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

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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...
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 cytopsin 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 × 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 × g for 10 min at 4°C to remove cellular debris. The supernatant was collected, and the bacteria were harvested at 10,000 × g for 20 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C. Ehrlichiae were purified by passage over a Sephadryl S-1000 column (Pharmacia, Uppsala, Sweden) with a bed volume of 5 ml (25). Bacteria were eluted with 2 min at 4°C.
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, the monoclonal antibodies R5E4, R1B10, and R5A9 had titers of 256, 256, and 128, respectively.

To determine whether the *Peromyscus* isolate was infectious for mice, 10^3 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 is uncertain. 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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