

## Human Disease in Europe Caused by a Granulocytic *Ehrlichia* Species

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**Human granulocytic ehrlichiosis (HGE) was recently described in North America. It is caused by an *Ehrlichia* species closely related to *Ehrlichia phagocytophila* and *Ehrlichia equi*, recognized to infect mostly ruminants and horses, respectively. The vector in North America is the tick *Ixodes scapularis*, which is also the vector of the Lyme disease agent, *Borrelia burgdorferi*. Previous serologic studies in patients with a diagnosis of Lyme borreliosis indicate that HGE may exist in Europe. We report the first documented case of HGE in Europe. The diagnosis was established by seroconversion to *E. equi* and the HGE agent and by PCR with sequence analysis of the gene encoding the HGE agent 16S rRNA. Interestingly, the patient presented with a self-limited but moderately severe illness. Thus, European physicians need to be aware that HGE exists in Europe and that the diagnosis should be considered in febrile patients with tick bites in areas where Lyme disease is endemic.**

Ehrlichioses are tick-transmitted febrile illnesses caused by gram-negative obligate intracellular bacteria that parasitize leukocytes. Ehrlichiae were first recognized in Algerian dogs as early as 1935 (8). In the United States, human disease caused by a monocytotropic *Ehrlichia* species was first described in 1987 (14). Recently, human infection with a granulocytotropic *Ehrlichia* species was described in the United States (3, 7). Seroepidemiological studies suggested that human infections with *Ehrlichia* species may also be present in Europe (6, 20). Only two acute cases of disease presumably caused by *Ehrlichia chaffeensis* have been described in Europe, and both were diagnosed by serology only (15, 17). To our knowledge no clinical cases of human granulocytic ehrlichiosis (HGE) have been reported outside the United States.

Slovenia is a small Central European country of 20,256 km<sup>2</sup> with a population of approximately 2 million. Since 1986 it has been recognized as a region where Lyme borreliosis is endemic, and borrelial infections have replaced tick-borne encephalitis as the most prevalent tick-borne illness (19). As in other European countries, the *Ixodes ricinus* tick is the main vector for the agents of both diseases. Outdoor recreation is favored by many Slovenians, and several tick bites per year are not unusual for a substantial part of the population. Every summer, numerous patients with febrile illnesses for which the etiology remains unresolved are examined at the University Medical Center, Ljubljana, Slovenia. This was a stimulus for a prospective study of the etiology of febrile illnesses occurring within 6 weeks after a tick bite. The patient described herein represents one of these patients and is the first confirmed clinical case of HGE in Europe.

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### CASE REPORT

In June 1996, a 70-year-old woman living in the northwest part of Slovenia presented to the Outpatient Clinic, Department of Infectious Disease, University Medical Center, Ljubljana, with a 2-day history of fever of up to 40.0°C, headache, nausea, vomiting, malaise, intense myalgias, and arthralgias. She recalled having sustained a tick bite on her abdomen 12 days prior to the onset of her illness while walking in the woods near her home; no skin lesions appeared at the site of a tick bite. Her past medical history was unremarkable. She had not been vaccinated against tick-borne encephalitis. She had not traveled outside Slovenia in the previous 15 years.

At presentation, her physical examination was unremarkable with the exception of conjunctivitis and slight cervical lymphadenopathy; rash and meningeal signs were not present. Laboratory tests revealed an erythrocyte sedimentation rate of 11 mm/h (normal range, 0 to 20 mm/h); a peripheral blood leukocyte count of  $6.0 \times 10^9$ /liter with 10% bands, 85% polymorphonuclear cells, 3% lymphocytes, and 2% monocytes; and a normal erythrocyte count. The platelet count was  $118 \times 10^9$ /liter (normal range,  $140 \times 10^9$  to  $340 \times 10^9$ /liter), the C-reactive protein concentration was 58.3 mg/liter (normal range <5 mg/liter), the serum lactate dehydrogenase level was 389.9 U/liter (normal range, 140 to 290 U/liter), and the serum creatine phosphokinase level was 239.9 U/liter (normal range, 42 to 124 U/liter). Serum transaminase and alkaline phosphatase activities were in the normal range. EDTA-anticoagulated blood and serum were obtained and stored frozen for later analysis.

The patient was instructed to rest at home. Three days later her fever diminished spontaneously and all the symptoms resolved. Examination 2 weeks after the first visit revealed no abnormalities. Laboratory values were in normal range with the exception of a mildly elevated erythrocyte sedimentation rate (38 mm/h). The patient was not given any antibiotics; the only medications that she used during her illness were antipyretics. During the 3-month follow-up, she remained well. Additional whole blood and serum samples were obtained when

TABLE 1. Antibody titers to different tick-transmitted agents in a Slovenian patient with HGE tested at different times after onset of the disease

Date	Antibody titer <sup>a</sup>									
	<i>E. equi</i> in equine neutrophils	<i>E. equi</i> in HL60 cells	Canine HGE agent (Cambridge)	<i>E. chaffeensis</i> (ULS-IFA/JHU-IFA) <sup>b</sup>	TBE IgG <sup>c</sup>	TBE IgM	<i>B. burgdorferi</i> IgG	<i>B. burgdorferi</i> IgM	<i>R. conorii</i>	<i>B. microti</i>
10 June 1996 <sup>d</sup>	<25	<80	<25	<64/<80	Neg	Neg	128	<64	<40	<16
21 June 1996	50	1,280	100	128/<80	Neg	Neg	NT <sup>e</sup>	NT	<40	<16
23 July 1996	100	≥2,560	200	128/<80	Neg	Neg	256	128	<40	<16
12 September 1996	>800	≥2,560	>800	128/<80	Neg	Neg	512	<64	<40	<16

<sup>a</sup> Antibody titers are reciprocals of the serum dilution.

<sup>b</sup> ULS-IFA, IFA performed at Medical Faculty, University of Ljubljana, Ljubljana, Slovenia; JHU-IFA, IFA performed at Johns Hopkins University School of Medicine, Baltimore, Md.

<sup>c</sup> TBE, tick-borne encephalitis virus; Neg, negative.

<sup>d</sup> Date of first examination (acute phase), day 3 of illness.

<sup>e</sup> NT, not tested.

the patient returned for follow-up at 2 weeks, 6 weeks, and 3 months after presentation and were stored frozen.

#### MATERIALS AND METHODS

**Serologic studies.** As a part of a prospective study on the etiology of febrile illnesses occurring within 6 weeks after a tick bite, which is ongoing at our institutions in Slovenia, several tests were performed. Serum samples were tested by an indirect immunofluorescence assay (IFA) for the presence of antibodies against *Ehrlichia equi* MRK in equine neutrophils, *E. equi* MRK propagated in HL60 promyelocyte cells, a canine isolate of the HGE agent (Cambridge isolate; courtesy Cambridge Biotech, Cambridge, Mass.), *Ehrlichia chaffeensis* Arkansas (MRL Diagnostics, Cypress, Calif.), *Borrelia burgdorferi* sensu lato (whole cells of a local isolate of *Burgdorferi afzelii*), and *Rickettsia conorii* (BioMerieux, Lyon, France). Fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (IgG; gamma heavy chain specific) was used as a conjugate at a dilution of 1:40 (Fluoline-G; BioMerieux, Marcy-l'Étoile, France). Antibodies to tick-borne encephalitis virus were determined by using an enzyme-linked immunosorbent assay kit (Immunozyne; Immuno AG, Vienna, Austria) according to the manufacturer's procedure. Detection of antibodies to *Babesia microti* was performed by IFA, courtesy of Lou Magnarelli, Connecticut Agricultural Experiment Station, New Haven.

**PCR analysis.** Blood samples were processed in a building where *Ehrlichia* species and their nucleic acids have never been present. Precautions were taken to prevent contamination of samples for PCR analysis including the use of aerosol barrier pipette tips. A 2-ml sample of whole blood was subjected to erythrocyte lysis with 10 ml of erythrocyte lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl<sub>2</sub> [pH 7.4]) for 3 min and then centrifuged at 4000 × g for 5 min. Erythrocyte debris was discarded and the leukocyte pellet was resuspended in 200 μl of sterile phosphate-buffered saline (PBS) and frozen at -70°C until DNA isolation. DNA was isolated by using a QIAamp Tissue kit (QIAGEN, Chatsworth, Calif.) according to the manufacturer's procedure. DNA was resuspended in 400 μl of 10 mM Tris-HCl (pH 9).

A total of 10 μl of DNA was used as a template in a 100-μl PCR with the HGE agent-specific primers ge9f and ge10r that produce a 919-bp product (7). Amplification was performed by using GeneAmp reagents in a thermal cycler (model 9600; Perkin-Elmer, Norwalk, Conn.), with 3 min of denaturation at 95°C, followed by 35 cycles consisting of 30 s of denaturation at 95°C, 30 s of annealing at 52°C, and 1 min of extension at 72°C for all cycles except the last one, during which extension was prolonged to 7 min (9). In addition, primers HE1 and HE3 specific for the *E. chaffeensis* 16S rRNA gene and primers FL6 and FL7 specific for the flagellin gene of *B. burgdorferi* were used with the same DNA samples (2, 16).

Multiple negative controls were processed in parallel. For analysis, 10 μl of amplified PCR product was subjected to electrophoresis on a 2% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, Maine). The gel was stained with ethidium bromide, and the bands were visualized with a UV transilluminator. The molecular sizes of the amplified bands were estimated by comparison with a molecular size marker (Gibco BRL, Paisley, Scotland).

**Sequence analysis.** Amplified DNA was used for sequence determination in two separate laboratories. Automated fluorescent sequencing was performed with an ABI model 377 instrument (Applied Biosystems, Inc., Foster City, Calif.), and all sequences were confirmed by cycle sequencing the forward and reverse strands. Solid-phase sequencing was performed by using an A.L.F. sequencer (Pharmacia, Uppsala, Sweden) by sequencing in both the forward and the reverse directions. The sequence data that were determined were compared for similarity with the sequences from other known *Ehrlichia* species in GenBank by using the sequence alignment program and Clustal program of PCGene software (Intelligenetics, Inc., Mountain View, Calif.).

#### RESULTS

**Blood smear examination and serology.** Despite exhaustive retrospective examination of a Giemsa-stained smear of the acute-phase blood (day 3 of illness) from the patient described here, ehrlichial inclusions (morulae) were not observed in any of the leukocytes. The results of the serological tests are presented in Table 1. Serologic tests with *E. equi* in equine neutrophils, with *E. equi* propagated in HL60 cells, and with the canine isolate of the HGE agent as the antigen all revealed final titers of ≥800, ≥2,560, and ≥800, respectively, in the 3-month convalescent-phase serum sample. Simultaneously, a fourfold increase in *B. burgdorferi* titer was observed. No antibodies against tick-borne encephalitis virus, *B. microti*, or *R. conorii* were detected in any sample.

**Amplification of 16S rRNA gene of the HGE agent.** Primers ge9f and ge10r amplified the 16S rRNA gene of the HGE agent in the acute-phase (day 3 of illness) blood sample to produce a fragment of the expected size (919 bp) (Fig. 1). No nucleic acids were amplified from negative controls (water) with primers specific for *E. chaffeensis* (HE1 and HE3) or with primers specific for *Borrelia* (FL6 and FL7) (data not shown).

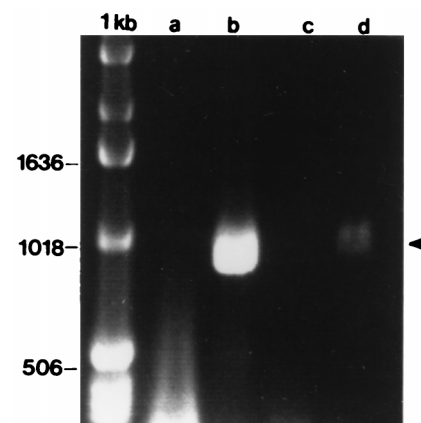


FIG. 1. PCR amplification of HGE agent DNA from the acute-phase blood nucleic acids of a Slovenian patient with HGE. Amplified DNA was separated by electrophoresis through agarose and was stained with ethidium bromide. Various nucleic acid templates were loaded onto the gel, as follows: lane a, blood from healthy human subject; lane b, purified HGE agent DNA; lane c, water only (no-DNA template control); lane d, blood from a Slovenian patient (note the presence of a band at approximately 919 bp [arrowhead]). The lane labeled 1 kb represents a 1-kb DNA ladder for estimation of molecular sizes. Estimated molecular sizes (in kilobases) are labeled on the left.

In addition, no amplification product was observed when blood obtained 3 months later was subjected to PCR with the same sets of primer pairs (data not shown).

**Sequence analysis.** Sequence analysis revealed a fragment of 869 bp without the flanking incorporated ge9f and ge10r primer sequences. The partial 16S rRNA gene sequence was 100% identical to that of the HGE agent (GenBank accession no. U02521) derived from a patient infected with the HGE agent in the upper midwestern United States and was identical to that of the agent of granulocytic ehrlichiosis in dogs, horses, and some cattle in Sweden (GenBank accession no. U010873). When the partial 16S rRNA gene sequence of the Slovenian HGE agent was compared with the GenBank sequences for *E. phagocytophila* (accession nos. M73220 and M73224) and *E. equi* (accession no. M73223), it was found to be 99.8% identical to each sequence, differing in sequence at only 2 nucleotide positions.

## DISCUSSION

In this report we describe the first case of human disease caused by a granulocytotropic *Ehrlichia* species in Europe. Although many reports indicate the presence of antibodies against ehrlichiae in Europe, no defined clinical presentation with objective laboratory confirmation has been described (4, 6, 12, 20). Indeed, infections of dogs, horses, and cattle caused by a granulocytotropic *Ehrlichia* species virtually identical to the HGE agent have been recently reported in Sweden (11).

Slovenia is a region known to be endemic for Lyme borreliosis as well as tick-borne encephalitis, but many febrile illnesses that occur after a tick bite have no proven etiology. The main vector of both diseases is *I. ricinus*, which is also the most frequently encountered tick in Slovenia (21).

The clinical characteristics of patients infected with HGE described in the United States include chills, fever, myalgias, headache, nausea, confusion, cough, and arthralgias. Laboratory data frequently show leukopenia, neutropenia, thrombocytopenia, lymphopenia, anemia, and elevated aspartate aminotransferase activity. The median age of patients with HGE is between 43 and 60 years (1, 5). The clinical presentation in our patient is similar to that described previously for patients with HGE, except that in our patient the course of illness was self-limited, with no specific antibiotic therapy. Negative results obtained from nucleic acid amplification of blood obtained 3 months after the onset of illness and normal findings on clinical follow-up indicate cure of HGE and reduction of the infectious agent to an undetectable level in blood.

Leukopenia and thrombocytopenia are common not only in the human ehrlichioses but also in some other tick-borne illnesses such as babesiosis and during the initial phase of tick-borne encephalitis, where it is found in 71% of patients (13). However, these hematologic abnormalities are seen only exceptionally in the course of early Lyme borreliosis (18). The presence of the typical clinical features, laboratory findings (thrombocytopenia and elevations in serum lactate dehydrogenase and C-reactive protein levels), the presence of HGE agent DNA in the acute-phase blood, and seroconversion to the agent of HGE strongly suggest that this patient had HGE. Although rising titers of IgG antibodies to *B. burgdorferi* sensu lato were detected, in the absence of any clear clinical signs or symptoms consistent with typical early Lyme borreliosis, it is unlikely that the illness was due to *B. burgdorferi* sensu lato infection. However, asymptomatic or clinically atypical *B. burgdorferi* coinfection or previous Lyme borreliosis cannot be excluded by these studies as an explanation for the serologic reactions with *B. burgdorferi*. Recently, Wormser et al. (22)

reported similar serologic reactivity to *B. burgdorferi* in 9 of 10 patients with well-defined HGE and no clear signs of Lyme disease. This finding has been supported by preliminary studies in an animal model of granulocytic ehrlichiosis in which immunoblots of serum from BALB/c mice experimentally infected with an HGE agent isolate, but not with *B. burgdorferi*, demonstrated reactivity to OspC and OspA antigens; 36-, 38-, and 93-kDa antigens; and other antigens (10). Alternately, seroconversion to *B. burgdorferi* may indicate the possibility of coinfection or prior infection with this agent, which is an intriguing possibility given that *Ixodes* species ticks are known vectors for the HGE agent and *B. burgdorferi*, as well as *Babesia* species and tick-borne encephalitis viruses (13, 19, 21).

We suspect that the dense population of deer and wild small rodents in Slovenia may represent a reservoir for the HGE agent as well as for other tick-borne agents of human disease. The *I. ricinus* tick is the most likely vector. Further studies will be required to determine the reservoir, vector(s), seroprevalence, and clinical presentation of the disease caused by the HGE agent in Europe.

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## REFERENCES

1. Aguero-Rosenfeld, M., H. W. Horowitz, G. P. Wormser, D. F. McKenna, J. Nowakowski, J. Munoz, and J. S. Dumler. 1996. Human granulocytic ehrlichiosis (HGE): a series from a single medical center in New York State. *Ann. Intern. Med.* **125**:904-908.
2. Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson. 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *J. Clin. Microbiol.* **29**:2838-2842.
3. Bakken, J. S., J. S. Dumler, S. M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker. 1994. Human granulocytic ehrlichiosis in the upper Midwest United States. A new species emerging? *JAMA* **272**:212-218.
4. Bakken, J. S., J. Krueth, R. L. Tilden, J. S. Dumler, and B. E. Kristiansen. 1996. Serological evidence of human granulocytic ehrlichiosis in Norway. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**:829-832.
5. 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.
6. Brouqui, P., J. S. Dumler, R. Lienhard, M. Brossard, and D. Raoult. 1995. Human granulocytic ehrlichiosis in Europe. *Lancet* **346**:782-783.
7. 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.
8. Donatien, A., and F. Lestoquard. 1935. Existence en Algérie d'une rickettsia du chien. *Bull. Soc. Pathol. Exot.* **28**:418-419.
9. Edelman, D. C., and J. S. Dumler. 1996. Evaluation of an improved PCR diagnostic assay for human granulocytic ehrlichiosis. *Mol. Diagn.* **1**:41-49.
10. Hofmeister, E. K., J. Magera, L. Sloan, C. Kolbert, J. Hanson, and D. H. Persing. 1996. *Borrelia burgdorferi* proteins are recognized by antibodies from mice experimentally infected with the agent of human granulocytic ehrlichiosis, abstr. E814. In Abstracts of the VIIth International Congress on Lyme Borreliosis.
11. Johansson, K. E., B. Pettersson, M. Uhlen, A. Gunnarsson, M. Malmqvist, and E. Olsson. 1995. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid-phase sequencing of PCR products from the 16S ribosomal-RNA gene. *Res. Vet. Sci.* **58**:109-112.
12. Lillini, E., G. Macri, P. Rombola, D. Grazioli, F. Russino, and M. Nuti. 1996. *Ehrlichia* seropositivity in Italian foresters, p. 335-339. In J. Kazár and R. Toman (ed.), *Rickettsiae and rickettsial diseases*. Slovak Academy of Sciences, Bratislava, Stara Lesna, Slovakia.
13. Lotric Furlan, S., and F. Strle. 1995. Thrombocytopenia—a common finding in the initial phase of tick-borne encephalitis. *Infection* **23**:203-206.
14. Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N. Engl. J. Med.* **316**:853-856.
15. Morais, J. D., J. E. Dawson, C. Greene, A. R. Filipe, L. C. Galhardas, and F.

- Bacellar.** 1991. First European case of ehrlichiosis. *Lancet* **338**:633–634.
16. **Picken, M. M., R. N. Picken, D. Han, Y. Cheng, and F. Strle.** 1996. Single-tube nested polymerase chain reaction assay based on flagellin gene sequences for detection of *Borrelia burgdorferi* sensu lato. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**:489–498.
  17. **Pierard, D., E. Levchenko, J. E. Dawson, and S. Lauwers.** 1995. Ehrlichiosis in Belgium. *Lancet* **346**:1233–1234.
  18. **Strle, F., J. A. Nelson, E. Ruzic Sabljic, et al.** 1996. European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. *Clin. Infect. Dis.* **23**:61–65.
  19. **Strle, F., A. Pejovnik Pustinek, G. Stanek, D. Pleterski, and R. Rakar.** 1989. Lyme borreliosis in Slovenia in 1986, p. 50–54. *In* G. Stanek, W. Kristoferitsch, M. Pletschette, A. G. Barbour, and H. Flamm (ed.), *Lyme borreliosis II*. Gustav Fischer Verlag, Stuttgart, Germany.
  20. **Sumption, K. J., D. J. M. Wright, S. J. Cutler, and B. A. S. Dale.** 1995. Human ehrlichiosis in the UK. *Lancet* **346**:1487–1488.
  21. **Tovornik, D.** 1988. The significance of the Roe-Deer (*Capreolus capreolus* Linne, 1758) as the host and disseminator of ixodid ticks in Slovenia. *Biol. Vestn.* **36**:85–94.
  22. **Wormser, G. P., H. W. Horowitz, J. S. Dumler, I. Schwartz, and M. Aguerro-Rosenfeld.** 1996. False-positive Lyme disease serology in human granulocytic ehrlichiosis. *Lancet* **347**:981–982.