

Isolation of the Etiologic Agent of Human Granulocytic Ehrlichiosis from the White-Footed Mouse (*Peromyscus leucopus*)

M. DANA RAVYN,¹ CARRIE B. KODNER,¹ SARAH E. CARTER,¹ JANET L. JARNEFELD,²
AND RUSSELL C. JOHNSON^{1*}

Department of Microbiology, University of Minnesota Academic Health Center, Minneapolis, Minnesota 55455,¹ and Metropolitan Mosquito Control District, St. Paul, Minnesota 55104²

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We examined white-footed mice (*Peromyscus leucopus*) from Minnesota for infection with the etiologic agent of human granulocytic ehrlichiosis (HGE). From April to September 1997, we collected *P. leucopus* from Washington County, Minnesota, an area enzootic for HGE. Blood was cultivated in HL60 cells for isolation of the HGE agent. Of 59 mice examined, only a single mouse was culture positive for the HGE agent. The 16S ribosomal DNA sequence of the isolate was determined to be identical to that of the HGE agent. The isolate was reactive with monoclonal antibodies to the 44-kDa antigen of the HGE agent and was infectious for laboratory mice.

Human granulocytic ehrlichiosis (HGE) is a recently described granulocytotropic infection first identified in the upper midwestern United States in 1994 (3). HGE is an acute febrile disease that may present as fever, myalgia, arthralgia, headache, and rigors (1, 4). Infection with the etiologic agent of HGE usually responds rapidly to treatment with tetracyclines. However, despite effective therapy, severe cases and some fatalities have occurred (9).

Sequences of 16S ribosomal DNA (rDNA) from the HGE agent are nearly identical to those of the granulocytotropic agents *Ehrlichia equi* and *E. phagocytophila* (7), which are responsible for zoonotic infections in horses and ruminants, respectively. Serological cross-reactivity between antibodies to the HGE agent and the antigens of *E. phagocytophila* and *E. equi* has been demonstrated (8). Infection of horses with the HGE agent follows a clinical course indistinguishable from that of equine granulocytic ehrlichiosis, and horses infected with the agent of HGE are protected against subsequent challenge with *E. equi* (5).

A family of 42- to 49-kDa surface proteins, designated P44, are capable of eliciting immunologic responses in patients with HGE (2, 11, 19). Genes encoding P44 proteins are members of the granulocytic ehrlichia–MSP-2 multigene family (15) and are present in multiple copies dispersed throughout the genome (24). The expression of P44 homologs has been postulated to be regulated at the level of transcription to maintain antigenic variability (24). P44 sequences from several isolates have been published (10, 15, 24), and these sequences may suggest that antigenic diversity exists among the species causing HGE.

Epidemiological, molecular, and transmission studies provide evidence that *Ixodes scapularis* is the vector of HGE in the

central and eastern United States (14, 18, 22; K. D. Reed, P. D. Mitchell, D. H. Persing, C. P. Kolbert, and V. Cameron, Letter, JAMA 273:23, 1995). Although the natural history of granulocytic ehrlichiosis is not clear, the white-footed mouse, *Peromyscus leucopus*, has been implicated as a reservoir of the HGE agent. It has been shown that white-footed mice collected from the wild are capable of transmitting ehrlichial organisms to laboratory-reared ticks (22). Serologic and molecular evidence of infection with the *E. phagocytophila* genomic group has been demonstrated for *P. leucopus* collected from regions endemic for HGE (13, 17, 23). Ehrlichial DNA has been amplified from the blood of wild mice, voles, chipmunks, and wood rats (16, 21, 23), and the sequences of ehrlichial 16S rDNA and DNA from the *groESL* operon are nearly identical to published sequences of the HGE agent. Isolates of the HGE agent have been obtained from the blood of three *P. leucopus* mice captured in Connecticut, and DNA from the HGE agent has been identified by PCR using primers for the P44 protein gene sequence (14, 21).

We report the results of the isolation and characterization of the first isolate of the etiologic agent of HGE obtained from *P. leucopus* in Minnesota.

Mammals were live trapped in Washington County, Minnesota, an area endemic for Lyme disease and located in the seven-county greater metropolitan area of Minneapolis-St. Paul. Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.) baited with peanut butter and rolled oats were placed on 91-m transects at 15-m intervals. Mice were collected daily and returned to the laboratory, where blood was harvested for cultivation.

Human promyelocytic HL60 (CCL 240) cells were grown in RPMI 1640 (Gibco, Grand Island, N.Y.) containing 20 mM sodium bicarbonate and 10% fetal bovine serum (Gibco) at 37°C with 5% CO₂. For isolation of the HGE agent, mice were killed by inhalation of CO₂, and blood was collected by cardiac puncture. Fifty microliters of whole blood was used to inoculate 25-cm² tissue culture flasks containing 5 ml of HL60 cells

* Corresponding author. Mailing address: University of Minnesota Academic Health Center, 420 Delaware St., S.E., Minneapolis, MN 55455-0312. Phone: (612) 624-5684. Fax: (612) 626-0623. E-mail: johnson@mail.ahc.umn.edu.

at a density of 10^5 cells per ml in RPMI 1640 containing 10% fetal bovine serum. Cultures were checked after 3, 5, and 7 days by microscopic examination of Giemsa-stained cytospin preparations.

Ehrlichiae were harvested when greater than 95% of the HL60 cells had visible morulae. Cultures were centrifuged in 100-ml volumes at $10,000 \times g$ for 20 min at 4°C. The supernatant was discarded, and the pellet was suspended in 5 ml of ice-cold, sterile 10 mM phosphate-buffered saline (PBS; pH 7.4). Infected HL60 cells were sonicated on ice using a model 550 Sonic Dismembrator (Fisher Scientific, Itasca, Ill.) set at a rate of 3 A by applying three 10-s pulses interspersed with 30-s rests. The resulting material was centrifuged at $500 \times g$ for 10 min at 4°C to remove cellular debris. The supernatant was collected, and the bacteria were harvested at $10,000 \times g$ for 20 min at 4°C. Ehrlichiae were purified by passage over a Sephacryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with $2 \times$ PBS, and the flowthrough volume was examined by spectrophotometry at 280 nm. Fractions were then combined, and bacteria were collected by centrifugation at $10,000 \times g$ for 20 min at 4°C. DNA was extracted from the partially purified bacteria using the guanidium isothiocyanate method (IsoQuick; ORCA Research Industries, Inc., Bothell, Wash.).

DNA was amplified in a reaction buffer containing 10 mM Tris (pH 8.3); 500 mM KCl; 15 mM $MgCl_2$; 200 μ M each dATP, dCTP, dGTP, and dTTP; and 1.25 U of sequencing-grade polymerase (Expand; Roche Biochemicals). The initial reaction was carried out using 50 pmol each of primer PL4 (5'-TCCTGGCTCAGAACGAACG-3') and primer PL6 (5'-CCATGTCAAGGAGTGGTAAGG-3'), which correspond to bases 1 to 20 and 943 to 925, respectively, of the 16S rDNA, based upon a comparison with the published sequence of the agent of HGE (7). Amplification was carried out using a model 4800 thermal cycler (Perkin-Elmer, Norwalk, Conn.), with denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and elongation for 3 min at 72°C. For sequencing, amplification was carried out in the same manner using primers PL1 (5'-TTATCGCTATTAGAGCCTATG-3'), corresponding to bases 118 to 211; PL3 (5'-ATGCATTAACCCCTCTG-3'), corresponding to bases 111 to 92; PL2 (5'-TCCTGGCTCAGAACGAACGC-3'), corresponding to bases 638 to 616; PL5 (5'-AAGCACTCCGCTGGGGACT-3'), corresponding to bases 818 to 837; and PL6 (5'-CCATGTCAAGGAGTGGTAAGG-3'), corresponding to bases 943 to 925.

All DNA sequences were determined by the detection of dye-labeled dideoxynucleotides (PRISM; Applied Biosystems, Foster City, Calif.) and obtained with an automated DNA sequencer (model 373; Applied Biosystems). Overlapping strands of DNA were sequenced in both directions.

For analysis of the P44 protein gene, DNA sample preparation and amplification were carried out by the methods described above using primers P44-1 (5'-AGCGTAATGATGTCTATGGC-3') and P44-2 (5'-ACCCTAACACCAATTCC-3'), which begin at positions 43 and 1322, respectively (GenBank accession number AF037599) (10). The conditions for amplification were identical to those described above. The resulting amplification products, which produced a single band in agarose gel electrophoresis, were then ligated into the pCR 2.1 cloning vector (Invitrogen, Carlsbad, Calif.). The vector

was used to transform *Escherichia coli* INV α F' cells (Invitrogen). A positive clone was selected, and the plasmid insert was sequenced by the dideoxy termination method using dye-labeled dideoxynucleotides (PRISM) and a model 373 DNA sequencer.

An immunofluorescence assay (IFA) was carried out with patient sera, sera generated in dogs, or monoclonal antibodies in hybridoma supernatant using previously described methods (19, 20). Briefly, sera were diluted in PBS (pH 7.4), and a 5- μ l volume was applied to each well of slides containing antigen. Slides were incubated for 1 h at an ambient temperature, rinsed twice in PBS (pH 7.4), and submerged in PBS for 5 min. After air drying, fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG), anti-mouse IgG, or anti-human IgG (heavy and light chain) antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was diluted to appropriate levels, as determined by checkerboard analysis, and applied to wells in 5- μ l volumes. Slides were incubated again at an ambient temperature for 1 h, rinsed twice in PBS (pH 7.4), and immersed for 5 min in PBS containing 0.005% Evans blue (Sigma, St. Louis, Mo.). Following a brief rinse with distilled water to remove excess stain, slides were overlaid with mounting medium, consisting of 10% PBS-buffered glycerol and 0.1% (wt/vol) diazibicyclooctane (Sigma). Controls included for each slide were sera from mice with culture-confirmed infection with the HGE agent or control mice that were injected with HL60 cells only. Titers were determined as the end point at which the fluorescence of *Ehrlichia* spp. in the cytoplasm of HL60 cells was no longer distinct. Western blotting was carried out as described previously, except that goat anti-dog IgG was used (19).

A total of 59 *P. leucopus* mice were collected from Washington County from April to September 1997. These mice were examined by cultivation of blood in HL60 cells. A culture from the blood of a single mouse captured in September resulted in the detection of morulae in Giemsa-stained cytospin preparations of HL60 cells.

DNA from cultures of the isolate (designated PL59) from *P. leucopus* was extracted, and rDNA was amplified using primers for 16S rDNA. Amplified DNA was then purified and sequenced. The resulting sequence corresponded to positions 1 to 922 of *E. coli* 16S rDNA (GenBank accession no. AF189153). Primers were designed to allow the sequencing of overlapping regions in both directions. The sequence of the PL59 16S rDNA was identical to the sequence of the 16S rDNA reported for an HGE agent isolate from humans (7). We next compared the sequence of the PL59 16S rDNA to two sequences of HGE agent rDNA PCR products derived from the blood of *P. leucopus* collected in Minnesota (23). Isolate PL59 was identical to one published sequence and differed by one nucleotide base from a second published sequence (23).

To examine the expression of antigens by isolate PL59 of the HGE agent, we used polyclonal and monoclonal antibodies. Antibodies from patients infected with *Coxiella burnetii* ($n = 1$), *Rickettsia typhi* ($n = 1$), *R. rickettsia* ($n = 2$), or *E. chaffeensis* ($n = 3$) did not react with the isolate at a significant titer (>80).

For studies of the reactivity of PL59 with polyclonal antibodies, four dogs were vaccinated with the HGE agent. Two dogs were vaccinated with human isolate HGE-2 (19), and two

dogs were vaccinated with the *P. leucopus* isolate. The sera generated in these dogs were used to determine the IFA reactivity of isolate PL59. The IFA titers of the dog sera raised against PL59 when the homologous antigen was used were 512 for both dogs, while the titers were 128 and 512 when HGE-2 was used as the antigen. In comparison, the antisera raised against human isolate HGE-2 reacted at titers of 128 and 256 when PL59 was used as the antigen. When HGE-2 was used as the antigen, the titers of the antisera raised against HGE-2 were 512 and 1,024. The preimmune sera from both dogs had titers of less than 64 when either PL59 or HGE-2 was used as the antigen.

Western immunoblot examination of the antigenic characteristics of isolate PL59 with these sera did not reveal any significant differences in the patterns of reactive polypeptides. Both PL59 and HGE-2 reacted similarly with the P44 antigen. We concluded from the IFA and Western blot results that isolate PL59 was antigenically similar to isolates of the HGE agent.

Monoclonal antibodies against the P44 protein of the HGE agent were reacted with PL59 antigen. Monoclonal antibodies R5E4, R1B10, and R5A9 (20) had titers of 512, 512, and 128, respectively. When HGE-2 was used as the antigen, monoclonal antibodies R5E4, R1B10, and R5A9 had titers of 256, 256, and 128, respectively.

To determine whether the *Peromyscus* isolate was infectious for mice, 10^5 HL60 cells infected with PL59 were injected into three strains of mice, C3H/HeN, BALB/c, and DBA/2. After 7 days, spleen tissues were examined by cultivation and PCR. All seven C3H/HeN mice were both culture positive and PCR positive for HGE 16S rDNA. Of the DBA/2 mice, five of six (83.3%) and six of seven (85.7%) were positive by culturing and PCR, respectively. Of the BALB/c mice, three of five (60.0%) and five of seven (71.4%) were positive by culturing and PCR, respectively. Mice injected with uninfected HL60 cells were negative by both culturing and PCR.

Studies of the role of *P. leucopus* in the ecology and epizootiology of HGE have suggested that, while this mammal is susceptible to infection by the HGE agent, its role as a reservoir may be limited. The percentage of mice found seropositive by IFA for anti-HGE antibodies has been reported to be in the range of 2 to 53% (6, 13, 16, 17, 21, 23). Dissimilarity in the prevalence of infection with the HGE agent among *I. scapularis* nymphal and adult stages provides evidence for the role of other mammals in the transmission of this agent to ticks (12). The prevalence of infection with the HGE agent in *Peromyscus* and the role of these mice as reservoirs may vary geographically (16). While cultivation from experimentally infected mice in our laboratory has been found reliable and sensitive, the sensitivity of cultivation from wild *P. leucopus* is uncertain. Consequently, we were unable to ascertain the exact prevalence of infection in the mice collected for this study. Studies by Stafford et al. have suggested that infection of *P. leucopus* by the HGE agent is transient in the wild (21). Further studies are necessary to determine the level of infectivity in *P. leucopus* in this geographical area.

The relationship of the 16S rDNA of isolate PL59 to the *Ehrlichia* sequences examined is consistent with the observations of others that *P. leucopus* mice are infected with the agent of HGE (23). It appears likely that a single species of the HGE

agent can be identified from wild mammals as well as humans in the United States.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of the gene for the P44 protein of the isolate described in this paper (PL59) is AF202317.

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