Experimental Infection of the Cotton Rat *Sigmodon hispidus* with *Rickettsia rickettsii*[1]

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Studies of experimental infection of the cotton rat, *Sigmodon hispidus*, with the virulent Sheila Smith (R type) and the avirulent Si 7 (U type) strains of *Rickettsia rickettsii* were undertaken to evaluate the role of this native wild mammal in the ecology of Rocky Mountain spotted fever. The Sheila Smith strain, which was highly lethal for guinea pigs, was nonpathogenic for cotton rats. Serial passage of the R-type strain in the cotton rat did not alter the virulence of the agent for cotton rats or guinea pigs. The U-type strain, which was originally recovered from a wild cotton rat, could not be maintained beyond the first passage in this animal host. Rickettsemia in the cotton rat occurred over a 24-hr period after inoculation of the virulent strain but was detected only 1 hr after inoculation of the avirulent strain. The short period of rickettsemia suggests that the cotton rat probably is not an important reservoir of *R. rickettsii*. Specific complement-fixing antibodies developed rapidly after infection with either strain, but the antibodies evoked by the R strain attained higher titers and persisted longer. Cotton rats previously infected with the Sheila Smith strain developed rickettsemia after reinfection with the same strain, even though relatively high levels of antibody were still present.

Evidence implicating certain species of wild mammals native to the United States in the biological survival of *Rickettsia rickettsii* is usually based upon the recognition of the occurrence of infection under natural conditions, and the known importance of the vertebrates as hosts for vector ticks. Although the recovery of rickettsiae from blood and tissues of the wild animal, or the finding of spotted fever antibodies in its serum, implies susceptibility, neither finding provides information about the importance of the species as a reservoir of infection. The cotton rat, *Sigmodon hispidus*, distributed throughout the southern United States (6), is a host for the immature stages of *Dermacentor variabilis* (2, 11), and a strain of spotted fever rickettsiae was recovered from one of this species trapped in southern Virginia (4). To evaluate the potential reservoir status of this animal, experimental infection with a highly virulent western and an avirulent eastern strain of *R. rickettsii* was in-

vestigated. Information about the following pertinent factors was obtained: (i) the occurrence of disease in the animals after infection; (ii) the onset and duration of rickettsemia; (iii) the sequence of invasion and persistence of rickettsiae in the tissues; (iv) the development and persistence of complement-fixing (CF) antibodies; (v) the susceptibility of immune animals to reinfection; and (vi) alteration in the virulence of the organism after serial passage in cotton rats.

MATERIALS AND METHODS

Cotton rats. Animals weighing 50 to 100 g were obtained from a colony which had been reared in captivity at Tumblebrook Farm, Brant Lake, N.Y. All animals were bled prior to use, and any rat whose serum was anticomplementary, or fixed complement nonspecifically in the presence of the rickettsial and control antigens, was discarded.

Strains of R. rickettsii. The Sheila Smith (SS) strain, obtained from the Rocky Moun. Laboratory, Hamilton, Mont., was used as the virulent strain. It was isolated in 1946 from a patient with Rocky Mountain spotted fever acquired in western Montana (1). Guinea pigs infected with the SS strain regularly developed fever which persisted for 5 to 10 days. Infection also caused a severe scrotal reaction with marked adhesions and subsequent necrosis of the scrotum, ears, and footpads. In most instances, the animals died by the 14th day.

The Si 7 strain, which was isolated in 1961 from a cotton rat trapped in Virginia, was employed as the avirulent strain (4). Guinea pigs infected with this strain showed no overt signs or symptoms of illness, but did develop spotted fever CF antibodies. According to the classification of virulence proposed by Price (7), the SS strain is an R type and the Si 7 a U type of *R. rickettsii*.

Although the virulence of strains of *R. rickettsii* for guinea pigs may be reduced by passage in certain wild and laboratory animals, as well as in ticks, maximal virulence for guinea pigs is attained after one to three serial egg passages and it remains unaltered for at least 12 subsequent yolk sac passages (8). Seed material was prepared for each strain by inoculating 6- to 8-day-old embryonated hens' eggs with a dilution of the rickettsial suspension which killed all embryos on the 4th or 5th day. After all eggs were dead, the yolk sacs were harvested aseptically. Smears prepared from each of the membranes and stained by Macchiavello's technique were examined microscopically, and those yolk sacs which contained the largest numbers of rickettsiae were pooled and homogenized with enough Snyder I diluent (3) to make a 20% (w/v) suspension. One-milliliter samples of the suspensions were quick-frozen in a dry ice-alcohol bath and stored at -65 C. The SS suspension was prepared from yolk sacs of the 7th egg passage, and the Si 7 suspension from the 3rd egg passage.

Each suspension was titrated by inoculation of 10-fold serial dilutions into 6- to 8-day-old embryonated eggs (6 per dilution) and into 450- to 700-g Japanese Hartley-strain male guinea pigs (5 per dilution). The eggs were injected with 0.2 ml via the yolk sac route, and the guinea pigs received 1.0 ml intraperitoneally. The 50% lethal dose (LD_{50}) and 50% infectious dose (ID_{50}) were calculated by the method of Reed and Muench (9). For the purpose of calculating ID_{50} titers, the demonstration of spotted fever CF antibodies in sera collected 21 to 28 days after inoculation was considered indicative of infection.

The LD_{50} titers of the SS and Si 7 suspensions in eggs were $10^{-5.4}$ and $10^{-5.2}$, respectively. In guinea pigs, the virulent SS strain had an LD_{50} titer of $10^{-6.5}$, which in most cases was equivalent to the ID_{50}; i.e., sera from guinea pigs which did not die after inoculation usually did not contain spotted fever antibodies. The ID_{50} titer of the avirulent Si 7 suspension for guinea pigs was $10^{-6.0}$.

Infection of cotton rats. The susceptibility of cotton rats to infection with the SS and Si 7 strains was determined by inoculating groups of six animals with 10-fold serial dilutions of the seed suspensions by different routes. Animals injected intraperitoneally or intracardially received 0.25 ml, and 0.03 ml was administered intracerebrally. They were observed daily for signs of illness and death. All survivors were bled 21 to 28 days after inoculation, and the sera were tested in CF tests for the presence of spotted fever antibodies.

In the studies of the pathogenesis of infection, the cotton rats were inoculated intraperitoneally with $10^{3.9}$ guinea pig LD_{50} of the SS strain or $10^{3.4}$ guinea pig ID_{50} of the Si 7 strain.

Detection of rickettsiae in cotton rats. The presence of rickettsiae in the blood or organs of infected cotton rats was determined by inoculating 10 to 20% tissue suspensions into either the yolk sacs of embryonated eggs or into eggs and guinea pigs. Each organ was washed in Snyder I diluent to remove exterior blood and fluids prior to preparing the suspensions for inoculation. The yolk sacs of embryos found dead after the 2nd day, as well as those from all eggs surviving 12 days, were smeared and stained by Macchiavello's stain and by indirect immunofluorescence. Rickettsiae were considered to be present when observed by either of the methods. Guinea pigs were examined for fever (≥ 104 F) and scrotal reaction daily for 14 days. Serum was obtained from survivors 21 to 28 days postinoculation and was tested for the presence of spotted fever CF antibodies.

Rickettsemia. Each of two cotton rats was bled from the heart 1, 6, 12, 18, 24, 36, and 48 hr after intraperitoneal inoculation of either the SS or Si 7 strain, and daily thereafter for 21 days. The heparinized blood from each animal, diluted to 10% with Snyder I diluent containing 100 units of penicillin and 100 μg of streptomycin per ml, was inoculated into five embryonated eggs to determine the presence of rickettsiae.

Appearance and persistence of rickettsiae in tissues. After infection by the intraperitoneal route with the spotted fever strain under study, two animals were exsanguinated daily for 21 days, and the brain, kidneys, liver, lungs, and spleen were removed aseptically from each. The presence of rickettsiae was determined by examining Giemsa- and immunofluorescent-stained smears prepared from the cut surface of each tissue, and by inoculation of eggs and guinea pigs, as previously described.

Development and persistence of spotted fever antibodies. Groups of 12 to 15 cotton rats were infected with each of the spotted fever strains. Each animal was bled every 3 days for 4 weeks and once a month thereafter by using the orbital bleeding technique (10). The sera were tested individually for the presence of spotted fever CF antibodies.

Susceptibility of immune animals to reinfection. A group of animals, which had been initially infected intraperitoneally 7 months before with $10^{3.9}$ guinea pig LD_{50} of the SS strain, was reinoculated intraperitoneally with the same dose of the SS strain. At the time of reinoculation, all of the animals had CF antibodies ranging in titer from 1:80 to 1:160. Another group, which had been infected 3 months prior with $10^{3.4}$ guinea pig ID_{50} of the avirulent Si 7 strain and which still had persisting antibody titers of 1:5 to 1:40, was reinoculated with the same dose of Si 7 organisms. The occurrence and duration of rickettsemia, as well as the appearance and persistence of rickettsiae in the different organs, were determined in the manner already described.

Serial passage of R. rickettsii in cotton rats. Two animals were infected intraperitoneally with yolk sac suspensions containing $10^{3.9}$ guinea pig LD_{50} of the SS strain. On the 4th day, both cotton rats were sacrificed and the livers and spleens were removed and pooled for preparation of a 20% (w/v) suspension for inoculation into two more cotton rats. This procedure was

repeated every 4 days. The 4-day interval was selected because it coincided with the time of maximal concentration of rickettsiae in the tissues and preceded the development of CF antibody. At selected passage levels, guinea pigs and additional cotton rats were inoculated with the rat liver-spleen passage material. These latter animals were observed daily for signs of illness, and serum collected from survivors 21 to 28 days postinoculation was tested for the presence of spotted fever antibodies.

Serial passage of the Si 7 strain in cotton rats was initiated with $10^{3.4}$ guinea pig ID_{50}. Since it had been shown previously that the Si 7 strain could not be maintained by serial passage in guinea pigs (4), the procedure for the serial passage of this agent in cotton rats was modified slightly from that just described. Groups of four animals were used at each passage level; two were sacrificed on the 4th day to provide tissues for the next passage, and the remaining animals were tested 21 to 28 days after inoculation for serological evidence of spotted fever infection.

Complement fixation tests. The cotton rat and guinea pig sera were tested with four units of soluble spotted fever group-reactive antigen prepared from yolk sacs infected with *R. rickettsii* (Bitterroot strain) or TT-118. The latter strain is a spotted fever group rickettsia recovered from a mixed pool of 62 *Ixodes* and *Rhipicephalus* larval ticks collected in Huai Mae Sanem, Thailand, in November 1962, by one of the authors (B. L. E.). The procedure of the test and method of preparation of diagnostic and control antigens have been described (4). The cotton rat sera were inactivated at 60 C for 20 min and the guinea pig sera at 56 C for 30 min prior to testing.

RESULTS

Susceptibility of cotton rats to spotted fever infection. The ID_{50} titers of SS and Si 7 seed suspensions titrated in cotton rats, inoculated intraperitoneally, were $10^{-6.4}$ and $10^{-5.6}$, respectively. Irrespective of the dose, all animals experienced only inapparent infections associated with the development of CF antibodies. The magnitude of the antibody response 21 to 28 days after inoculation was not related to antigenic mass administered, i.e., antibody titers in the sera of the cotton rats inoculated with the higher dilutions were the same as in those which received the 10% suspensions.

Intracerebral or intracardial inoculation also caused only inapparent infection. These latter routes were used in an attempt to find a method of producing demonstrable illness or death of uninfected animals, in order to be able to assay immunity by resistance to challenge.

Rickettsemia. Rickettsiae were detected in the blood of one or both of the cotton rats infected with $10^{3.9}$ guinea pig LD_{50} of the SS strain 6, 18, and 24 hr after inoculation. Thus, four of ten animals developed rickettsemia during the first day after infection with the virulent strain. Of the

ten animals examined during the first 24 hr after infection with $10^{3.4}$ guinea pig ID_{50} of the avirulent Si 7 strain, rickettsiae were present in the blood of only one animal, taken 1 hr after inoculation. The level of rickettsemia after infection with both strains was of low magnitude as rickettsiae were found only in a portion of the embryonated eggs inoculated with a 1:10 dilution of the infected blood. Rickettsemia was not demonstrated after the first day of infection with either strain of *R. rickettsii.*

Occurrence and persistence of rickettsiae in tissues. After intraperitoneal infection with the SS strain, rickettsiae were present in the kidneys, liver, lungs, and spleen on the first day and persisted in these organs for almost 3 weeks (Table 1). Organisms were not detected in the brain until the 7th day. The Si 7 organisms persisted for a much shorter time; they were found in the liver, lungs, and spleen from the 1st through the 3rd day after inoculation, in the kidneys on days 2 and 3, and in the brain on day 3 only.

Although rickettsiae were more readily detected in the smears of tissues stained by the immunofluorescent technique than in Giemsa-stained smears, neither method was as sensitive as inoculation of the specimens into eggs. When organisms were seen by either method of microscopic examination, rickettsiae were always recovered in eggs.

Development and persistence of spotted fever antibodies. All SS-infected cotton rats developed detectable antibodies ranging in titer from 1:5 to 1:40 between the 3rd and 6th day after inoculation (Fig. 1). On the 12th day, the majority of the rats had titers of 1:640 or greater, and the lowest titer obtained at that time was 1:160. The peak geometric mean titer, 1:600 or greater, was obtained during the 3rd and 4th weeks, after which there was a slight decline. The titers ranged from 1:160 to 1:640 for 8 months and then dropped fourfold in the 9th and 10th months, when the experiment was terminated. As in the animals inoculated with the SS strain, antibodies in the sera of rats infected with the Si 7 organisms were first detected between the 3rd and 6th day after inoculation, with the titers ranging from 1:5 to 1:80. The maximal mean titer of 1:61 was found on the 9th day. The antibody levels fell during the ensuing months and were undetectable at a 1:2.5 dilution of serum after 9 months.

Susceptibility of immune animals to reinfection. Rickettsiae were detected in the yolk sacs of eggs inoculated with each of the bloods of the two SS-infected rats which were bled 12 hr after reinfection with $10^{3.9}$ guinea pig LD_{50} (Table 2). There was no evidence of rickettsemia, however, in the rats reinfected with the avirulent strain.

TABLE 1. *Time of appearance and persistence of rickettsiae in tissues of cotton rats after primary infection with Rickettsia rickettsii*

Strain	Tissue	Day postinoculation																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	28
Sheila Smith	Brain......	$-^a$	−	−	−	−	−	$+^b$	+	+	−	+	+	+	+	−	+	+	+	−	−	−	−
	Kidneys...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−
	Liver......	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−
	Lungs.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
	Spleen.....	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	−	−
Si 7	Brain......	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Kidneys...	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Liver......	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Lungs.....	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Spleen.....	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

^{a} − = No rickettsiae in tissue of either of two animals sacrificed.
^{b} + = Rickettsiae detected in the tissue of one or both animals sacrificed.

Because of the limited number of SS-immune animals available for reinoculation, two animals were sacrificed 2, 6, 10, and 14 days after reinfection to determine the presence of organisms in various tissues. Rickettsiae were recovered in the eggs inoculated with the lung suspension from immune cotton rats sacrificed on day 6, from the livers harvested on the 14th day, and from the kidneys taken on days 2, 6, and 10. Organisms were not recovered from the brain and spleen, nor were they seen in stained smears of the peritoneum. Two reinfected Si 7 rats were sacrificed every other day from day 1 through day 21, and at no time were rickettsiae detected in any of the tissues.

Serial passage of R. rickettsii in cotton rats. Cotton rats infected with the SS strain did not produce detectable CF antibodies during the 4 days prior to autopsy, and they did not develop any overt clinical manifestations throughout 15 serial passages. The only gross abnormality observed at necropsy was that the spleens of most of the rats were slightly enlarged and were covered with a gray exudate. Each of the rat liver-spleen suspensions from the 5th, 10th, and 15th passages was inoculated into two guinea pigs, and the 10th and 15th passage materials were injected also into three to six additional cotton rats. All of the guinea pigs developed symptoms typical of infection with a highly virulent strain of spotted fever and died within 6 to 12 days. No signs of disease were observed in the cotton rats, but all developed spotted fever CF antibodies with titers ranging from 1:160 to 1:640 or greater.

The avirulent Si 7 strain could not be maintained by serial passage in cotton rats. Four rats were infected initially with $10^{3.4}$ guinea pig ID_{50}
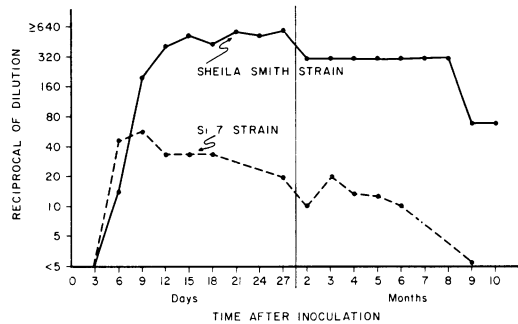

FIG. 1. *Geometric mean complement-fixing antibody titers of 12 to 15 cotton rats after infection with R. rickettsii. Sera tested with 4 units of spotted fever group reactive antigen.*

of yolk sac material. Two of the animals were sacrificed 4 days after inoculation, and the remaining two were held 28 days for testing for serological evidence of infection. These latter two animals had antibody titers of 1:20 and 1:40. The liver-spleen suspension from the first-passage rats had an ID_{50} titer of 10^{-2} in guinea pigs. However, the second-passage cotton rats which received 20 guinea pig ID_{50} of this suspension failed to develop complement-fixing antibodies. Similarly, sera from guinea pigs inoculated with the tissue suspension from the second-passage cotton rats were negative. In two subsequent passages, no serological evidence of infection was found in either cotton rats or guinea pigs inoculated with the cotton rat liver-spleen suspensions.

DISCUSSION

In light of the current knowledge of the ecology of Rocky Mountain spotted fever, two basic

TABLE 2. *Circulation of rickettsiae in blood of cotton rats after reinfection[a]*

Strain	Time after first infection (months)	Range of persisting complement-fixing antibody titer	Time of rickettsemia (hr)				
			1	6	12	18	24
Sheila Smith	7	1:80 to 1:160	0[b]/2	0/2	2/2	0/2	0/2
Si 7	3	1:5 to 1:40	0/2	0/2	0/2	0/2	0/2

[a] Sheila Smith-immune cotton rats received $10^{3.9}$ guinea pig LD_{50} of the same strain. Si 7-immune cotton rats received $10^{3.4}$ guinea pig ID_{50} of the Si 7 strain.

[b] Numerator = no. of animals in which rickettsiae were detected in the blood. Denominator = no. of animals sacrificed at indicated time interval.

mechanisms are important for the biological survival of *R. rickettsii:* one involves the transovarial and transstadial transmission of the agent in vector ticks, and the other is the cyclic transmission of rickettsiae from infected to uninfected ticks through the medium of a vertebrate reservoir. A valid assessment of the role a mammalian species may play in the perpetuation of spotted fever rickettsiae can be made only after certain aspects of the host-parasite relationship are known. In order to function as a reservoir host in Rocky Mountain spotted fever, susceptible vertebrate species must be capable of transmitting the infection to vector ticks that feed upon them. Rickettsiae are discharged from infected ticks while feeding and may circulate in the blood of susceptible animals. The relative efficiency of the mechanism of direct transfer of rickettsiae from infected to uninfected ticks at the time of coincident feeding on different species of animals is not known. Rickettsemia also may occur during the initial period of active infection. Where there is persistence of organisms in the tissues long after the acute stages of infection have subsided, rickettsemia may recur if recrudescence of the infection occurs. Although recrudescence of latent infection is known to occur with epidemic typhus in man, and with Q fever in domestic animals, it remains to be shown that wild animals infected with spotted fever or other rickettsiae can experience similar recurrences. Species to species variation in relative effectiveness as a reservoir can be expected but, as the present investigation shows, within a single species the virulence of the infecting strain is an additional factor that influences the ability of animals to serve as a source of infection for vector ticks.

It is generally accepted that, on the basis of the clinical response of guinea pigs, strains of *R. rickettsii* recovered in the western part of the United States are more virulent than those isolated in the east. Strains are classified as R, S, T, or U types; the most virulent is R type and the least virulent is U type (7). There is, however, a certain degree of variation in the virulence among strains in the two regions (8).

S. hispidus was susceptible to experimental infection with the R-type virulent strain, SS, and with the U-type avirulent Si 7 strain. The 50% infectious dose of suspensions of each of the strains was virtually the same for cotton rats and guinea pigs. Although the SS strain was highly lethal for guinea pigs, it was nonpathogenic for cotton rats, irrespective of the dose of rickettsiae or the route of inoculation. Throughout 15 serial passages of the SS strain in cotton rats, the strain remained nonpathogenic for this host, and virulence for guinea pigs was not altered. By contrast, previous studies with cottontail rabbits, dogs, and opossums showed that R-type strains decreased in virulence to S- and T-type strains after 8 to 11 tissue passages in the respective animals. On the other hand, no change in virulence was noted when R strains were passaged in Columbian ground squirrels, chipmunks, and meadow voles (7). Less virulent strains of the S and T types could not be maintained in cottontail rabbits, meadow voles, or dogs beyond 8 or 9 serial passages. The U-type Si 7 strain in the present study, which was originally recovered from a wild cotton rat, could not be maintained beyond the first passage in this species, nor, as has been previously reported, in guinea pigs (4).

Significant differences were found between the antibody response of cotton rats to infection with the R-type and the U-type strains. Peak antibody titers in animals infected with the SS strain were at least eightfold higher than those infected with the Si 7 strain, and the antibodies persisted at higher levels for a much longer period of time. Because the maximal age of cotton rats in the wild is probably less than 1 year (12), animals infected in nature with virulent strains would be expected to maintain demonstrable levels of CF antibodies throughout their natural life. Failure to find antibodies would not exclude the possibility of prior infection with strains of low virulence.

Burgdorfer (5) evaluated the role of various species of small mammals native to western Montana as sources for infecting larval *D. andersoni* with a highly virulent strain of *R. rickettsii*. He estimated that 10 to 100 guinea pig ID$_{50}$ per 0.5 ml of blood was required to infect 50% of the larvae feeding on susceptible vertebrate hosts. The threshold dose of virulent or avirulent spotted fever rickettsiae required to infect *D. variabilis*, the principal vector in the eastern United States, is not known. However, in studies of the experimental infection of *Microtus pennsylvanicus*, subadult stages of *D. variabilis* which fed upon animals inoculated with a low-virulent T-type strain became infected only during the first week. This interval coincided with the period of rickettsemia which was demonstrated by recovery of the organisms from blood. Rickettsiae were recovered from certain tissues of the meadow vole for about 1 month after infection (7).

In the present investigation, it was not possible to determine if the level of rickettsemia which developed in cotton rats was sufficient to infect subadult ticks, as a colony of *D. variabilis* known to be free of spotted fever infection was not available. If the susceptibility of *D. variabilis* is presumed to be similar to that of *D. andersoni*, transmission of the virulent strain would have occurred only during the first day after initial infection. The occurrence of rickettsemia only 1 hr after inoculation of the avirulent strain would have provided even less opportunity for infection of ticks. Since the virulent strain persisted in the tissues for only 3 weeks, and the avirulent strain for not longer than 3 days, it is unlikely that possible reactivation of a latent infection in the cotton rat contributes to the maintenance of spotted fever.

It is usually presumed that, once an animal is infected, the immunity that develops precludes a further role in the maintenance of enzootic rickettsioses. However, following the inoculation of the virulent SS strain, rickettsiae were recovered from the blood and tissues of cotton rats that had been infected 7 months previously with the same strain. The course of the second infection was somewhat curtailed, as was indicated by the shorter period of rickettsemia and the limited distribution of organisms in the tissues. Nonetheless, the demonstration of rickettsiae circulating in the blood even in the presence of relatively high levels of antibody implies that "immune" members of animal populations may be still capable of serving as a reservoir host.

The evidence provided by this study of the course of *R. rickettsii* infection in the cotton rat indicates that this species has the capacity to make only a minor contribution in the survival of virulent strains of Rocky Mountain spotted fever in nature. It probably plays little, if any, role as a reservoir host for the perpetuation of avirulent strains.

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