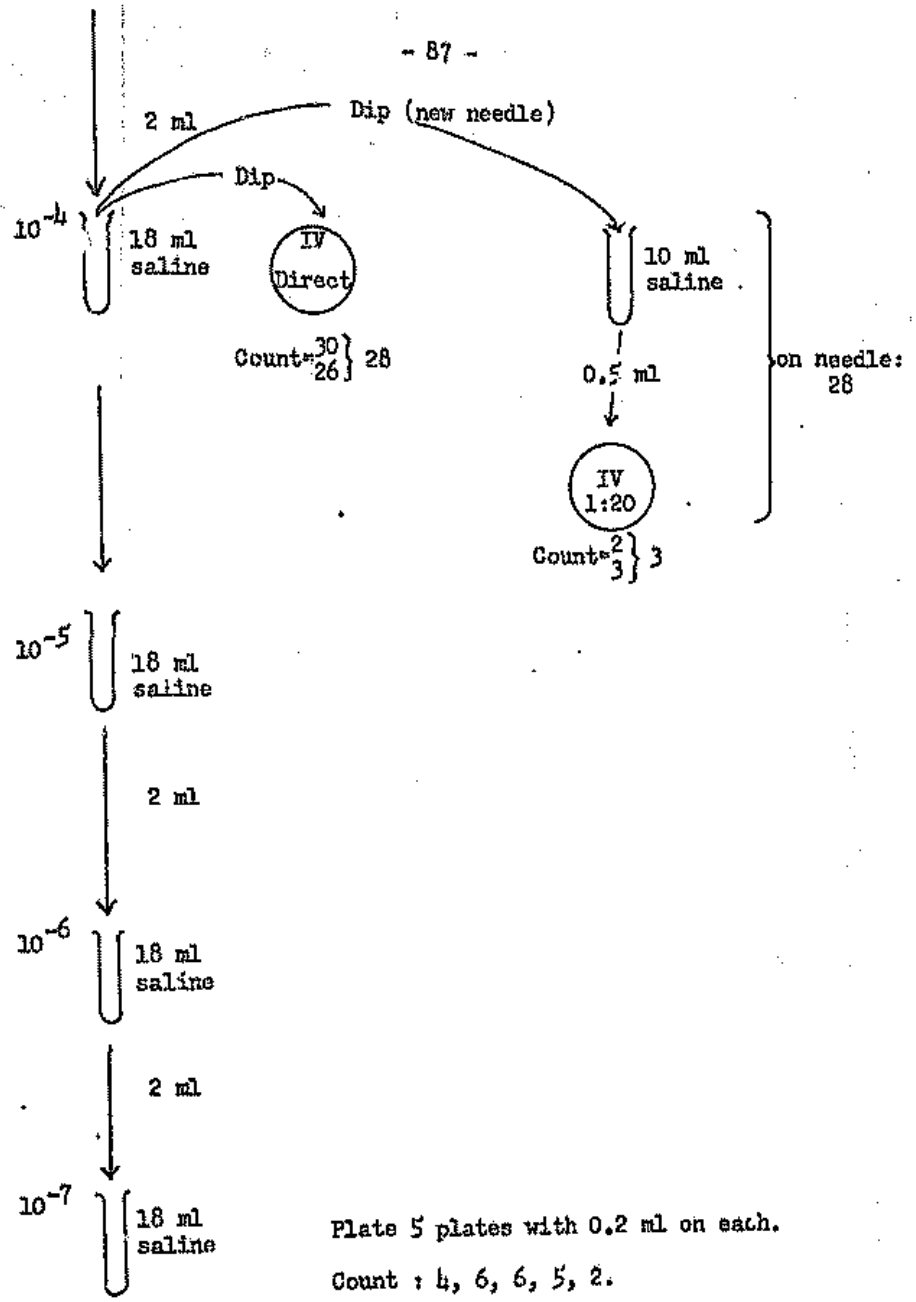


Plate 5 plates with 0.2 ml on each.

Count : 4, 6, 6, 5, 2.

Total = 23 organisms per ml in 10^{-7} dilution.

Total count = 2.69×10^9 per ml in pool

$$\frac{2.3 \times 10^8}{2.68 \times 10^9} \times 100 = 8.6\% \text{ viability.}$$

Dilution of inoculation	Inoculum viable count per ml	Viable count on needle	% needle inoculum
10^0(pool)	23×10^7	28×10^4	0.122
10^{-1}	23×10^6	66×10^3	0.287
10^{-2}	23×10^5	26×10^2	0.113
10^{-3}	23×10^4	860	0.374
10^{-4}	23×10^3	28	0.122
			Average = 0.203%

This average of 0.203% was then applied to each dilution of the inoculum count to obtain uniformly graded values.

Thus: *Highest dosage group* = 23×10^7 *per ml* x 0.00203

= 466,900

and thus:

Dilution of inoculum	Averaged viability actually injected
10^0(pool)	466,900
10^{-1}	46,690
10^{-2}	4,669
10^{-3}	467
10^{-4}	47

The animals were observed for cutaneous reactions for 5 days after vaccination, but the skin remained healthy in all. There were no fatalities after vaccination.

After 28 days these Praomys were challenged with 8,420 virulent P. pestis strain F357 inoculated subcutaneously in the right hind leg. Within 10 days after challenge all the animals vaccinated with 4,669 EV51f bacilli intracutaneously had died of acute septicaemic plague. There was a survival rate of 30% and 60% in the groups inoculated with 46,690 and 466,900 organisms respectively. Results of the virulent

challenge are shown in the following table:

Vaccine dose (intracutaneous)	Challenge dose (day 29)	Mortality after challenge	Average day of death	A.P.I.
466,900	8,420	4/10	4.0	10
46,690	8,420	7/10	3.4	4.9
4,669	8,420	10/10	3.4	3.4
467	8,420	10/10	3.1	3.1
47	8,420	10/10	3.2	3.2

Post-mortem examination of the animals which died after challenge revealed a typical picture of death due to plague, with haemorrhages and oedema at the site of inoculation and subcutaneous tissue, and enlarged inguinal lymph nodes, liver and spleen. Involvement of the lungs appeared to be more severe in animals which died after the third day. In these animals there was marked hyperaemia and haemorrhages, oedema and pleural fluid, whereas lung oedema and pleural fluid were absent in the early deaths.

The rate of protection against virulent challenge given by the vaccine inoculated intracutaneously is similar to that given by the lyophilised EV51f vaccine inoculated subcutaneously in Praomys. Doses of 1,700 viable organisms and lower inoculated subcutaneously, gave no resistance to challenge, whereas 17,000, 170,000 and 1,700,000 organisms protected 17%, 66% and 60% of animals respectively.

Although there were no apparent changes at the site of inoculation after intracutaneous vaccination the involvement of lymph nodes could not be determined by palpation on account of the small size of the animal. Inoculation of lyophilised EV51f live vaccine via the intracutaneous

route in doses between 466,900 and 47 viable organisms failed to cause any vaccination fatalities. This was not unexpected however as 1,700,000 viable organisms inoculated subcutaneously caused 10% mortality and animals which received doses of 170,000 and lower survived the vaccination. As high doses of the vaccine could not be attained by single inoculations using bifurcated needles, any differences in the pathogenicity of the vaccine could not be detected in Praomys using the intracutaneous route.

CHAPTER 6.

Vaccination studies with a culture derived from the original EV 76 strain, stored in Paris

The observation that the residual virulence of an EV76 strain which had been passaged through iron treated guinea pigs, could be readily detected in Praomys warrented the testing of the original EV76 strain which had been used extensively for human inoculation. A culture of this strain had been maintained at the Institut Pasteur in Paris by regular agar subculture. A subculture of this strain was obtained by Dr. K.F. Meyer through the courtesy of Dr. Girard and was made available in a lyophilised state and designated the EV76 (Paris) strain. Tested at the George Williams Hooper Foundation, this culture was found to be non-pathogenic for guinea pigs in doses up to 26,300,000 viable organisms given subcutaneously and even by intravenous inoculation in a dose of 100,000 organisms the EV 76 (Paris) strain was non-pathogenic for white mice. The 50% protective dose (PD_{50}) for white mice was 38 determined by the intraperitoneal route and 2,800 by the subcutaneous route. In addition to the immunogenic ability, the 'granuloma' forming properties in guinea pigs, specified for the original isolate in 1932, and the biochemical characteristics were retained during its maintainance at the Institut Pasteur for nearly 35 years. The following characteristics of the strain were determined (Meyer, 1970); Pigment -, Fl+, OA dependence +, Uracil dependence -, Pesticin I -, Pesticin II +, Fibrinolysin-.

1. Subcutaneous inoculation of a single dose of lyophilised EV76 (Paris) vaccine in Praomys.

Five vials of the lyophilised vaccine EV76 Lot No. G-2- MFG Date Aug

1969, Walter Reed were pooled in a 100 ml Erlenmeyer flask after adding 10 ml normal saline to each vial. A 1:10 dilution of this pool in 1.5% formol saline was prepared and a total count done in a counting chamber. Tenfold dilutions of the vaccine pool were then prepared in normal saline for inoculation in Praomys. Plate counts were done on the 10^{-4} and 10^{-5} dilutions by adding 0.2 ml of the dilution to each of 5 blood agar plates and counting the colonies after incubation at 28°C for 72 hours. Sixty Praomys were divided into 6 groups of 10 animals. Each of the ten animals was inoculated subcutaneously in the right hind leg with 0.5 ml of the appropriate vaccine dilution. The following total and viable numbers of organisms were injected:

Group 10 animals each	Total number of organisms per dose	Number of viable organisms per dose
I	3.1×10^8	2.2×10^7
II	3.1×10^7	2.2×10^6
III	3.1×10^6	2.2×10^5
IV	3.1×10^5	2.2×10^4
V	3.1×10^4	2.2×10^3
VI	3.1×10^3	2.2×10^2

The animals were inspected daily for 3 weeks.

Pathogenicity of the vaccine.

Four of the 10 Praomys inoculated with 22,000,000 viable EV76 (Paris) organisms died after 3 (two animals), 4 and 6 days. P. pestis could only be isolated from the inguinal lymph nodes on blood agar. At post-mortem the site of inoculation was not abnormal, there being no haemorrhages or oedema and the subcutaneous tissue also showed an absence of pathological changes. The inguinal lymph nodes of two

animals were enlarged, the spleens of the other two were small in size and the adrenals were haemorrhagic. The lungs showed severe pathological changes in the form of hyperaemia, haemorrhages and oedema and clear fluid was present in the pleural cavities of all four animals.

Animals inoculated with vaccine doses of 2.2×10^6 to 2.2×10^2 viable organisms survived.

Protection against virulent challenge.

All the surviving animals were challenged 23 days after vaccination with 21,500 virulent *P. pestis* strain MP6 grown at 28°C for 48 hours, inoculated subcutaneously in the right hind leg in 0.5 ml amounts. The following deaths were recorded after challenge:

Vaccine dose	Deaths	% mortality	Average day of death	Active Protection Index
2.2×10^7	0/6	0	> 21	> 210
2.2×10^6	4/10	40	6.3	15.7
2.2×10^5	8/10	80	6.1	7.6
2.2×10^4	8/10	80	4.0	5.0
2.2×10^3	10/10	100	2.8	2.8
2.2×10^2	10/10	100	2.9	2.9

The PD_{50} calculated by the method of Thompson is 927,600 viable EV76 (Paris) organisms for Praomys.

Post-mortem appearances of the animals which died were typical of acute septicaemic plague. Oedema and haemorrhages were present at the site of inoculation and in the subcutaneous tissue. The inguinal lymph nodes were enlarged as was the liver and the spleen. The cortex of the kidneys was pale and there was extensive lung involvement in the

form of haemorrhages and oedema. Pleural exudates were most often present. P. pestis was isolated from all the tissues cultured.

That the original EV76 strain has a residual virulence in animals is confirmed by this experiment. This characteristic is not revealed in normal mice even by intravenous inoculation, although some local and systemic reactions were observed in three primate species after intradermal inoculation of 26,800,000 viable EV 76 (Paris) organisms (Meyer, 1970).

The realisation that the immunogenic potency of the live EV plague vaccine deteriorated in the lyophilised state (Meyer, personal communication and observations on the EV51f vaccine in Praomys) prompted the testing of agar-grown cultures of these vaccines.

2. Pathogenicity and immunogenic efficacy of an agar-grown EV76 (Paris) vaccine in Praomys.

An isolate of the lyophilised EV76 (Paris) vaccine, stored in gelatine-agar pellets by the method of Stamp (1947), was grown on trypticase soy agar slopes at 37°C for 48 hours. The culture was then washed off in normal saline and total and viable counts were done.

Six dilutions of the vaccine were inoculated subcutaneously in 0.5 ml amounts in the right hind leg of Praomys using 10 animals per dilution.

The following numbers of organisms were inoculated into each animal:

	Total number of organisms per dose	Number of viable organisms per dose
I	6.4×10^8	5.5×10^8
II	6.4×10^7	5.5×10^7
III	6.4×10^6	5.5×10^6
IV	6.4×10^5	5.5×10^5
V	6.4×10^4	5.5×10^4
VI	6.4×10^3	5.5×10^3

The residual virulence of an agar-grown culture of EV76 (Paris) was reflected by the following deaths occurring after vaccination of Praomys:

Vaccine dose	Vaccination fatalities	Days of death	Average day of death
5.5×10^8	6/10	2, 3(2), 6, 8, 13	5.9
5.5×10^7	6/10	3(6)	3.0
5.5×10^6	2/10	2, 3	2.5
5.5×10^5	1/10	17 (intercurrent)	
5.5×10^4	1/10	17 (intercurrent)	

Autopsy and bacteriological findings were typical of bubonic-septicaemic plague: oedema and haemorrhages were present at the site of inoculation and in the subcutaneous tissue - most cases but not all, non-haemorrhagic lymphadenopathy was present in all but 5 animals, necrosis and enlargement of the liver and spleen, extensive lung involvement with haemorrhagic pleural exudates. P. pestis was isolated from inguinal lymph nodes, spleen, liver, kidneys, lungs and heart blood.

These vaccination fatalities clearly attest to the fact that the original EV76 isolate is invasive; it causes 20% fatalities when 5,500,000 organisms are inoculated or as high as 60% mortality with 55 million or 550 million viable organisms.

The survivors of this vaccine trial were challenged subcutaneously in the right hind leg 28 days later with 37,000 viable P. pestis strain MP6. Ten control unvaccinated Praomys were inoculated simultaneously. The agar-grown EV76 culture proved to be highly immunogenic in these doses, there being mortalities only in the groups inoculated with the

three lowest vaccine doses:

Vaccine dose	Vaccination fatalities	Deaths after challenge	% mortality after challenge	T* (days)	API
550 million	5/10	0/5	0	> 21	> 210
55 million	6/10	0/4	0	> 21	> 210
5.5 million	2/10	0/8	0	> 21	> 210
550,000	0/10	2/9	23	10.5	45.6
55,000	0/10	3/9	34	9.0	27.0
5,500	0/10	6/10	60	7.3	12.1

* T = mean time of death in days.

Death due to bubonic-septicaemic plague in the animals which died was confirmed bacteriologically and by autopsy. A PD_{50} value could not be calculated from these results following the high protection conferred by the large doses.

The agar-grown culture in this experiment possessed a slightly higher residual virulence and greater protective capacity against virulent challenge, than the lyophilised isolate. Whether this greater virulence of the agar-grown vaccine was real or merely coincidental had to be confirmed by a second experiment.

3. Comparative study of the pathogenicity and immunogenic efficacy of an agar-grown EV76 (Paris) vaccine in Praomys and white mice.

It has been shown by Meyer (1970) and others that white mice are not adversely affected by the subcutaneous inoculation of live attenuated plague vaccines of the EV strain. Whether the white mice used routinely at the South African Institute for Medical Research were similarly

unaffected had to be determined. The reaction of these animals could also simultaneously be compared with that of Praomys to the live EV plague vaccine.

An EV76 (Paris) culture was grown on agar at 37°C for 48 hours and suspended in normal saline. Tenfold dilutions were then prepared and 0.5 ml of the appropriate dilution was inoculated into the right hind leg of each of 15 Praomys and 10 white mice.

The doses inoculated into each animal were as follows:-

Group	Total number of organisms per dose	Number of viable organisms per dose
I	6.7×10^6	4.7×10^6
II	6.7×10^5	4.7×10^5
III	6.7×10^4	4.7×10^4
IV	6.7×10^3	4.7×10^3
V	6.7×10^2	4.7×10^2
VI	67	47

After vaccination 4 Praomys inoculated with 4,700,000 viable EV76 (Paris) died (days 2 (two animals), 3 and 4).

At post-mortem there was oedema at the site of inoculation without necrosis and one, which died on day 2, had haemorrhages. The subcutaneous tissue was hyperaemic and two had haemorrhages. The inguinal lymph node of only one, which died on day 2, was enlarged. The spleen, liver and kidneys were normal in appearance but the adrenals were haemorrhagic. The lungs were hyperaemic, haemorrhagic and oedematous, and there was straw coloured fluid in the pleural cavities. P. pestis was cultured on blood agar from the inguinal

lymph node of one, which died on day 2, and from the spleens of two others. Cultures of all other organs were negative. One animal had putrified and P. pestis could not be cultured.

Histological examination of the tissues revealed congestion of the liver with some small granulomatous areas of macrophages and a few polymorphonuclear leucocytes (fig. 23). Spleen sinuses were dilated with red cells and monocytes; giant cells were also present (fig. 24). Kidneys had marked glomerular congestion and necrosis of cells in the convoluted tubules (fig. 25). The lungs were congested, being particularly marked in areas below the pleura; air sacs filled with red cells, oedema fluid in groups of air sacs in lobular arrangements; monocytes and some polymorphonuclear infiltration into alveolar walls (fig. 26).

Five intercurrent deaths were recorded; groups I (2) (day 26), IV (day 28), V (day 26) and VI (day 29). All the white mice survived the vaccination.

Ten animals in each group were kept for virulent challenge and the remainder were bled for serological test on day 14 and day 30, after which they were sacrificed for autopsy and bacteriological culture. The serological response of Praomys to vaccination as determined by the haemagglutination test is shown by the following titres:

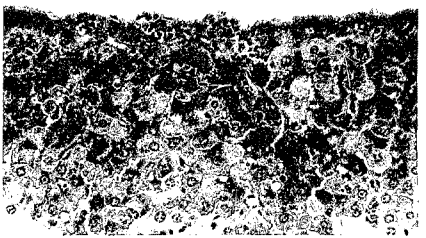


Fig. 23. Section of the liver of a Praomys (Mastomys) natalensis which died after subcutaneous injection of attenuated P. pestis strain EV76 (Paris). Showing a granulomatous area of macrophages and polymorphonuclear leucocytes; x 240.

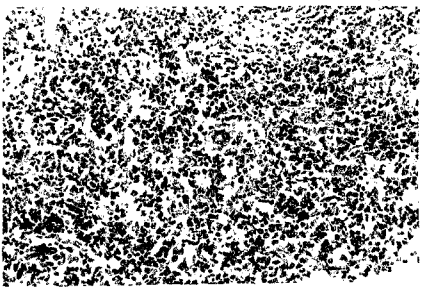


Fig. 24. Section of the spleen of a Praomys (Mastomys) natalensis which died after subcutaneous injection of attenuated P. pestis strain EV76 (Paris). Showing congestion of the sinuses and a giant cell; x 240.

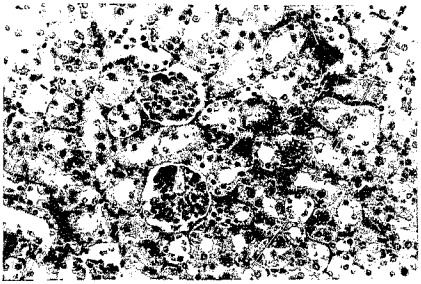


Fig. 25. Section of the kidneys of a Praomys (Mastomys) natalensis which died after subcutaneous injection of attenuated [illegible] strain EV76 (Paris). Showing congestion of the glomeruli and capillaries; x 240.

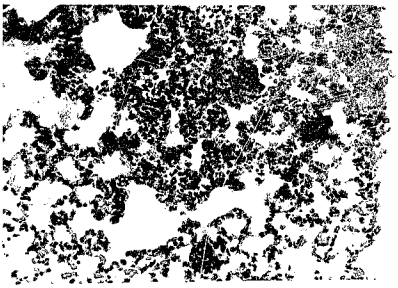


Fig. 26. Section of the lung of a Praomys (Mastomys) natalensis which died after subcutaneous injection of attenuated [illegible] strain EV76 (Paris). Showing congestion below the pleura, air face filled with red cells and oedema fluid and mononuclear and polymorphonuclear leucocyte infiltration into the alveolar walls; x 240.

Vaccine dose	Animal number	Reciprocal of HA titre 14 days after vaccination	Reciprocal of Geometric mean titre (day 14)	Reciprocal of HA titre 30 days after vaccination	Reciprocal of Geometric mean titre (day 30)
4,700,000	I:15	256	256	-	-
470,000	II:11	negative		128	
	II:12	256		256	
	II:13	64		32	
	II:14	32		256	
	II:15	256	42	0	48
47,000	III:11	64		128	
	III:12	64		512	
	III:13	0		256	
	III:14	0		32	
	III:15	0	5	512	194
4,700	IV:12	256		256	
	IV:13	64		0	
	IV:14	0		0	
	IV:15	0	11	32	9
470	V:12	0		0	
	V:13	0		0	
	V:14	0		0	
	V:15	0	0	0	0
47	VI:12	0		-	
	VI:13	0		-	
	VI:14	0		0	
	VI:15	0	0	0	0

Generally the serological titres were low and few animals inoculated with doses of 4,700 viable EV76 (Paris) or lower were positive. There was not much variation between the titres on day 14 and day 30, but it is difficult to explain the higher titres on day 30 than day 14 in animals inoculated with 4,700 organisms. Multiplication of the EV strain would not be expected after that time and the bacteriological cultures of tissues were negative in animals examined two weeks after vaccination.

Bacteriological cultures of the tissues of these animals bled for serology were negative when they were sacrificed 30 days after vaccination. There were no pathological changes in the tissues at post-mortem except the lungs of some which were hyperaemic and haemorrhagic in patches, which probably resulted from the anaesthesia.

Thirty days after vaccination ten white mice and ten Praomys in each vaccine group except the highest vaccine dose group in which there were only 8 survivors, were challenged with 25,000 virulent P. pestis strain MP6. The inoculum was suspended in 0.5 ml normal saline and injected subcutaneously into the right hind leg. Ten unvaccinated white mice and ten Praomys were included as controls. After challenge the following deaths of Praomys and white mice were recorded:

Praomys

Vaccine dose	Deaths after challenge	% mortality	Average day of death	API
4,700,000	2/8	25	8.5	34.0
470,000	1/10	10	6.0	60.0
47,000	4/10	40	9.0	22.5
4,700	5/10	50	4.2	8.4
470	8/10	80	3.0	3.7
47	10/10	100	2.5	2.5
Nil	10/10		3.5	3.5

White Mice

Vaccine dose	Deaths after challenge	% mortality	Average day of death	API
4,700,000	1/10	10	4.0	40
470,000	0/10	0	> 21	> 210
47,000	4/10	40	6.2	15.5
4,700	6/10	60	5.9	9.8
470	5/10	50	3.2	6.4
47	9/10	90	3.1	3.4
Nil	10/10	100	4.7	4.7

Bacteriological and autopsy findings of unvaccinated control white mice were typical of bubonic septicaemic plague. Haemorrhages and oedema were present at the site of inoculation; the inguinal lymph nodes were enlarged, as was the spleen and liver. Post-mortem findings of the vaccinated white mice which died after challenge differed from the controls in the pathology of the lungs. Hyperaemia, haemorrhages and in some cases oedema and a pleural exudate were present in the vaccinated animals whereas these changes were absent in the unvaccinated controls.

Autopsy findings of vaccinated Praomys which died after virulent challenge were similar to those of the white mice. Histological examination of the tissues showed the following pathological changes:

Liver: Congested and degenerative changes in the liver cells and some large granulomatous areas of polymorphonuclear leucocyte and macrophage infiltration and P. pestis masses (fig. 27).

Spleen: Marked congestion of venous sinuses and pulp spaces. Small areas of hyperplasia with necrosis surrounded by groups of leucocytes with large clusters of P. pestis (fig. 28).

Kidneys: Congestion of the glomeruli with few P. pestis in the blood

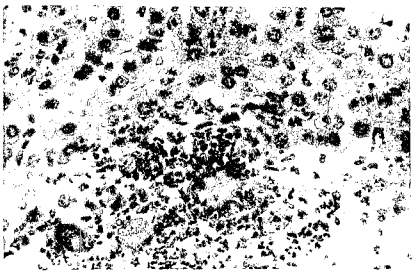


Fig. 27. Section of the liver of a Praomys (Mastomys) natalensis which had been immunised with the attenuated P. pestis strain EV76 (Paris), and died after virulent challenge. Showing a large granulomatous area of polymorphonuclear leucocyte and macrophage infiltration and P. pestis masses; x 375.

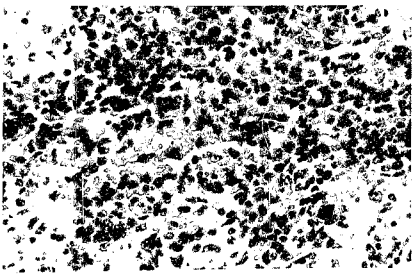


Fig. 28. Section of the spleen of a Praomys (Mastomys) natalensis which had been immunised with the attenuated P. pestis strain EV76 (Paris), and died after virulent challenge. Showing congestion of the sinuses, leucocytes and clusters of P. pestis; x 375.

vessels; necrosis of the convoluted tubular cells (fig. 29).

Lungs: Congestion, air sacs filled with cells and clumps of P. pestis surrounded by alveoli with homogeneous oedema fluid (fig. 30).

Heart: Large myocardial infarct seen in one specimen with masses of P. pestis spreading from the coronary vessel to the myocardial muscle fibres (fig. 31).

The 50% protective dose calculated by the method of Thompson was 4,700 viable EV76 (Paris) for white mice and 9,072 for Pregomys. The lower active protection index of the highest vaccine dose (4,700,000 viable organisms) compared with the second highest dose (470,000 viable organisms) in both white mice and Pregomys can probably be explained by the toxic effect of these large vaccine doses which left the animals in a slightly weakened state, possibly by damage of the immunological mechanism or blockage of the reticulo-endothelial system. They were then overcome by the virulent challenge dose.

4. Two doses of lyophilised EV76 (Paris) vaccine inoculated subcutaneously.

Immunisation of humans with live plague vaccines has mainly been done using a single dose, while two doses of killed vaccine are usually given with boosters periodically at 6 - 12 month intervals (Meyer, 1970, Girard, 1963, Grasset, 1962). Although it has been demonstrated that large doses of live EV vaccine immunise Pregomys these may be pathogenic to the extent of being lethal. Doses lower than 100,000 viable EV organisms inoculated in a single dose give only slight protection against virulent challenge. Although low doses were not immunogenic in Pregomys it was not known whether the animals were sensitized. A two dose schedule of the live EV 76 (Paris) vaccine was therefore inoculated in

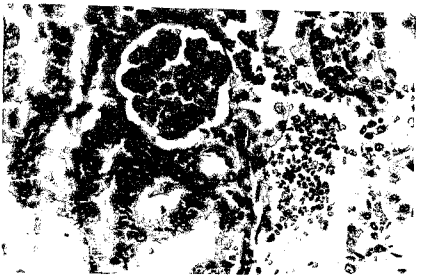


Fig. 29. Section of the kidney of a Pregwre (Mastomys) natalensis which had been immunized with the attenuated B. pertis strain BP6 (Paris), and died after virulent challenge. Showing congestion of the glomerulus and blood vessels; x 375.

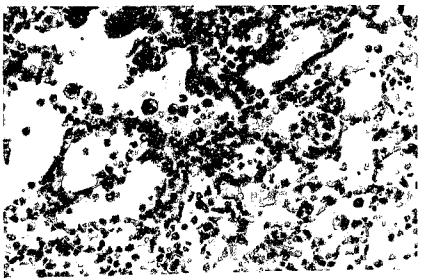


Fig. 30. Section of the lung of a Pregwre (Mastomys) natalensis which had been immunized with the attenuated B. pertis strain BP6 (Paris), and died after virulent challenge. Showing air sacs filled with cells and clumps of B. pertis and oedema; x 375.



Fig. 31. [illegible] of the heart of a Pracays (Mastomys) natalensis [illegible] been immunized with the attenuated P. pestis [illegible] (Paris) and died after virulent challenge. Showing myocardial infarct with masses of P. pestis spreading to the myocardial muscle fibres; x 375.

Praomys.

Five vials of BCG (Paris) vaccine Lot No. C-2 MFG Date Aug 1969 Walter Reed were pooled by the addition of 10 ml normal saline to each. After a total count on a 1:10 dilution in formol saline was done, dilutions of the vaccine were prepared in normal saline. Plate counts were done and 10 Praomys were inoculated subcutaneously in the right hind leg with 0.5 ml of each dilution. The numbers of organisms inoculated were as follows:

Group	Total count per dose	Viable count per dose
I	4.6×10^8	1.2×10^7
II	4.6×10^7	1.2×10^6
III	4.6×10^6	1.2×10^5
IV	4.6×10^5	1.2×10^4
V	4.6×10^4	1.2×10^3
VI	4.6×10^3	1.2×10^2

After vaccination one animal inoculated with 12,000,000 viable organisms died on day 2 and one inoculated with 12,000 died on day 7. All the others survived.

Fourteen days after primary vaccination the schedule was repeated using approximately the same doses. Actual counts of the second inoculation were as follows:

Group	Total count per dose	Viable count per dose
I	3.1×10^8	2.2×10^7
II	3.1×10^7	2.2×10^6
III	3.1×10^6	2.2×10^5
IV	3.1×10^5	2.2×10^4
V	3.1×10^4	2.2×10^3
VI	3.1×10^3	2.2×10^2

After this inoculation one animal vaccinated with 22,000,000 and one vaccinated with 22,000 viable organisms died on day 5 and the others survived.

The post-mortem appearance of the animal which died after the first inoculation of 12,000,000 EV76 (Paris) was typical of acute plague, with oedema and haemorrhages at the site of inoculation and in the subcutaneous tissue. The liver was enlarged but the spleen and kidneys appeared normal. The lungs were hyperaemic with some haemorrhages but there was no exudate in the pleural cavity. P. pestis was isolated from the liver, spleen and inguinal lymph nodes. Death of the single animal after primary inoculation of 12,000 EV76 (Paris) was intercurrent as all the tissues appeared normal and P. pestis was not isolated.

The pathological changes of the animal which died 5 days after the second inoculation of 22,000,000 viable EV76 (Paris)were less marked. The inguinal lymph nodes were enlarged and the subcutaneous tissue was hyperaemic and haemorrhagic. Other tissues appeared normal including the site of inoculation, liver, spleen and kidneys, although the lungs were slightly hyperaemic. P. pestis was isolated from the inguinal lymph nodes. The death on day 5 of the animal vaccinated with 22,000 organisms was intercurrent.

All the surviving animals were challenged 23 days after the second vaccination with 21,500 virulent P. pestis strain MP6 suspended in 0.5 ml normal saline inoculated subcutaneously in the right hind leg. The following deaths were then recorded:

Vaccine dose	Vaccination deaths	Deaths after challenge	% mortality	Average day of death	API
12,000,000 22,000,000	2/10	0/8	0	>21	>210
1,200,000 2,200,000	0/10	1/10	10	>21	>210
120,000 220,000	0/10	6/10	60	6.1	9.8
12,000 22,000	0/10	7/8	88	3.3	3.7
1,200 2,200	0/10	9/10	90	3.1	3.4
120 220	0/10	10/10	100	3.4	3.4

The animals which died after virulent challenge had gross pathological and bacteriological manifestations of bubonic-septicaemic plague.

As it can be assumed that the active protection index is directly related to the mass of protective antigen(s) available for antibody production (Burrows and Bacon, 1958), it is evident from the above experiments with sirgle and two EV76 (Paris) vaccine doses that low numbers of these organisms were unable to multiply significantly in Praomys. A single dose of 2,200,000 viable bacilli reconstituted from the lyophilised state, conferred some protection (API=15.7) and this was greatly increased by a second dose of approximately the same number of micro-organisms (API raised to>210). A low degree of immunity was conferred on Praomys by a single dose of 220,000 viable bacilli with a resultant API of 7.6. After a second dose of approximately the same number of bacilli, the active protection index was

raised to 9.8. Lower doses of the vaccine than these however, protected very few Praomys against virulent challenge.

5. Vaccination with a live lyophilised EV76 (Paric) vaccine after primary immunisation with a single dose of killed vaccine.

One of the main disadvantages of a live attenuated plague vaccine is the unpleasant local and systemic reactions which they cause. Girard (1963) however, attributes the high immunogenicity of the EV strain to its partial virulence and the reactions it causes. This low pathogenicity has been shown to be readily detectable in Praomys by death after subcutaneous inoculation.

The following experiment was done to determine whether the pathogenicity of the EV76 strain could be reduced by primary immunisation with a single dose of killed plague vaccine. The USP plague vaccine (E medium) Bulk Lot No. M037961 was diluted 1:5 in normal saline, to contain 84 million organisms per 0.2 ml. This dose was then inoculated subcutaneously in the right hind leg of each of 10 Praomys. Two weeks later 5 vials of the live vaccine EV76 - Girard Lot No. G-2 MFG Date Aug 1969 Walter Reed were reconstituted with 10 ml normal saline per vial. A 1:10 dilution was done in 1.5% formol saline for a total count and tenfold dilutions were done in normal saline for plate counts. 0.5 ml of the neat suspension was then inoculated subcutaneously into the right hind leg of each of the 10 Praomys. This dose, which contained 3.1×10^8 total and 2.2×10^7 viable organisms, caused 10 - 40% mortality in non-vaccinated Praomys.

After the inoculation of the live vaccine 2 Praomys died on the second and third day. The organisms were only isolated from the lymph nodes

and at autopsy there were few pathological changes. There were no signs of haemorrhages or oedema at the site of inoculation or in the subcutaneous tissue and the liver, spleen and kidneys were normal in appearance. The inguinal lymph nodes were enlarged and the lungs were severely affected; hyperaemia, haemorrhages, oedema and pleural exudates were present.

The death of these animals reveals that 85,000,000 killed P. pestis in a single inoculation will not protect Praomys when, on the 14th day they are reinoculated with a live EV vaccine in a dose which causes 10 - 40% mortality in non-vaccinated Praomys. The pathological changes indicated that immunity was not sufficient to inhibit the invasiveness of the organisms and death due to severe toxaemia in the lungs ensued.

The 8 surviving Praomys were challenged 23 days later with 21,500 viable virulent P. pestis strain M76. One animal died on day 9 of bubonic-septicaemic plague; the remainder survived.

CHAPTER 7.

Immunisation of Praomys with a live attenuated plague vaccine using combined oral and subcutaneous routes of administration.

Attempts to reduce the reactions to inoculation of live plague vaccines in man and experimental animals have been made by investigating various routes of inoculation. Thus the intracutaneous route has been used in the U.S.S.R. and in the U.S.A. in experimental animals and human trials. The reactions caused by the live plague vaccines are less severe when administered by this route than when they are inoculated subcutaneously (Korobkova, 1955). Meyer (1970) has found however, that these vaccines are still sufficiently invasive to cause death in susceptible animals when administered intradermally.

Administration via the oral route has been used to a more limited extent in experimental animals. It has been shown that large doses of up to 1,000 million organisms of live vaccine administered orally are harmless to guinea pigs (Korobkova et al., 1970). The administration of EV vaccine strains via the oral route or in combination with intracutaneous inoculation conferred solid protection on guinea pigs.

The purpose of the present experiment in Praomys was to determine whether the EV51f plague strain is pathogenic in this animal species when administered orally and whether immunity could be established in them by this technique, and also to investigate the effectiveness of combined oral administration and subcutaneous inoculation.

An EV51f culture grown on agar at 37°C for 48 hours was suspended in normal saline and two dilutions containing 5×10^8 and 1.5×10^9 viable organisms per ml were prepared. One hundred and ten Praomys were

divided into 11 groups of 10 animals and placed in separate cages. The two vaccine dilutions were then administered orally in 0.2 ml amounts, using 18 gauge 2 inch Luer-lock hypodermic syringe needles of which the ends had been rounded off. The vaccine was introduced slowly into the buccal cavity at the back of the mouth so the animal swallowed. Each Praomys in four groups of 10 received 100 million bacilli orally and each animal of 4 other groups received 300 million bacilli orally.

Three of the groups which received 100 million bacilli orally and three which received 300 million, were inoculated subcutaneously immediately afterwards with dilutions containing 100,000; 10,000 or 1,000 viable bacilli. These dilutions were then also inoculated into each of 10 animals which had not received an oral administration of the vaccine.

All the animals to which 100 million bacilli had been administered orally, including those animals which received combined oral and subcutaneous inoculations, were given second and third doses of 100 million bacilli orally on the two following days. These were freshly grown cultures for the second and third oral administrations.

The doses inoculated are summarised in the following table:

Group (10 animals in each)	Dose	Number of inoculations	Route
1	100 million	3	oral
2	300 million	1	oral
3	100,000	1	subcutaneous
4	100 million 100,000	3 1	oral subcutaneous } combined
5	300 million 100,000	1 1	oral subcutaneous } combined

Group (10 animals in each)	Dose	Number of inoculations	Route	
6	10,000	1	subcutaneous	
7	100 million	3	oral	combined
	10,000	1	subcutaneous	
8	300 million	1	oral	combined
	10,000	1	subcutaneous	
9	1,000	1	subcutaneous	
10	100 million	3	oral	combined
	1,000	1	subcutaneous	
11	300 million	1	oral	combined
	1,000	1	subcutaneous	

Eleven animals in the following groups died after vaccination:

Group	Vaccination deaths		Days of death after first inoculation (average)
	Number	Percentage	
1	1/10	10	6
2	1/10	10	5
3	2/10	20	5,7 (6)
7	2/10	20	2,7 (4.5)
8	3/10	30	3,5,7 (5)
10	1/10	10	1
11	1/10	10	7

Post-mortem findings of those animals which died after vaccine administration.

a) Deaths after oral administration

Group 1 (100 million) and Group 2 (300 million)

The subcutaneous tissue of the abdomen and thorax was hyperaemic and haemorrhagic. Inguinal lymph nodes were not enlarged but on culture produced a confluent growth of *P. pestis*. Spleens

were hyperaemic and grossly enlarged to about twice the normal size. Histologically there was severe congestion of the sinusses and P. pestis in large bacterial masses (fig. 32). Confluent growths of P. pestis were cultured. Livers were normal in size, pale and mottled in appearance.

Histologically there was congestion of the sinusoids, granulomatous areas of monocyte infiltration and P. pestis in large bacterial masses (fig. 33). Bacteriological culture showed confluent growths of P. pestis. Kidneys were pale and flabby and histologically there was congestion of the blood vessels, but no P. pestis were seen. From each kidney more than 50 P. pestis colonies were cultured.

The lungs were normal in appearance at autopsy and on histological examination, but more than 50 P. pestis colonies were cultured from each lung. Cultures of the heart blood produced 30 P. pestis colonies from one animal and a confluent growth from the other.

b) Deaths after subcutaneous inoculation (Group 3 - 100,000 organisms)

The subcutaneous tissue was hyperaemic and haemorrhagic and the inguinal lymph nodes were slightly enlarged.

Bacteriological culture of the latter resulted in a confluent growth of P. pestis. The spleens were grossly enlarged and flabby. Histologically there was severe congestion of the red pulp with a large increase in mononuclear cells, and clumps of P. pestis were present throughout the red pulp. A confluent growth of P. pestis was cultured. The livers were pale and mottled in appearance and sections showed there was an increase in monocytes

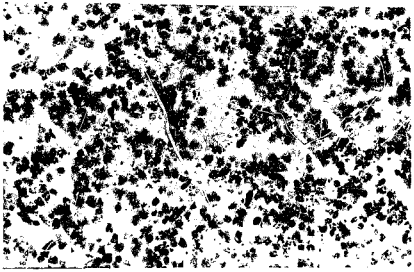


Fig. 32. Section of the spleen of a Praomys (Mastomys) natalensis which died after oral administration of 100 million attenuated P. pestis strain EV51f. Showing congestion of the sinuses and large bacterial masses; x 375.

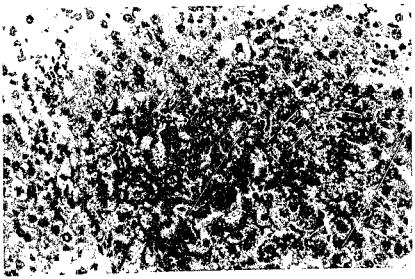


Fig. 33. Section of the liver of a Praomys (Mastomys) natalensis which died after oral administration of 100 million attenuated P. pestis strain EV51f. Showing congestion of the sinusoids and a granulomatous area; x 240.

and Kupffer cells. Bacterial masses of P. pestis were also seen in the sinusoids. Bacteriological culture showed a confluent growth of P pestis.

The kidneys were pale and flabby. Histologically there was congestion of the vessels and glomeruli but no P. pestis were seen. More than 50 P. pestis colonies were cultured per inoculum. The lungs were hyperaemic and haemorrhagic in patches. Some was present but no fluid was seen in the pleural cavities. Histology: some oedema fluid in air spaces, but lungs almost completely consolidated by macrophage and polymorphonuclear cell infiltration. Many red blood cells amongst nucleated cell masses. Clumps of P. pestis amongst the nucleated cells. Culture: confluent growth of P. pestis.

c) Deaths after combined oral administration and subcutaneous inoculation

Group 10. (100 million oral combined with 1,000 subcutaneous)

Death of the animal in Group 10 on day 1 may have been the result of pneumonic infection resulting from aspiration of the vaccine. The lungs were filled with bloodstained fluid at post-mortem and counts of P. pestis colonies from the tissues were as follows: inguinal lymph node 2, spleen 50, kidneys 8, liver 50, lungs confluent, heart blood confluent.

Groups: 7 (100 million oral combined with 10,000 subcutaneous)
8 (300 million oral combined with 10,000 subcutaneous)
11 (300 million oral combined with 1,000 subcutaneous)

The subcutaneous tissue of the thorax and abdomen was hyperaemic, and some oedema was present. Inguinal lymph nodes were slightly

enlarged and bacteriological cultures were confluent growths of P. pestis in all except Group 11, which was negative. Spleens were slightly enlarged or normal in size and histologically there was congestion of the sinuses and an increase in mononuclear cells. Cultures showed confluent growth of P. pestis in all except Group 11, which was negative. Kidneys were pale in colour and flabby in texture. In section there was congestion of the glomeruli with red blood cells and mononuclear cells. There were confluent growths of P. pestis on culture in all except Group 11, which was negative. Livers were pale and mottled in appearance and histologically there was slight congestion of the sinusoids, with an increase in monocytes. The cultures showed confluent growths of P. pestis in all except Group 11, which was negative. Lungs were hyperaemic and had patchy haemorrhages, with oedema present in all. Sections showed oedema fluid was present in the air spaces. Cultures of all the animals had confluent growths of P. pestis. Heart blood cultures showed confluent growths of P. pestis.

Live attenuated P. pestis strain EV51f is invasive and causes toxic-bacteraemic deaths in Praomys even when administered via the oral route and not merely on subcutaneous inoculation. The organisms spread from the buccal cavity, probably via the submaxillary or retropharyngeal lymph nodes to the blood stream and hence to all the tissues. In a laboratory experiment with subhuman primates (Macacus rhesus and M. cynomolgus philippinensis), Meyer and Larson (1960) showed that virulent P. pestis could enter the upper respiratory tract through the lymphatic tissues of the oropharynx. The afferent lymphatics then carried the plague

bacilli to the regional lymph nodes and induced primary buboes, and septicaemia followed rapidly. This is also the most likely route of invasion of the EV51f strain in Praomys after oral administration. No pathological differences could be detected between those Praomys which died after subcutaneous inoculation of the EV51f strain and those which died after oral administration.

Twenty one days after administration of the vaccine all the animals were challenged subcutaneously with the virulent P. pestis strain MP6 in a dose of 18,000 viable organisms, and ten unvaccinated controls were challenged simultaneously.

Results of the challenge are tabulated as follows:

Group	Vaccine dose	Number of inoculations	Route	Challenge survivors Number	%
1	100 million	3	oral	9/9	100
2	300 million	1	oral	6/9	88
3	100,000	1	subcut.	7/8	87
4	100 million + 100,000	3 1	oral subcut.	10/10	100
5	300 million + 100,000	1 1	oral subcut.	10/10	100
6	10,000	1	subcut.	5/10	50
7	100 million + 10,000	3 1	oral subcut.	7/8	87
8	300 million + 10,000	1 1	oral subcut.	7/7	100
9	1,000	1	subcut.	4/10	40
10	100 million + 1,000	3 1	oral subcut.	9/9	100
11	300 million + 1,000	1 1	oral subcut.	7/9	78

All the deaths were confirmed as bubonic-septicaemic plague by autopsy and bacteriological culture.

This experiment revealed that a dose of 100 million live attenuated P. pestis strain EV51f will confer solid protection on Praomys against virulent challenge, when vaccinated via the oral route on three successive days. The same total dose when given in one inoculation orally gives slightly less protection (88%). A single dose of 100,000 EV51f organisms inoculated subcutaneously will confer the same protection on Praomys as 300 million organisms administered orally. The subcutaneous inoculation of 100,000 or 10,000 organisms given simultaneously to the oral dose of 300 million, will raise the protection of the oral dose from 88% to 100%. A dose of 1,000 organisms, inoculated subcutaneously does not enhance the protection of a single oral dose of 300 million organisms.

Summary of results of oral and subcutaneous administration of attenuated P. pestis strain EV51f in Praonys.

Group	Basic vaccination	Simultaneous inoculation	Followed by second and third oral admin. on successive days	Post vaccination fatalities	Results of challenge Survivors/Total	(%
2	300 million oral	none	no	1/10	8/9	(88
5	300 million oral	100,000 subcutaneous	no	none	10/10	(100
8	300 million oral	10,000 subcutaneous	no	3/10	7/7	(100
11	300 million oral	1,000 subcutaneous	no	1/10	7/9	(78
1	100 million oral	none	100 million each dose	1/10	9/9	(100
4	100 million oral	100,000 subcutaneous	100 million each dose	none	10/10	(100
7	100 million oral	10,000 subcutaneous	100 million each dose	2/10	7/8	(87
10	100 million oral	1,000 subcutaneous	100 million each dose	* 1/10	9/9	(100
3	100,000 subcutaneous	none	no	2/10	7/8	(87
6	10,000 subcutaneous	none	no	none	5/10	(50
9	1,000 subcutaneous	none	no	none	4/10	(40

* Probable aspiration pneumonia.

CHAPTER 8.

Vaccination of Praomys with two attenuated P. pestis strains lacking different single virulence determinants.

The importance of the virulence determinants in the immunogenicity of P. pestis has been thoroughly investigated by Burrows and Bacon (1958). From their work they concluded that Fl antigen is of great importance in conferring immunity against experimental infection with virulent Fl+ strains. In addition it was shown that VW antigens are not less important for this purpose. Maximum protection was achieved by the use of vaccines containing both Fl and Vw antigens. They also concluded that the ability of strains to form pigmented colonies (P+) may be associated with a product which may be antigenic and play an important role in immunity.

Considering the importance of these three virulence factors in immunity to plague, it was decided to immunise Praomys with two attenuated P. pestis strains, which together possessed the characters Fl+, VW+ and P+, to determine whether these would induce a higher level of immunity than a sirgle attenuated strain lacking either VW or P.

The two attenuated P. pestis strains EV76 (Paris) and Tjiwidej S, both of which have been used extensively for human immunisation, were selected for this investigation. EV76 (Paris) is Fl+, VW+ and P- (Burrows and Bacon, 1958) but a certain percentage of stock culture populations are known to lack other virulence determinants, such as pesticin . (Surgalla et al., 1970), and may also be uracil dependent (Meyer personal communication). The EV76 (Paris) strain used in this experiment was a subculture obtained by Dr. K.F. Meyer, of the original EV76 strain maintained at the Institut Pasteur, Paris. Tjiwidej S is

Fl+, VW- and P+. The classical strain "Tjiwidej" Smooth described by Otten (1936) was originally obtained from a plague rat at Tjiwidej in Netherlands East Indies. A culture of this strain has been repeatedly purified at the Microbiological Research Establishment, Porton by single colony selection and maintained in the dry state (Burrows and Bacon, 1953a). Through the courtesy of Dr. T.W. Burrows, a subculture of this strain was obtained in October 1968.

The 50% protective dose of the Tjiwidej strain was first determined in Praomys by subcutaneous inoculation of 6 dilutions of a culture grown on agar at 37°C for 48 hours.

The following numbers of organisms were inoculated:

Group	Number of animals	Total number of organisms	Number of viable organisms
1	10	1.5×10^7	1.3×10^7
2	10	1.5×10^6	1.3×10^6
3	10	1.5×10^5	1.3×10^5
4	10	1.5×10^4	1.3×10^4
5	10	1.5×10^3	1.3×10^3
6	10	1.5×10^2	1.3×10^2

The animals were examined daily and all except one in group 3, which died of an intercurrent infection on day 18, survived the vaccine inoculation. After 21 days all these animals were challenged subcutaneously with the virulent P. pestis strain EP6. This culture was incubated at 28°C for 24 hours and suspended in normal saline.

From the total count it was calculated that each animal received a dose of 38,000 organisms in a 0.5 ml amount. Ten non-vaccinated

control Praomys were also inoculated simultaneously.

After challenge the following deaths were recorded:

Group	Vaccine dose	Survivors/ total	Percent mortality	Average day of death	API
1	13,000,000	9/10	10	10.0	100
2	1,300,000	9/10	10	7.0	70
3	130,000	4/9	44	4.8	10.8
4	13,000	6/10	40	4.0	10.0
5	1,300	3/10	70	4.7	6.7
6	130	2/10	80	3.2	4.0
Control	nil	0/10	100	2.9	2.9

Deaths were confirmed bacteriologically and by autopsy as due to bubonic-septicaemic plague. No difference could be seen in the autopsy appearances of the animals inoculated with different vaccine doses. The 50% protective dose calculated by Thompson's moving averages was 23,120 viable organisms.

Vaccination of Praomys with two attenuated P. pestis cultures lacking different single virulence antigens was carried out as follows: cultures of Tjiwidej and EV76 (Paris) were inoculated onto trypticase soy agar and incubated at 37°C for 48 hours. These were then suspended in normal saline and total counts done.

Comparison of the protection conferred by inoculating two attenuated P. pestis cultures lacking different single virulence antigens with that of two doses of the same strain, and also single doses of each strain was carried out according to the following summary:

First inoculation	Second inoculation (one month later)	Virulent challenge (one month after second vaccination)
EV76 (Paris)	EV76 (Paris)	MP6
TS	TS	MP6
TS	EV76 (Paris)	MP6
TS	-	MP6
EV76 (Paris)	-	MP6
	TS	MP6
	EV76 (Paris)	MP6

Cultures of the Tjiwidej and EV76 (Paris) P. pestis strains were planted onto trypticase soy agar and incubated at 37°C for 48 hours. These were then suspended in normal saline and total counts done. Tenfold dilutions of each culture were then prepared in normal saline and plate counts done on the highest dilutions.

Thirty Praomys were inoculated subcutaneously with the 10^{-4} dilution of the Tjiwidej culture containing 30,000 organisms, of which 15,000 were viable. In addition, the other dilutions were inoculated subcutaneously into each of 5 Praomys per dilution for serological test. Thus the numbers of animals inoculated with the Tjiwidej strain were as follows:

Group	Total number of organisms	Number of viable organisms	Number of Praomys inoculated
1	3.0×10^8	1.5×10^8	5
2	3.0×10^7	1.5×10^7	5
3	3.0×10^6	1.5×10^6	5
4	3.0×10^5	1.5×10^5	5
5	3.0×10^4	1.5×10^4	35
6	3.0×10^3	1.5×10^3	5

At the same time each of twenty Praomys were inoculated subcutaneously with 9,600 EV76 (Paris) organisms.

The 5 Praomys in each group were then bled 28 days after vaccination and the sera tested for HA antibodies to the Fraction 1 antigen. The titres obtained are as follows:

Group	Number of viable Tjiwidej organisms	Animal number	Reciprocal of HA titre	Reciprocal of Geometric mean titre
1	150,000,000	1	1024	
		2	512	
		3	1024	
		4	1024	
		5	2048	7024
2	15,000,000	6	1024	
		7	512	
		8	128	
		9	1024	
		10	256	445
3	1,500,000	11	128	
		12	256	
		13	128	
		14	256	
		15	128	169
4	150,000	16	32	
		17	128	
		18	256	
		19	32	
		20	128	64
5	15,000	21	8	
		22	0	
		23	8	
		24	64	
		25	8	8
6	1,500	26	4	
		27	16	
		28	8	
		29	8	
		30	4	7

The serological response of each animal was not strictly related to the dose inoculated; however there was an association between the size of the vaccine dose and the geometric mean titre. Doses of 15,000 viable organisms or less resulted in very few serological reactors, but higher doses resulted in geometric mean titr s of 1:84 or over.

Twenty eight days after the first inoculation, booster doses were given. The EV76 (Paris) and Tjiwidej strains were cultured on trypticase soy agar at 37°C for 48 hours. The culture was suspended in normal saline, total counts done, and tenfold dilutions prepared. Plate counts were done on the highest dilution. Ten Praomys previously inoculated with the EV76 (Paris) vaccine were reinoculated subcutaneously with 13,000 organisms of the same strain. Ten Praomys previously inoculated with the Tjiwidej vaccine were also reinoculated with the same EV76 (Paris) dose and ten previously uninoculated animals were vaccinated to act as booster controls. Ten Praomys previously inoculated with Tjiwidej vaccine were reinoculated subcutaneously with 11,000 viable organisms of the same strain and ten uninoculated animals were vaccinated with this dose as booster controls. Ten Praomys inoculated with the Tjiwidej strain and ten vaccinated with the EV76 (Paris) strain were not revaccinated.

All the animals survived the vaccine inoculations. Twenty eight days after the second vaccination, they were challenged subcutaneously with a virulent culture of strain MP6 in a dose of 25,500 viable organisms. This culture was grown at 28°C for 24 hours on trypticase soy agar. Ten unvaccinated control Praomys were also challenged simultaneously. After challenge the following deaths were recorded:

Vaccination schedule: First inoculation / Second inoculation	EV76 / EV76	TS / TS	TS / EV76	TS / -	EV76 / -	- / TS	- / EV76	Control
Deaths after challenge	3/10	3/10	1/10	5/10	8/10	4/10	5/10	10/10
Percent mortality	30	30	10	50	80	40	50	100
Average day of death	14.3	10.0	5.0	6.0	5.0	7.8	8.0	3.1
A.P.I.	47.7	33.3	50.0	12.0	6.3	19.5	16.0	3.1

The 50% protective dose of an agar-grown EV76 (Paris) culture in Praomys as determined by previous experiments was 4,700 viable organisms inoculated subcutaneously. When a dose of 13,000 organisms was inoculated into Praomys in this experiment and these animals were challenged one month later, 50% of the animals survived, the A.P.I. being 16.0. When 9,600 organisms were inoculated and the animals challenged 2 months later, there was 80% mortality and an A.P.I. of 6.3. The immunity conferred by this vaccine had therefore declined over these two months.

The 50% protective dose of an agar-grown Tjiwidej culture was 23,120 organisms by subcutaneous inoculation in Praomys. When a dose of 11,000 organisms of this culture was inoculated subcutaneously, 40% of the animals succumbed to virulent challenge one month later and resulted in an A.P.I. of 19.5. A dose of 15,000 viable organisms conferred 50% protection against virulent challenge 2 months later, with an A.P.I. of 12.0. Some reduction in the immunity conferred by this vaccine therefore also appeared to have taken place over these two months.

Two doses of the EV76 (Paris) vaccine given one month apart protected 70% of Praomys against virulent challenge, the A.P.I. being 47.7. The second dose had a booster effect on the immunity and raised the protection index considerably above those of the single dose.

A second dose of the Tjiwidej strain had a similar booster effect on the immunity. Comparison of the protection conferred by two doses of the Tjiwidej strain with that of two doses of the EV76 (Paris) strain shows that the latter resulted in a higher protection index but the percent mortality was the same.

Immunisation with an EV76 (Paris) vaccine one month after an initial dose of Tjiwidej vaccine resulted in the highest rate of protection (90%) and the A.P.I. (50.0) was higher than that using two doses of EV76 (Paris) vaccine (47.7) or two doses of the Tjiwidej strain (33.3).

CHAPTER 9.

Vaccination of Praomys with an attenuated non-pigmented, Fraction 1-negative P. pestis strain.

It was reported by Janssen and Surgalla (1969) that a non-capsulated variant of P. pestis was more immunogenic in Rhesus monkeys (Macaca mulatta) than other live vaccines used for human inoculation. The isolate did not cause serious illness to Rhesus monkeys, even when relatively large numbers of organisms were administered and protected these animals against challenge with typical virulent plague strains. The non-pigmented variant was selected from the non-capsulated strain N23 described by Burrows (1957) and possessed the following characteristics:

pigment -, F1-, Purine dependence -, Pesticin 1+, Pesticin 11+, Fibrin + (Meyer, 1970).

A freeze-dried preparation of the non-pigmented N23 strain was manufactured by the Walter Reed Institute for Medical Research, Washington and made available for testing in Praomys. Tests done by Meyer (1970) showed that the freeze-dried preparation was well tolerated by guinea pigs in doses of 27 million organisms inoculated by the intraperitoneal and subcutaneous routes. The PD_{50} values in these animals were 1,300 following intraperitoneal and 2,200 following subcutaneous vaccination. The same preparation as used in the guinea pigs proved lethal to 50% of mice when inoculated intravenously in doses of 500 viable organisms.

Five vials of the vaccine NPM - 23V Lot No. NPM -2 Dec 68 were pooled by rehydrating each vial with 10 ml normal saline and adding the total amount to a 100 ml Erlenmeyer flask. A total count was then done on a

tenfold dilution in 1.5% formol saline using a counting chamber. Tenfold dilutions of the vaccine pool were made in normal saline and plate counts were done.

Fifty Praomys were divided into 5 groups of 10 and each animal placed in a separate cage. The vaccine dilutions were inoculated subcutaneously in 0.5 ml amounts and each animal received the following number of organisms calculated from the total and plate counts:

Group (10 Praomys in each)	Total number of organisms inoculated	Number of viable organisms inoculated
I	1.1×10^9	1.7×10^8
II	1.1×10^7	1.7×10^6
III	1.1×10^6	1.7×10^5
IV	1.1×10^4	1.7×10^3
V	1.1×10^3	1.7×10^2

Three animals inoculated with 170 million viable non-pigmented M23 organisms died; one on day 2 and two on day 4. All the remaining animals survived. A post-mortem of the animal which died on the second day after vaccination showed hyperaemia and haemorrhages at the site of inoculation and subcutaneous tissue with an absence of oedema. The inguinal lymph nodes were enlarged and the adrenals were enlarged and haemorrhagic. The lungs were hyperaemic but there was no oedema of pleural fluid. Cultures of the inguinal lymph nodes, spleen and lungs were positive for P. pestis. Pathological changes in the animals which died on the fourth day after vaccination were more extensive than those which died on the second day. Again there was an absence of oedema at the site of inoculation and in the subcutaneous tissue but the spleens were enlarged and the lungs were hyperaemic, haemorrhagic and oedematous. Muco-haemorrhagic fluid was present in the pleural cavities.

P. pestis was isolated from the liver, spleen and kidneys of one of these animals and from the inguinal lymph node of the other.

The non-pigmented M23 strain is sufficiently invasive in Praomys to cause mortality when inoculated in high doses. The viable doses of non-pigmented M23 bacilli inoculated in this experiment were the same as those of the lyophilised EV51f strain inoculated in Praomys (Chapter 3). Comparing the results of these two experiments the non-pigmented M23 strain may have a slightly lower pathogenicity than the lyophilised EV51f; the non-pigmented M23 strain caused 30% vaccination fatalities in a dose of 170 million but no mortality in lower doses and the EV51f strain caused 30% and 10% vaccination fatalities in doses of 170 million and 1.7 million respectively.

Twenty eight days after vaccination the Praomys were challenged with virulent P. pestis strain MP6. The culture was grown for 18 hours at 37°C on trypticase soy agar and suspended in normal saline. A total count was done in a counting chamber and tenfold dilutions of the neat suspension prepared in normal saline. Plate counts were done on 5 blood agar plates. Each vaccinated Praomys and 10 control unvaccinated Praomys were inoculated subcutaneously in the right hind leg with 0.5 ml of the 10^{-5} dilution of the virulent P. pestis suspension, which contained a total of 3,900 organisms of which 2,150 were viable. After virulent challenge all but one animal died; the deaths are tabulated as follows:

Vaccine dose (viable non-pigmented M23 organisms)	Vaccination mortality	Average day of death after vaccination	Challenge mortality	Average day of death after challenge
170,000,000	3/10 = 30%	3.3	6/7 = 86%	4.0
17,000,000	0/10	0	10/10 = 100%	3.7
170,000	0/10	0	10/10 = 100%	3.5
1,700	0/10	0	10/10 = 100%	4.5
170	0/10	0	10/10 = 100%	3.9
unvaccinated controls	-	-	10/10 = 100%	3.1

Post-mortem appearances and bacteriological findings were typical of bubonic-septicaemic plague.

The strain M23 originally studied by Burrows and Bacon (1958) did not produce F1 antigen (F1-), produced V and W antigens (VW+), formed pigmented colonies (P+) and was independent of purines for growth (Pu+). This isolate was fully virulent in white mice. A mutant (strain M15) of this strain which was non-pigmented but retained the other characteristics, had attenuated virulence in white mice but was non-protective against virulent challenge.

Meyer (1970) inoculated the non-pigmented M23 mutant described by Janssen into three species of non-human primates; Hanuman langurs (Presbytis entellus), vervets (Cercopithecus aethiops) and macaques (Macaca mulatta). Striking differences in the susceptibility of these three primate species to the inoculation of this mutant were observed. All the macaques survived the vaccination but 2 of the 6 vervets and all the langurs died between the seventh and nineteenth day after inoculation, as a result of invasion of the organisms. When the strain was inoculated into Praomys in a dose of 170 million viable organisms, 3 out of 10 animals

died, but all those inoculated with lower doses survived. After virulent challenge, one of 4 Cercopithecus and all of four Macaca mu atta vaccinated with the non-pigmented M23 mutant survived (Meyer, personal communication). Only one of 57 Praomys vaccinated with these organisms survived the challenge dose.

These results strongly emphasise the importance of the animal species in investigating plague vaccines. Macaque monkeys were only slightly affected by inoculation of the non-pigmented M23 strain and were completely protected against virulent challenge; 2 of 6 vervet monkeys died after inoculation of the strain and one of the survivors was protected; all of 5 langurs died after inoculation of the strain. There was an attenuated virulence of the strain in Praomys and only slight protection of the survivors. The behaviour of the strain in Praomys was similar to that of strain M18 in white mice described by Burrows (1956). This strain has the same genetic constitution as the non-pigmented M23 mutant.

Until further work is done on the infection of various animal species with non-pigmented, F1- P. pestis strains, one can only speculate on the reasons for the pathogenicity in some and immunogenicity in other animals. The difference in invasiveness of the non-pigmented M23 strain in the 3 primate species and Praomys may be attributable to differences in availability of iron in these animals. This is known to be a limiting factor for the virulence of non-pigmented P. pestis strains (Jackson and Burrows, 1956b). The pigmented parent strain of this vaccine is fully virulent in mice. Compensation for the P- factor in the mutant, with differences in availability of iron, may result in a manifestation of differences in virulence of this vaccine, as observed in these animal species. The low rates of protection of vervet monkeys

and Praomys are in agreement with results of previous work on vaccine strains which lack the F1 antigen. The solid protection conferred in macaques is unexplained and will have to be clarified by further work on this species as suggested by Meyer (1970).

The absence of peritoneal and pleural transudates in fatal M23 vaccinations in langur monkeys, in contrast to EV51f infections, was noted by Meyer (1970). In Praomys septicaemic death, following subcutaneous inoculation of the non-pigmented M23 strain, was characterised by an absence of oedema in the lungs and subcutaneous tissues and also the absence of fluid in the pleural cavity.

Meyer suggested that these observations in langurs were associated with changes in the water metabolism as a result of non-specific diarrhoea and the inability, or unwillingness, of the animals to replace the loss by drinking water. As the absence of oedema was common to both langurs and Praomys, one may speculate that these pathological changes are attributable to a characteristic of the attenuated P. pestis strain, notably the absence of F1 antigen. This is certainly an aspect of the pathology of plague which requires further investigation.

CHAPTER 10.

Immunogenic efficacy of killed *plague vaccines in Praomys*.

The present status of killed plague vaccines has been summarised by Meyer (1970). Although numerous killed vaccines have been developed for use in experimental animals, the one used most extensively for human inoculation is the American USP plague vaccine. The local and systemic reactions in man following initial basic inoculation are with few exceptions relatively mild. Controlled field trials have not been conducted to assess the efficacy of this vaccine. However the experience of U.S. Army personnel in Vietnam, where a high frequency of plague outbreaks exists, reveals that persons repeatedly vaccinated with USP vaccine enjoy almost complete freedom from plague, despite frequent exposure to the disease (Meyer, 1970).

The USP plague vaccine is an aqueous suspension of virulent P. pestis 195/P isolated in 1948 from a case of septicaemic plague in India. The stock culture is kept on blood agar in the cold in screw top bottles, with occasional passage through guinea pigs in order to maintain the virulence at a LD_{50} of 3 - 15 organisms for mice. The culture is grown on agar (E medium, low in levels of Anti A and Anti B blood group substances) in Roux bottles at 37°C for 48 hours. The organisms are harvested in physiological saline and killed by adding formalin to an overall concentration of 0.5%. Viability tests are then done. The culture is then diluted in a final phenol concentration of 0.5%, and the standardised suspension is supplied in 20 ml vaccine vials, each ml containing 2×10^9 bacilli.

Standardised procedures have been adopted to evaluate the vaccine. Toxi-

city and safety tests are performed on guinea pigs and white mice. Each of three guinea pigs (weighing 300 - 400 gm) is inoculated intraperitoneally with the human dose (1 ml = 2 x 10^9 organisms). A series of white mice are likewise inoculated intraperitoneally with 0.5 ml of the vaccine. All animals must survive without significant symptoms or weight loss. The immunogenicity of the vaccine is determined by the mouse potency test which is a modification of the procedure developed by Sokhey (1939). Each lot of the vaccine is tested on 100 white mice, 20 mice for each of five graded threefold dilutions. Dilutions are inoculated intraperitoneally in two doses of 0.2 ml each, with a seven day interval between injections. On the seventh day after the second inoculation of vaccine, the mice are challenged subcutaneously with 0.2 ml of a suspension containing 2,000 - 4,000 virulent P. pestis. This dose must kill 100% of ten control mice inoculated simultaneously. All the mice are observed for 14 days after challenge and dead mice are autopsied and smears made from the spleens. A reference vaccine is tested simultaneously in the same number of white mice. This reference vaccine is a formalin-killed preparation lyophilised in the amount representing 1 ml of vaccine, sealed in vials and maintained in the cold away from light. The content of a vial is reconstituted with 1 ml sterile water, and as it contains a stabilizer it is uniformly suspendable. The threefold dilutions are prepared from this suspension in saline, inoculated intraperitoneally and the animals challenged as with the vaccine being tested.

The efficacy of the USP killed vaccine and the reference vaccine was tested in Praomys using the standard mouse potency test and also by subcutaneous inoculation.

Subcutaneous inoculation.

Plague vaccine (E medium) Bulk Lot No. EO 37961 containing 2.1×10^9 organisms per ml was diluted in normal saline as follows: 1:5, 1:15, 1:45, 1:135, 1:405, 1:1,215. These dilutions were inoculated subcutaneously in the right hind leg in 0.2 ml amounts, into 6 groups of Praomys using ten animals for each dilution. Fresh dilutions of the vaccine were then prepared 14 days later and the same dilutions prepared. The animals were then reinoculated with the same doses of the vaccine. The Praomys were kept under observation for two weeks: all survived. Twenty three days after the second inoculation these Praomys and 10 unvaccinated controls were challenged with 21,500 virulent P. pestis strain MP6 inoculated subcutaneously in the right hind leg in 0.5 ml amounts. This was a twenty four hour culture grown on trypticase soy agar at 37°C.

After challenge the animals were kept under observation for 21 days and the following deaths were recorded:

Vaccine	Number of organisms in each of 2 inoculations	Number of deaths after challenge	% mortality	Average day of death	A.P.I.
1/5	84×10^6	4/10	40	9.2	23
1/15	28×10^6	6/10	60	6.3	10.5
1/45	9.3×10^6	7/10	70	4.8	6.9
1/135	3.1×10^6	8/10	80	4.6	5.7
1/405	1.0×10^6	9/10	90	5.1	5.7
1/1,215	3.5×10^5	8/10	80	4.2	5.3
nil		10/10	100	3.0	3.0

Autopsies were done on all the animals which died, and the spleen and heart blood were cultured on blood agar. Pathological changes were typical of bubonic-septicaemic plague and P. pestis was isolated from all specimens. The 50% protective dose calculated by Thompson's moving averages was 64×10^6 organisms given in two doses of 0.2 ml amounts.

The USP vaccine was shown in this experiment to be protective in Praomys when inoculated subcutaneously. When inoculated intramuscularly in 1 ml amounts into *Langur monkeys* (Presbytis entellus) and given boosters of 0.2 ml intramuscularly 86 days later, this vaccine gives 60% protection against a severe subcutaneous virulent challenge (Meyer, 1970).

Intraperitoneal inoculation.

The average 50% protective dose of the USP vaccine in Swiss Webster white mice by the intraperitoneal route is 30×10^6 organisms given in two 0.2 ml doses one week apart, the challenge dose being administered one week after the second inoculation (Meyer, personal communication).

Having established that Praomys can be immunised with the USP vaccine by the subcutaneous route, the standard mouse potency test was done to determine whether the immunising dose was similar to that in Swiss Webster mice.

The USP plague vaccine "E medium", batch No. MD37961 (total count = 2.1×10^9 organisms per ml) was diluted as follows in normal saline: 1:5, 1:15, 1:45, 1:135, 1:405 and 1:1,215. The reference plague vaccine lot No. 2, Division of Biologics Standards, N.I.H., Bethesda, Md. (total count = 2.1×10^9 organisms per ml) was also similarly

diluted. These were the standard dilutions used for the test in Swiss Webster mice. For the inoculation of each of these vaccines, 100 Praomys were divided into 5 groups of 20 and put in separate cages. The vaccine dilutions from 1:15 to 1:1,215 were then inoculated intraperitoneally in 0.2 ml amounts. Each of these animals was then revaccinated with the same vaccine dilution one week later. All the animals were then challenged one week after the second inoculation with 5,200 P. pestis strain MP6, inoculated subcutaneously on the inner side of the right hind leg in 0.2 ml amounts. A virulence titration of this challenge dose was also done in unvaccinated control Praomys using tenfold dilutions. The following deaths were recorded:

		USP plague vaccine "E medium"				Reference vaccine			
Vaccine dilution	Total No. of organisms	Number of deaths after challenge	% mortality	Ave. day of death	API	Number of deaths after challenge	% mortality	Ave. day of death	API
1/15	28×10^6	15/20	75	4.2	5.6	13/20	65	3.5	5.1
1/45	9.3×10^6	15/20	75	4.4	5.9	18/20	90	3.4	4.7
1/135	3.1×10^6	20/20	100	2.5	2.5	19/20	95	2.9	3.2
1/40	1.0×10^6	20/20	100	2.4	2.4	19/20	95	2.8	3.1
1/1,215	3.5×10^5	20/20	100	2.5	2.5	20/20	100	2.8	2.8

Unvaccinated controls

Challenge dose	Number of deaths	% mortality	Average day of death
5,200	10/10	100	2.7
520	10/10	100	3.6
52	10/10	100	4.1
5.2	8/10	80	4.4

Autopsies and bacteriological cultures confirmed the deaths as bubonic-septicaemic plague.

Intraperitoneal inoculation of Praomys with the same doses of vaccine as used in the standard mouse potency test resulted in a higher mortality rate than in Swiss Webster mice. A 50% protective dose could therefore not be calculated. However, the protection indices are similar when the vaccine is administered by the subcutaneous or intraperitoneal routes.

The virulence titration of the challenge dose in unvaccinated Praomys confirmed the high susceptibility of the species to plague. A dose of 5.2 organisms inoculated subcutaneously killed 8/10 animals.

A repeat experiment was then done to determine the 50% protective dose of the USP vaccine in Praomys by intraperitoneal inoculation, using higher doses of the vaccine. Threefold dilutions of the USP plague vaccine "E medium" (total count = 2.1×10^9 organisms per ml) were prepared in normal saline. These were then inoculated by the intraperitoneal route in 0.2 ml amounts using 10 Praomys for each dilution. One week later the same dilutions of the vaccine were prepared and the animals revaccinated. A culture of virulent P. pestis strain MP6 was grown on trypticase soy agar at 28°C for 24 hours. The vaccinated Praomys were challenged subcutaneously on the inner side of the right hind leg, one week after the second vaccine dose with 6,300 viable organisms. Ten unvaccinated control Praomys were also challenged simultaneously.

The animals were observed for 21 days and the following deaths recorded:

Vaccine dilution	Total number of organisms	Number of deaths after challenge	Percent mortality	Average day of death	A.P.I.
Neat	2.1×10^9	3/10	30	6.3	21.0
1/3	7.0×10^8	2/10	20	4.5	22.5
1/9	2.3×10^8	5/10	50	9.8	19.6
1/27	7.8×10^7	7/10	70	8.1	11.5
1/81	2.6×10^7	9/10	90	5.3	5.9
1/243	8.6×10^6	9/10	90	5.7	6.3
1/729	2.9×10^6	9/10	90	4.7	5.2
1/2187	1.0×10^6	8/10	80	5.6	7.0
Control	nil	10/10	100	4.0	4.0

Deaths were confirmed to be due to bubonic-septicaemic plague by autopsy and bacteriologically. The 50% protective dose calculated by Thompson's method was 80×10^6 organisms given in two doses of 0.2 ml amounts. This value is similar to that obtained by subcutaneous inoculation of this vaccine in Praomys. It is of importance to note that the protection indices of live vaccines were higher than those of the killed vaccines, when similar doses were inoculated either once or twice.

CHAPTER 11.

Discussion.

In the foregoing pages the experimental results of an investigation into the use of live and killed plague vaccines in some animal species were presented. Emphasis was placed on vaccines which have been or are in use for human inoculation against plague. The significance of these results will now be discussed in relation to previous experimental work and the present knowledge of the immunogenicity of plague vaccines.

Live plague vaccines.

Although live vaccines were advocated very early in the history of plague immunisation by Strong (1906) and later by many others, at present they are used only on a limited scale. These vaccines were shown to confer greater protection against virulent challenge in experimental animals and the advocates of these vaccines claimed there was also better protection in man. Live plague vaccines have been used extensively throughout the world in the past but fell into disrepute as a result of the local and generalised reactions which they caused, although it has been repeatedly claimed that these reactions are mild. Today live vaccines (EV strain) appear only to be used in Vietnam (Meyer, 1970) where a high frequency of plague has existed since 1962 (Cavanaugh et al., 1968).

Attempts to find a plague vaccine of greater immunogenicity have recently again focused attention on the experimental use of live attenuated strains. A highly effective vaccine is needed mainly for plague control when other well known means can no longer be applied, such as under the hostile conditions existing in Vietnam. It is also important for the

protection of laboratory and field personnel investigating plague outbreaks. The EV76 strain is the live vaccine which has been most widely used for human immunisation and is the live plague vaccine of choice recommended by the World Health Organization (1970).

The principle aims of recent experimental work have been to develop standard techniques for the preservation of immunogenicity and tests for measuring the degree of protection conferred, and develop methods of reducing the reactions caused by the inoculation of these live organisms.

Evaluation of the EV51f strain.

A subculture of the EV76 vaccine strain taken to the U.S.A. from Madagascar in 1951 was passaged through guinea pigs previously inoculated with non-toxic doses of iron salts to stabilise the vaccine antigenically and to increase the immunogenic capacity. This EV51f culture was then preserved by lyophilisation and made available for experimental use. The 50% protective dose (PD_{50}) by the subcutaneous route was 3,600 viable organisms for mice and 5 for guinea pigs. The preparation proved highly pathogenic for vervet monkeys (Cercopithecus aethiops) and langurs (Presbytis entellus), but not for macaques (Meyer, 1970).

Meyer (personal communication) conducted a trial of the EV51f vaccine in 14 human volunteers, 10 of whom had not previously been vaccinated or exposed to plague, and 4 who had been vaccinated with the original EV76 strain. Inoculation was done intracutaneously with bifurcated needles and caused pustules and blisters exceeding 10 cm in diameter. No sharply defined differences in extent and intensity of the reactions were noted between previously vaccinated and previously non-vaccinated

individuals. However, there was a distinct dose-reaction relationship; 2 million organisms (4 punctures) caused slightly more marked inflammation than 530,000 bacilli (1 puncture). Axillary lymph nodes were never palpable. Systemic reactions were absent but the white blood cell counts were slightly elevated in 8 of the 14 men 24 - 48 hours after inoculation. The doses did not stimulate significant serological titres and no booster effect was noted. In humans the EV51f strain therefore produces definite inflammatory reactions but no invasion into lymph nodes when inoculated in small doses.

The pathogenicity of the EV51f plague strain in Cercopithecus aethiops was confirmed in the present work. Doses between 101 and 10^8 viable organisms inoculated subcutaneously caused a total of 13 deaths (26%) in 50 vervet monkeys. Even doses as low as 101 organisms were able to invade, multiply and cause death in this animal species. When inoculated subcutaneously into Praomys (Mastomys) natalensis this vaccine strain caused death of the animals only in high doses between 1.1×10^6 and 1.7×10^8 viable organisms. Fifty guinea pigs inoculated with the same doses of the vaccine as the Cercopithecus, survived.

In C. aethiops the EV51f strain produced high HA antibody titres to the F1 antigen in all doses between 101 and 1.0×10^8 bacilli, but in Praomys doses of 1,700 viable organisms or lower failed to stimulate antibodies.

The vaccine conferred a high rate of protection in guinea pigs to virulent challenge on day 28. Even a dose of 100 viable EV51f protected 7/10 guinea pigs and higher doses conferred complete protection. The serological reactions in guinea pigs were not determined. Seven

of 10 Cercopithecus resisted a subcutaneous challenge inoculation of 8,770 virulent P. pestis 170 days after vaccination, but there did not appear to be a relationship between the rate of protection and dose of the vaccine. The three animals which succumbed to virulent challenge had received 1.009×10^8, 1.009×10^6 or 1,009 viable EV51f organisms.

The protection of Praomys was directly related to the vaccine dose, as shown by the percentage mortality and the protection index. Some relationship also existed between the serological response and the rate of protection. A dose of 1,700 organisms which did not stimulate circulating F1 antibodies, protected 17% of Praomys and the protection index was 4.8. 170,000 organisms stimulated antibodies in all the animals tested and conferred protection on 66% with a protection index of 18. The same or only slightly higher protection was conferred by higher vaccine doses.

Striking differences therefore exist in the pathogenicity and rate of protection of the live EV51f plague vaccine in these three animal species.

It is know that maximum virulence of P. pestis can be expressed only by those bacilli possessing all of a number of different, independently determined characters, which include: F1 antigen (F1+), V and W antigens (VW+), the ability to produce pigmented colonies on a defined medium containing haemin (P+) and the ability to synthethise purines (Pu+). Others, which may also be of importance are: toxigenicity (T+), pesticinogenicity (Pg+) and antigen 4 (4+) (Burrows, 1963). With most attenuated strains their avirulence can be attributed to the absence of one or more of these characters.

It is known that P- derivatives of virulent strains display the high

virulence of their P+ parents in mice which had been injected with small, non-toxic amounts of iron salts (Jackson and Burrows, 1956b). The injected iron not only reduces the LD_{50} value to that of virulent strains, but also permits growth in vivo to the large population characteristic of virulent infections. Burrows (1963) concluded that injected iron provides an essential element not sufficiently available to P- bacilli in vivo.

It has been shown (Jackson and Burrows, 1956b) that the attenuated strain EV76 behaves as a fully virulent strain in mice previously inoculated with ferrous sulphate. The passage of this strain through iron treated guinea pigs would, therefore, select a population of bacilli lacking only the virulence determinant for which the iron compensates, i.e. the ability to form pigment on a defined medium (P+). Differences in the pathogenicity of the strain designated EV51f in different animal species would therefore most likely be a reflection of the available iron. In Cercopithecus this is apparently such that low doses of these bacilli are able to multiply and invade and cause death of a certain proportion (26%) of individuals, while the others become ill, as reflected by a rise in temperature and white cell count. Multiplication of the strain was confirmed by positive blood cultures in monkeys 4 to 15 days after subcutaneous inoculation of doses as low as 101 bacilli (table 1). This would explain why all doses of the vaccine cause similar rises in serological titres and similar rates of protection against virulent challenge after 6 months. It is likely that the availability of iron in guinea pigs is less than in Cercopithecus but sufficient for multiplication of the organisms to be immunogenic, but not sufficient for the bacilli to reach a lethal dose for the animal, which is estimated to be 1.6×10^{11} virulent organisms (Cocking et al., 1960). A low dose of 100 organisms which protected

70% of the individuals after 28 days may not have been able to multiply sufficiently to reach a fully immunogenic population, whereas a dose of 1,000 organisms or more conferred complete protection, possibly by a greater degree of multiplication.

The availability of iron is probably the lowest in Praomys of the three species. If this is so, it may explain why low doses of the EV51f strain are unable to multiply in this species and therefore do not stimulate serological F1 antibodies and do not protect against virulent challenge. High doses between 1.7 million and 170 million EV51f bacilli do however invade and cause mortality in this animal species after subcutaneous inoculation.

It was shown by Albizo and Surgalla (1970), that virulent P. pestis Alexander strain produces a lipopolysaccharide endotoxin which exhibits classical endotoxic properties and possesses sufficient toxicity to contribute or account for death in small laboratory animals. The LD_{50} for white mice by intraperitoneal inoculation was 444 µg. Approximately two LD_{50} of the endotoxin for both guinea pigs and white mice could be extracted from 1.8×10^{11} organisms. The lethal dose of EV51f bacilli in Praomys therefore approaches the dose from which a lethal dose of toxin is extractable, assuming this strain contains the same amount of endotoxin as a fully virulent strain. The EV51f strain would therefore only require slight multiplication in Praomys to reach the lethal dose of mice. The availability of iron in different animal species in vivo is a field of investigation which requires further study. It would be of importance to know to what extent and in what form iron is available to P- strains, especially in man, for the investigation of strains which may be considered as candidates for use as vaccines.

Although high vaccine doses stimulate a serological response in Praomys the impact on the immunity mechanism is lower than in Cercopithecus or guinea pigs, as only 33% - 40% protection is conferred against virulent challenge. An intermediate dose of 170,000 bacilli is sufficient to give the same protection rate as the high doses (33%) but not sufficient to cause mortality.

These experiments using the EV51f vaccine were carried out with a lyophilised preparation. This method of preservation is the generally accepted method for preservation of stock cultures of bacteria and viruses, but has not always been useful for P. pestis (Heckly et al., 1958). These workers found a large decrease in the viability of 10 P. pestis strains investigated and also the virulence of the highly virulent strain 139L. They suggested that lyophilisation and storage in the dried state adversely affect the virulence of the bacilli without destroying their viability in vitro. Such cells require some time to rehydrate or repair sublethal damage and when injected into an animal the majority of such damaged cells are destroyed by the host before they become fully reactivated and functional as infectious units. Similar reductions in viability of the EV76 strain after lyophilisation have been encountered by Girard (1963) and were also experienced in the present work. High viabilities were however found by Quan, Chen and Meyer (1956) and by Berman et al., (1970), who used a number of attenuated strains of P. pestis which had either been used in the production of live vaccines, or were considered as potential vaccine strains. These discrepancies in viability of vaccine strains after lyophilisation have not yet been explained, but the recognition and demonstration that lyophilisation impairs viability and immunogenicity of the EV strain must be taken into account in determining the potency and shelf-

life of a live vaccine. This is of considerable importance when effective live plague vaccines must be delivered anywhere and at any time, especially to areas remote from the production laboratory.

On account of the drop in viability experienced in the lyophilised strains received for test in Praomys, experiments were conducted using cultures freshly grown on agar. These preparations caused a higher mortality rate in Praomys than the lyophilised cultures, but all white mice inoculated subcutaneously with the same doses survived. It was again established that doses of 1,000 EV51f organisms or less do not stimulate circulating HA antibodies, but the protection rate against virulent challenge was greater than the lyophilised preparation in the higher vaccine doses. Lyophilization therefore does have an adverse effect on the attenuated EV51f strain, as the ability to invade and cause death in high doses was reduced and the immunity against virulent challenge was less. Preservation of live attenuated plague vaccines in dried gelatin pellets according to the method of Stamp (1947), may be a more desirable method than lyophilisation.

It was shown by Soviet authors and by Meyer (1970), that live attenuated plague vaccines produce less severe local and systemic reactions when administered by the intradermal route. This technique of administration of the live EV51f vaccine was used in Praomys but the high doses attained by subcutaneous inoculation could not be reached by single intradermal puncture, and all the animals survived the vaccination. The pathogenicity of the vaccine could therefore not be determined by the intradermal route. The protection conferred against virulent challenge by the highest dose (466,900 viable EV51f bacilli) inoculated intracutaneously (40% mortality, active protection index = 10) was comparable with that achieved by a similar dose (170,000 viable

EV51f bacilli) inoculated subcutaneously (33% mortality, active protection index = 18).

Although the intradermal route may be advantageous for reducing the local and systemic reactions to the vaccine in man, a higher rate of protection using intermediate doses of the vaccine could not be achieved by this route than by subcutaneous inoculation in Praomys.

Evaluation of the EV76 strain.

Investigation of the original EV76 culture kept at the Institut Pasteur, Paris showed this culture to have approximately the same PD_{50} value in white mice as the EV51f strain by subcutaneous inoculation (Meyer, 1970). When inoculated intradermally in a dose of 26,800,000 viable organisms into three non-human primate species, the EV76 culture also proved to be highly invasive and pathogenic (Meyer, 1970). Local reactions were observed in four langurs (Presbytis entellus) in the form of erythematous indurated lesions. Fever, anorexia and loss of aggressiveness were noted in all. Systemic reactions were less marked in 6 vervets (Cercopithecus aethiops) though slight rises in temperature for one or two days were recorded and positive blood cultures were obtained on the fifth day from two animals. Local reactions in 4 macaques (Macaca mulatta) were confined to pinhead pink nodules. One macaque died on the 8th day after vaccination and EV76 organisms were isolated from the heart blood and other tissues. A second animal contracted an intercurrent Streptococcus infection and died while being held for virulent challenge. All the animals challenged, survived the infection.

A clinical trial of this vaccine was done in human volunteers by Meyer (personal communication). Of 5 men with no history of previous plague

vaccination, 2 were inoculated intradermally with 406,000 and 3 with 1,600,000 organisms. Small erythematous nodules developed at the site of inoculation but there were no febrile reactions, and no axillary lymph adenopathy developed. None of the men developed serum antibodies to the F1 antigen. Four men previously vaccinated with the USP killed vaccine were also included in this trial and three of these received 1,600,000 and one received 406,000 EV76 (Paris) organisms. There was no serological booster effect in these four men.

A higher dose of 28 million viable EV76 (Paris) organisms was then inoculated intradermally into these nine men and four others who had no previous contact with plague vaccine. The local reactions produced in all these volunteers were stated to be unacceptable by Public Health workers. No elevations in body temperature or changes in the white blood cell count were recorded. The serological results in antibody titres and mouse protection indices reflected low titres in response to cutaneous multiple puncture vaccination. Meyer suggested that the local reaction immobilised the inoculated P. pestis in the skin and prevented transport of the organisms into the lymph nodes. Thus few antibody producing cells were brought into contact with antigen. He suspected that the antigenic mass reaching the circulation was inadequate and therefore failed to exert a definite booster effect on the mouse protective antibodies, but in some individuals raised the haemagglutinins.

To overcome the cutaneous resistance to live plague vaccines the EV76 (Paris) strain was inoculated subcutaneously in a dose of 56 million viable organisms into 12 men, of whom 5 had been previously vaccinated with USP vaccine and 7 had had no previous contact with plague vaccine. Local and systemic reactions were quite marked and the opinion was that

the side effects were more pronounced than those previously experienced with the USP vaccines. In the previously uninoculated men the serological response showed a rise in haemagglutinating antibodies, but with one exception there were no mouse protective antibodies. In those men who had previously been vaccinated with the USP vaccine there was a booster effect in both the haemagglutinating and mouse protective antibodies. It was therefore established that the live EV76 (Paris) vaccine exerts a significant booster-dose effect in previously vaccinated human subjects, while the antibody response in the non-vaccinated is similar to that induced by a single dose of USP killed vaccine on the 28th day after inoculation.

Subcutaneous inoculation of the lyophilised EV76 (Paris) vaccine into Praomys in a dose of 22 million viable bacilli proved fatal to 4 of 10 animals but not in lower doses. In comparison, the lyophilised EV51f vaccine in a dose of 1.7×10^8 bacilli caused 3 deaths in 10 and one death in 10 Praomys inoculated with 1.7×10^6 bacilli. The lyophilised EV76 (Paris) vaccine conferred complete protection on Praomys in a dose of 2.2×10^7 viable organisms, whereas the highest dose of lyophilised EV51f (1.7×10^6 bacilli) protected 66% of Praomys. The protection rates of lower doses of these two vaccines were similar.

Subcutaneous inoculation of freshly grown agar cultures of the EV76 (Paris) vaccine proved to be more pathogenic in Praomys than the lyophilised preparation. A dose of 22 million lyophilised bacilli caused 40% mortality, whereas the agar-grown culture caused fatalities in 20% of animals inoculated with 5.5 million, and 40% of animals inoculated with 6.7 million. Doses of 55 million and 550 million

bacilli each resulted in 60% mortality. A similar mortality rate was obtained after subcutaneous inoculation of the agar-grown EV51f vaccine (33% - 1.1×10^7 bacilli; 20% - 1.1×10^6 bacilli). Higher rates of protection were achieved with the agar-grown EV76 (Paris) vaccine than the lyophilised preparation. Doses of 4,700 agar-grown EV76 (Paris) failed to stimulate significant HA titres in Praomys, but the geometric mean titres 30 days after subcutaneous inoculation of 47,000 and 470,000 bacilli were 1:194 and 1:48 respectively. This investigation of lyophilised and agar-grown preparation of the EV76 (Paris) strain in Praomys confirms the deterioration of the vaccine after freeze drying.

The EV76 (Paris) vaccine, used for the mass vaccination of humans is pathogenic in Praomys in large doses, causes local and systemic reactions in subhuman primates and local reactions in human volunteers. The strain is however nonpathogenic for guinea pigs and white mice, even by intravenous inoculation of the latter (Meyer, 1970).

The subcutaneous inoculation of a large dose of EV76 (Paris) vaccine failed to protect Praomys against the pathogenicity of a second large dose inoculated two weeks later. The time interval between the two doses was probably too short and did not allow time for recovery of the reticulo-endothelial system. Two low doses of the vaccine failed to booster the protection when the animals were challenged 23 days after the second dose. However two doses of 1.2 million and 2.2 million viabl organisms raised the protection index from 15.7 to 210. Inoculation of 2.2 million viable EV76 (Paris) organisms two weeks after primary immunisation with 85 million UV killed vaccine organisms failed to protect all the Praomys after virulent challenge. This primary immunisation also did not protect the animals against the residual viru-

lence of the EV organisms, but again the second inoculation may have been too early.

Although no local reaction could be detected at the site of inoculation of the EV vaccine in Praomys, small doses of organisms are eliminated without having the opportunity of stimulating immunity. There is some indication that this clearing of organisms may be more efficient if inoculation is done intracutaneously.

Large doses of the attenuated EV strain administered subcutaneously in Praomys resulted in sufficient invasion of organisms to stimulate antibodies and protect the animals against virulent challenge. A second large dose of the vaccine resulted in boosting the immunity conferred by an initial large dose of EV vaccine. Basic immunisation with killed vaccine failed to reduce the harmful effects of live vaccine administered two weeks later; a similar observation was recorded in human volunteers.

Invasiveness of attenuated and virulent P. pestis in Praomys.

Investigation of the invasiveness of the EV5lf strain in Praomys revealed that from the site of inoculation an immunogenic dose of organisms is transported via the afferent lymphatics to the regional lymph nodes within 6 hours. Organisms spread to the blood stream probably via the efferent lymphatics and thoracic duct and reached the liver and spleen within 12 hours. Clearance from these sites is effected after 1 - 2 days, but the organisms may persist in the regional lymph nodes for 2 - 3 days. Infection by fully virulent organisms is established without an efficient barrier provided by the regional lymph nodes. As the virulent bacilli could not be detected in the tissues for a longer period than attenuated organisms, it is suspected that

virulent P. pestis initially remains localised at the site of inoculation where it multiplies. After 24 to 48 hours the bacilli spread throughout the body and death usually follows on the third or fourth day.

The spread of virulent P. pestis inoculated subcutaneously into immune Praomys appear to resemble an attenuated strain in normal animals, but differs in the rate of spread. In some cases the organisms reach the regional lymph nodes within 6 hours and gradually spread to the liver and spleen. Invasion throughout the body is effected within 6 - 9 days but the numbers of organisms remain at a low order. Clearance from the body is gradual and virulent P. pestis can persist in the regional lymph nodes and spleen for at least 2 - 3 weeks.

The mode of infection in Praomys with attenuated P. pestis agrees with that described by Jawetz and Meyer (1944a) and Faibich (1964) for white mice, confirming the sequence of lymphatic to haematogenous spread. They also established that the same route of invasion applies for virulent organisms and that this infection differed only in extent, but not in kind from an attenuated strain in white mice. The infection of Praomys however with virulent P. pestis does not show the rapid dissemination of these organisms throughout the body as in white mice, but the later sequence of lymphatic to haematogenous spread appears to be the same in these two rodents.

The rapid clearance of the attenuated strain from normal Praomys is in agreement with the findings of Jawetz and Meyer (1944a), who could not correlate the immunogenic potency of an attenuated strain with the duration of infection produced by such a strain. Markenson and Ben-Efraim (1969) however found that attenuated plague strains persisted in

the livers and spleens of non-immunised mice and guinea pigs in direct proportion to their protective power. They showed that strains W9 and EV76 persisted the longest. The intensity of infection in Praomys as shown by generalised spread throughout the body may account for the adequate antigenic stimulus and subsequent protection. This is in agreement with the concept of Korobkova and Smoilova (1962), who stress the importance of the intensity of infection by live vaccine strains.

Investigation of the invasiveness and persistence of attenuated P. pestis in Praomys agrees with the statement of Jawetz and Meyer (1944a) that the immunogenic activity does not only depend on the survival of a plague organism, but also on its antigenic make-up. The intensity of invasion in Praomys does, however appear to play an important role. In spite of the rapid clearance there is enough exposure of antigens to antibody producing cells to provide an adequate immune response. The importance of the antigenic structure was demonstrated by the enhanced protection given by two live vaccines (EV76 and Tjiwidej) lacking different virulence antigens but together having the full complement of antigenic virulence determinants.

Combined oral and subcutaneous administration of a live vacci .

Combined oral and intracutaneous administration of the EV strain in guinea pigs was investigated by Korobkova et al., (1970). These workers showed that a small dose of the vaccine inoculated intracutaneously by itself is not very effective in stimulating immunity, but it enhances the resistance produced by oral vaccination. Their results showed that after intracutaneous immunisation with 500 vaccine organisms 27% of guinea pigs survived a virulent challenge and a single oral dose of 3000 million bacilli protected 44% of guinea pigs against the challenge infec-

tion. An oral dose of 3,000 million EV organisms combined with simultaneous intracutaneous inoculation of 500 bacilli resulted in protection of 60% of guinea pigs against virulent challenge. This rate of protection was raised to 90% when oral administration of the vaccine was given in 3 doses of 1000 million and combined with simultaneous intracutaneous inoculation of 500 vaccine organisms.

In Praomys the EV51f strain is sufficiently invasive to cause mortality even when inoculated via the oral route in doses of 300 million organisms. The portal of entry was most likely the submaxillary and retropharyngeal lymph nodes similar to a virulent infection of the upper respiratory tract of monkeys demonstrated by Meyer and Larson (1960). The liver, spleen and other organs were then invaded, probably via the afferent lymphatics. Repeated doses of the vaccine given orally on three successive days conferred better protection against virulent challenge than the same number of organisms administered in a single dose. This triple administration ensured the penetration of a greater number of organisms. It is likely that only a small proportion of the bacilli administered orally invade the tissues, as a single dose of 100,000 EV51f inoculated subcutaneously confers the same protection on Praomys as one dose of 300 million organisms given orally. In view of the invasive properties of the EV vaccine strain via the oral route in Praomys, it is unlikely that this route will be used for human immunisation.

Assessment of the immunogenic effect of known virulence determinants in vaccine strains.

Investigation of the immunity of P. pestis strains lacking different virulence determinants by Burrows and Bacon (1958) confirmed the importance of the Fraction 1 antigen, which had previously been established

by Meyer and his associates (Baker et al., 1952, Chen, 1952, Walker et al., 1953 and Chen and Meyer, 1955), and VW antigens (Burrows and Bacon 1956a). The ability to form pigmented colonies on a defined medium (P+) was also found to be of importance, in that it increased the immunogenicity in mice of strains having the determinants F1+ and/or VW+, but was ineffective in their absence. P. pestis contains other antigens (Burrows, 1963), some of which are important in determining the virulence of the bacillus and may also be important in immunity by enhancing the immunogenicity of F1 and VW antigens, or possibly also be immunogenic by themselves.

Praomys were immunised with two live attenuated P. pestis strains which together possess the three virulence determinants which have been established to be important in immunity to plague. This was an attempt to determine whether these would confer greater immunity than a single strain. The strains used were: EV76 (Paris), which is F1+, VW+ and P-; and Tjiwidej which is F1+, P+ and VW-.

Subcutaneous inoculation of the strain Tjiwidej proved to be nonpathogenic in Praomys in doses up to 150 million viable organisms. The strain was also highly immunogenic in this animal species, but the 50% protective dose (23,120 viable bacilli) was higher than the EV76 (Paris) strain (9,072 viable bacilli). The serological response as determined by the average HA titre for each dose, was in proportion to the dose of organisms inoculated. Doses of 15,000 organisms and lower failed to stimulate significant HA titres.

The protection against virulent challenge conferred in Praomys by both the Tjiwidej and EV76 (Paris) strains declines after two months, but a booster inoculation of the 50% protective dose one month after the

primary inoculation, gives greater protection than a single dose. Alternate inoculations with both these strains in the same animal stimulates greater immunity than two inoculations of the same strain. This confirms the importance of the antigenic constitution of the strain in immunity to plague.

The live plague vaccines used in South Africa for human immunisation by Grasset and Pirie consisted of a mixture of the EV and Tjiwidej strains, in amounts of 500 million organisms of each strain per dose (Grasset, 1942). In this way the virulence determinants, not known to be important in immunity to plague at the time, were inoculated fortuitously and may have partly contributed to the success of the immunisation programme. During two plague outbreaks in 1941, 206 persons who were close contacts of pneumonic and bubonic cases were immunised with the mixed vaccine. Six of these persons contacted plague and 5 died. The one case which recovered was immunised 5 - 6 days before contact and those which died were vaccinated after contact.

The serological response to one dose of live EV76 (Paris) vaccine in man is similar to that induced by a single dose of USP killed vaccine on the 28th day after immunisation. In order to attain a mouse protection index indirectly expressing satisfactory protection against plague, a second booster inoculation is required (Meyer, personal communication). As two doses of live vaccine are required to induce significant immunity, evidence from the study in Praomys indicates that it might be advantageous to use the Tjiwidej and EV76 (Paris) strains in successive inoculations one month apart for human immunisation. These preliminary results in Praomys using two live attenuated strains lacking different virulence determinants are encouraging and worthy of future investigation, possibly in primates and this may even-

tually lead to trials in human volunteers.

Importance of the Fraction 1 antigen.

A live attenuated plague strain designated M23, deficient in F1 antigen (F1-) and the ability to form pigmented colonies on a defined medium (P-), protected macaques (Macaca mulatta) against aerosol challenge when inoculated intradermally and caused only slight local reactions (Janssen and Surgalla, 1969). This strain was subsequently shown to be highly virulent in langurs (Presbytis entellus) by intradermal inoculation, less virulent in vervets (Cercopithecus aethiops) but [illegible] were only slightly affected (Meyer, 1970). This vaccine has not been tested in human volunteers. The pathogenicity of the non-pigmented M23 strain in Pracomys proved to be similar to the EV51f and EV76 (Paris) strains. The pigmented parent M23 strain which lacks the F1 antigen (F1-) is highly virulent in mice (Burrows, 1958). It is to be expected therefore that the pathogenicity of a non-pigmented mutant of this strain would be similar to other non-pigmented P. pestis strains in laboratory animals. This is confirmed by the experimental findings in subhuman primates and in Pracomys. The protection against virulent challenge afforded by the non-pigmented M23 strain was low in Cercopithecus but all five macaques challenged 207 days after vaccination with this strain survived (Meyer, 1970). Pracomys were not protected by this strain even in doses of 170 million viable organisms, but as F+ P- attenuated P. pestis strains protect this animal species, F1 is an important antigen in the immunity of Pracomys against plague. The protection of macaques by this non-pigmented M23 strain, when inoculated intradermally, against lethal aerosol or subcutaneous challenge was unexpected. Immunity to infection in these animals

is probably established by antigens of this strain other than Fraction 1. The organisms multiply sufficiently in macaques to establish an immunogenic population, but not sufficiently to reach a lethal dose. The inability of the non-pigmented H23 strain to establish immunity in Praomys was similar to the behaviour of genetically identical P. pestis strains in white mice (Burrows and Bacon, 1958).

Vaccination of man with the H23 P- strain would probably be tolerated as well as or better than the EV51f or EV76 (Paris) vaccines, but is unlikely to be equally protective, as it lacks the F1 antigen which has been shown to be important in all experimental animals except macaques.

Killed vaccines.

Standard tests on laboratory animals have shown that killed plague vaccines are relatively ineffective in protecting guinea pigs against virulent challenge although adequate for mice (Jawetz and Meyer, 1943 and Girard, 1955). The normal human dose of 3×10^9 USP vaccine organisms only protected 10 - 20% of guinea pigs against virulent challenge (Meyer, 1970). However when the vaccine is administered in large repeated doses or with adjuvants, the protection rate can be raised to 80 - 100% in guinea pigs. High rates of protection in guinea pigs were also achieved by Smith and Packman (1966) with a non-toxic vaccine prepared from cell wall material of the attenuated P. pestis strain EV76.

Intramuscular inoculation of 1 ml of USP plague vaccine in Hanuman langurs (Presbytis entellus) produced no local or systemic reactions (Meyer, 1970). After booster intramuscular inoculations with 0.2 ml

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