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Susceptibility of Owl Monkeys (*Aotus nancymaae*) to Experimental Infection with *Bartonella bacilliformis*

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Bartonellosis, caused by *Bartonella bacilliformis*, is a clinically significant disease in parts of South America, where it is characterized by fever and hemolytic anemia during the often-fatal acute stage and warty skin eruptions during chronic disease. In this study, we evaluated owl monkeys (*Aotus nancymaae*) as a potential model for studying the immunogenicity and pathology of bartonellosis. Two groups of animals (n = 3 per group) received either 9.5×10^7 CFU *B. bacilliformis* by the ID route or 1.1×10^6 CFU by the IV route and were followed for 140 d. Animals were evaluated by physical exam, complete blood count or hematocrit (or both); infection was confirmed by Giemsa staining of blood smears, PCR amplification, and blood culture. On days 7 and 21, Giemsa-stained blood smears from both groups contained organisms (1% to 4% of erythrocytes). All blood cultures and PCR tests were negative. Complete blood counts and chemistry panels showed no difference from baseline. Serology revealed a greater than 4-fold increase in the IgM titer (compared with baseline levels) in the 3 animals from the ID group and 1 animal from the IV group. On day 35, a dermal lesion was excised from the inguinal region of 1 monkey from each group, with a second lesion excised on day 84 from the same monkey in the IV group. However the histopathology and immunostaining of these samples were not consistent with *B. bacilliformis*. The present study shows that owl monkeys can be infected with *B. bacilliformis*, but additional dosage studies are necessary to evaluate the usefulness of this species as a disease model for human bartonellosis.

Abbreviations: CBC, complete blood count

Bartonellosis (Carrion disease, Oroya fever, verruga peruana) is a complicated, multistage infectious disease caused by the bacterium *Bartonella bacilliformis*. *B. bacilliformis*-associated disease is limited almost exclusively to the Andes mountain region of South America because of the limited habitat of its sand fly vector, *Lutzomia verrucarum*. In the valleys of the Andes, approximately 60% of the human population is seropositive for the bacterium, and 5% to 10% of the population are active carriers of the disease.[9] For 1997 through 2005, the Peruvian Ministry of Health reported a 10-fold increase in the incidence of bartonellosis (948 to 10,390 cases).[16] Oroya fever is the hemolytic, immunosuppressive manifestation of acute infection[1] and has a case fatality rate of as high as 90% if left untreated; death is often associated with bacterial and protozoal superinfections.[2,20] The chronic stage of bartonellosis produces disfiguring warty, vascular nodules on the skin and is termed verruga peruana (Peruvian wart disease), which has a prolonged course but ultimately resolves and seldom results in death.[4] To date, the organism has failed to be isolated from an animal reservoir, suggesting that eradication of the disease could be achieved by successful vaccination of the human population where the disease is endemic.

Rhesus macaques can be infected with *B. bacilliformis* and develop Oroya fever (without the severe anemia) and granulomatous nodules resembling verruga peruana.[14] Later experiments with mice, hamsters, guinea pigs, rabbits, and rhesus monkeys revealed their susceptibility to infection and found that the rabbit was the most susceptible.[19] In these previous studies, however, the animals were inoculated by direct injection with biologic samples from infected patients, and no dose quantitation was performed. The purpose of the present study was to inoculate owl monkeys (*Aotus nancymaae*) with a defined inoculum of *B. bacilliformis* to determine their susceptibility to infection. We selected owl monkeys because they are native to Peru and are representative of New World nonhuman primates; previous studies with Old World primates produced conflicting results.[14,19] Small size, ease of handling, and lack of *Cercopithicine herpesvirus 1* make owl monkeys an ideal nonhuman primate to use as an animal model. With the establishment of a nonhuman primate model that approximates the disease in humans, new strategies for the prevention and treatment of bartonellosis could be developed.

Materials and Methods

Animals. Animal studies were approved by the Naval Medical Research Center Detachment Institutional Animal Care and Use Committee (protocol no. NMRCD06-4) and the Department of the Navy Bureau of Medicine and Surgery. Captive-born owl monkeys (*A. nancymaae*) were purchased from the Instituto Veterinario de Investigaciones Tropicales y de Altura (University of San Marcos, Peru). We randomly selected 3 male and 3 female owl monkeys (weight, 1 to 1.5 kg; age, 2 to 9 y) from the nonhuman primate issue pool at our facility, which is accredited by AAALAC International; all husbandry and experimental proce-

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dures were performed in compliance with the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals*.[13] Animals were pair-housed in standard metal cages with nest boxes and perches. A commercially formulated monkey diet (New World Primate Diet 8794N, Harlan Teklad, Madison, WI) was fed daily. The diet was supplemented with a variety of fresh fruits and monkey biscuits purchased from the animal vendor. Distilled water was provided ad libitum. Animals were provided a reverse 12:12-h light:dark cycle that is offset from the normal day so that monkeys could be observed during their active time.

Bacteria. The inoculum was prepared from a fatal clinical isolate of *B. bacilliformis* that was collected and characterized as part of an epidemiological study by our facility (COIN600-01; GenBank accession no., AY114119).[1] The inoculum was grown in F1 biphasic media (solid phase [w/v]: 2% Bacto Agar (Becton, Dickinson, Sparks, MD), 0.5% NaCl, 0.1% dextrose, 2% Bacto Proteose Peptone Number 3 (BD, Sparks, MD); liquid phase [v/v]: 91% RPMI 1640, 9% inactivated FBS) for 7 d at 28 °C.[11] A 100-µl aliquot of the liquid phase was used to inoculate each of 25 Columbia Blood Agar plates (1% pancreatic digest of casein, 0.5% Bacto™ Proteose Peptone Number 3, 0.5% yeast extract, 0.3% beef heart digest, 0.1% corn starch, 0.5% NaCl, 1.5% agar, 10% sheep blood [w/v]; Difco (BD, Sparks, MD)), and the plates were incubated at 28 °C for 10 d. The bacteria was harvested and serially diluted into PBS to an OD of 0.235, which was determined to be equivalent to 1×10^4 CFU/ml by previous plating of serial dilutions. The dilutions calculated containing 1×10^8 and 1×10^6 CFU/ml, were used to inoculate the group I (ID) and group II (IV) animals, respectively. Postinfection plating of the inoculum determined that the actual concentrations were 9.5×10^7 CFU/ml for the ID group and 1.1×10^6 CFU/ml for the IV group.

Animal infection and evaluation. We randomly assigned 6 owl monkeys to 2 groups of 3 animals each. Group I animals each received ten 0.1-ml injections of *B. bacilliformis* inoculum ID (9.5×10^7 CFU total dose) over the lower back using a 26-gauge needle after the hair had been removed with electric clippers using a #10 blade. Group II received 1.1×10^6 CFU of *B. bacilliformis* IV into the femoral vein by use of a 26-gauge needle. We chose ID inoculation to simulate the natural route of infection from sandfly bites, whereas the IV route was used as a comparison to determine whether direct systemic inoculation increased the severity or onset of disease. Animals were followed for 140 d after inoculation. Animals were restrained manually, and body temperature was recorded daily for 7 wk to determine the presence of fever. Animals received weekly physical exams and weight monitoring for the duration of the study. Blood was collected from febrile animals (temperature exceeding 40.5 °C, which is upper end of the normal range for our colony) or displaying clinical signs of disease (that is, pallor, lymphadenopathy, lethargy, or anorexia) for culture, Giemsa staining of smears, and PCR assay to detect the presence of *B. bacilliformis*. Complete blood count (CBC) or hematocrit was performed on alternate weeks for 7 wk to detect the presence of anemia. Serum chemistry panels were evaluated every other week for 7 wk after inoculation. Blood culture, PCR analysis, serology, and Giemsa-stained blood smears were evaluated weekly after inoculation until day 49; thereafter, CBC, chemistry panels, blood culture, PCR analysis, serology, and Giemsa-stained blood smears were evaluated on days 77, 112, and 140.

PCR assay. 50-100 µl of whole blood was collected onto filter paper (no. 1, Whatman International, Maidstone, Kent, UK) and allowed to dry. DNA was extracted by using the FTA protocol (Whatman). PCR amplification was performed by using the Ampli*Taq* Gold PCR Master Mix (Applied Biosystems, Foster City, CA) and primers 781P and 1137N specific for the *gltA* citrate synthase gene.[15]

Blood culture. Whole blood (1 ml) was inoculated into 30-ml (25-cm^2) bottles of F1 biphasic media and incubated for 14 d at 28 °C. Cultures were tilted briefly every 3 d so that the liquid phase washed over the solid media. Positive blood culture was determined by the presence of colonies on the solid media.

Giemsa blood smear. A single drop of whole blood was smeared onto a glass slide and allowed to dry for 30 min. Slides were fixed in absolute methanol and allowed to dry, after which they were stained with Giemsa (1:20 v/v) for 15 min and washed briefly with distilled water. Slides were read under light microscopy (magnification, ×400), and the level of parasitemia was calculated as the average percentage of infected erythrocytes in 10 fields of view.

ELISA. ELISA was performed as previously described[21]; 96-well microtiter plates were coated with Pap31 (0.5 µg per well), a dominant *B. bacilliformis* antigen, diluted in 100 µl PBS and incubated overnight at 4 °C, rinsed 3 times with PBS + 0.1% Tween 20 and blocked with skim milk. Animal sera were diluted 1:100 in PBS + 0.02% sodium azide, and 100 µl of the diluted sera was added to each well of the ELISA plates. Plates were incubated for 1 h at room temperature and washed 4 times with PBS + 0.1% Tween 20. Peroxidase-conjugated mouse antihuman IgG (1:8000 dilution; Fc specific; Accurate Chemical and Scientific, Westbury, NY) and goat antihuman IgM (1:1000 dilution; µ-chain specific; Kirkegaard and Perry, Gaithersburg, MD) were added to wells. After 1 h of incubation at room temperature, the plates were washed 4 times with PBS + 0.1% Tween 20, with a final wash with PBS only before the addition of ABTS (Kirkegaard and Perry, Gaithersburg, MD). Optical density (OD_{430}) was measured after 15 min of incubation at room temperature.

Clinical chemistry. Complete blood counts were obtained by using EDTA-anticoagulated whole blood in an automated hematology instrument (Coulter JT, Coulter, Hialeah, FL). Serum was analyzed for glucose, BUN, creatinine, calcium, albumin, total protein, alanine aminotransferase, aspartate transaminase, γ-glutamyl transferase, alkaline phosphatase, total bilirubin, and amylase by use of an automated clinical chemistry instrument (Piccolo, Abaxis, Sunnyvale, CA).

Histology. Excised dermal lesions were immersion-fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, sectioned at 4 to 5 µm, stained with hematoxylin and eosin, and evaluated with light microscopy (Department of Pathology, Naval Medical Research Center, Silver Spring, MD). Immunofluorescent staining was performed as described previously.[10]

Results

On day 2 after inoculation, erythematous, dermal indurations were evident at the sites of ID injection in all animals from Group I. Figure 1 shows the appearance of these lesions on day 7. Injection-site reactions persisted in all animals through day 49 postinoculation before resolving and did not appear to cause the animals any discomfort. On day 5 after inoculation, an ID-inoculated animal presented with a body temperature of 41.7 °C; this fever lasted 1 d. Day 5 Giemsa-stained blood smears, PCR assays, and blood cultures from this animal were negative for *B. bacil-*

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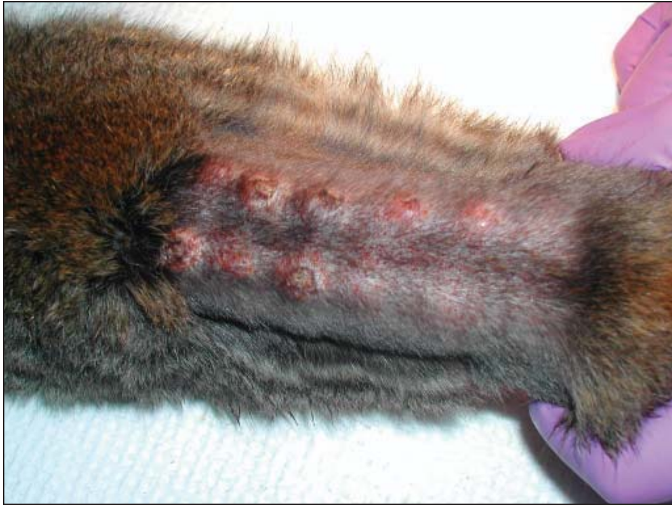

Figure 1. Day 7 after inoculation. ID injection sites are swollen, firm, and erythematous. The reaction to the injections persisted through day 49 postinoculation.

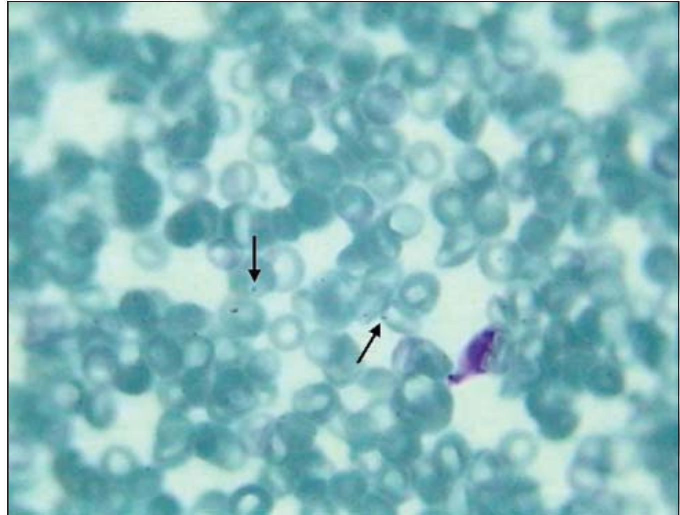

Figure 2. Day 21 after inoculation. Giemsa blood smear from ID-inoculated monkey. *B. bacilliformis* organisms are indicated with arrows. Parasitemia was graded as 4% of erythrocytes infected.

liformis. All of the ID-inoculated animals gained weight by Day 140, whereas only 1 IV-inoculated animal gained weight during the study. Weight loss in the remaining 2 IV-inoculated animals was not clinically significant.

Parasitemia, monitored as percentage of erythrocyte infection, was visualized by use of Giemsa-stained thin blood smears on days 7 and 21 postinfection. On day 72, animals in the ID- and IV-inoculated groups demonstrated parasitemia at 1% and 3% (quantified as the percentage of erythrocytes containing bacteria in 10 consecutive microscopic fields), respectively. On day 21, 2 IV-inoculated animals were parasitemic at 1% each, and 2 ID-inoculated animals were affected at 1% and 4% each (Figure 2). Further erythrocyte invasion was not noted after the 21-d period, and blood smears from the other animals remained negative throughout the study. Serologic analysis of animals on days 7, 21, 35, 49, 77, and 112 demonstrated at least 4-fold increase of IgM titers over baseline (day 0) levels in all 3 of the ID-infected animals (5.8- to 7.5-fold increase) and 1 of the IV-infected animals (7.5-fold increase). PCR assays and blood cultures were negative at all time points in the study. CBC, hematocrit, and serum chemistry values did not change significantly from baseline throughout the observation period. Study results are summarized in Table 1.

Dermal lesions present on day 35 were excised from the inguinal region of 1 monkey from each group, and an additional lesion from the same ID-inoculated monkey was biopsied on day 84. Histopathologic diagnosis from hematoxylin-and-eosin–stained slides suggested staphylococcal dermatitis. In addition, immunostaining of lesions was negative for *B. bacilliformis*.

Discussion

We demonstrated that owl monkeys (*A. nancymaae*) can be infected with *B. bacilliformis*, as indicated by low-level parasitemia (1% to 4% of erythrocytes infected in 4 of 6 animals) and a 4-fold–increase in IgM titers, despite the absence of clinical signs that commonly develop in humans. Our findings contrast with results from work done in the 1920s,[14] in which young rhesus macaques displayed intermittent fever and decreased erythrocyte number and hemoglobin concentration after IV inoculation, although the number of organisms inoculated is not clear. In addition, animals inoculated in the earlier study[14] developed positive peripheral blood cultures and injection-site granulomatous nodules that were rich in capillaries and from which the organisms could be recovered. We noted areas of induration at the sites of ID inoculation on day 2 after injection; these findings were similar to those at 10 to 14 d postinfection in the earlier study.[14] Some of these disparate results can be attributed to differences in experimental procedure. Of particular note is the manner in which the inoculum was prepared. In the previous study,[14] verrugous lesions from infected humans were excised, homogenized, and injected into the study animals without quantification of the infectious dose. Despite interspecies differences in pathogenesis, the degree of erythrocyte parasitemia observed experimentally in nonhuman primates is far less than that seen in human infections, where levels can reach 100%.[6,7,17]

Consistent with our findings, a previous study in which normal and splenectomized rhesus monkeys were inoculated with 1×10^9 CFU by the IV and ID routes did not result in organisms in peripheral blood cultures or elicit hematocrit changes or clinical signs of infection consistent with verruga peruana. [19] Blood films stained with Giemsa were negative for the organism, and the animals remained grossly normal with no complications.[19]

PCR is generally regarded as a highly sensitive method for detection of pathogenic organisms. Nonetheless, our negative findings should not be considered definitive. In providing laboratory support for numerous human outbreaks over the past 5 y, we have learned that the particular PCR assay we used is not consistently sensitive to parasitemias lower than 10%.[5] Therefore the failure of PCR to detect bartonella organisms in peripheral blood is not unexpected. Another measure of bartonella infection is the IgM–IgG ELISA. Postinfection sera from 4 of the 6 animals had a greater than 4-fold increase in IgM antibodies over baseline values. This magnitude of increase typically indicates active infection,[22] and these results thus strengthen our claim that owl monkeys can be infected by *B. bacilliformis.* The observation that all of the animals infected by the ID route mounted an immune response whereas only 33% of the IV-infected animals did so warrants mention,

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Table 1. Summary of results from owl monkeys inoculated with *B. bacilliformis* by the IV (1.1×10^6 CFU) and ID (9.5×10^7 CFU) routes

Route of infection	Fever	Blood culture	Giemsa smear[a]	PCR assay	Serology	Skin lesions	Weight change (g)	CBC	HCT	Chemistry
IV	–	–	+ (3%)	–	–	+	–100	NR	NR	NR
IV	–	–	+ (1%)	–	+	–	+10	NR	NR	NR
IV	–	–	–	–	–	–	–10	NR	NR	NR
ID	–	–	+ (1%)	–	+	–	+270	NR	NR	NR
ID	+	–	+ (4%)	–	–	+	+280	NR	NR	NR
ID	–	–	–	–	+	–	+60	NR	NR	NR

NR, not remarkable.
[a]Proportion of erythrocytes with organisms is given in parentheses.

considering the ID route is a closer model of natural transmission. Given in vitro studies involving cultured human endothelial cells, current opinion holds that after the bacilli are introduced into the host through the bite of the infected arthropod, they colonize the vascular endothelium.[3,8,12] From this niche, *Bartonella* are believed to be seeded repeatedly into the blood stream to infect erythrocytes. Continuous seeding of the bloodstream may, therefore, be necessary for initiation of an immune response; IV infection may preclude the establishment of this intracellular reservoir of bacteria, instead allowing rapid clearance from the host. Furthermore, the *vir* locus of *Bartonella*, which codes putative virulence factors and a type IV secretion system, are induced only in intracellular bacteria.[18]

Although the animals we experimentally inoculated with *B. bacilliformis* did not develop clinical illness similar to that observed in natural human infection, our results do support the conclusion that further development of *A. nancymaae* as a viable animal model of infection is warranted. The lack of pronounced clinical disease may be attributed to the small sample size of animals evaluated in this pilot study or to the dose or virulence of the strain of *B. bacilliformis* used. The isolate we selected for this study originated from a 1999 case of fatal acute bartonellosis in a human and represents, according to the clinical progression of disease, the most virulent strain in our possession. Admittedly, multiple passage of the strain in the laboratory might have attenuated the virulence of the organism. As such, further analysis of the concentration of inoculum needed to induce an infection representative of human disease and the use of multiple isolates from cases of acute human disease will be required to determine the full potential of this animal model. Regardless, this report is the first description of the use of a defined concentration of *B. bacilliformis* to infect a New World nonhuman primate and represents a step toward establishing a defined model for studying the pathogenesis and immunity of Carrion disease.

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The views, opinions, and findings contained in this report are those of the authors and do not reflect official policy or positions of the Departments of the Navy or Army, Department of Defense, or the United States Government. We are military service members. This work was prepared as part of our official duties. Title 17 USC § 105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 USC § 101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties.

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