J Vet Diagn Invest 11:55–59 (1999)

Evaluation of C3H/HeJ mice for xenodiagnosis of infection with *Ehrlichia chaffeensis*

J. Mitchell Lockhart, William R. Davidson

Abstract. Because mice are experimentally susceptible to infection with *Ehrlichia* species, C3H/HeJ mice were evaluated as a potential xenodiagnostic model for detection of infection with and isolation of *E. chaffeensis*. Intraperitoneal inoculation of mice with *E. chaffeensis*-infected DH82 cell cultures produced seroconversion, with peak serum antibody titers of 1:256, at high dosages ($>1.9 \times 10^4$ infected cells) but not at low dosages (1.9 or 1.9×10^2 infected cells). *Ehrlichia chaffeensis* was not reisolated from blood samples collected from inoculated mice on postinoculation day 21. Nested polymerase chain reaction (PCR), using primers specific for *E. chaffeensis*, was positive for only 2/70 (2.9%) tissue samples. A field evaluation in which C3H/HeJ mice were inoculated with blood and lymph node suspensions from 5 seropositive white-tailed deer, including 3 deer that were PCR positive for *E. chaffeensis*, failed to produce seroconversion in mice. The lack of seroconversion at low dosages, the failure to reisolate at any dosage, and the inability to confirm infection in PCR-positive field samples suggests C3H/HeJ mice are not a sensitive model for xenodiagnosis or detection of *E. chaffeensis*.

Since first described in 1986, more than 400 cases of human monocytic ehrlichiosis have been documented by serologic analysis at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia.[7,8] To date, however, only 3 isolates of the causative agent, *Ehrlichia chaffeensis*, have been obtained from humans using DH82 canine macrophage cell cultures.[2,3,6] Isolation of *E. chaffeensis* and related ehrlichae is considered difficult.[1,14]

A human isolate of *E. chaffeensis* has been successfully reisolated from experimentally infected dogs[4] and white-tailed deer (*Odocoileus virginianus*), the latter being a suspected reservoir host.[5] Researchers reisolated *E. chaffeensis* 6 times from the blood of 2 deer.[5] However, attempts to culture ticks and blood or other tissues from wild white-tailed deer have encountered problems such as cell culture contamination with exogenous bacteria and trypanosomes (*Trypanosoma cervi*).[9]

Laboratory rodents have been used to study the immunopathology of ehrlichial infections of veterinary medical importance such as *E. risiticii*.[11,15] Laboratory C3H/HeJ strain mice, which are unable to undergo macrophage activation,[12] were susceptible to infection with *E. risticii*. In a recent study,[13] C3H/HeJ mice and white-footed deer mice (*Peromyscus leucopus*), but not LVG strain hamsters or red-backed voles (*Clethrionomys gapperi*), were susceptible to experimental infection with *E. chaffeensis*.

Because of complications and contaminants associated with performing culture work with wild white-tailed deer, a pilot study was conducted to evaluate C3H/HeJ mice as a xenodiagnostic tool for detection of *E. chaffeensis* infection. The specific aims of this study were to use serologic, polymerase chain reaction PCR, and cell culture techniques to monitor the response of C3H/HeJ mice to inoculation with *E. chaffeensis* and to apply this xenodiagnostic procedure in a pilot study that utilized tissues from an *E. chaffeensis*-infected white-tailed deer population.

Materials and methods

Experimental inoculation of C3H/HeJ mice. Five 6-wk-old C3H/HeJ strain mice were obtained[a] and housed at animal resources facilities, College of Veterinary Medicine, University of Georgia. *Ehrlichia chaffeensis* was cultured in DH82 canine macrophage cells as previously described.[3] Uninfected DH82 cells served as the negative control inoculum.

An *E. chaffeensis* inoculum was prepared from 1 25-cm^2 culture flask. Cells were harvested when >80% where infected with *E. chaffeensis* as determined by direct fluorescent antibody (FA) analysis using a human anti-*E. chaffeensis* conjugate obtained from the CDC and diluted 1:50 in phosphate-buffered saline (PBS) (pH 7.2). Cells were collected using a cell scraper, concentrated by centrifugation, and resuspended in 1 ml of minimum essential medium.[b] A portion of the suspension was used to quantify the number of cells, using a Coulter counter. Serial 10-fold dilutions were made in PBS from the original infected cell suspension for inoculation into mice.

Each mouse was given an intraperitoneal 0.2-ml inoculation of *E. chaffeensis*-infected cells. Two mice received 1.9

From the Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA 30602 (Lockhart, Davidson), and the D. B. Warnell School of Forest Resources, University of Georgia, Athens, GA 30602 (Davidson).

Received for publication February 3, 1998.

$\times 10^6$ cells, 2 received 1.9×10^5 cells, 3 received 1.9×10^4 cells, 3 received 1.9×10^2 cells, and 3 received 1.9 cells. A single control mouse received an inoculum of 2.7×10^6 uninfected DH82 cells.

Blood was collected retroorbitally from all mice prior to inoculation, and all mice were marked by ear punch for identification. Mice that received the same inoculation dilution were cohoused and provided a liberal food and water supply. Mice were euthanized with CO_2 on postinoculation day (PID) 21, and blood was collected-via cardiac puncture. Blood was divided into 2 fractions; 1 was placed in heparinized tubes, and 1 was placed in tubes without an anticoagulant. Serum was collected for serologic examination, and heparinized blood was prepared for culture and PCR analysis.

Indirect FA tests were performed as previously described.[3] Serum was screened at a dilution of 1:32 in PBS on spot slides of *E. chaffeensis*-infected DH82 cells. Samples exhibiting fluorescence were evaluated at serial 2-fold dilutions. Results were recorded as the reciprocal of the highest dilution at which specific staining of the ehrlichial organisms was made using fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG[c] diluted 1:50 in PBS (pH 7.2).

Isolation of *E. chaffeensis* from C3H/HeJ mice was attempted as described previously.[5] Whole blood collected in heparin was mixed 1:3 with lysing buffer (150 mM NH_4Cl, 0.7 mM KH_2PO_4, and 3 mM ethylenediaminetetraacetic acid [EDTA]-Na_2) and incubated at room temperature for 5 min with frequent inversion. The suspension was centrifuged at $1,500 \times g$ for 5 min to concentrate leukocytes. This procedure was repeated 2 more times with lysing buffer as a wash. Uninfected DH82 canine macrophage cells from a 25-cm^2 flask were suspended in 5 ml of fresh medium and added to the pellet. The entire suspension was then put back into the flask. Twice weekly, the supernatant from the culture flask was decanted, and 5 ml of fresh medium was added.

Starting at day 5, cultures were examined twice weekly for evidence of infection using direct FA staining as previously described.[4] Culture supernatant was collected and centrifuged at $1,500 \times g$ for 5 min, and cells were resuspended in 1 ml of medium. Approximately 0.1 ml of the suspension was cytocentrifuged onto glass slides. Slides were allowed to air dry for 15 min, fixed in acetone for 15 min, and dried at room temperature for 15 min. Conjugate for the direct FA consisted of FITC human anti-*E. chaffeensis* IgG.

Cells for PCR assays were isolated from the blood, mesenteric lymph nodes, spleen, lung, liver, and kidney of mice. A small section of tissue from each organ was removed aseptically, crushed with the edge of a scalpel blade, and mixed with sterile PBS prior to addition of lysing buffer. Following removal of red blood cells, isolated cells were concentrated by centrifugation and resuspended in 0.5 ml PBS (pH 7.2). DNA was extracted from cell samples using an isolation matrix[d] according to the manufacturer's directions. Nested PCR was performed as described previously.[5] For the outside amplification, a 100-μl reaction mixture containing 1 μl of DNA template, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 5 μM tetramethylammonium chloride, 2.5 U of *Taq* DNA polymerase, and 1 μM (each) primers ECB (5′-CGTATTACCGCGGCTGCTGGCA-3′) and ECC (5′-AGAACGAACGCTGGCGGC AAGCC-3′) was assembled and overlain with mineral oil. These primers amplify all known *Ehrlichia* spp. and a few other bacteria. The following temperature profile was run for 40 cycles: 1 min at 94 C, 2 min at 45 C, and 30 sec at 72 C, with 1 sec added to each successive 72 C extension step.

For the nested PCR, 1 μl of each outside reaction product was amplified in a second 100-μl reaction mixture with *E. chaffeensis*-specific primers HE1 (5′-CAATTGCTTATAACCTTTTCCTTATAAAT-3′) and HE3 (5′-TATAGGTACCGTCATTATCTTCCCTAT-3′). The following temperature profile was run for 40 cycles: 1 min at 94 C, 2 min at 55 C, and 15 sec at 72 C, with 1 sec added to each successive 72 C extension step. All products from the nested reactions were analyzed by electrophoresis in 1.5% agarose, stained with ethidium bromide, and examined by ultraviolet illumination.

Inoculation of deer tissue into C3H/HeJ mice. Five white-tailed deer were collected on September 21, 1994, from Piedmont National Wildlife Refuge (NWR), Jasper and Jones Counties, Georgia (33°07 ′N, 83°40 ′W). Blood was obtained aseptically via cardiac puncture and collected in clot tubes for serologic examination, in heparin for inoculation into mice, and in EDTA for use in PCR analysis. Prescapular lymph nodes and spleen samples were collected aseptically, placed in sterile bags,[f] and transported on ice to the laboratory. Serologic examination was performed as above except the secondary antibody conjugate consisted of FITC-labeled rabbit anti-deer IgG[c] diluted 1:100.

Cells were harvested from deer blood and prescapular lymph nodes within 12 hr and processed for culture as described above. Isolated cells from the blood, prescapular lymph nodes, and spleen were evaluated as above by PCR for the presence of *E. chaffeensis*.

A fraction of cells isolated from blood and prescapular lymph node from each deer was resuspended in 0.25 ml PBS and inoculated intraperitoneally into 1 C3H/HeJ mouse (1 mouse per tissue, 3 mice/deer). Blood was collected retroorbitally on PID 15, and on PID 30, mice were euthanized by CO_2 and blood was collected by cardiac puncture. Serum was separated for indirect FA analysis. Any tissue-inoculated mice that seroconverted were euthanized, and cardiac blood, mesenteric lymph nodes, and spleen samples were collected for confirmatory in vitro isolation attempts and for PCR assays as previously described.

Results

Experimental inoculation of C3H mice with E. chaffeensis. Mice that received the 3 highest dilutions of *E. chaffeensis*-infected cells seroconverted by PID 21 (Table 1). The geometric mean titers for mice that received 1.9×10^6, 1.9×10^5, and 1.9×10^4 cells were 181 (titers = 256, 128), 128 (titer = 128), and 81 (titers = 128, 64, 64), respectively. Mice in the 2 lowest dilution and negative control groups were all seronegative at 1:32 on PID 21. One of the 2 mice that received 1.9×10^5 *E. chaffeensis*-infected cells was

Table 1. Results of inoculation of C3H/HeJ mice with *Ehrlichia chaffeensis*-infected DH82 canine macrophages.

No. mice	Inoculum*	Serology†	PCR‡					
			B	LN	SP	LU	LI	KI
2	1.9×10^6	128, 256	0/1	0/2	1/2	0/2	0/2	0/2
1	1.9×10^5	128	0/1	0/1	0/1	0/1	0/1	0/1
3	1.9×10^4	64, 64, 128	0/3	0/3	0/3	0/3	0/3	0/3
3	1.9×10^2	<32, <32, <32	0/3	0/3	0/3	1/3	0/3	0/3
3	1.9	<32, <32, <32	0/3	0/3	0/3	0/3	0/3	0/3
1	2.7×10^6	<32	ND	0/1	ND	ND	0/1	0/1

* *E. chaffeensis*-infected DH82 canine macrophages determined to be >80% infected by results of direct FA analysis and counted with a Coulter counter. Negative control was inoculated with uninfected cells.

† Reciprocal of dilution of serum that produced specific fluorescence of ehrlichial organisms using an indirect FA test.

‡ Nested PCR analysis using ECB/ECC and HE1/HE3 primer pairs and analyzed with gel electrophoresis. No. positive/no. tested. B = blood; LN = lymph node; SP = spleen; LU = lung; LI = liver; KI = kidney; ND = not done.

cannibalized by cage mates at PID 4 and was excluded from the study.

Ehrlichia chaffeensis was not reisolated from the blood from any of the mice at PID 21. Cultures from 1 mouse inoculated with 1.9×10^6 cells, 1 with 1.9×10^5 cells, and 1 with 1.9×10^4 cells were discontinued at PIDs 5, 5, and 9, respectively, because of bacterial contamination. All other cultures were maintained until day 63.

A total of 70 tissue specimens from 12 inoculated mice were examined by PCR for the presence of *E. chaffeensis* DNA (Table 1). Two tissues, spleen from 1 mouse that received 1.9×10^6 cells and lung from 1 mouse that received 1.9×10^2 cells, were positive by PCR using *E. chaffeensis*-specific primers HE1 and HE3 (Fig. 1).

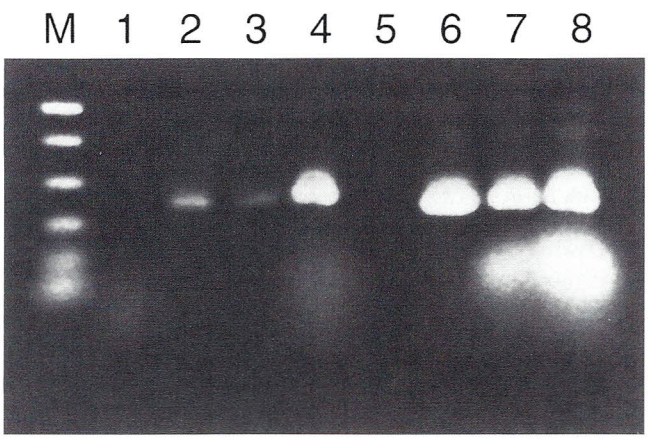

Figure 1. Agarose gel electrophoresis of products amplified from C3H/HeJ mice and from white-tailed deer blood and lymph node using a nested PCR with ECB and ECC as outside primers and HE1 and HE3 as inside primers. Lane 1 = negative control; lane 2 = positive control (cell tissue culture-maintained Arkansas strain of *Ehrlichia chaffeensis*); lane 3 = spleen of C3H/HeJ mouse inoculated with 1.9×10^6 *E. chaffeensis*-infected cells; lane 4 = lung of C3H/HeJ mice inoculated with 1.9×10^2 *E. chaffeensis*-infected cells; lane 5 = blank; lanes 6–8 = positive reactions from wild white-tailed deer collected from Piedmont National Wildlife Refuge: lane 6 = deer 2 blood, lane 7 = deer 4 blood, and lane 8 = deer 5 lymph node. M = Molecular weight markers of 1,000, 750, 500, 300, 150, and 50 bp.[e]

Inoculation of deer tissue into C3H/HeJ mice. Deer collected from Piedmont NWR had *E. chaffeensis*-reactive antibody titers of 128–512 (Table 2). *Ehrlichia chaffeensis* was not isolated from blood or lymph node of any of the deer. The culture from the lymph node of deer 3 was discontinued at PID 12 because of bacterial contamination. All remaining cultures were examined weekly by direct FA and maintained until PID 68. Blood from 2 deer and lymph node from a third deer were positive by PCR for *E. chaffeensis* (Fig. 1). All other blood, lymph node, and spleen samples were negative by PCR for *E. chaffeensis*.

None of the C3H/HeJ mice inoculated with blood, lymph node, and spleen samples from white-tailed deer developed *E. chaffeensis*-reactive antibodies, including 3 mice inoculated with tissues that were positive for *E. chaffeensis* by PCR. The negative control mouse remained seronegative. Because none of the

Table 2. Serology and PCR results on white-tailed deer collected September 21, 1994, from Piedmont National Wildlife Refuge, Jasper and Jones counties, Georgia.

Deer No.	Serology*	PCR†		
		B	LN	SP
1	512	–	–	–
2	512	+	–	–
3	128	–	–	–
4	512	+	–	–
5	256	–	+	–

* Reciprocal of dilution of serum that produced specific fluorescence of ehrlichial organisms using an indirect FA test.

† Nested PCR analysis using ECB/ECC and HE1/HE3 primer pairs and analyzed with gel electrophoresis. B = blood; LN = lymph node; SP = spleen.

mice seroconverted, no further testing was done on their tissues.

Discussion

Inoculation of C3H/HeJ mice produced equivocal results with regard to susceptibility to *E. chaffeensis*. Although mice that received higher doses seroconverted, the inability to reisolate *E. chaffeensis* and detection of *E. chaffeensis* DNA in only 2 of 12 inoculated mice suggests that replication of *E. chaffeensis* was minimal and presumably transient. Antibody titers paralleled the number of infected cells injected, suggesting that the serologic responses may have been simply a function of the number of organisms in the inocula. Conversely, the detection of *E. chaffeensis* DNA in the lung of 1 mouse dosed with 1.9×10^2 infected cells could be interpreted as evidence that replication occurred in that animal.

The present results are similar to those obtained by other researchers[13] who also inoculated C3H/HeJ mice with *E. chaffeensis*. In that study, mice developed *E. chaffeensis*-reactive antibodies within 21 PIDs, were infrequently positive when tested by PCR within 30 PIDs, and were difficult to confirm as infected via reisolation (a single reisolation at PID 11). Other research[13] indicated that C3H/HeJ become persistently infected and it was proposed that C3H/HeJ mice may be a useful animal model for the study of protective immunity against *E. chaffeensis*. Although the present data do not provide support for the concept of persistent infection, they do suggest that C3H/HeJ mice may be suitable for immunity studies.

Regardless of the differing results and interpretations between this and the previous study,[13] both studies show that C3H/HeJ mice are markedly less susceptible to this infection than are white-tailed deer and dogs, the only nonrodent mammals that have been experimentally inoculated with *E. chaffeensis*.[4,5] In those studies, *E. chaffeensis* was consistently reisolated from the blood of needle-exposed dogs from between PIDs 7 and 26 and from deer between PIDs 13 and 24.

The interest in utilizing mice as a xenodiagnostic aid arose because isolation of ehrlichiae in cell culture generally has proved difficult and, more specifically, because prior attempts to isolate *E. chaffeensis* from deer blood often had been confounded by contamination with *T. cervi*.[9] The utility of C3H/HeJ mice for this purpose is best evaluated based on the results from field samples. Mice inoculated with deer tissue that was PCR positive for *E. chaffeensis* uniformly failed to seroconvert. Use of C3H/HeJ mice in an identical manner in another field survey also yielded negative results for 8 PCR-positive tissue suspensions and, more importantly, failed to detect infection in 5 deer blood samples from which *E. chaffeensis* was isolated in cell culture.[10] Thus, C3H/HeJ mice apparently are not a sensitive xenodiagnostic tool for the detection of *E. chaffeensis* from field samples. Nevertheless, as proposed in previous studies,[13] they may be useful as an experimental model for the study of immune response to *E. chaffeensis*.

Acknowledgements

This research was conducted through sponsorship from the fish and wildlife agencies of Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia. Funds were provided by the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and through Grant Agreement 14-45-0009-94-906, National Biological Survey, US Department of the Interior. We thank SCWDS personnel for field and technical assistance.

Sources and manufacturers

a. Harlan Sprague Dawley, Indianapolis, IN.
b. HyClone Laboratories, Logan, UT.
c. Kirkegaard & Perry Laboratories, Gaithersburg, MD.
d. InstaGene Purification Matrix, BIO-RAD Laboratories, Hercules, CA.
e. Promega, Madison, WI.
f. Whirl-paks, Fisher Scientific, Pittsburgh, PA.

References

1. Anderson BE, Dawson JE, Jones DC, et al.: 1991, *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J Clin Microbiol 29:2838–2842.
2. Chen SM, Yu XJ, Popov VL, et al.: 1997, Genetic and entigenic diversity of *Ehrlichia chaffeensis*: comparative analysis of a novel human strain from Oklahoma and previously isolated strains. J Infect Dis 175:856–863.
3. Dawson JE, Anderson BE, Fishbein DB, et al.: 1991, Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol 29:2741–2745.
4. Dawson JE, Ewing SA: 1992, Susceptibility of dogs to infection with *Ehrlichia chaffeensis,* causative agent of human ehrlichiosis. Am J Vet Res 53:1322–1327.
5. Dawson JE, Stallknecht DE, Howerth EW, et al.: 1994, Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis,* the etiologic agent of human ehrlichiosis. J Clin Microbiol 32:2725–2728.
6. Dumler JS, Chen SM, Asanovich K, et al.: 1995, Isolation and characterization of a new strain of *Ehrlichia chaffeensis* from a patient with nearly fatal monocytic ehrlichiosis. J Clin Microbiol 33:647–655.
7. Eng TR, Harkess JR, Fishbein DB, et al.: 1990, Epidemiologic, clinical, and laboratory findings of human ehrlichiosis in the United States, 1988. J Am Med Assoc 264:2251–2258.
8. Fishbein DB, Dawson JE, Robinson LE: 1994, Human ehrlichiosis in the United States, 1985 to 1990. Ann Intern Med 120: 736–743.
9. Lockhart JM, Davidson WR, Dawson JE, et al.: 1996, Natural history of *Ehrlichia chaffeensis* in the Piedmont physiographic province of Georgia. J Parasitol 83:887–894.
10. Lockhart JM, Davidson WR, Stallknecht DE, et al.: 1996, Confirmation of wild white-tailed deer (*Odocoileus virginianus*) as natural reservoir hosts of *Ehrlichia chaffeensis.* J Clin Microbiol 35:1681–1686.

11. Park J, Rikihisa Y: 1991, Inhibition of *Ehrlichia risticii* infection in murine peritoneal macrophages by gamma interferon, a calcium ionophore, and concavalin A. Infect Immun 59:3418–3423.
12. Ruco LP, Meltzer MS: 1978, Macrophage activation for tumor cytotoxicity: tumoricidal activity by macrophages from C3H/HeJ mice requires at least two activation stimuli. Cell Immunol 41:35–51.
13. Telford SR III, Dawson JE: 1996, Persistent infection of C3H/HeJ mice by *Ehrlichia chaffeensis*. Vet Microbiol 52:103–112.
14. Walker DH, Dumler JS. 1996, Emergence of the ehrlichioses as human health problems. Emerg Infect Dis 2:18–29.
15. Williams NM, Timoney PJ. 1994, Variation in susceptibility of ten mouse strains to infection with a strain of *Ehrlichia risticii*. J Comp Pathol 110:137–143.