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EXPERIMENTAL INFECTION OF COTTON RATS WITH THREE NATURALLY OCCURRING *BARTONELLA* SPECIES

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ABSTRACT: The kinetics of infection and humoral immune response of laboratory-bred cotton rats (*Sigmodon hispidus*) challenged with three *Bartonella* spp. recovered from the blood of naturally infected cotton rats captured in Georgia (USA) are described. *Bartonella* spp. infection, as determined by bacteremia, occurred in all 18 cotton rats inoculated with live *Bartonella* of each species at either a low dose, 10^3 colony-forming units (CFU's), or high dose, 10^7 CFU. Cotton rats inoculated with lower doses of *Bartonella* spp. developed higher bacteremia that persisted for longer periods than in those inoculated with high doses. Peak bacteremia varied among *Bartonella* spp, ranging from 10^4 to 10^6 CFUs per 1.0 ml of blood. Antibody measured by immunofluorescence assays using species-specific antigens indicated more rapidly rising and higher antibody titers in cotton rats challenged with high doses vs. low doses and with inactivated bacteria vs. live bacteria. Each group of rats produced high IgG titers to the homologous challenge antigen; low or unmeasurable cross-reactivity was detected to heterologous *Bartonella* antigens. Exposure of cotton rats to a specific *Bartonella* sp. resulted in protection, as measured by detectable bacteremia, in eight of nine animals challenged with the same *Bartonella* sp. used initially; no evidence of resistance to secondary challenge with different *Bartonella* spp. was obtained. Cross-protection between *Bartonella* spp., isolated from the same rodent species, may not occur.

Key words: Animal model, bacteremia, *Bartonella* spp., cotton rat, experimental infection, immune response, rodents, *Sigmodon hispidus.*

INTRODUCTION

Bacteria of the genus *Bartonella* (former *Rochalimaea*) are responsible for a variety of distinct diseases in humans. *Bartonella quintana* is the etiologic agent of trench fever, and *B. henselae* is the causative agent of cat scratch disease. In addition to specific diseases, these organisms have been associated with various syndromes, such as endocarditis and bacillary angiomatosis, that may be caused by multiple etiologic agents (Anderson and Neuman, 1997; Koehler, 1996). Although nonhuman vertebrate reservoirs for all the *Bartonella* spp. that cause human disease are believed to exist, only the domestic cat has been conclusively demonstrated to act as a primary reservoir, as in the case of *B. henselae.*

Within the last several years, field and laboratory investigations have demonstrated that *Bartonella* spp. exist in wildlife, and several novel species have been isolated from rodents (Birtles and Harrison, 1994; Birtles et al., 1995; Kosoy et al., 1997). Rats of the genus *Rattus* from North and South America are infected with *Bartonella* sp. that is identical or closely related by DNA analyses to *B. elizabethae* (Birtles and Raoult, 1996; Ellis et al., 1998), an organism associated with human endocarditis (Daly et al., 1993). The ability of other *Bartonella* spp. isolated from rodents to act as human pathogens is undefined. Research on the natural occurrence of *Bartonella* spp. in rodent communities of the southeastern United States has indicated that various *Bartonella* bacteria are widely distributed and highly prevalent in rodents. All 47 *Bartonella* isolates obtained from cotton rats (*Sigmodon hispidus*) were clustered based on sequence analysis of the citrate synthase gene (*glt*A) in three well-supported phylogenetic groups (Kosoy et al., 1997). Those phylogenetic groups presumably represent unique and novel species, as the levels of similarity in sequence homology between them are comparable with the

levels between well-characterized and named species, *B. henselae, B. quintana, B. vinsonii,* and *B. elizabethae* (Birtles and Raoult, 1996). Immunologic comparison of sera obtained from BALB/c mice inoculated with the three *Bartonella* spp. isolated from cotton rats demonstrated a high degree of specificity to the homologous antigen (Kosoy et al., 1996). However, rodents tested from natural populations, whether bacteremic or not, have no detectable antibody or only low antibody titer, as measured by immunofluorescence using homologous antigens (Kosoy et al., 1997).

An obstacle in investigations of the natural history of *Bartonella* spp. infection and transmission has been the lack of a suitable animal model or natural host for establishing experimental infection. Until the discovery of *B. henselae* and its association with the domestic cat, attempts to produce *Bartonella* spp. infections in laboratory animals were unsuccessful or limited in scope. Inbred CD-1 mice have been used to produce immune sera to *B. henselae* and *B. quintana* (Slater et al., 1992), and *B. henselae* has been isolated from the blood, liver, and spleen of previously immunocompromised mice at 3 to 15 days postinoculation (p.i.) (Brouqui et al., 1996).

Cats inoculated with *B. henselae* develop persistent bacteremia of as long as 3 mo duration. These animals also develop a high-titered antibody response within about 2 wk following infection (Greene et al., 1996; Regnery et al., 1996). Although the development of serum antibody is not temporally associated with clearance of bacteremia, animals do not develop bacteremia upon rechallenge. In contrast to the situation with natural rodent populations, serosurveys have demonstrated antibody prevalence as high as 80% in natural cat populations (Childs et al., 1994). Although cats are an important reservoir for *B. henselae* and laboratory infections are readily produced, their size and associated cost of maintenance limits their use by many investigators.

We describe here experimental infections in laboratory-bred cotton rats with *Bartonella* spp. which occur naturally in this rodent host. The objectives of this study were to describe the dynamics of *Bartonella* spp. infection as measured by bacteremia and the development of antibody response, investigate challenge exposure in cotton rats, and develop additional immunologic and biological criteria to define new *Bartonella* spp.

MATERIALS AND METHODS

Three *Bartonella* spp., as determined on the basis of sequence analysis (Kosoy et al., 1997), serologic typing (Kosoy et al., 1996), and phenotypical description (M. Y. Kosoy, unpublished data), were used for the experimental study. All the microorganisms were isolated from the blood of cotton rats captured in Georgia (USA). The *Bartonella* spp., conditionally designated as A, B, and C, were closely related, with percent nucleotide sequence homologies of 92.3% (between A and B), 88.7% (A and C), and 90.2% (B and C) based on a 338-base-pair (bp) sequence of *glt*A (Kosoy et al., 1997). The *glt*A sequences obtained from the species have the following GenBank accession numbers: A-U84372, B-U84375, and C-U84377 (Kosoy et al., 1997).

The bacteria were cultivated on heart infusion agar plates supplemented with 5% rabbit blood (Becton Dickinson Microbiology System, Cockeysville, Maryland, USA) at 32 C in an aerobic atmosphere with 5% carbon dioxide. Species A had been passed five times, and species B and C had been passed three times. Each isolate was grown on an agar plate until confluent and then suspended in sterile phosphate-buffered saline, pH 7.6 (PBS). Serial 10-fold dilutions in PBS were plated on agar to calculate inoculum dose.

Cotton rats were purchased from Virion System, Inc. (Rockville, Maryland, USA). The breeding colony was established in 1989 with inbred stock obtained from the National Institutes of Health (Bethesda, Maryland, USA) and has been maintained on an inbred/outbred basis since then. Thirty cotton rats, 15 males and 15 females, approximately 40-day-old, were used. Blood cultures and serum antibody titers to *Bartonella* spp. antigens were negative for all cotton rats prior to experimental infection. Each cotton rat was housed separately in cages

TABLE 1. Experimental design of primary inoculations and secondary challenge to 30 cotton rats with three *Bartonella* spp.

Cotton rat[a] (record, sex)	Primary *Bartonella* spp. inoculum		Boost[d] with irradiated *Bartonella* spp.,[b] dose[c]	Secondary challenge[e] with live *Bartonella* spp.,[b] dose[c]
	Species,[b] dose[c]	Irradiated, krads		
9, female	A, 1,000	no	no	A, 1,000
19, male	A, 1,000	no	no	none
28, male	A, 1,000	no	no	C, 1,000
13, female	A, 10,000,000	no	no	none
22, male	A, 10,000,000	no	no	A, 10,000,000
23, male	A, 10,000,000	no	no	C, 10,000,000
1, female	A, 10,000,000	704	A, 10,000,000	A, 1,000
2, female	A, 10,000,000	704	A, 10,000,000	C, 1,000
16, male	A, 10,000,000	704	A, 10,000,000	none
4, female	B, 1,000	no	no	B, 1,000
10, female	B, 1,000	no	no	A, 1,000
20, male	B, 1,000	no	no	none
14, female	B, 10,000,000	no	no	none
24, male	B, 10,000,000	no	no	B, 10,000,000
25, male	B, 10,000,000	no	no	A, 10,000,000
3, female	B, 10,000,000	704	B, 10,000,000	B, 1,000
5, female	B, 10,000,000	704	B, 10,000,000	none
17, male	B, 10,000,000	704	B, 10,000,000	A, 1,000
11, female	C, 1,000	no	no	C, 1,000
12, female	C, 1,000	no	no	A, 1,000
21, male	C, 1,000	no	no	none
15, female	C, 10,000,000	no	no	A, 10,000,000
26, male	C, 10,000,000	no	no	C, 10,000,000
27, male	C, 10,000,000	no	no	none
6, female	C, 10,000,000	704	C, 10,000,000	C, 1,000
7, female	C, 10,000,000	704	C, 10,000,000	A, 1,000
18, male	C, 10,000,000	704	C, 10,000,000	none
19, male	PBS	no	no	none
29, male	PBS	no	no	B, quintana, 10,000,000
30, male	PBS	no	no	B, elizabethae, 10,000,000

[a] Cotton rats were from an inbred colony at Virion System, Inc., Rockville, Md.
[b] *Bartonella* spp. were isolated from the blood of cotton rats collected in Georgia, USA. The species A, B, and C have the following GenBank accession number: U84372, U84375, and U84377, respectively.
[c] Colony-forming units per 1.0 ml of inoculum.
[d] Inoculation given 7 wk p.i.
[e] Challenged 11 wk p.i.

covered with microisolator tops and was given food and water ad libitum.

The nine cotton rats in the low-dose group were inoculated with 1.0 ml of PBS containing 10^3 colony-forming units (CFUs) of each *Bartonella* sp. (three rats per species) via a combination of intraperitoneal (0.5 ml) and subcutaneous routes (0.5 ml distributed at three dorsal sites), using 22-ga needles. Similarly, nine cotton rats in the high-dose group were inoculated with 10^7 CFU's of the same species (Table 1). Three cotton rats received only diluent (PBS) as negative controls. The weight and length of cotton rats were measured at day 0 (before inoculation), at 9 wk p.i. when most rats had cleared their bacteremia, and at the termination of the study (22 wk p.i.). All protocols were reviewed and approved by the Centers for Disease Control and Prevention Animal Care and Use Committee (CDC, Atlanta, Georgia, USA).

Nine cotton rats (three animals per species) were inoculated with 10^7 CFUs of irradiated *Bartonella* spp. suspended in 1.0 ml of PBS. Seven weeks later, all of the cotton rats were boosted with the same irradiated suspension (Table 1). The route of administration was identical as that used for live *Bartonella* spp. Suspensions of 10^7 CFU's of the *Bartonella* spp. were exposed to 704 krads of gamma ra-

diation (Gammacell, model 220, Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada). This Gammacell contained Cobalt-60 with an activity of 23,477 rads/min on the day of the inoculum irradiation. The samples were kept frozen on dry ice during irradiation. A suspension (0.1 ml) of each isolate of the irradiated *Bartonella* spp. was plated on agar to assess inactivation.

As a preliminary effort to assess immunity and resistance to both heterologous and homologous challenge, 18 cotton rats that received either live or irradiated initial inoculum were challenged at 11 wk p.i. with live *Bartonella* spp. In each group of three animals that received the same primary inoculum, one animal was challenged with the same species as the primary inoculum, a second animal was challenged with a different species, and the third animal was not challenged (Table 1).

For blood bleeding animals were anesthetized by intraperitoneal administration of a 1:10 mixture of xylazine (Rompun©, The Butler Company, Norcross, Georgia, USA) and ketamine hydrochloride (Ketaset©, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA). Blood samples (0.3 to 0.5 ml) were collected from animals by retroorbital bleeding, using sterile heparinized capillary tubes at weekly intervals through the first month p.i. and at 2 wk intervals for the next 6 mo. Blood samples were stored at −70 C until use.

Blood was diluted 1:4 in brain heart infusion medium, and 0.1 ml was applied to heart infusion agar plates supplemented with 5% rabbit blood (Becton Dickinson Microbiology System). Cultures were examined daily for bacterial growth and CFU's were assessed at 7 to 10 days. When CFU's were too high to be counted after primary plating, serial fourfold dilutions of the original sample were plated until an accurate count could be determined. The minimal threshold bacteremia was equivalent to 40 CFU/ml.

Cultures of *Bartonella* spp. recovered from cotton rats were confirmed by polymerase chain reaction (PCR) and sequence analysis as previously described (Kosoy et al., 1997). The cultures were prepared for PCR by using QIAamp Tissue Kit (Qiagen, Inc., Chatsworth, California, USA). Primers homologous to *glt*A gene of *B. henselae* were used to amplify a 379-bp product. Sequences were analyzed by STADEN and Wisconsin Sequence Analysis Package 8.1 Unix (Genetic Computer Group, Inc., Madison, Wisconsin, USA) and aligned with species A, B, and C used for inoculation and challenge.

Indirect fluorescence antibody test (IFA) was performed as previously described (Kosoy et al., 1997). The antigens were prepared from three *Bartonella* spp. (A, B, and C) grown on Vero E6 cell monolayer at 32 C in an aerobic atmosphere for 4 days. Wells of 12-well spot slides were dotted with antigens, fixed with acetone, and overlaid with blood dilutions. The slides were incubated at 37 C for 30 min and washed twice in PBS for 15 min. A mixture of anti-mouse and anti-hamster conjugate (Organon Teknika Corporation, Durham, North Carolina, USA) labeled with fluorescein isothiocyanate was used. Whole blood was initially diluted at 1:32 and titrated in twofold dilutions to endpoint (maximum of 1:16,384). The antibody titers were expressed as the reciprocal of the highest dilution showing specific fluorescence of *Bartonella* spp.

Differences in antibody titer and CFU counts among groups of rats were examined among species (A, B, and C) and between doses (low, 1×10^3 CFU's versus high, 1×10^7) by week p.i. Statistical comparisons were made across a period of 11 wk p.i. when most cotton rats had been non-bacteremic for at least 2 wk and before rechallenge. Because of differences identified in exploratory analysis, separate two-way analyses of variance (ANOVA) were performed comparing cotton rat responses to inoculation with different *Bartonella* spp. and doses. In each analysis the main effects were weeks p.i. and cotton rat response to either dose or species of inoculum, as measured by CFUs of bacteremia or antibody titer. When the interaction term was statistically significant at the $P < 0.05$ level, or near significance ($P < 0.08$), separate 1-way ANOVA's were used to examine week-by-week differences in CFU counts or antibody titers among the different groups of three rats. For statistical calculation of antibody titer mean, antibody titers below the 1:32 cutoff were arbitrarily set at 1:8, a level four-fold below the dilution considered positive. Values of $P < 0.05$ were considered significant, and all values reported were for two-tailed tests of significance.

RESULTS

All of the cotton rats inoculated with live *Bartonella* spp. (A, B, or C) became bacteremic (Fig. 1). The highest level of bacteremia was detected in cotton rats inoculated with genotype A (peak ranges for three animals, 250,000 to 800,000 CFU/ml for low dose and 7,600 to 800,000 CFU/ml for high dose). The number of *Bartonella* colonies recovered from cotton rats inoculated with species C were lower (peak

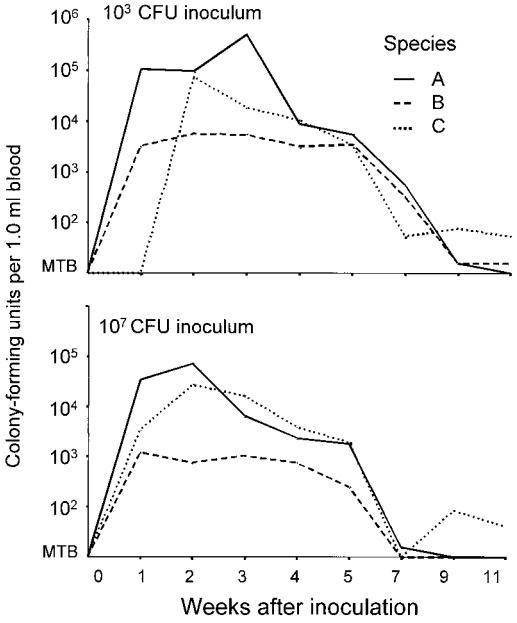

FIGURE 1. The development of bacteremia, as measured by counting colony-forming units (CFU), in six groups of cotton rats. The level of bacteremia varied according to *Bartonella* spp. (A, B, or C) and the challenge dose (10^3 and 10^7 CFU). Minimal threshold bacteremia (MTB) was equivalent to 40 CFU/ml (using 0.1 ml of 1:4 diluted blood as an initial inoculum for plating).

range, 21,000 to 160,000 CFU/ml). The lowest bacteremia was found in rats inoculated with species B (peak range, 1,200 to 10,000 CFU/ml). Inoculates of species A and B at either dose produced bacteremia in 100% of rats within 1 wk p.i., while cotton rats inoculated with the low dose of species C did not develop detectable bacteremia until second week p.i (Fig. 1). Bacteremia typically persisted for approximately 5 wk in rats receiving the high-dose inoculum and for 7 wk in rats receiving the low dose (Fig. 1). However, cotton rats inoculated with species C were bacteremic for a longer period, with one rat (Table 1, animal 21) remaining bacteremic at 15 wk p.i.

For two *Bartonella* spp. (A and B), CFU counts among groups of cotton rats varied significantly by both inoculum dose and week p.i. ($P < 0.001$). When CFU counts between groups of rats receiving the different inoculum doses were compared by week, the group of cotton rats inoculated with the low dose of species A had significantly higher colony counts compared with animals receiving the high dose at week 3 (mean 550,000 and 12,500 CFU/1 ml, respectively) and at week 7 (mean 630 and 13 CFU/1 ml, respectively). For rats inoculated with species B, the low-dose group had significantly higher colony counts compared with the high-dose group at weeks 3 (mean 5,870 and 1,030 CFU/1 ml, respectively), at week 5 (mean 4,370 and 480 CFU/1 ml, respectively), and at week 7 (mean 347 and 0 CFU/1 ml, respectively). For rats receiving species C, colony counts varied significantly by the week p.i., but the two doses produced indistinguishable mean CFU's.

Similar analyses compared CFU's obtained at different time intervals from groups of cotton rats infected with different *Bartonella* spp. but at either the high or low dose (Fig. 2). At the low dose, differences were apparent both among the three species and at the different sampling intervals, resulting in significant week-by-species interactions for all comparisons. When mean CFU counts for the low dose groups of rats were compared by week p.i., rats infected with species A had significantly higher CFUs compared with those exposed with species B at weeks 2 and 3 p.i. and species C at weeks 1 and 3 (Fig. 1).

With groups of rats inoculated with the high dose, significant differences were apparent among the species and at different sampling intervals ($P < 0.001$), but no species-by-week interaction was noted ($P = 0.18$). Overall, rats inoculated with high doses of species A had a significantly higher mean CFU over the period of study and developed peak bacteremia more rapidly than the other rats receiving high doses of B and C species (Fig. 1).

Infected cotton rats had no detectable illness and no animals died following infection. There was no weight loss associated with infection. Groups of animals in-

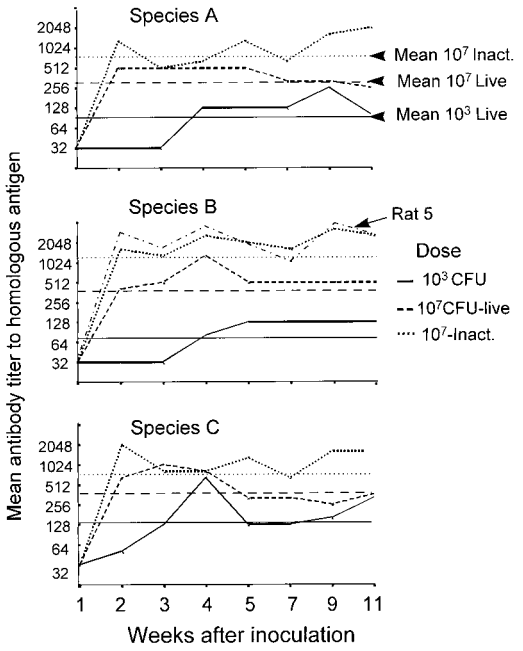

FIGURE 2. The development of antibody responses in nine groups of cotton rats varied according to the dose [10^3 and 10^7 colony-forming units (CFU)] and irradiated status (live or inactivated) of the inoculum of *Bartonella* spp. (A, B, or C). Animals receiving the live high dose of species A or B had significantly higher mean titers than the animals receiving the low dose of the same *Bartonella* spp. averaged over the entire 11 wk (as indicated by the dashed and solid lines). Animals receiving the irradiated high dose had significantly greater titers than the animals receiving either dose of live organisms (indicated by the dotted line). Rat number 5 in the species B panel received an improperly inactivated high dose of irradiated *Bartonella* spp. and reacted as if it had been inoculated with a high dose of inactivated bacteria.

oculated with each of the live *Bartonella* species were indistinguishable from each other and from control animals (rats receiving PBS or irradiated *Bartonella* spp.). Mean weights for females and males were 98 g and 116 g, respectively, for infected cotton rats and 98 g and 118 g, respectively, for control groups at 9 wk p.i. Mean weights for females and males were 113 g and 160 g, respectively, for infected cotton rats and 125 g and 149 g, respectively, for the control group at 22 wk p.i. Although not specifically weighed, the spleens of infected animals were indistinguishable in size from those of control animals.

No antibody reactive to the *Bartonella* spp. antigens (A, B, and C) was detectable in cotton rats prior to inoculation. Cotton rats inoculated with the high dose of live bacteria of each *Bartonella* spp. showed marked antibody responses by the second week p.i., with 100% of animals having titers above the baseline at 32, ranging from 128 to 2,048 to the homologous antigen (Fig. 2). When heterologous antigens of other *Bartonella* spp. isolated from cotton rats were used, low titers at 32 (baseline) were detected in two rats; all other rats were seronegative.

Animals receiving the low dose of *Bartonella* spp. were slower to develop measurable titers, and titer values never achieved the same levels as for animals inoculated with either of the high doses of live or irradiated *Bartonella* spp. (Fig. 2), with the highest peak titer observed at 516. By week 2, rats inoculated with the high dose of species A had significantly higher antibody titers than rats inoculated with the low dose ($P < 0.01$; Fig. 2); this pattern continued through week 9, at which time differences in titer became non-significant. The identical pattern occurred with the species B; significant differences were apparent between groups of rats inoculated with different doses by week 2 p.i. and continued through week 5 p.i. ($P < 0.05$ at each interval). This pattern was again the same for the species C; greater titers were observed for the high inoculum groups of rats throughout the 11-wk period.

Groups of rats challenged with the three *Bartonella* spp. showed significantly different antibody titers depending on the week p.i. and on the different doses of the inoculum (Fig. 2). At low doses, groups of rats inoculated with species C showed the most rapid antibody rise and had significantly higher titers (mean 171) than those inoculated with species A or B at week 4 p.i. (mean 12). At week 9, rats inoculated with the low dose of species A had higher titers (mean 64) than rats with species B

(mean 32; $P < 0.05$), but were not significantly different from rats with species C.

Antibody titers among groups of rats inoculated with the high doses of *Bartonella* spp. were indistinguishable through week 7. At week 9, rats inoculated with the high dose of species B had a significantly higher titers (mean 128) than rats inoculated with species C (mean 64), but not those inoculated with species A (mean 85). Similarly, at week 11, rats inoculated with the high dose of species B had a significantly higher titers (mean 128) than those receiving genotype A (mean 64), but were indistinguishable from rats receiving species C (mean 96).

All cotton rats immunized with high doses of inactivated *Bartonella* spp. developed high antibody titers to the homologous antigen (Fig. 2). In each case, antibody responses in rats inoculated with the inactivated organisms were significantly higher ($P < 0.05$) than those receiving the high dose of live organisms. Overall, mean titers for species A were 265 versus 132 for inactivated and high dose live bacteria, respectively; for species B were 520 versus 192, respectively; and for species C were 337 versus 158, respectively (Fig. 2).

No bacteremia was detected in rats receiving the irradiated *Bartonella* spp. at any time point for inoculates of species A and C. However, one rat (Table 1, rat 5) inoculated with the irradiated species B did develop bacteremia and seroconverted specifically to species B (rat 5 in Figs. 2). The kinetics of this animal's response to infection were an interesting mix; the antibody response was marked and indistinguishable to that of rats receiving high doses of irradiated *Bartonella* of species B (Fig 2, middle panel), while the bacteremia was relatively high and similar to that of animals receiving a low-dose inoculum of species B. We conclude that the irradiation process left a small number of live bacteria that were undetectable when irradiated material (0.1 ml) was plated, but sufficient to produce infection in the rat with 1.0 ml of the inoculum.

Of the 18 cotton rats reinoculateded after spontaneous loss of initial bacteremia, reinfection with resulting bacteremia was demonstrable in all nine animals challenged with *Bartonella* spp. that were different from the primary inoculum (Table 2). Peak bacteremia was in the same range (12,000 to 800,000 CFU/ml) as those produced by the primary inoculation and the dose or irradiation/live status of the primary inoculation had no influence on reinfection with a different species.

Cotton rats previously inoculated with live or inactivated bacteria of species A and B and later challenged with a homologous species failed to become bacteremic during the 10 wk of follow up. A single animal of three rats initially inoculated with species C did become bacteremic when challenged with the same species (Table 2, rat 6). Challenge with the homologous species boosted specific antibody titers to the homologous antigen in five of nine animals. None of the nine animals challenged with a heterologous species developed an enhanced antibody response to the species used in the primary infection, but eight produced antibody, with titers ranging from 32 to 1,024, to the heterologous antigen present in challenge inoculum (Table 2).

DISCUSSION

This study describes the course of infection in laboratory-reared cotton rats inoculated with three *Bartonella* spp. originally isolated from field-captured rodents of the same species. The study monitored the kinetics of the infection by bacteremia at weekly or biweekly intervals and determined the kinetics of the humoral immune response as measured by IFA, using both homologous and heterologous *Bartonella* spp. antigens. The resulting data suggest the cotton rat is a useful animal for the study of the ecological features of *Bartonella* spp. infections in a natural animal reservoir. Furthermore, the results indicate that infections induced by different *Bartonella* spp., all naturally occurring and iso-

TABLE 2. Bacteremia and IgG antibody responses in cotton rats after secondary challenge reinfection with homologous and heterologous *Bartonella* spp.

Cotton rat,[a] record	Primary *Bartonella* spp. inoculum		Secondary *Bartonella* spp. challenge		
	Species,[b] dose,[c] irradiation status[d]	Peak antibody response[e]	Species,[b] dose,[c] irradiation status[d]	Bacteremia, CFU/1.0 ml; species[f]	Peak antibody response[e]
9	A, low, live	32 to A	A, low, live	<40	512 to A
22	A, high, live	256 to A	A, high, live	<40	1,024 to A
1	A, high, inactive	1,024 to A	A, low, live	<40	2,048 to A
4	B, low, live	32 to B	B, low, live	<40	<32
24	B, high, live	512 to B	B, high, live	<40	512 to B
3	B, high, inactive	1,024 to B	B, low, live	<40	2,048 to B
11	C, low, live	256 to C	C, low, live	<40	256 to C
26	C, high, live	256 to C	C, high, live	<40	512 to C
6	C, high, inactive	1,024 to C	C, low, live	21,000; C	256 to C
28	A, low, live	64 to A	C, low, live	48,000; C	64 to A; 32 to C
23	A, high, live	128 to A	C, high, live	12,000; C	256 to C; 32 to A
2	A, high, inactive	1,024 to A	C, low, live	60,000; C	512 to A; 32 to C
10	B, low, live	32 to B	A, low, live	140,000; A	128 to A
25	B, high, live	256 to B	A, high, live	80,000; A	256 to A; 128 to B
17	B, high, inactive	512 to B	A, low, live	800,000; A	128 to B; 32 to A
12	C, low, live	128 to C	A, low, live	14,000; A	<32
15	C, high, live	256 to C	A, high, live	80,000; A	512 to A; 128 to C
7	C, high, inactive	1,024 to C	A, low, live	120,000; A	256 to C; 64 to A

[a] Cotton rats were from an inbred colony at Virion System, Inc., (Rockville, Maryland, USA)
[b] *Bartonella* spp. were isolated from the blood of cotton rats collected in Georgia, USA. The species A, B, and C have the following GenBank accession numbers of nucleotide sequence of citrate synthase gene: U84372, U84375, and U84377, respectively.
[c] Doses: low—1,000 colony-forming unit (CFU), high—10,000,000 CFU.
[d] *Bartonella* spp. were exposed to 704 krads of gamma radiation.
[e] Reciprocal of peak antibody titer to antigens of *Bartonella* spp. A, B, and C.
[f] *Bartonella* spp. were confirmed by sequencing of 379 bp of the citrate synthase gene.

lated from the same population of wild cotton rats, show variation in their development and that resistance to challenge appears to be predominantly species specific.

The time required for reproduction of bacteria to a detectable level was short, as after 1 wk p.i., *Bartonella* spp. were found in the blood of all the experimental animals except those inoculated with the low dose of species C (Fig. 1). The cotton rats that were inoculated with species A, at either low or high doses, had the highest concentration of microorganisms in their blood after 1 wk, and typically their bacteremia remained higher than those produced by other species. These observations are consistent with the observed growth characteristics of the different *Bartonella* spp. in *vitro.* After primary plating of blood collected from wild-captured cotton rats, colonies later confirmed as *Bartonella* sp. A were visible after 4 days, while species C growth typically required 7 days before becoming visible (M. Y. Kosoy, unpubl. data).

Cotton rats inoculated with the lower doses of *Bartonella* spp. eventually developed higher CFUs of bacteremia, regardless of the inoculated species, than those initially inoculated with higher doses. Bacteremia was persistent and was detectable in rats receiving low dose inoculum for approximately 2 wk longer (9 wk; Fig. 1) than in those receiving higher doses (7 wk). Two cotton rats receiving species C remained bacteremic for 15 wk. The long-term persistence of *Bartonella* in the blood of rodents acting as their natural hosts was not unexpected. Infections in cats chal-

lenged with *B. henselae* also spontaneously resolve at about 8 wk p.i. (Regnery et al., 1996). In addition, the high prevalence of bacteremia in natural populations of cotton rats indicated that either the duration of bacteremia or the incidence of infection had to be considerable to result in standing infections of 80% (Birtles and Harrison, 1994; Kosoy et al., 1997).

Antibody was detectable in cotton rats inoculated with either the high dose of live or inactive bacteria of the three *Bartonella* spp. by 2 wk p.i. (Fig. 2). At high-inoculum doses, high-titered and species-specific IgG responses to homologous antigens were apparent. Antibody responses to the low dose inoculum were slower to develop and never achieved the titers associated with animals receiving the high dose inoculum. In some rats, antibody responses were detectable throughout the 22 wk observation period, with gradual declines in unboosted animals after 7 to 10 wk p.i. Low or unmeasurable cross-reactivity was detected to heterologous *Bartonella* spp. antigens.

The development of humoral immunity in these experimental animals was of particular interest because of our infrequent finding of antibody-positive rodents in naturally infected populations. Serologic screening of cotton rats from locations where 80% of the animals were bacteremic has demonstrated the lack or extremely low prevalence of animals with specific IgG titers higher of >32 (Kosoy et al., 1997). These findings, plus the recovery of *Bartonella* spp. from embryonic tissues and the blood of newborn wild-captured cotton rats, are suggestive of vertical transmission (Kosoy et al., 1998). The lack of antibody may be indicative of immune tolerance to the infection in this natural host, or it may indicate some immunosuppressive or other characteristic of the organism to evade the immune response. The dose-dependent antibody response found in our experiments suggests that if inoculum doses near the 50% infectious dose (ID_{50}) were identified, then little antibody would be detectable. However, antibody responses after challenges with low doses of live inoculum reached titers as high as 512, that are almost never observed among wild-captured animals.

Exposure of cotton rats to one *Bartonella* sp. with either live or inactivated bacteria resulted in evident resistance to secondary challenge with a homologous species, as measured by lack of bacteremia among eight of nine animals (Table 2). The reinfection of animals with different *Bartonella* sp. resulted in levels of bacteremia comparable with those following initial inoculation. These results suggest that cross-protection between *Bartonella* spp., isolated from the same rodent species, may not occur. A high degree of specificity in the immune response to these different *Bartonella* spp., the lack of cross-protection between them, and different kinetics of growth provide support for the genetic data (Kosoy et al., 1997) indicating that these *Bartonella* spp. deserve species status.

The cotton rat has served as an experimental model for an extensive list of human and animal pathogens, such as poliovirus, typhus rickettsiae, diphtheria toxin, human parainfluenza virus, respiratory syncytial virus, human and bovine adenoviruses, and filarie (Faith et al., 1997; Mayer and Mayer, 1944; Prince, 1994). The present study indicates that cotton rats also may offer opportunities to investigate persistence, transmission mechanisms, and immunological control of *Bartonella* spp. infections in *vivo.*

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