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Natural Infection of Small Mammal Species in Minnesota with the Agent of Human Granulocytic Ehrlichiosis

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The natural reservoirs for the agent of human granulocytic ehrlichiosis (HGE) are suspected to be the small mammals that host immature stages of *Ixodes scapularis* ticks. To determine if such small mammals are naturally infected, we collected blood and serum samples from small mammal species in rural and suburban areas of Minneapolis and St. Paul, Minn. Samples were collected from white-footed mice (*Peromyscus leucopus*), eastern chipmunks (*Tamias striatus*), southern red-backed voles (*Clethrionomys gapperi*), and insectivorous shrews (*Blarina brevicauda* and *Sorex cinereus*). Blood samples were tested by PCR for active infection with the HGE agent, and sera from *P. leucopus* mice were tested for serologic evidence of infection by indirect immunofluorescence. PCR analyses revealed the presence of HGE agent DNA in 20 of the 190 samples (10.5%) tested. Of the 119 *P. leucopus* mouse serum samples that were analyzed, 12 (10.1%) contained *Ehrlichia equi* antibodies. In 3 of 119 (2.5%) *P. leucopus* mice from which both blood and serum were collected, HGE agent DNA and antibodies against *E. equi* were present. Animals with evidence of infection with the HGE agent are widely distributed around the Minneapolis-St. Paul area in regions with known *I. scapularis* tick activity. Small mammals that are frequent hosts for larval *I. scapularis* ticks and that are found in areas where HGE occurs are likely to be a major reservoir from which infected ticks that bite humans are derived.

Human granulocytic ehrlichiosis (HGE), an emerging tick-borne rickettsial infection, has been implicated as an agent of significant disease throughout the Northeast and upper midwestern United States (1, 3). This acute febrile illness is associated with mild to severe signs and symptoms, and in some cases infected patients have died (3). The majority of cases are associated with tick exposure or tick bites and occur during the months of April through September. The most likely tick vector of this agent are nymphal *Ixodes scapularis* ticks, the same vector that transmits the Lyme disease spirochete *Borrelia burgdorferi* (12, 15). The reservoir of infection has not, however, been conclusively determined. The role of white-footed mice (*Peromyscus leucopus*) as potential reservoirs for the HGE agent is supported by successful acquisition of infection by xenodiagnostic *Ixodes* ticks on white-footed mice collected on Nantucket Island in Massachusetts (16). Moreover, the HGE agent can be transmitted among laboratory mice in a tick-mouse cycle (16). Additional evidence for natural infection of *P. leucopus* is the presence of antibodies against the HGE agent in serum samples collected from white-footed mice in Connecticut, where focally high rates of tick infection have been demonstrated (11, 12). Tyzzer's (18) original description of a morphologically similar transmissible agent indicated that other small mammals, including voles, may also be natural reservoirs. In this study, a natural infection with the agent of HGE in small mammal species is demonstrated, suggesting that these animals act as reservoirs by participating as hosts for larval stage *I. scapularis* ticks and perpetuating infection to other susceptible mammals.

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MATERIALS AND METHODS

Small mammals and sample collection. Blood samples (approximately 100 to 500 μl) were collected from 158 white-footed mice (*P. leucopus*), 23 eastern chipmunks (*Tamias striatus*), 6 southern red-backed voles (*Clethrionomys gapperi*), 2 northern short-tailed shrews (*Blarina brevicauda*), and 1 masked shrew (*Sorex cinereus*) in 51 wooded rural and suburban areas surrounding Minneapolis-St. Paul, Minn., between 31 May and 30 June 1995. After processing, serum was available from only 119 of the *P. leucopus* mice. The animals were collected as part of an ongoing *I. scapularis* surveillance effort by using baited Sherman traps, and blood was collected by cardiac puncture after the animals were euthanized by CO_2 inhalation.

Nucleic acid preparation, PCR, and 16S rRNA gene sequence analyses. DNA was isolated from 100- to 200-μl aliquots of whole clotted blood by adding 30 μl of a mixture of proteinase K (200 μg/ml) in 10 mM Tris–1 mM EDTA buffer with 1% Tween 20 directly to the tubes into which the clotted blood was stored. These samples were then incubated at 37°C overnight. Released nucleic acids were purified by phenol-chloroform extraction and precipitation of nucleic acids with sodium acetate and ethanol (4, 9). The precipitated DNAs were resuspended in 100 μl of sterile water. For PCR, 5 μl of the DNA template was used in a 50-μl PCR mixture containing the HGE agent primers ge9f and ge10r (4, 8). The results of a single round of amplification were analyzed by agarose gel electrophoresis with ethidium bromide staining. A second round of amplification with 5 μl of the first-round product was performed with all samples for which the result for the first round was negative in order to increase the sensitivity of the procedure. All PCRs were performed under strict conditions to minimize the risk of amplicon contamination, including sample preparation in a dedicated PCR hood, and all post-PCR analysis was performed in a separate laboratory. Second-round reamplification templates were aliquoted in a third separate laboratory prior to amplification. Each PCR included a known positive control HGE agent DNA (from a human or an experimentally infected horse) and several controls with no template DNA (water only). PCR products from two *P. leucopus* mice were chosen to corroborate the identity of the amplified DNA by nucleic acid sequencing by a fluorescent, automated dideoxynucleotide method (model 373; Perkin-Elmer, ABI, Foster City, Calif.). Sequences were then analyzed for maximal similarity to other small-subunit prokaryotic rRNA gene sequences by using the Similarity Analysis program of the Ribosomal Database Project (13) and by BLAST sequence analysis of the sequences in the GenBank database. By using the PCGene nucleic acid alignment program (Intelligenetics, Mountain View, Calif.), the 16S rRNA gene sequences derived from the white-footed mouse blood were then aligned for maximal similarity and to calculate the index of similarity with other closely related ehrlichial sequences in GenBank, including the HGE agent (accession number U02521), *Ehrlichia equi* (accession number M73223), *Ehrlichia phagocytophila*, Old Sourhope and feral goat strains (acces-

TABLE 1. PCR and indirect immunofluorescence assay results with blood and serum collected from different species of small mammals during late spring and early summer in Minnesota

Species	No. of samples PCR positive/ no. tested (%)	No. of samples *E. equi* antibody positive/no. tested (%)	No. of samples PCR positive and *E. equi* antibody positive/no. tested (%)	No. of samples PCR positive and *E. equi* antibody positive/total no. antibody positive (%)
P. leucopus	18/158 (11.4)	12/119 (10.1)	3/119 (2.5)	3/12 (25)
T. striatus	1/23 (4.3)	ND[a]	ND	ND
C. gapperi	1/6 (17)	ND	ND	ND
B. brevicauda	0/2	ND	ND	ND
S. cinereus	0/1	ND	ND	ND
Total	20/190 (10.5)	12/119 (10.1)	3/119 (2.5)	3/12 (25)

[a] ND, not done.

sion numbers M73220 and M73224), respectively, and the white-tailed deer ehrlichia, Oklahoma isolate no. 1 (accession number ESU27102) (5).

Antibody detection. Serum samples from 119 *P. leucopus* mice were tested for *E. equi* antibodies by a modification of an indirect immunofluorescence technique (6). Antigens were prepared by purifying leukocytes derived from an experimentally infected horse (courtesy John Madigan, University of California, Davis) as described. The secondary antibody was fluorescein isothiocyanate-labeled goat anti-*P. leucopus* immunoglobulins (Kierkegaard & Perry Laboratories, Gaithersburg, Md.). All sera that reacted at the screening dilution of 1:80 were titrated to the endpoint. Each run included a positive control serum sample (courtesy of Lou Magnarelli, New Haven, Conn.) and a negative control serum sample. No attempt was made to test other small mammals for antibodies because of the general unavailability of appropriate conjugates and control sera.

Nucleotide sequence accession numbers. The nucleotide sequences of the two PCR products detected in this study have been submitted to GenBank and have been given accession numbers U72878 and U72879, respectively.

RESULTS

PCR and sequencing. Results of PCR analyses of blood from small mammals are presented in Table 1. Of the 190 whole-blood samples tested by PCR, 20 (10.5%) were positive for DNA of the HGE agent. Specifically, 18 *P. leucopus* mice, 1 *T. striatus* chipmunk, and 1 *C. gapperi* vole were found to contain HGE agent DNA. All PCR-positive results were obtained during the first round of amplification. Initial sequence similarity searches revealed a high degree of similarity of both white-footed mouse-derived ehrlichia sequences to that of *Ehrlichia* species, especially the agent of HGE. Sequence analysis of the two PCR products revealed 100 and 99.9% sequence identities with that of the HGE agent, a 99.8% sequence identity with those of both *E. equi* and *E. phagocytophila*, and 96.3% sequence identity to that of the white-tailed deer ehrlichia 16S rRNA gene. The only nucleotide difference between the two sequences derived from the white-footed mouse blood was a single deletion of a T at position 153 of the HGE agent 16S rRNA gene sequence. This position is conserved among the HGE agent, *E. equi*, *E. phagocytophila*, and the white-tailed deer ehrlichia.

Detection of HGE agent antibodies. Of the 119 serum samples from *P. leucopus* mice available for testing, 12 (10.1%) were found to contain antibodies against *E. equi*, as indicated in Table 1. A range of antibody titers was found; 4 of the 12 seropositive samples had a titer of 80, 2 had a titer of 160, 2 had a titer of 320, 3 had a titer of 640, and 1 had a titer of 1,280. Of the 119 *P. leucopus* mice for which serologic results were determined, 3 were found to contain both HGE agent DNA and antibodies against *E. equi* (Table 1). These three samples were from among the 15 *P. leucopus* mice with positive PCR results for which serologic tests were performed and among 12 mice that had *E. equi* antibodies.

Geographic distribution. Blood and serum samples were obtained for testing from 51 sites mostly in the northern and eastern parts of the Minneapolis-St. Paul area. The sampling sites included six sites not in or near areas where *I. scapularis* is known to be endemic. Seventeen sites spread over a diameter of 60 miles had animals with evidence of active or prior HGE agent infection. *I. scapularis* tick activity is documented in 14 of these areas, and 2 of the 3 remaining areas are within 1 to 3 miles of regions with known *I. scapularis* activity (14).

DISCUSSION

Human, canine, and equine granulocytic ehrlichioses caused by an agent or agents similar to *E. equi* and *E. phagocytophila* are increasingly being diagnosed in the northeastern and upper midwestern United States (1, 3, 4, 17). These regions are also well known as areas where *I. scapularis* ticks transmit the agents of Lyme borreliosis and babesiosis to humans. *P. leucopus*, the white-footed mouse, is a favored host for larval *I. scapularis* ticks and is a reservoir for both *B. burgdorferi* and *Babesia microti*, the agents of Lyme disease and human babesiosis, respectively (2). Both of these diseases occur with a high prevalence in the Northeast and the upper midwestern United States, where HGE has been identified. Many patients with HGE describe deer tick bites preceding infection (3), and naturally infected *I. scapularis* ticks have been identified in both geographic regions (11, 15). Experimental evidence of the competence of *I. scapularis* ticks at transmitting and maintaining the HGE agent in an infectious tick-rodent cycle has recently been described, along with evidence that 5 of 15 white-footed mice from Nantucket, Mass., might have been naturally infected with an agent morphologically similar to the HGE agent (16).

A new *Ehrlichia* species has been proposed to occur in white-tailed deer from Oklahoma and Georgia (5). This new species, the white-tailed deer ehrlichia, was found to share approximately 96.5% of its 16S rRNA nucleotide sequences with that of the HGE agent and could theoretically be amplified with the primers ge9f and ge10r. Since it is unknown whether the white-tailed deer-derived ehrlichiae may also infect small mammals and, thus, lead to false-positive PCR results with the ge9f-ge10r primer set, we performed sequence analysis of the amplified PCR products from two *P. leucopus* mice with PCR evidence of HGE infection. This limited sequence analysis showed definitive evidence of infection in these mice by an *Ehrlichia* species identical or nearly identical to the HGE agent and no evidence of infection by the white-tailed deer ehrlichia.

The finding that 10.5% of wild rodents captured in the rural and suburban areas of Minneapolis-St. Paul are naturally infected suggests that these animals are reservoirs of the HGE agent in the upper Midwest and contribute to an enzootic tick-mammal cycle of ehrlichial maintenance. The production of antibodies in 10.1% of the rodents also supports this hypothesis; however, the limitations of serologic analyses limit interpretation since antibodies are simply a marker of current or previous infection and imply little about active infection. The geographic coincidence of small mammals with evidence of HGE agent infection and regions where *I. scapularis* ticks are known to be endemic provides additional evidence that this tick is likely to be the major vector responsible for natural transmission and maintenance of the bacteria in these ecologic niches.

White-footed mice that contained HGE agent DNA, a find-

ing that suggests the occurrence of active, ongoing ehrlichial replication in the mammalian hosts, could be identified during the late spring and early summer. The presence of seropositive, PCR-negative white-footed mice also indicates that at least some of these rodents suppress or recover from infection after the induction of immunity. A relatively large percentage (20 to 25%) of all *P. leucopus* mice with any evidence of active or previous infection were simultaneously PCR and antibody positive. This finding suggests that either persistent infection occurs or that there is an interval during which antibodies and immunity are strengthening prior to the elimination or suppression of ehrlichial infection. The exact interval of infectivity or ehrlichemia during which ticks may acquire an infectious blood meal cannot be ascertained by this work; however, by extrapolating from equine and human infections, an infectious interval of at least several weeks could be expected (3, 7, 10), a period that occupies a significant proportion of the life span of *P. leucopus* mice.

A bimodal seasonal distribution of HGE cases has been demonstrated in the upper midwestern United States, similar to that seen for Lyme disease (3). The majority of cases occur during the months of May through July, with a secondary minor peak in October and November, a pattern that is typical for transmission by nymphal-stage *I. scapularis* ticks earlier in the calendar year and adult *I. scapularis* ticks later. Since *P. leucopus* mice and other small mammals that are suitable hosts for the larval *I. scapularis* ticks sustain active infection during the early summer, it is likely not only that these species contribute to producing infected nymphal and adult *I. scapularis* ticks that might bite humans and transmit the agent of HGE but also that these species are the natural reservoir hosts for the HGE agent.

By understanding the tick-mammal cycles by which these ehrlichiae are established and maintained under natural conditions, it is possible to predict the geographic regions where human populations are at the highest risk for HGE. These findings also underscore the need for a more comprehensive evaluation of the ecological factors that contribute to the enzootic cycle, including tick, small mammal, and deer populations. With this evaluation will come a more comprehensive understanding of the factors that contribute to human risk and exposure to this potentially fatal, tick-borne zoonotic pathogen.

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REFERENCES

1. **Aguero-Rosenfeld, M. E., H. W. Horowitz, G. P. Wormser, D. F. McKenna, H. Nowakowski, J. Munoz, and J. S. Dumler.** 1996. Human granulocytic ehrlichiosis (HGE): a case series from a single medical center in New York State. Ann. Intern. Med. **125:**904–908.
2. **Anderson, J. F., R. C. Johnson, L. A. Magnarelli, F. W. Hyde, and J. E. Myers.** 1986. *Peromyscus leucopus* and *Microtus pennsylvanicus* simultaneously infected with *Borrelia burgdorferi* and *Babesia microti*. J. Clin. Microbiol. **29:**2779–2783.
3. **Bakken, J. S., J. Krueth, C. Wilson-Nordskog, R. L. Tilden, K. Asanovich, and J. S. Dumler.** 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA **275:**199–205.
4. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
5. **Dawson, J. E., C. K. Warner, V. Baker, S. A. Ewing, D. E. Stallnecht, W. R. Davidson, A. A. Kocan, M. Lockhart, and J. G. Olson.** 1996. *Ehrlichia*-like 16S rDNA sequence from wild white-tailed deer (*Odocoileus virginianus*). J. Wildl. Dis. **82:**52–58.
6. **Dumler, J. S., K. M. Asanovich, J. S. Bakken, P. Richter, R. Kimsey, and J. E. Madigan.** 1985. Serologic cross reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granulocytic ehrlichia. J. Clin. Microbiol. **33:**1098–1103.
7. **Dumler, J. S., and J. S. Bakken.** 1996. Human granulocytic ehrlichiosis in Wisconsin and Minnesota: a frequent infection with the potential for persistence. J. Infect. Dis. **173:**1027–1030.
8. **Edelman, D. C., and J. S. Dumler.** 1996. Evaluation of an improved PCR diagnostic assay for human granulocytic ehrlichiosis. Mol. Diagn. **1:**41–49.
9. **Greig, B., K. M. Asanovich, P. J. Armstrong, and J. S. Dumler.** 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J. Clin. Microbiol. **34:**44–48.
10. **Madigan, J. E., P. J. Richter, R. B. Kimsey, J. E. Barlough, J. S. Bakken, and J. S. Dumler.** 1995. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1141–1144.
11. **Magnarelli, L. A., K. C. Stafford, T. N. Mather, M.-T. Yeh, K. D. Horn, and J. S. Dumler.** 1995. Hemocytic rickettsia-like organisms in ticks: serologic reactivity with antisera to ehrlichiae and detection of DNA of agent of human granulocytic ehrlichiosis by PCR. J. Clin. Microbiol. **33:**2710–2714.
12. **Magnarelli, L. A., J. F. Anderson, K. C. Stafford III, and J. S. Dumler.** Antibodies to multiple tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in white-footed mice. J. Wildl. Dis., in press.
13. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. Nucleic Acids Res. **22:**2485–3487.
14. **Neitzel, D. F., J. L. Jarnefeld, and R. D. Sjogren.** 1993. An *Ixodes scapularis* (deer tick) distribution study in the Minneapolis-St. Paul, Minnesota area. Bull. Soc. Vector Ecol. **18:**67–73.
15. **Pancholi, P., C. P. Kolbert, P. D. Mitchell, K. D. Reed, J. S. Dumler, J. S. Bakken, S. R. Telford III, and D. H. Persing.** 1995. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1007–1012.
16. **Telford, S., J. E. Dawson, P. Katavolos, C. K. Warner, C. P. Kolbert, and D. H. Persing.** 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. USA **93:**6209–6214.
17. **Telford, S. R., III, T. H. Lepore, P. Snow, and J. E. Dawson.** 1995. Human granulocytic ehrlichiosis in Massachusetts. Ann. Intern. Med. **123:**277–279.
18. **Tyzzer, E. E.** 1938. *Cytoecetes microti* n. gen. n. sp. A parasite developing in granulocytes and infection in small rodents. Parasitology **30:**242–257.