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EXPERIMENTAL PLAGUE IN ROCK SQUIRRELS, *SPERMOPHILUS VARIEGATUS* (ERXLEBEN)

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ABSTRACT: Experimental infections with *Yersinia pestis* were followed in groups of rock squirrels. Development of coagulopathy and pneumonia were observed in 2–4% and 11–12% of the test animals, respectively. Susceptibility to experimental infection was heterogeneous with some animals surviving inoculation with large numbers of organisms and others succumbing after inoculation with small numbers. Production and longevity of serum antibody titers, as measured by passive hemagglutination tests, were variable as well, and apparently unrelated to dose. The data presented attest to the need for care in interpreting serologic test results for individual animals.

INTRODUCTION

Rock squirrels play a major role in the epizootic amplification of bubonic plague among rodents and its transmission to humans in the southwestern United States. During the period 1974–1980, 44 (42%) of 105 human cases reported from the United States were epidemiologically associated with rock squirrels (Barnes, 1982). Another 11 (11%) were linked to the California ground squirrel, *Spermophilus beecheyi* (Richardson), a closely related species in the Pacific States.

Plague was isolated from rock squirrels and their fleas for the first time in 1936, and a human case was linked to the same host/flea complex in Utah in 1938 (Eskey and Haas, 1940). McCoy and Smith (1910) experimentally infected five rock squirrels captured in New Mexico long before plague first was reported in that state and found them "highly susceptible." Holdenreid and Quan (1956) challenged nine rock squirrels and found them "moderately resistant." Marchette et al. (1962) tested nine rock squirrels from New Mexico and three from Utah, classifying the former as resistant and the latter as susceptible.

Our observations during epidemiologic investigations in recent years have shown that while rock squirrel populations suffer high mortality rates during plague epizootics, survivors typically remain after the epizootic has passed. Survivors frequently, but not always, have antibodies to *Yersinia pestis* detectable by the passive hemagglutination test (PHA), proving their prior infection and survival, and suggesting their possible clinical resistance to plague.

Intrinsic and extrinsic factors that interact to determine the roles of rodent species and species populations in the epizootiology of plague (*Y. pestis* infection) and their importance in the transmission of the agent to humans are many, varied, and sometimes obscure. However, the susceptibility of populations to plague in terms of morbidity and mortality rates has obvious and fundamental importance and, therefore, deserve attention from plague workers. In this report, we present results of studies involving the responses to experimental plague infection of rock squirrels to gain insight into the dynamics of plague in natural populations.

METHODS AND MATERIALS

Animals: Three groups of animals were used in three separate experiments conducted in 1976–1978. The first group was comprised of 12 wild-caught animals collected in Santa Fe County, New Mexico, in May 1976 at a site where plague had occurred in 3 successive

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years—1974–1976. Two of these animals had antibodies to *Y. pestis* with PHA titers of 1:16 and 1:256, respectively. The 12 were challenged in four groups of three animals each with log dilutions of *Y. pestis* ranging from 2×10^3 to 2×10^6.

The second group of 64 animals was obtained on the Isleta Indian Reservation, Valencia County, New Mexico in September 1976 from a 1.6-km-long transect paralleling an irrigation canal. The population sampled had been under surveillance by the Indian Health Service for several years and had no history of plague. None were seropositive when captured.

The 63 animals in the third group were born of females which were pregnant when captured in July 1977 from the Isleta Indian Reservation site mentioned above and were reared in a wild animal facility at the CDC Laboratory in Fort Collins. They were experimentally challenged when 6–7 mo of age.

All wild-caught animals (Groups I and II and dams of group III) were bled for subsequent serologic tests and their ectoparasites removed at the collection site. Both animals and transport cages were treated with an aerosol insecticide containing 0.6% pyrethrins and 1.4% piperonyl butoxide by weight. Before being allowed in the laboratory, wild caught animals were kept for at least 60 days in the separate wild animal facility where they were given food and water ad lib. After 60 days, wild-caught animals were removed to the laboratory, where they were held for a least 3 wk before being subjected to experimental procedures. Animals in Group III also were transferred to the laboratory 3 wk prior to initiation of tests.

Six-wk-old laboratory mice (NIH general purpose strain) reared in the Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control specific pathogen-free colony at Fort collins were used as experimental controls.

Infectious challenges: Yersinia pestis strain 766259 was originally isolated from fleas (*Diamanus montanus* and *Hoplopsyllus anomalus*) of a *S. variegatus* collected in Sandoval County, New Mexico. The strain was fully virulent and typical in all tested characteristics at the time of original isolation. The culture was grown on blood agar at 28 C for 48 hr when five to 10 typical colonies were transferred to 10 ml sterile brain–heart infusion (BHI, Difco Laboratories, Detroit, Michigan 48201, USA). After 24 hr at 28 C, 0.1 ml of the culture suspension was inoculated subcutaneously into laboratory mice. When the mice died 3–4 days after inoculation, spleens and livers were excised. Portions of the tissues were cultured on blood agar at 28 C to recover the inoculated strain of *Y. pestis*. Five to 10 typical colonies of *Y. pestis* were selected after 48 hr incubation and transferred to 10 ml sterile BHI. The broth culture was incubated at 28 C for 24 hr when 0.5 ml was transferred to 10 ml sterile BHI. The second broth was incubated at 28 C for 24 hr, at which time it was assumed to contain about 1×10^8 *Y. pestis* cells per ml. Doses for inoculation into test animals were prepared by logarithmically diluting this BHI culture in sterile physiological saline. Individual animals were inoculated with 0.1 ml of the appropriate dilution subcutaneously in the inguinal region. Bacterial counts were made by spreading 0.1-ml portions of appropriate dilutions on duplicate blood agar plates. Colony counts were made after 48 to 72 hr incubation at 28 C, and the averaged counts were used to determine actual doses given.

Six-wk-old male white laboratory mice were inoculated subcutaneously with 0.1 ml of the same dilutions used to challenge the squirrels to ensure standard culture virulence. After inoculation, all animals were observed at least twice daily for morbidity and mortality for 21 days. Animals that died after being inoculated were necropsied, and their tissues were excised for bacteriologic culturing to verify cause of death as plague. Surviving animals were bled on days 10, 21, and 30 and at monthly intervals thereafter.

Sera from surviving animals were tested for antibody to *Y. pestis* Fraction 1 by the PHA test following standard methods (W.H.O., 1970).

Mean lethal doses (LD_{50}) and mean infectious doses (ID_{50}) were calculated by the method of Reed and Muench (1938). The number of animals infected was defined as the number dead plus the number of survivors demonstrating seroconversion.

RESULTS AND DISCUSSION

The responses of *S. variegatus* to experimental plague infection were recorded (Table 1). Actual doses of *Y. pestis* inoculated in the three separate experiments were very close (2–2.45 × log dilution) and all of the suspensions used exhibited acceptable levels of virulence as measured by LD_{50} determination in laboratory mice (LD_{50} = 1–10 organisms by subcutaneous inoculation).

Overall results were consistent among the three experiments. Heterogeneity of

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TABLE 1. Responses of rock squirrels to experimental infection with *Yersinia pestis*.

Group no. [calc. LD_{50}] (source of squirrels)	Subcutaneous dose	No. D/ no. I[a]	Mean TTD (days)[b]	No. surv. with aby./ total surv.[c]	Highest titer[d]	Longevity of meas. titer (days)[e]	No. infected/ no. inoc.[f]
I. [<2,000]	2.00×10^6	3/3	5.3	—	—	—	3/3
(wild-caught)	2.00×10^5[g]	2/3	4.5	1/1	—	—	3/3
	2.00×10^4	0/3	—	1/1[i]	256	540	1/1
	2.00×10^3[g]	3/3	6.0[j]	—	—	—	3/3
II. [1,816]	2.45×10^7	8/8	4.4	—	—	—	8/8
(wild-caught)	2.45×10^6[g]	6/8	4.8	0/2	—	—	6/8
	2.45×10^5	7/8	5.3	1/1	16	120	8/8
	2.45×10^4	7/8	6.3	1/1	64	≥120	8/8
	2.45×10^3	8/8	6.5[j]	—	—	—	8/8
	2.45×10^2	5/8	8.2	1/3	128	120	6/8
	2.45×10^1	0/8	—	0/8	—	—	0/8
	2.45×10^0[h]	1/8	8.0	0/7	—	—	1/8
III. [1,149]	2.40×10^7	8/8	4.1	—	—	—	8/8
(laboratory-reared)	2.40×10^6	8/8	5.0	—	—	—	8/8
	2.40×10^5	8/8	6.0	—	—	—	8/8
	2.40×10^4[g]	7/8	5.9	1/1	32	60	8/8
	2.40×10^3	7/8	7.0[j]	1/1	16	60	8/8
	2.40×10^2[h]	4/8	14.8	2/4	128	150	6/8
	2.40×10^1	0/8	—	0/8	—	—	0/8
	2.40×10^0	0/7	—	0/7	—	—	0/7

[a] No. D/no. I = number dead/number inoculated.
[b] Mean TTD = average time to death (days).
[c] No. surv. with aby./total surv. = number survivors producing antibody/total number of survivors.
[d] Highest titer = highest positive titer measured for any survivor in dose group.
[e] Longevity of titer = number of days following single laboratory challenge that an anti-plague titer was still detectable. ≥titer still present at day stated = individual animal died or otherwise not sampled beyond day stated.
[f] No. infected = number dead + number survivors demonstrating seroconversion.
[g] Highest dose permitting survival of individual animals.
[h] Lowest dose causing death in individual animals.
[i] ⅔ animals had prechallenge titers—representing residual antibody from prior field infection(s). Data given for the single animal with a negative baseline serum.
[j] Average time to death at LD_{50} dose.

individual responses to plague infection is perhaps the most remarkable feature of the results, as demonstrated in death/survival rates and LD_{50} determinations, time until death among animals that succumbed to infection, qualitative and quantitative production of antibody to *Y. pestis* among survivors, and longevity of measurable *Y. pestis* antibody titers after one laboratory challenge.

The estimated LD_{50} values for adult wild-caught animals with unknown exposure histories but that had no detectable plague antibodies were less than 2,000 organisms in the pilot study and 1,816 organisms in the larger study. Young adult, laboratory-reared squirrels were somewhat more susceptible, demonstrating an LD_{50} of 1,149 organisms. Individual animals, however, survived doses of 10^6 organisms, while at least one other succumbed to a dose of as few as 10^0 organisms. The mean time until death for squirrels that succumbed to plague infection varied approximately in relation to the numbers of *Y. pestis* inoculated (Table 2); at about the LD_{50} dose the mean time until death was 6–7 days.

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TABLE 2. Range and mean times to death among rock squirrels following experimental inoculation with *Yersinia pestis*.

Dose	Pilot test		Wild-caught adult		Lab-reared subadult	
	Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)
10^7	ND[a]	ND	3–6	4.4	3–5	4.1
10^6	4–6	5.3	3–7	4.8	4–6	5.0
10^5	4–5	4.5	4–6	5.3	4–10	6.0
10^4	—[b]	—	4–8	6.3	5–6	5.9
10^3	5–7	6.0[c]	5–11	6.5	5–10	7.0
10^2	ND	ND	6–13	8.2	7–29	14.8
10^1	ND	ND	—	—	—	—
10^0	ND	ND	8	8	—	—

[a] ND = not done.
[b] — = not applicable, no deaths in group.
[c] *n* = approximate LD_{50} level results.

Among wild-caught animals that succumbed to laboratory infection, 12% (6 of 49) developed pneumonitis and bled from the nose during terminal stages; others (2 of 49, 4%) developed extensive petechial hemorrhages, especially noticeable on the chest and abdomen, indicative of coagulopathy. Five of 43 (12%) of the laboratory-reared animals that succumbed demonstrated pneumonia while one other (2%) had petechial hemorrhaging.

Development of these lesions is at about the same frequency as that seen in human patients and suggests the rock squirrel as a possible experimental host for laboratory study of plague pneumonia and possibly of plague coagulopathies.

The 50%-infectious doses could be calculated only for the two larger experiments with rock squirrels; these levels were 52 organisms for wild-caught and 51 organisms for the laboratory-reared animals. The LD_{50}'s were 1,816 and 1,149 organisms, respectively, for wild-caught and laboratory-reared animals.

The responses of rock squirrels to experimental plague infection are comparable to those reported by Williams et al. (1979) for the California ground squirrel as shown in Table 3. The differences seen may be attributed to minor differences in laboratory methodology or to minor variability between the plague strains used. Alternatively, the differences seen may reflect developing resistance to plague by California ground squirrels, but this hypothesis needs further study.

One rock squirrel inoculated with 245 plague organisms died after being bled on the 29th day postinoculation. Initially it was felt that death may have been caused by handling and bleeding techniques, but cultures of spleen and liver at necropsy grew plague organisms. Blood was not cultured, therefore the bacteremic status of the animal is unclear. Recovery of plague cells from the animal 29 days after inoculation suggests the possibility that fleas may become infected by feeding on hosts surviving prolonged periods and serve to extend the epizootic period. The finding also suggests the need to provide

TABLE 3. Comparative LD_{50} and ID_{50} responses of rock squirrels and California ground squirrels to experimental plague infection.

Parameter	Rock squirrels	California ground squirrels[a]
LD_{50}	1,149–1,816	6,070
ID_{50}	51–52	256
Average days to death	6–7	7.9

[a] Data from Williams et al. (1979).

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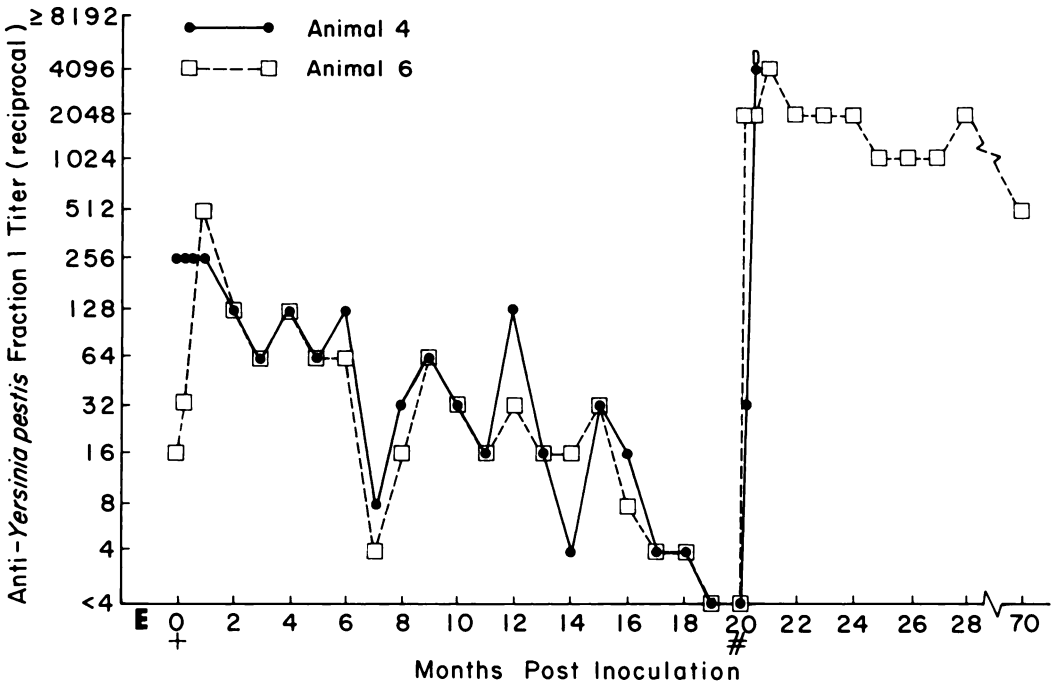


FIGURE 1. Serological responses of two rock squirrels repeated by challenge with *Yersinia pestis*. E = Prior field exposure(s). + = Subcutaneous inoculation with *Y. pestis*: (i) 2×10^4 organisms, animal number 4, ●——●; (ii) 2×10^5 organisms, animal number 6, □- - -□. # = Second challenge with 1×10^6 *Y. pestis* organisms. D = Animal 4 died.

effective flea control measures for at least a month in those areas where plague in rock squirrels requires control.

Serologic responses: Except for two animals used in the pilot study, none of the rock squirrels had detectable antibodies to *Y. pestis* before experimental laboratory inoculation. Those two animals, for which response data have been excluded from those of other animals, had titers of 16 and 256, indicative of prior infection(s) with *Y. pestis* (Fig. 1). Both animals survived laboratory challenge with 2×10^4 *Y. pestis* organisms. Their (anamnestic) serologic responses were followed at monthly intervals to 570 days postinoculation when *Y. pestis* antibodies were no longer detectable. A second laboratory challenge of 1×10^6 *Y. pestis* organisms was administered to both animals at about the 600th day after the first challenge, and their *Y. pestis* antibody titers were measured periodically thereafter. One animal died 21 days after the second laboratory challenge from causes unrelated to plague infection. The surviving squirrel still had detectable antibodies 50 mo after the second laboratory challenge.

Formation of detectable antibody after inoculation with *Y. pestis* was as varied in these tests with rock squirrels as reported for California ground squirrels by Williams et al. (1981). For rock squirrels, however, antibody production apparently was not related to dose. Thirty animals survived doses of 10^0 and 10^1 organisms. None of the 30 produced antibody, which may indicate that these challenges were not infectious doses. However, no antibody was detected in sera from the two squirrels surviving 10^6 organisms nor in sera from four of seven that survived 10^2 organisms. Antibody was detected in the sera of only 10 squirrels, nine of which

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survived inoculation with 10^2 to 10^5 organisms. (One animal had an antibody titer of 4 on the 10th postinoculation day but died of plague before the 21st postinoculation day.) Only one of the nine remaining seropositive animals developed a high antibody titer (8,192), and antibody remained detectable in serum specimens from this animal for 450 days after laboratory challenge of 10^5 organisms. The maximum titer detected in the other eight seropositive squirrels was 128 (geometric mean maximum titer [GMMT] for the eight squirrels was 38; if the 9th survivor's maximum titer is included, the GMMT is 69). Serum antibody was detectable, on the average for the eight squirrels, for more than 94 days; on inclusion of the 9th survivor's data, the average antibody longevity increases to >133 days postinoculation.

Antibody production was seen by the 10th postinoculation day in three squirrels, one of which died of plague infection before the scheduled serum sampling on the 21st day. The data for this animal, which was not considered a survivor, were excluded from further discussion. One of the remaining two animals had a titer of 32, and the other had the highest titer seen (8,192) among all surviving squirrels. This early, high response and its prolonged longevity described above, offer evidence that this animal also may have had prior infection with *Y. pestis* but previous antibody had decreased below detectable levels (or never developed) by the time of laboratory studies. Of the remaining seven animals, antibody was detected for the first time at day 21 for two, day 30 for one and day 60 for four. Sera from three of the four squirrels that had no antibodies before the 60-day sample, were again negative a month later; the titers achieved by these animals were low—4, 16, and 32, respectively.

These data again are similar to those of Williams et al. (1979) for California ground squirrels and attest to the need for care in interpreting serologic results for individual animals—a titer is still indicative of the past infection, but a lack of titer in both rock squirrels and California ground squirrels, at least, is a less reliable indicator of absence of recent infection.

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