Human Granulocytic Ehrlichiosis in Northern California: Two Case Descriptions with Genetic Analysis of the Ehrlichiae

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We report two cases of human granulocytic ehrlichiosis (HGE) that occurred in northern California in summer 1998. Patients had fever, malaise, and myalgia, reported tick bites, had moderate thrombocytopenia, and had normal or slightly elevated liver enzyme activities. Ehrlichial inclusions were observed in the blood of one patient, and HGE-agent DNA was amplified by PCR from both patients. Genetically, the strains resembled horse isolates from northern California. The close spatial and temporal proximity of the two new cases may be due to a nidus of infection in the area or heightened surveillance by local physicians.

Human granulocytic ehrlichiosis (HGE) is an emerging rickettsiosis in Midwestern and North Atlantic states [1, 2]. Clinical manifestations range from mild flu-like disease to death, with most patients describing myalgia, headache, and fever [3, 4]. Thrombocytopenia, leukopenia, and abnormal liver enzyme activities are common [5], and infection may cause immunosuppression [6].

The epidemiology and ecology of HGE have not been completely described; the agent of HGE may be closely related to or synonymous with Ehrlichia equi of domestic animals [7, 8]. Like E. equi, the HGE agent is transmitted by Ixodes species ticks [9, 10]. However, because antibody assays cannot distinguish between the agent of HGE and E. equi [9], serological studies to identify reservoir and alternative hosts have not elucidated whether a single species of Ehrlichia infects humans and animals or whether there are two or more strains or species with differing host tropisms. In regions where HGE is endemic, seropositive hosts include wood rats [10] and deer mice [10, 11], eastern chipmunks and southern red-backed voles [11], horses [12], dogs [13], black bears [14], mule deer and tule elk [15], mountain lions [16], and cats, skunks, and coyotes (Foley and Madigan, unpublished data).

Despite numerous equine and canine cases of granulocytic ehrlichiosis and the presence of an appropriate tick vector (Ixodes pacificus) in California, there have previously been only two cases involving humans in the state, both in Santa Cruz County, near predominantly redwood forest [17, 18]. Nevertheless, we suspected that more cases would be identified with increased surveillance. Herein we present two additional cases of HGE that occurred in Humboldt County, California.

Case Report

Patient 1, a 55-year-old previously healthy woman, presented in June 1998 because of a 3-day history of fever (maximum temperature, 40.5°C), night sweats, headache, and malaise. She had been bitten by a tick on the abdomen 10 days previously. Blood tests revealed leukopenia (1.9 × 10^3 leukocytes/mL; reference range, 4.0–10.2 × 10^3/mL) and thrombocytopenia (96 × 10^3 platelets/mL; reference range, 140–440 × 10^3/mL). Levels of γ-glutamyltransferase, alanine aminotransferase, and alkaline phosphatase were within normal limits. She was treated with doxycycline (100 mg po b.i.d. for 10 days), and defervescence occurred within 24 hours.

Patient 2, a 45-year-old previously healthy man, presented in early July 1998 because of a 6-day history of an oral temperature of 37.2°C, headache, malaise, night sweats, and arthralgia (knees, elbows, and hands). He recalled two tick bites in the preceding 10 days. One day after presentation, laboratory tests revealed leukopenia (3 × 10^3 leukocytes/mL), thrombocytopenia (139 × 10^3 platelets/mL), and mildly elevated levels of γ-glutamyltransferase (66 IU/mL; reference range, 2–65 IU/mL), alanine aminotransferase (60 IU/mL; reference range, 3–50 IU/mL), and alkaline phosphatase (185 IU/mL; reference range, 30–128 IU/mL). He was treated with doxycycline (100 mg po b.i.d. for 10 days). Clinical recovery occurred within 1 week.

Materials and Methods

Serology and western blotting. Immunofluorescence assay (IFA) for IgG antibodies to E. equi was performed as described...
Blood culture and animal inoculations. Culture of Ehrlichia was attempted with use of HL-60 cells by a method described previously [20], with the following modifications. Cell cultures at a density of 2 × 10⁵ cells/5 mL were inoculated with 200 µL of whole blood from the patients and maintained at 37°C and 5% CO₂. After 2 days, the culture was subdivided by removing 2 mL and replacing it with 2 mL of fresh medium. After 2 additional days, 2 mL of culture was replaced with 1 × 10⁶ uninfected HL-60 cells. Infection was assessed by Wright staining and PCR.

Propagation of the HGE agent was attempted by ip inoculation of 0.25 mL of EDTA-anticoagulated blood from patients 1 and 2 into two Peromyscus maniculatus deer mice per patient. In addition, 0.5 mL of whole blood from patient 2 was inoculated ip into two cats (Felis catus). The animals were observed daily after inoculation for lethargy, depression, and (in cats only) elevated rectal temperature. Blood was collected every other day until day 20 postinfection for whole blood smear (deer mice) or buffy coat smear (cats) and PCR. A final sample from day 24 was collected for serology, which was performed as described above except with use of conjugated antibodies to Peromyscus leucopus or cat IgG (Kirkegaard-Perry Laboratory, Gaithersburg, MD).

DNA extraction and PCR. Extraction of DNA and PCR were performed independently for both patients in the laboratories of the California Department of Health Services (Berkeley) and at UC Davis. DNA was extracted either from 100 µL of buffy coat with use of guanidine lysis [4] or from 100 µL of whole blood by lysis in buffer (10 mM Tris-HCl, pH of 8.0; 1 mM EDTA; and 1% [w/v] sodium dodecyl sulfate) at 37°C for 1 hour. The lysate was incubated at 37°C for 30 minutes with RNase A (10 µg/mL) and then incubated overnight at room temperature with proteinase K (50 µg/mL). Genomic DNAs were purified with use of Phase Lock Gel I Light (5 Prime–3 Prime, Boulder, CO).

The PCR reactions were performed as described in [21] for E. chaffeensis and in [22] for the E equi/HGE agent. Amplification of a 517-bp fragment from the 3’ end of the 16S rRNA gene was performed with primers EE-5F and EE-6R (table 1). All reactions were run with a water negative control. The products were separated by electrophoresis through 1.5% agarose minigels and visualized with ethidium bromide.

DNA sequencing and phylogenetic comparison. Products of PCR reactions were cloned with a kit (TA Cloning Kit; Invitrogen, San Diego). Inserts were verified by PCR using specific primers. Two clones of each sample were arbitrarily chosen to sequence the forward and reverse strands. Preparations of plasmid DNA for sequencing were purified with a kit (Quantum Prep; Bio-Rad, Hercules, CA). Extracted DNA was sequenced with M13 forward and reverse primers and internal primers EE-5F and EE-6R (table 1). All reactions were run on a 5% LongRanger gel with use of an ABI Prism 377 DNA Sequencer (ABI Prism). The products were separated by electrophoresis through 1.5% agarose minigels and visualized with ethidium bromide.

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Big dye terminator-cycle DNA sequencing was performed according to the manufacturer’s instructions (ABI Prism, Foster City, CA). Reactions were run on a 5% LongRanger gel with use of an ABI Prism 377 DNA Sequencer (ABI Prism). The DNA sequences were aligned and compared with the CLUSTAL W multiple sequence alignment program, version 1.60 [23], and MACAW multiple alignment construction and analysis workbench, version 2.05 Win 32i [24]. The DNA sequences were compared to sequences maintained in GenBank (Bethesda, MD) by means of the BLAST algorithm [25] from the National Center for Biotechnology Information. GenBank accession numbers for sequences included for comparison were U02521, U23038, U23039, M73223, AFO36645, AFO36647, AFO36646, M73220, AFO57707, U77389, U10873, U72878, U72879, and AF084907. The two Humboldt patients’ fragments were
submitted to Genbank under accession numbers AFO93788 (CAHU-HGE1) and AFO93789 (CAHU-HGE2).

Results

Clinical testing. A summary of antibody testing and evaluations of smears of blood from the two California patients is presented in table 2. Initial serology of patient 1 was negative for *E. equi*, *E. chaffeensis*, and the agent of HGE. Serology at 2 weeks was negative for *E. chaffeensis* and positive for *E. equi* at a dilution of 1:128 and for the HGE agent at 1:640. Serology for patient 2 at presentation was negative for *E. chaffeensis*, positive for *E. equi* at 1:2,048, and positive for HGE agent at ≥1:2,560. Reexamination of the blood smear of patient 1 from the initial presentation revealed one characteristic *Ehrlichia* morula in a neutrophil. No morulae were observed in the blood smear of patient 2 (the specimen used was >1 week old).

Both patients had strong 44-kDa HGE-characteristic bands on western blots (figure 1). Results of PCR for both patients were strongly positive for HGE and negative for *E. chaffeensis*. Attempted culture of blood on HL-60 cells and inoculation of patient blood into deer mice and cats were not successful, on the basis of clinical status, examination of blood smears, PCR of blood, and serology.

DNA sequencing. Nucleic acid sequences of 1,395 DNA bases coding for 16S rRNA were compared for the two patients and for previously reported granulocytic ehrlichiae. The two California HGE-agent strains were identical in the region sequenced. There was an A at position 40 for both patients, California horses, and an *Ehrlichia phagocytophila* strain (M73220), while other reported strains had a G at that position, including an HGE strain (U02521) and strains isolated from the blood of small mammals (U72878 and U72879).

Discussion

Two cases of granulocytic ehrlichiosis involving humans were diagnosed within 1 month in the vicinity of one town in northern California. The other previously confirmed cases of HGE in California both occurred ~275 miles south of that town, in Santa Cruz County, in 1993. The patients’ clinical signs were typical for HGE, including headache, fever, and

![Figure 1](image-url)
myalgia, but biochemical and hematologic parameters were either normal or only moderately altered. Only one ehrlichial morula was observed in the blood smear of patient 1, and none in that of patient 2; the morula was found only on a third review of the smear by an ehrlichial specialist (Dr. Dumler). Other diagnostic test results, such as the strong 44-kDa immunodominant band on western blots and positive PCR, were consistent with results for patients in regions of HGE endemicity east of the Rocky Mountains.

Confirmation of HGE was accomplished by DNA sequencing of PCR products because we were unable to obtain a viable isolate either in blood culture or in vivo by passage into laboratory animals. Blood from both patients was at least 10 days old before it was available for culture. It is not known how long the HGE agent remains viable in EDTA for animal inoculation or how effective *P. maniiculatus* is as a host for the agent, despite previous reports of infections with HGE in *P. leucopus*. However, previous inoculations of cats were successful in experiments by Lewis et al. [26], using *E. equi*, and in our laboratory with use of the HGE-agent strain BDS (Foley, Madigan, and Pedersen, unpublished data).

The genetic analysis of these strains revealed that the two new human agents are identical in the conserved 16S rRNA gene. In position 40, the California HGE strains differed from other HGE strains but were identical to a sequence from horse “Alice,” which acquired ehrlichiosis as part of an experiment in which unfed adult *I. pacificus* ticks were acquired by flagging in Mendocino County and were fed on horses [27]. Two additional horses were also infected with ticks from the same location but had ehrlichial sequences in the 16S rRNA gene that differed by 1–2 bases. This suggests that analyses of 16S rRNA gene data are already at a poor for *I. pacificus*.

Clearly, it would be useful to compare the ecological factors supporting ehrlichiosis in California with those in the upper Midwest and Atlantic coast. While we have definitely documented that HGE occurs in California, it is not known whether the low frequency of HGE in California results from the presence of strains with low pathogenicity, ecological factors, underreporting, or other causes. However, because there are few clinical or routine laboratory findings that are diagnostic for HGE, it is important that physicians be aware of emergence of this disease in their areas.

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References