Evaluation of Live Attenuated Plague Vaccines in *Praomys* (Mastomys) natalensis

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A live attenuated *Yersinia pestis* vaccine designated EV76-51f, which had previously been shown to be pathogenic in vervet monkeys but not in guinea pigs, was tested in the multimammate mouse *Praomys* (Mastomys) natalensis. Doses of $10^6$ viable organisms inoculated subcutaneously as either a lyophilized suspension or an agar-grown culture resulted in vaccination fatalities in *Praomys* but not in white mice. Hemagglutinating antibodies to the fraction 1 antigen were not stimulated by doses lower than $10^4$ viable organisms. Agar-grown cultures of the vaccine gave better protection against a virulent *Y. pestis* challenge than did a lyophilized suspension. All *Praomys* vaccinated with a dose of $10^6$ agar-grown EV76-51f protected against a virulent challenge, whereas even doses up to $10^8$ lyophilized bacilli failed to give complete protection. The pathogenicity of a live attenuated plague vaccine derived from the *Y. pestis* EV76 vaccine strain can be detected in *Praomys* (Mastomys) natalensis, a rodent species highly susceptible to plague. This animal species may therefore be valuable for the testing of live attenuated plague vaccines before they are tested in costly nonhuman primates.

Killed plague vaccines have been used extensively in animal experiments and humans to confer immunity to *Yersinia pestis* infection. The value of these vaccines was, however, seriously questioned at an early date (17, 26), and the greater efficacy of live attenuated *Y. pestis* strains in conferring immunity to virulent challenge was demonstrated in guinea pigs and monkeys by Strong in 1906 (29, 30). In humans, the live attenuated plague vaccine used most extensively has been the *Y. pestis* EV strain (11, 13), but it produces unpleasant side effects (19, 20, 22). Plague vaccines derived from the original EV76 strain were recently shown to be virulent in the highly susceptible vervet monkey *Cercopithecus aethiops* by subcutaneous inoculation (14, 19, 22). When administered orally to these nonhuman primates, the live attenuated *Y. pestis* vaccine designated EV76 (Paris) F was nonfatal, but caused a transient gastrointestinal disturbance in some animals (6).

Highly susceptible vervets are sensitive indicators of the pathogenicity of live attenuated *Y. pestis* strains, but are costly and difficult to obtain. These considerations prompted the investigation of the multimammate mouse *Praomys* (Mastomys) natalensis as a possible test animal for live attenuated plague vaccines. This wild rodent species had previously been adapted to the laboratory at the South African Institute for Medical Research, Johannesburg, and, because of its high susceptibility to infection, is used as the standard test animal for routine plague surveillance.

*Praomys* (Mastomys) natalensis plays an important role in the plague cycle in southern Africa (9) and has recently emerged as a reservoir host of the arenavirus disease Lassa fever in West Africa (10). The taxonomy, ecology, distribution, and disease vector status of this rodent species have been studied by numerous workers, but little is known of its immunological response to infection. Laboratory colonies of *Praomys* have been established in a number of countries to study patterns of neoplastic, autoimmune, and degenerative lesions as models of human diseases (10). It has also been used experimentally for the study of several infectious agents, including arboviruses (18), *Listeria* (23), *Mycobacteria* (31), *Borrelia* (33), *Schistosoma* (5, 12, 24, 25, 27, 32), and *Plasmodium vincke* (16). Amies (2) tested the immunogenicity of *Y. pestis* capsular antigens in *Praomys* and concluded that this animal could be used for the assay of antigens intended for human prophylaxis.

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**MATERIALS AND METHODS**

**Animals.** The *Praomys* (Mastomys) natalensis colony was built up from animals caught in Johannesburg in 1946 and maintained at the South African
Institute for Medical Research since that time. The animals are about twice the size of a white mouse, the body weight at maturity being between 50 to 70 g. For experimental work, they were used when they were 9 to 12 weeks old at a body weight of 25 to 30 g. Food consisted of standard mouse cubes supplemented with carrots, potatoes, and cabbage once a week. Water was supplied by a drop bottle. Animals under experiment were caged singly in cages 13 by 13 by 18 cm. Care had to be taken in handling Praomys as they are agile and bite viciously.

White mice were noninbred conventional animals from a colony maintained at the South African Institute for Medical Research for various purposes. The animals were fed on standard mouse cubes daily, and water was supplied from a drop bottle. The mice were 7 to 11 weeks old with an average weight of 20 g when used experimentally.

Vaccine. Vials of lyophilized EVV76-51f Y. pestis were kindly supplied by the late K. F. Meyer. Details of this vaccine preparation have been described previously (19). After reconstitution, the strain was stored in the laboratory at 4°C in dried gelatin pellets by the method of Stamp (28) for the preparation of agar-grown vaccines.

Serial dilutions of all vaccines were made in normal saline, and total bacterial counts of inocula were done in a counting chamber after dilution in 1.5% formal saline. Viable counts were done by inoculation of 0.2 ml of serially diluted suspensions onto each of five blood agar plates and spreading with metal spotters. These were then incubated at 28°C for 72 h before counting the colonies. All vaccines and virulent challenge cultures were checked for purity and confirmed biochemically to be Y. pestis after inoculation.

Challenge organisms. Two virulent Y. pestis strains were used for the challenge of vaccinated and control animals. A dose of 6,000 viable organisms of the virulent strain F357, which has an average lethal dose of less than eight bacilli for guinea pigs (15), was used in the initial experiments with the lyophilized vaccine. This strain was also shown to be highly virulent in Praomys (Table 1). For challenge of animals vaccinated with the agar-grown vaccine, a dose of 22,000 viable organisms of the virulent strain MP6 (3) was used. The average lethal dose of this strain for white mice and guinea pigs is less than 10 bacilli (4) and was kindly supplied by T. W. Burrows.

Vaccination procedure. Each of five vials of the lyophilized EVV76-51f vaccine was rehydrated with 10 ml of sterile normal saline and pooled in a 100-ml Erlenmeyer flask. Tenfold dilutions were made in normal saline, and total counts were then done. Tenfold dilutions of the vaccine suspension were prepared in sterile normal saline up to 10⁻⁷, and plate counts were done on dilutions of 10⁻⁶ and 10⁻⁷ by adding 0.2 ml to each of the blood agar plates.

Fifty Praomys were divided into five groups of ten, and each animal was injected subcutaneously on the inner side of the right hind leg with 0.5 ml of viable doses varying from 10² to 10⁴ organisms.

Agar-grown vaccines were prepared from dried gelatin pellets by incubation of the pellet on blood agar at 37°C for 1 h to melt the gelatin. The inoculum was then spread over the agar surface and incubated at 28°C for 18 h. This culture was then seeded onto Trypticase soy agar in MacCourtney bottles and incubated at 37°C for 48 h. The growth was harvested in sterile normal saline, using glass beads to produce a uniform suspension. Tenfold dilutions were then made in normal saline, and total and viable counts were done. 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 doses that varied from 10² to 10⁴ viable organisms.

**Virulent challenge procedure.** After vaccination, the Praomys and white mice were challenged subcutaneously with a virulent agar-grown Y. pestis culture. The challenge inoculum was cultured on Trypticase soy agar at 37°C for 48 h and then suspended in sterile normal saline. Tenfold dilutions were prepared in normal saline, and total counts were then done. Plate counts were done on the lower dilutions by planting 0.2 ml onto each of five blood agar plates and counted after incubation at 28°C for 72 h. Each animal was challenged with 0.5 ml of the virulent culture subcutaneously in the right hind leg, and 10 unvaccinated controls were challenged at the same time. After a minimum observation period of 3 weeks, the protection conferred by the vaccine was determined by calculation of the active protection index (API) (4). The API takes both the percentage of deaths and the times of survival of challenged animals into account and was calculated as follows: API = (mean time of death in days × 100)/percent deaths. This value bears a direct relationship to the degree of protection, in contrast to the inverse relationship of the mouse protection index (21).

**Autopsies and serology.** Autopsies on 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 streaked onto blood agar plates and incubated at 28°C for 48 h. Pieces of tissue for culture were removed by use of sterile instruments for each organ. Growth on the plates was identified biochemically.

Tissues removed at autopsy from animals that died were fixed in 10% neutral formal saline for histological section. Sections 4 µg thick were cut and stained with hematoxylin and eosin.

Blood for serology was collected aseptically by heart puncture from Praomys as previously described (14). The sera were tested for antibodies to the fraction 1 antigen by the passive hemagglutination (HA) test, using the microtechnique (7).

**Table 1. Pathogenicity of virulent Y. pestis strain F357 in unvaccinated Praomys.**

<table>
<thead>
<tr>
<th>Viable dose*</th>
<th>Survivors/total</th>
<th>Avg day of death</th>
<th>Heart blood culture for Y. pestis</th>
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<tr>
<td>800</td>
<td>0/5</td>
<td>3</td>
<td>+</td>
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<tr>
<td>80</td>
<td>1/5</td>
<td>4</td>
<td>+</td>
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<tr>
<td>8</td>
<td>3/5</td>
<td>4.5</td>
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*Inoculated subcutaneously.
RESULTS

Response of Praomys to the lyophilized EV76-51f vaccine. Local lesions were not observed in any of 50 Praomys inoculated with the lyophilized EV76-51f vaccine in doses varying from $10^2$ to $10^6$ viable bacilli. Those animals inoculated with $10^6$ organisms or more had positive plague HA titers up to 1:1,024, whereas the lower vaccine doses failed to stimulate a serological response (Table 2).

Four vaccination fatalities were recorded after the inoculation of high doses (Table 2). On postmortem examination these animals showed hemorraghes, gelatinous edema, and necrosis at the site of inoculation; hyperemia, hemorrhage, and gelatinous edema of the abdominal and thoracic subcutaneous tissue; and enlargement of the inguinal lymph nodes. In one animal the spleen, liver, and adrenals were enlarged, but not in the others. None of the spleens or kidneys showed signs of necrosis, but the liver of one had a mottled appearance. The lungs were hyperemic and edematous in all animals, and there was straw-colored fluid in the pleural cavities.

Y. pestis was seen in direct smears and cultured from the heart blood and lungs of all animals and also seen in direct smears and cultured from the inguinal lymph node, spleen, liver, and kidneys of one animal.

The histological appearance of the tissues was as follows. In livers, there was severe congestion of the sinusoids and venules, with a marked increase in monocytes and polymorphonuclear cells in the venules and an increase of macrophages in the sinusoids. Clusters of Y. pestis were distinct in the venules of one animal. In spleens, there was engorgement of the red pulp with erythrocytes and a marked increase in mononuclear cells in all animals, with large numbers of Y. pestis throughout the section of one animal. In lungs, many alveolar spaces were filled with edema fluid, and some contained monocytes and erythrocytes. Alveolar walls were thickened and contained many mononuclear cells. No organisms could be seen.

Persistence of the vaccine was studied in 10 animals, 2 from each group, by sacrificing on day 27 after vaccination. The lymph nodes of two animals were enlarged, and the liver of one was slightly mottled. The lungs of two animals showed slight focal necrosis and hyperemia. Cultures done on blood agar of liver, spleen, kidneys, lungs, heart blood, and inguinal lymph nodes of these animals after maceration of the tissues were negative for Y. pestis.

Response of vaccinated Praomys to virulent challenge. With one exception, all of the Praomys inoculated with the two lowest vaccine doses died of acute septicemic plague after a virulent challenge 34 days after vaccination. Y. pestis was seen in smears and cultured from all tissues examined. Six of 17 animals vaccinated with higher doses survived the virulent challenge (Table 2). All 10 controls died, the average time of death being 4.6 days.

The serological titers of the survivors at 7 days before challenge varied from 0 to 1:1,024, but there was a relationship between the geometric mean titer of each vaccine dose and the API.

Response of Praomys and white mice to an agar-grown EV76-51f culture. Six groups of 15 Praomys and six groups of 10 white mice were inoculated subcutaneously in the right hind leg with doses from $10^2$ to $10^7$ viable EV76-51f bacilli. Ten Praomys and ten white mice in each group were kept for virulent challenge, and the other Praomys survivors were bled on day 29 for plague HA antibody tests.

The number of vaccination fatalities in this experiment (Table 3) indicates that an agar-grown culture of the EV76-51f strain has the same virulence in Praomys as does the lyophilized vaccine. The postmortem and histological

<table>
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<tr>
<th>Table 2. Response of Praomys to lyophilized EV76-51f Y. pestis vaccine and virulent challenge a</th>
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<td>Group</td>
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a Virulent challenge dose = 6,000 viable Y. pestis F357 at 34 days after vaccination.

b Six animals in each group.

c One died of an intercurrent staphylococcal infection.
appearances were also similar to those of the vaccination fatalities after inoculation of the lyophilized preparation. All of the white mice survived the vaccination.

Only the Praomys inoculated with doses of $10^6$ agar-grown EV76-51f bacilli or higher reacted serologically at 28 days after vaccination. The highest titer recorded was 1:512, with a dose of $10^6$ bacilli. These results are also comparable to those obtained with the lyophilized vaccine.

Response to virulent challenge of Praomys and white mice vaccinated with agar-grown EV76-51f culture. Greater protection was conferred in Praomys against a virulent Y. pestis MP6 challenge at 28 days after vaccination with the agar-grown EV76-51f culture than with the lyophilized EV76-51f vaccine (Table 3). The highest protection conferred by the lyophilized preparation was 66% after a dose of $10^6$ organisms, the active protection indexes being 33, 20, and 18 after doses of $10^5$, $10^4$, and $10^3$, respectively. The inoculation of $10^2$ and $10^3$ agar-grown EV76-51f bacilli, however, resulted in the complete protection of Praomys against a virulent challenge. Doses of $10^4$ and $10^5$ agargrown EV76-51f bacilli protected 90 and 60% of individuals, respectively, with active protection indexes of 80 and 13.2 (Table 3). The animals that died had postmortem and bacteriological manifestations of bubonic (septicemic) plague.

**DISCUSSION**

Live attenuated plague vaccines that pass safety tests in guinea pigs and white mice have been found to be virulent in nonhuman primates (15, 22). The expense and difficulty in obtaining these animals prompted an investigation of the reaction of Praomys (Mastomys natalensis, a highly susceptible rodent species, to live attenuated Y. pestis vaccines.

The Y. pestis EV76-51f strain (22) previously shown to be nonvirulent in guinea pigs and white mice by subcutaneous inoculation but virulent in African green vervet monkeys (Cercopithecus aethiops) (15, 19, 22) was received from the late K. F. Meyer as a lyophilized preparation. This vaccine strain is fatal to Praomys (Mastomys natalensis) when inoculated subcutaneously in doses of $10^6$ or higher, either as a lyophilized suspension or as an agar-grown culture. The lesions produced are those of typical plague, and Y. pestis could be cultured from various tissues, indicating that the strain is invasive in this rodent species.

The serological response of Praomys to the vaccine strain is poor compared with Cercopithecus aethiops (15). Only high doses stimulated plague HA antibodies, the highest titer recorded being 1:1,024 (geometric mean titer = 239). Low doses of $10^2$ or $10^3$ viable bacilli failed to stimulate antibodies.

Differences in the pathogenicity of the EV76-51f Y. pestis strain in different animal species are probably reflections of the availability of iron (15). In African green vervet monkeys this is apparently such that low doses of these bacilli are able to multiply and invade and may be fatal. It is likely that the availability of iron in guinea pigs is less than in vervet monkeys, but not sufficient for the bacilli to reach a lethal level in the animal, which is estimated to be $1.6 \times 10^{11}$ virulent organisms (8). 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 EV76-51f strain are unable to multiply in this species and therefore do not stimulate fraction 1 antibodies or protect against virulent challenge. High doses between...
10⁶ and 10⁹ viable bacilli do, however, invade and cause mortality in this rodent after subcutaneous inoculation, whereas in white mice they do not. This indicates that some multiplication of the vaccine strain occurs in Praomys in high doses but not in white mice. The multiplication in Praomys is sufficient to produce a lethal dose of lipopolysaccharide endotoxin, of which two mean lethal doses for white mice can be extracted from 1.8 × 10¹¹ virulent Y. pestis (1). Only a slight multiplication of a high dose of the EV76-51f strain in Praomys would be required to reach this level, assuming this strain contains the same amount of endotoxin as does a fully virulent strain.

The protection conferred against a virulent challenge, as determined by the API, was greater in Praomys than in white mice. This difference in the animal species may be attributed to greater invasiveness of the EV76-51f strain in the former than in the latter species. This would result in a greater impact of the organisms on the immune mechanism in Praomys.

This study has shown that Praomys (Mastomys natalensis), a rodent species that is highly susceptible to plague, is also susceptible to large doses of a live attenuated Y. pestis strain, which lacks the virulence determinant for which iron compensates. The pathogenicity of such strains is not shown by subcutaneous inoculation in guinea pigs or white mice. Praomys are, however, less susceptible than vervet monkeys, but are cheaper to maintain and more readily available. This rodent species may, therefore, be valuable for the investigation of live attenuated plague vaccines before they are tested in costly nonhuman primates.

ACKNOWLEDGMENTS
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