Experimental *Ehrlichia chaffeensis* infection in beagles

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A canine model for human monocytic ehrlichiosis was used to assess persistent infection and antigenic variation of *Ehrlichia chaffeensis*. Two beagle dogs were infected subcutaneously with *E. chaffeensis* Arkansas strain. The dogs were observed for 6 months after inoculation for clinical signs, blood chemistry changes, antibodies to *E. chaffeensis* and presence of *E. chaffeensis* in the blood. Both dogs developed thrombocytopenia, but exhibited normal body temperatures during the entire course of infection. In one dog, *E. chaffeensis* was cultivated for up to 74 days post-inoculation and *E. chaffeensis* DNA was detected in the dog's blood for up to 81 days. In the other dog, *E. chaffeensis* was cultured for up to 102 days and *E. chaffeensis* DNA was detected in the blood for up to 117 days. PCR amplification and DNA sequence analysis indicated that there was no genetic variation in the 120 kDa outer-membrane glycoprotein gene of *E. chaffeensis* during infection of the dogs. The dogs developed antibodies to the immunodominant proteins of *E. chaffeensis*, including the 175, 140, 120, 80, 50 and 28 kDa proteins, starting in the fifth week post-inoculation. The dogs maintained high antibody titres throughout the 6-month study period. These results indicate that dogs become carriers of *E. chaffeensis* for 2–4 months after infection without exhibiting signs of clinical disease, suggesting that dogs may serve as a natural host for *E. chaffeensis*.

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INTRODUCTION

Ehrlichia chaffeensis is the pathogen of the emerging infectious disease human monocytic ehrlichiosis. *Ehrlichia* spp. are small, obligately intracellular, Gram-negative bacteria that reside within endosomes of host cells. The genus *Ehrlichia* is a member of the family *Anaplasmataceae*, which also includes the genera *Anaplasma* and *Neorickettsia* (Dumler *et al.*, 2001). *Anaplasma* and most *Ehrlichia* species usually cause persistent infection in their natural animal hosts (Andrew & Norval, 1989; Telford *et al.*, 1996; Breitschwerdt *et al.*, 1998; Harrus *et al.*, 1998). Persistent ehrlichial infection in humans has also been documented (Dumler *et al.*, 1993; Roland *et al.*, 1995; Dumler & Bakken, 1996; Horowitz *et al.*, 1998). Experimentally infected white-tailed deer (*Odocoileus virginianus*) carry *E. chaffeensis* for up to 3 months (Davidson *et al.*, 2001). *E. chaffeensis* DNA has been detected in naturally infected dogs and *E. chaffeensis* has been cultured from experimentally infected dogs (Dawson & Ewing, 1992; Breitschwerdt *et al.*, 1998). Although these studies show that *E. chaffeensis* can infect dogs, the experiments did not determine whether dogs were persistently

infected, either because experimentally infected dogs were observed for a short period of 28 days or because blood was obtained only once from naturally infected dogs. In this study, we examined the persistence of *E. chaffeensis* infection in two experimentally infected dogs.

Another aim of this study was to investigate whether persistence of infection by *E. chaffeensis* was associated with antigenic variation of a major outer-membrane protein, gp120. One striking feature of gp120 is that it contains tandem repeats. The repeat regions contain both hydrophilic and hydrophobic domains, which is typical of transmembrane proteins (Yu *et al.*, 1997). The *E. chaffeensis* gp120 protein is an O-linked glycoprotein, in which carbohydrates account for about 50 % of the molecular mass (McBride *et al.*, 2000). The gp120 protein is expressed differentially on the surface of the dense-core cell form of *E. chaffeensis* and accumulates in intramolecular fibrillar structures (Popov *et al.*, 2000). Antigenic variability of gp120 has been observed in *E. chaffeensis* strains. The number of repeats in clinical isolates of *E. chaffeensis* varied from two to five (Chen *et al.*, 1997; Sims *et al.*, 2000; Standaert *et al.*, 2000). Other than difference in the number of repeats, no other mutation has been found in the gp120 gene of *E. chaffeensis* isolates (Chen *et al.*, 1997).

Abbreviations: IFA, immunofluorescence assay; UTMB, University of Texas Medical Branch.

METHODS

Inoculation of dogs with *E. chaffeensis*. *E. chaffeensis* (Arkansas strain) was obtained from Jacqueline Dawson (Centers for Disease Control and Prevention, Atlanta, GA, USA). *E. chaffeensis* stock used in this study was isolated originally from dog blood after laboratory inoculation of *E. chaffeensis* in our laboratory and was passaged three times in DH82 cells after reisolation from a dog.

Sera from four 6-month-old male beagle dogs were obtained from Convince. The sera were tested for antibodies to *E. chaffeensis* and *Ehrlichia canis* by immunofluorescence assay (IFA). All dogs were seronegative to *E. chaffeensis* and *E. canis*. These four dogs were subsequently purchased and used in this study. Dogs ACC and AFJ were inoculated subcutaneously with 10^6 *E. chaffeensis*-infected DH82 cells (80 % infected) as described by Dawson & Ewing (1992). Two dogs (AZY and ATL) were sham-inoculated with PBS to act as controls.

The study was approved by the Institutional Care and Animal Use Committee of the University of Texas Medical Branch (UTMB) and the dogs were housed in accordance with the *Guide of the Care and Use of Laboratory Animals* in UTMB's Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility (<http://research.utmb.edu/iacuc/>).

Dog blood collection and blood chemistry analysis. After inoculation with *E. chaffeensis*, 10 ml blood was obtained from each dog at 1-week intervals for 3 months and then at 2–3-week intervals for 3 months. Heparin anti-coagulated blood was used for cell culture. EDTA anti-coagulated blood was used for PCR and blood chemistry analysis. Complete blood counts and blood chemistry analysis were performed in the UTMB clinical laboratory and the veterinary laboratory at Texas A&M University.

Isolation of *E. chaffeensis* from dog blood. Dog blood (1 ml) was lysed in ACK lysis buffer (0.15 M NH_4Cl , 10.0 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.2) (Coligan *et al.*, 1992) at room temperature for 10 min. Leukocytes were collected by centrifugation at 400 *g* for 10 min. Cells were washed twice by centrifugation at 400 *g* for 10 min in PBS. Leukocytes were suspended in 1 ml Eagle's minimum essential medium (MEM) supplemented with 5 % bovine serum and inoculated onto a DH82 cell monolayer in a T25 flask. Flasks were rocked for 1 h at room temperature and 4 ml of 5 % MEM was then added. Flasks were incubated at 37 °C with periodic media changes and monitoring of *E. chaffeensis* growth by Diff-quick staining and PCR detection of *E. chaffeensis* DNA. Samples were considered negative if *E. chaffeensis* morulae were not observed and if PCR detection of *E. chaffeensis* DNA was also negative in DH82 cells 3 months post-inoculation of dog blood.

DNA preparation. Dog-blood DNA was extracted by using a QIAamp DNA Mini kit (Qiagen). *E. chaffeensis* genomic DNA was prepared by using an IsoQuick Nucleic Acid Extraction kit (ORCA Research) according to the instructions of the manufacturer.

PCR amplification. PCR was used to detect *E. chaffeensis* DNA from dog blood and DH82 cells. The repeat region of the *gp120* gene was amplified by using primers pxc3b (5'-CAGCAAGAGCAAGAAGATGAC-3') and pxar5 (5'-ATCTTTCTCTACAACAACCGG-3') as described previously (Yu *et al.*, 2000a). The *gp120* gene was amplified for 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. A nested PCR assay was used to amplify the 16S rRNA gene of *E. chaffeensis* from dog-blood samples. The primary PCR was 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min, using primers ECC (5'-AGAACG AACGCTGGCGGCAAGCC-3') and ECB (5'-CGTATTACCGCG GCTGCTGGCA-3'). The nested PCR was 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min, using primers HE1 (5'-CAATTGCTTATAACCTTTGGTTATAAAT-3') and HE3 (5'-TA-

TAGGTACCGTCATTATCTTCCCTAT-3') (Swift & Thomas, 1983; Zaugg *et al.*, 1986; Anderson *et al.*, 1992).

DNA sequencing. PCR products were purified by using a QIAquick PCR Purification kit (Qiagen) and were sequenced directly by using PCR primers. An ABI PRISM DNA sequencer (Perkin-Elmer Applied Biosystems) was used to sequence the DNA by the UTMB Protein Chemistry Laboratory.

IFA. Indirect IFA was used to titrate *E. chaffeensis* antibodies in the dog sera. Sera were diluted, starting at 1 : 8, in twofold increments. A dilution of 1 : 64 was used as the cut-off for a positive titre. Antigen slides were prepared by using *E. chaffeensis*-infected L929 cells (rather than DH82 cells) to avoid cross-reaction with conjugate, as DH82 is a canine histiocyte cell line. FITC-labelled anti-dog IgG (H+L-chains) was purchased from Kirkegaard & Perry Laboratories.

Protein immunoblotting. Antigens were prepared from *E. chaffeensis*-infected L929 cells. L929 cell monolayers were inoculated with *E. chaffeensis*. When 100 % of cells were infected with ehrlichiae, cells were centrifuged at 17 400 *g* for 20 min. Pellets were disrupted with a Braun-Sonic 2000 sonicator at 40 W for 30 s twice on ice. The suspension was centrifuged at 200 *g* for 10 min. The resulting pellet, which consisted primarily of host-cell debris, was discarded and the supernatant was centrifuged at 17 400 *g* for 20 min. The pellet was suspended in SPK (sucrose/potassium phosphate) buffer and was centrifuged through 30 % sucrose solution at 17 400 *g* for 30 min.

The repeat region of the *E. chaffeensis gp120* gene was expressed in *Escherichia coli* as described previously (Yu *et al.*, 1996, 2000b). The *p28-15* gene, a member of the p28 multigene family, was amplified by PCR using primers 19f (5'-GAAGCGCAATATCCAACCTCCTC-3') and 19r (5'-TGACCAATAAACACAGAAG-3'). The PCR product was cloned into pCRT7/CT-TOPO vector (Invitrogen) and the recombinant plasmid was used to transform *E. coli* BL21 to express the p28-15 protein.

Proteins were separated on NuPAGE SDS-PAGE gels (Invitrogen) and electroblotted onto nitrocellulose membranes. Alkaline phosphatase-labelled anti-dog IgG (H+L-chains) was purchased from Kirkegaard & Perry Laboratories.

RESULTS

Clinical manifestation and laboratory results of the dogs

During the 6-month observation period, neither dog manifested a fever or exhibited loss of body mass. However, thrombocytopenia was observed in both infected dogs, but not in sham-infected dogs. Thrombocytopenia within infected dogs appeared by the second week after subcutaneous inoculation with *E. chaffeensis* and was still present at the end of the study period, despite the fact that *E. chaffeensis* was not demonstrable within the blood at that time.

Culture of *E. chaffeensis* from dog blood

E. chaffeensis was cultured from blood samples of dog ACC from days 23–102 post-inoculation. *E. chaffeensis* was isolated from blood samples of dog AFJ from days 23–74 post-inoculation (Tables 1 and 2). *E. chaffeensis* was not isolated from the sham-infected dogs at any time.

Table 1. Detection of *Ehrlichia chaffeensis* and antibodies from blood samples of dog ACC

Test	Time post-inoculation (days)																			
	8	17	23	30	38	46	53	60	67	74	81	88	95	102	110	117	131	145	159	180
PCR	—	—	+	+	+	+	+	+	+	+	—	+	+	+	+	+	—	—	—	—
Culture	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
IFA	—	—	128	512	1024	4096	16384	8192	8192	8192	4096	4096	8192	8192	8192	8192	8192	4096	2048	8192
Western blotting:																				
gp120	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p28	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Detection of *Ehrlichia chaffeensis* and antibodies from blood samples of dog AFJ

Test	Time post-inoculation (days)																			
	8	17	23	30	38	46	53	60	67	74	81	88	95	102	110	117	131	145	159	180
PCR	—	—	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
Culture	—	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
IFA	—	—	64	512	8192	16384	16384	16384	16384	16384	16384	16384	8192	8192	16384	16384	16384	16384	16384	16384
Western blotting:																				
gp120	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p28	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

PCR amplification of *E. chaffeensis* DNA from dog blood

E. chaffeensis DNA was amplified from dog-blood samples by nested PCR (Tables 1 and 2). DNA sequence analysis confirmed that PCR-amplified DNA was that of the *E. chaffeensis* 16S rRNA gene. Dog ACC was PCR-positive for approximately 4 months (117 days) and dog AFJ was PCR-positive for 81 days.

Indirect IFA

Pre-inoculation dog sera and sera from the first week to the sixth month after inoculation of *E. chaffeensis* were analysed for antibodies to *E. chaffeensis* antigens by IFA. Both infected dogs developed antibodies to *E. chaffeensis* (Tables 1 and 2). Antibodies to *E. chaffeensis* were detected at the fourth week post-inoculation (day 23) at a titre of 1:64 or higher. Peak titres in both dogs were 1:16384 at the seventh or eighth week post-inoculation. Titres remained high throughout the remainder of the study. Uninfected dogs did not seroconvert to *E. chaffeensis*.

Protein immunoblotting of dog sera

Major proteins of *E. chaffeensis* that reacted with dog sera were the 175, 140, 120, 80, 50 and 28 kDa proteins (Fig. 1). Antibodies to major *E. chaffeensis* proteins appeared on the fifth to the seventh week post-inoculation and lasted until the end of the study period. Reactions of the sera with gp120 and p28 were confirmed by using recombinant proteins (Fig. 2).

Genetic variation of the *gp120* gene

The repeat region of *gp120* was amplified from all *E. chaffeensis* isolates from dogs ACC and AFJ. PCR products had identical sizes to that of the parental strain of Arkansas, which contained four repeat units of 240 bp each (Fig. 3). DNA sequencing indicated that there was no change in the repeat region of the *gp120* gene among the isolates of *E. chaffeensis* from both dogs. This indicates that any antigenic variation of the gp120 protein did not originate from insertion or deletion of repeat units within the *gp120* gene.

DISCUSSION

The life-cycle of *Ehrlichia* involves a tick vector and a mammalian host. Mammals are infected with *Ehrlichia* by the bite of infected ticks; non-infected ticks acquire *Ehrlichia* by taking a blood meal from an infected mammal. *Ehrlichia* are not transmitted transovarially from one generation of ticks to the next (Stich *et al.*, 1989); therefore, mammalian hosts are essential for maintenance of *Ehrlichia* in the environment. Carrier animals serve as reservoirs for *Ehrlichia* organisms (Swift & Thomas, 1983; Zaugg *et al.*, 1986). Our results indicate that dogs carry *E. chaffeensis* for 3–4 months with no clinical symptoms; thus, dogs can be considered a carrier for *E. chaffeensis*. Currently, no suitable animal model is available for human monocytic ehrlichiosis. Immunocompetent mice are not susceptible to *E. chaffeensis* infection and

Fig. 1. Protein immunoblotting of *E. chaffeensis* antigens reacting with sera of dogs ACC (top) and AFJ (bottom). Day, days post-inoculation of *E. chaffeensis*.

immunodeficient mice would not be suitable for studying *E. chaffeensis* infection. Our results indicate that canines may serve as a model for the study of *E. chaffeensis* infection.

The persistence of infection by *Ehrlichia* may be the result of antigenic variation of surface proteins, especially the two major outer-membrane proteins of *E. chaffeensis*, gp120 and p28. The p28 protein is encoded by a multigene family (Reddy *et al.* 1998; Yu *et al.*, 2000b). The *gp120* gene of *E. chaffeensis* contains a tandem-repeat region; each repeat is 240 bp in length. Clinical isolates of *E. chaffeensis* contain two to five repeats. Immune escape mediated by antigenic variation of the number of repeats in outer-membrane protein genes is a well-established feature of many bacteria. Group B streptococcal α C protein, a protective surface protein antigen, contains a maximum of 16 tandem repeats. Loss of repeats from the α C protein of bacteria used in a challenge inoculation resulted in decreased protection of the vaccinated host (Gravekamp *et al.*, 1996). Variants of group B streptococci that contain fewer tandem repeats have been isolated from neonates; in the presence of α C protein-

Fig. 2. Protein immunoblotting of sera of dogs ACC and AFJ reacting with recombinant *E. chaffeensis* gp120 and p28-15 proteins. Day, days post-inoculation.

Fig. 3. PCR amplification of *gp120* repeat region of *E. chaffeensis* isolated from blood samples of dogs ACC (top) and AFJ (bottom). Day, days post-inoculation of *E. chaffeensis*; ch, parental strain of *E. chaffeensis*; M, molecular marker (bp).

specific antiserum, human polymorphonuclear leukocytes killed the maternal, but not the neonatal, isolates (Madoff *et al.*, 1996). Deletion of repeat(s) in the M6 protein of group A streptococci alters the ability of certain antibodies, produced originally in response to sequences of the parent M protein, to bind to the mutant M protein (Jones *et al.*, 1988). Change in the repeats causes change in epitopes; presumably, such conformational changes in the protein give micro-organisms the ability to escape the host immune system. However, our results indicate that the repeat region of gp120 was stable during a 3–4-month period of infection in dogs. It is most likely that the number of repeats in the *gp120* gene of *E. chaffeensis* is relatively stable in natural infection over time.

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