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CLINICAL ARTICLES

Human Granulocytic Ehrlichiosis in Europe: Clinical and Laboratory Findings for Four Patients from Slovenia

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Febrile illnesses following a tick bite in patients from Slovenia were evaluated for an ehrlichial etiology. A case of acute human granulocytic ehrlichiosis (HGE) was confirmed by seroconversion to the HGE agent or molecular identification of ehrlichial organisms. Acute infection with the HGE agent was confirmed in four patients. None of the patients had detectable antibodies to the HGE agent at their first visit, but polymerase chain reaction analysis was positive for three patients. All four patients subsequently seroconverted to the HGE agent as shown by high titers of antibody. Clinical features and laboratory findings were similar to those in reports from the United States, although the disease course was relatively mild in the Slovenian cases. All patients recovered rapidly and without sequelae, although only two received antibiotic therapy (of whom only one was treated with doxycycline). HGE is an emerging tick-borne disease in the United States and should now be included in the differential diagnosis of febrile illnesses occurring after a tick bite in Europe.

Human granulocytic ehrlichiosis (HGE) is a tick-borne zoonosis recently described in the United States that is caused by an *Ehrlichia* species closely related or conspecific to the known animal pathogens *Ehrlichia phagocytophila* and *Ehrlichia equi* [1]. In the United States, white-tailed deer and small mammals may serve as primary reservoirs of the HGE agent, and the tick *Ixodes scapularis* has been shown to be a competent vector [2–5].

The presence of antibodies to *Ehrlichia* antigens has been documented in sera from individuals from Switzerland [6], the United Kingdom [7], Italy [8], Norway [9], and Sweden [10]. However, clinical description of acute HGE in Europe is limited to a single case documented in June 1996 in Slovenia [11]. Slovenia is a region in which several tick-borne diseases are endemic. The annual incidence of tick-borne encephalitis and

Lyme borreliosis, both transmitted by *Ixodes ricinus* ticks, in Slovenia is among the highest in Europe [12, 13]. However, each summer, numerous patients develop a febrile illness after a tick bite for which an etiology has never been established.

The aim of this prospective study was to determine if infection with an *Ehrlichia* species was associated with febrile illnesses occurring after a tick bite. Herein we report the clinical presentation and laboratory confirmation of the first four consecutive cases of HGE occurring outside of the United States.

Patients and Methods

A study to assess the potential ehrlichial etiology of febrile illnesses after a tick bite was conducted at the Department of Infectious Diseases, University Medical Centre, Ljubljana, Slovenia, between March 1995 and December 1996. Patients with a febrile illness were referred for evaluation to our institution by primary care physicians; only patients with a history of a tick bite in the preceding 6 weeks were eligible for the study. Patients were examined at the time of their acute illness and 14 days, 6–8 weeks, 6 months, and 12 months later. Suspected acute cases of HGE were confirmed by seroconversion with a minimum fourfold rise in titer of antibody to HGE agent antigen or by PCR analysis with subsequent sequencing of the amplicons. One patient (no. 1, table 1) has been described previously [11].

At the first visit, blood samples were obtained for determination of a complete blood cell count and serum chemistry profile, including concentrations of serum aspartate aminotransferase,

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Informed consent was obtained from all patients.

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Table 1. Demographic characteristics and clinical presentation of four patients with human granulocytic ehrlichiosis in Slovenia.

Patient no.	Age (y)/sex	Incubation period* (d)	Temperature† (°C)	Chills	Headache	Vertigo	Nausea	Malaise	Myalgia	Arthralgia	Other	Antibiotic treatment	Duration of fever (d)
1	70/F	12	40	—	+	—	+	+	+	+	Vomiting	None	5
2	59/F	21	41	+	+	+	+	+	+	+	—	Doxycycline‡	4
3	43/M	7	40	+	+	—	—	+	+	+	Dry cough	Azithromycin§	9
4	55/F	30	39.3	—	+	+	+	+	+	+	Vomiting	None	7

NOTE. + = present; — = absent.

* Time from tick bite to the onset of illness.

† Maximum body temperature recorded.

‡ Dosage, 100 mg b.i.d. for 12 days; treatment was instituted 2 days after spontaneous defervescence.

§ Dosage, 250 mg b.i.d. on the first day, followed by 250 mg q.d. for the following 4 days; treatment was instituted on the second day of illness by a primary physician because of suspicion of atypical pneumonia.

alanine aminotransferase, serum bilirubin, alkaline phosphatase, lactate dehydrogenase, creatine phosphokinase, and C-reactive protein. Clinical laboratory procedures were repeated at sequential examinations. Giemsa-stained peripheral blood smears were examined by use of light microscopy for the presence of ehrlichial morulae within WBCs.

Acute- and convalescent-phase serum samples were tested by use of an indirect immunofluorescence assay (IFA) for the presence of specific antibodies to the HGE agent (with use of the USG3 strain, which was propagated in HL60 promyelocyte cells) [14]. This test uses a polyvalent conjugate (goat antibody to human IgG with heavy and light chains) and will detect human IgG and any other immunoglobulin classes with similar light chains. A similar IFA is also conducted with use of *Ehrlichia chaffeensis* antigen (MRL Diagnostics, Cypress, CA). IgM and IgG antibodies to other tick-borne agents were also assessed by IFA for *Borrelia burgdorferi* sensu lato (whole cells of a local isolate of *Borrelia afzelii*) and *Rickettsia conorii* (bioMérieux, Lyon, France) or by use of an ELISA kit (Immunozyne, FSME, Immuno AG, Vienna) for the presence of antibody to tick-borne encephalitis virus.

DNA was extracted from WBCs in the buffy coat and used as a template for PCR reactions with 16S rRNA gene primers GE9f and GE10r, specific to the *E. phagocytophila* genogroup (*E. phagocytophila*, *E. equi*, and the HGE agent) [1], and primers HE1 and HE3, specific to *E. chaffeensis* [15]. These samples were also tested with a nested PCR method [16] designed to detect *E. chaffeensis* and members of the *E. phagocytophila* genogroup by amplification of part of the *groESL* heat shock operon. Primers HS1 and HS6 were used in primary PCR reactions. Samples (1 μ L) of products of the primary reactions were used as a template for nested reactions with primers HS43 and HS45 [16]. Primers HS43 and HS45 span a 442-bp region that includes the intergenic spacer between the *groES* gene and the *groEL* gene and a 392-bp region of the amino coding terminus of the *groEL* gene. Primers HS43 and HS45 were used in the initial PCR testing because they also amplify the homologous region of the *E. chaffeensis groESL* operon.

A variable region of the *Ehrlichia groEL* gene was amplified in additional nested reactions with use of primers HS43 or HSVF (5'-CAATAAGCTCCGTGGTGGCCTTC) paired with primer HSVR (5'-CTCAACAGCAGCTCTAGTAGC). The nucleotide sequences for primers HSVF and HSVR were not reported previously, but the cycling parameters were as previously described [16]. Primers HSVF and HSVR span a 395-bp region of the *groEL* gene that includes nucleotides 812 through 1,206 when adenine of the putative *groEL* translation initiation codon is designated nucleotide 1. PCR products were separated and detected by electrophoresis in agarose gels containing ethidium bromide. Amplified bands of DNA were excised and purified from the gel for sequencing. Purified PCR products were sequenced by using the Prism Ready Reaction dideoxy cycle sequencing kit (Applied Biosystems, Foster City, CA). Both strands of each product were sequenced with use of sets of primers from conserved regions [16].

Results

The diagnosis of acute HGE was confirmed in four patients (table 1). Three patients were female, and the mean age of the patients was 57 years. All the patients presented between June and August 1996 and recalled a tick bite 7 to 30 days before the onset of their symptoms.

Two patients required hospitalization, while two were treated as outpatients. In all patients, the highest temperature was $\geq 39.3^{\circ}\text{C}$. Fever abated spontaneously in all patients, including two who had received no antibiotic therapy and one who was treated with doxycycline 2 days after defervescence. With the exception of fever, clinical examinations revealed few significant abnormalities: one patient presented with conjunctivitis and mildly enlarged cervical lymph nodes, and one had pneumonia that was demonstrated radiographically. This patient (no. 3, table 2) had clinical characteristics of atypical pneumonia and was seronegative at all times for *Coxiella burnetii*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Mycoplasma pneumoniae*. Rash was not present in any of the patients. In all four

Table 2. Summary of initial principal laboratory data and physical examination findings for patients with acute human granulocytic ehrlichiosis in Slovenia.

Patient no.	Day of illness	Physical examination finding		Laboratory finding							
		Temperature (°C)	Other	ESR (mm/h)	WBC count* ($\times 10^9/L$)	Thrombocyte count† ($\times 10^9/L$)	CRP level (mg/L)	AST level (U/L)	ALT level (U/L)	LDH level (U/L)	CPK level (U/L)
1	3	39.5	Conjunctivitis, cervical lymphadenopathy	11	6.0	118	58	30	14	390	240
2	6	39.8	—	24	3.4	37	179	38	37	372	60
3	5	39.0	—†	54	10.9	219	83	100	107	214	120
4	7	39.3	—	11	1.9	60	12	16	13	209	102

NOTE. ALT = alanine aminotransferase (normal value, 0–42 U/L); AST = aspartate aminotransferase (0–36 U/L); CPK = creatine phosphokinase (42–124 U/L); CRP = C-reactive protein (<5 mg/L); ESR = erythrocyte sedimentation rate (<20 mm/h); LDH = lactate dehydrogenase (140–290 U/L); — = absent.

* Normal value, $4.0\text{--}10.0 \times 10^9/L$.

† Normal value, $150\text{--}350 \times 10^9/L$.

‡ During later examinations, pneumonia was demonstrated radiographically.

patients, the clinical course was favorable: none of them died, and no long-term consequences were found during a follow-up period of 1 year.

The most common laboratory abnormalities were an elevated concentration of serum C-reactive protein (4 patients), thrombocytopenia (3), elevated liver enzyme levels (3), leukopenia (2), and an elevated erythrocyte sedimentation rate (2) (table 2). Lymphopenia was found in only one patient. This patient also had granulocytosis with a marked left shift. None of the

patients had elevated serum creatinine and blood urea nitrogen levels or anemia (data not shown).

Despite intensive examination, intracytoplasmic morulae were not seen in any leukocytes on the blood smears.

Acute infection with the HGE agent was indicated by seroconversion from a negative titer of antibody (<1/64) to a high titer of polyvalent antibody to HGE agent antigen in all four patients (table 3). In addition, patient 2 developed a high titer of antibody to *E. chaffeensis* antigen, and patient 1 had a stand-

Table 3. Results of PCR analysis and titers of antibody to different tick-transmitted agents for four Slovenian patients tested at different times after the onset of HGE.

Patient no., day from onset of the illness	PCR analysis*		IFA for antibody to HGE agent	IFA for IgG to <i>Ehrlichia chaffeensis</i>	IgM/IgG to TBEV	IgM/IgG to <i>Borrelia burgdorferi</i>	IgG to <i>Rickettsia conorii</i>
	rRNA gene	<i>groESL</i> operon					
1							
3	+	+	—	128	—/—	—/128	—
14	—	NT	256†	128	NT	NT	NT
46	—	NT	512	128	—/—	128/256	—
2							
11	+	+	—	—	—/+	—/—	—
24	—	NT	1,024	>4,096	NT	NT	NT
67	—	NT	2,048	1,024	—/+	—/—	—
3							
5	—	—	—	—	—/—	—/—	—
20	—	NT	256	—	NT	NT	NT
54	—	NT	256	—	—/—	—/—	—
4							
7	+	+	—	—	—/—	—/—	—
20	—	NT	512	—	NT	NT	NT
55	—	NT	1,024	—	—/—	—/—	—

NOTE. HGE = human granulocytic ehrlichiosis; IFA = indirect immunofluorescence assay; NT = not tested; TBEV = tick-borne encephalitis virus; + = positive; — = negative.

* With use of GE9f and GE10r primers specific to the HGE agent and *groESL* nested primers.

† Reciprocal value of serum dilution.

ing titer of antibody to *E. chaffeensis* antigen. Patient 1 developed only IgM antibodies to *B. burgdorferi* sensu lato.

PCR analysis with primers GE9f and GE10r generated products of the predicted molecular size (919 bp) from DNA extracted from blood samples from three of four patients that were obtained at their first hospital visit. Similar results were obtained with PCR analysis with the *groESL* primers (table 3). Amplification products were not obtained when material collected during the follow-up period was subjected to the same PCR assay. No product was observed at any time when primers specific to *E. chaffeensis* were used.

The nucleotide sequence of the 16S rRNA gene amplicon from the agent identified in patient 1 was reported [11] to be identical to the original sequence of DNA from the HGE agent [1]. Two regions of the *groESL* operon were amplified and sequenced from DNA extracted from blood samples from patients 1, 2, and 4. The nucleotide sequences of DNA from the HGE agents identified in all three patients were identical to one another and to previously reported sequences of DNA from the HGE agent that were from the region of the *groESL* operon amplified with primers HS43 and HS45 [16]. Sequences were found to be more variable in the region amplified by primers HSVF and HSVR in a previous comparison, and there were five nucleotide positions in which the sequences of DNA from North American and European HGE agents consistently differed [16]. The sequences of DNA from the HGE agents identified in all three Slovenian patients were identical in this variable region and were most similar to sequences previously amplified from DNA from *E. phagocytophila*, including sequences of PCR products from DNA from *E. phagocytophila* naturally infecting a Swiss horse (100% similarity) and from DNA from *E. phagocytophila* experimentally infecting a goat (99.4%) and sheep (99.5%) [16].

The sequences of DNA from the HGE agents identified in the Slovenian patients matched the sequences of DNA from European HGE agents in the five positions in which consistent differences were previously detected. A 99.2% similarity to sequences of DNA from North American HGE agents, including those identified in two patients, a tick, and a horse, was found. Finally, a 1,256-bp region was amplified from DNA from the HGE agent identified in patient 2 by using primers HS43 and HSVR. This sequence was deposited in GenBank (Bethesda, MD; accession number AF033101). The sequence of this longer product differed from that of the DNA from the HGE agent identified in the Swiss horse by one nucleotide (99.9% similarity). Sequence variation at the nucleotide level did not change the predicted amino acid sequence for the portion of the *groEL* gene included in the PCR products. The predicted amino acid sequences were identical for all of these sequences.

Discussion

To our knowledge, this is the first series of European patients with acute HGE to be described. Each patient had received a

tick bite while in central Slovenia, and none had traveled outside of Slovenia during the indicated incubation period. In this region, tick-borne encephalitis and Lyme borreliosis are also endemic, and etiologic agents of both infections are transmitted by *I. ricinus* [12, 13]. This species is also known as the vector of tick-borne fever due to *E. phagocytophila* in ruminants in Europe [17]. Because *I. ricinus* is the most widespread tick species in this part of Europe, it appears likely that it is a vector of the HGE agent in Europe.

The clinical features of and laboratory parameters for our four patients were similar to those reported in cases from the United States [18–20]. However, the severity of the illness in Slovenia was mild to moderate relative to HGE in some patients in the United States, where deaths were reported in two of 41 cases [19]. In general, all laboratory abnormalities were of short duration and had resolved by 14 days after the initial presentation. Symptoms abated without the use of antibiotics in two patients, and the only patient to receive doxycycline, the drug of choice for therapy for HGE [21], was treated after defervescence. None of the patients died, and no long-term consequences were found during a follow-up of 1 year.

In our study, acute infection with the HGE agent in three patients was confirmed by PCR analysis early in the course of illness when IgG antibody could not be detected. Other researchers who used the same primers, detected ehrlichial DNA in 43% of acute-phase blood samples [19]. Serology for IgG was useful for the retrospective confirmation of infection. Patient 1 could have been coinfecting with *B. burgdorferi*, although no clinical signs compatible with early Lyme borreliosis were present. Cross-reactive antibodies to *B. burgdorferi* could have resulted from infection with the HGE agent as described previously [22]. The findings of a standing titer of antibody to *E. chaffeensis* in one patient and seroconversion to *E. chaffeensis* in another patient are of potential interest, although dual reactivity to *E. chaffeensis* antigens has been shown in 8% to 17% of HGE cases in the United States [19, 20]. The possibility of additional *Ehrlichia* species causing human illness in Europe has not been ruled out.

PCR assays targeting the 16S rRNA gene have been useful in detecting ehrlichial infections in humans. The *E. phagocytophila* genogroup, as defined by the similarities among sequences of the 16S rRNA gene [1], contains several closely related and possibly conspecific taxa. In previous reports, only three base positions within the 1,433-bp amplified region of the 16S rRNA gene allowed differentiation of these previously identified species. Thus, we chose to amplify and sequence portions of the *groESL* heat shock operon to provide additional resolution. A 395-bp region of the *groEL* gene from members of the *E. phagocytophila* genogroup has been shown to contain a distinct pattern of bases in several positions that has consistently (to date) separated the sequences into Old World and New World groupings [16]. In this study, nucleotide sequences for this variable region from DNA from the HGE agents identified in three of our patients were identical to each other and

were similar to other sequences clustering in the Old World group.

This initial study should prompt clinicians and practitioners to be aware of the potential for ehrlichial infection in febrile patients in Europe. In most of Europe, the main differential diagnosis of HGE would be a viral infection, particularly the initial phase of tick-borne encephalitis (which has a similar clinical presentation and is usually accompanied by leukopenia, thrombocytopenia, and, in some patients, elevated liver enzyme levels [23]).

Although the clinical and laboratory findings in this small series of patients may not represent the complete spectrum of disease, our findings indisputably demonstrate the presence of HGE in Slovenia. HGE should be now included in the differential diagnosis of febrile illnesses in European patients presenting during the months when tick bites occur.

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