Human granulocytic ehrlichiosis (HGE) is a recently described rickettsiosis in the United States transmitted by *Ixodes* species ticks. In Europe, only a few studies on HGE exist. Two hundred Bulgarian patients with tick bites and 70 healthy blood donors were tested for HGE using an immunofluorescence assay with the HGE agent as an antigen. Elevated antibody titers (≥ 1:80) were found in 14 (9.7%) of 145 patients with erythema migrans, two (8%) of 25 tick-exposed patients with lymphadenopathy only, one (20%) of five patients with tick bite with fever, chills, and headache, one (4%) of 25 healthy tick-exposed patients, and two (2.9%) of 70 blood donors. These results show for the first time that HGE is probably common in southeastern Europe. The study provides evidence of coinfection or concurrent infection of patients with Lyme disease and HGE, thus supporting the possible role of *I. ricinus* for transmitting the HGE agent.

### Abstract.

Human granulocytic ehrlichiosis (HGE) is a recently described rickettsiosis in the United States transmitted by *Ixodes* species ticks. In Europe, only a few studies on HGE exist. Two hundred Bulgarian patients with tick bites and 70 healthy blood donors were tested for HGE using an immunofluorescence assay with the HGE agent as an antigen. Elevated antibody titers (≥ 1:80) were found in 14 (9.7%) of 145 patients with erythema migrans, two (8%) of 25 tick-exposed patients with lymphadenopathy only, one (20%) of five patients with tick bite with fever, chills, and headache, one (4%) of 25 healthy tick-exposed patients, and two (2.9%) of 70 blood donors. These results show for the first time that HGE is probably common in southeastern Europe. The study provides evidence of coinfection or concurrent infection of patients with Lyme disease and HGE, thus supporting the possible role of *I. ricinus* for transmitting the HGE agent.

### Materials and Methods

**Subjects.** Patients with tick bites and healthy blood donors were tested for the presence of antibodies against the HGE agent. All sera were obtained from the patients between two and three weeks after documented tick bites that occurred during the spring and summer of 1997. The subjects were divided into five groups. Group 1 included patients with clinically defined erythema migrans, the characteristic sign of early Lyme borreliosis. Group 2 represented patients who recalled previous tick bite and had only enlarged lymph nodes but no erythema migrans. Group 3 consisted of patients with fever, chills and headache who recalled a tick bite and had no clinical sign of Lyme disease. Group 4 included persons who had a tick bite but no clinical sign(s). Group 5 consisted of healthy Bulgarian blood donors serving as controls. These studies were approved by the National Center of Infectious and Parasitic Diseases of Bulgaria. Informed consent was obtained from each patient prior to obtaining clinical data and samples for serologic tests.

**Human granulocytic ehrlichiosis agent IFA.** Serum samples were tested by a modification of an indirect immunofluorescence technique. Antigen was prepared from HL60 cells infected with the HGE agent (Webster strain). Serum samples were diluted 1:80 in 0.1 M phosphate-buffered saline (PBS), pH 7.4, 0.5% nonfat dry milk. Diluted serum samples were added to each well on Teflon-coated slides. After a 1-hr incubation at room temperature in a moist chamber, the slides were washed three times in PBS. The secondary fluorescein isothiocyanate–conjugated goat anti-human IgG or IgM antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to the wells at a 1:50 dilution. Slides were again incubated for 1 hr in a moist chamber at room temperature, washed twice in PBS, and counterstained with 0.025% Evans blue for 5 min. The slides were then rinsed with distilled water and air-dried. All sera that reacted at the screening dilution of 1:80 were titrated to their endpoint. Previously identified positive and negative control sera were tested with each run.

**Borrelia burgdorferi ELISA.** Serum samples reactive with the HGE agent at the screening dilution were tested further for antibodies against *B. burgdorferi sensu lato*. A flagellar ELISA kit was used according to instructions of the manufacturer (Dako, Dakopatts, Denmark).
**RESULTS**

**Patient group 1.** Antibodies reactive with the HGE agent were found in 14 (9.7%) of 145 serum samples from patients with clinically defined erythema migrans (\(P = 0.06\); Table 1). Three (2.0%) had IgM anti-HGE agent antibodies, 10 (6.9%) had IgG antibodies and 1 (0.7%) had both IgM and IgG antibodies. Titers of IgM antibodies ranged from 1:80 to 1:320 (one patient) to 1:160 (three patients) and for IgG antibodies from 1:80 to 1:320 (one patient with 1:80, two with 1:160, and seven with 1:320). Twelve (85.7%) of 14 seropositive for the HGE-agent were also positive for anti-*B. burgdorferi* IgM. Three of 12 patients had both IgM and IgG antibodies reactive with *B. burgdorferi*. (Table 2).

**Patient group 2.** Antibodies against the HGE-agent were found in two (8.0%) of 25 patients with tick bites whose only clinical sign was enlarged regional lymph nodes (\(P = 0.05\)). Both seropositive patients had IgG antibodies (titer = 1:640). Serologic testing for Lyme borreliosis revealed a weak positive reaction for IgM anti-*B. burgdorferi* antibodies in one of the two samples.

**TABLE 1**

<table>
<thead>
<tr>
<th>Serum sample group</th>
<th>No. of positive serum samples</th>
<th>% of positive serum samples</th>
<th>No. of anti-HGE agent-positive serum samples</th>
<th>% of anti-HGE agent-positive serum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema migrans (group 1)</td>
<td>145</td>
<td>14</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Lymphadenopathy (group 2)</td>
<td>25</td>
<td>2</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Fever, chills, headache (group 3)</td>
<td>5</td>
<td>1</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>No clinical signs (group 4)</td>
<td>25</td>
<td>1</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects (group 5)</td>
<td>70</td>
<td>2</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>270</td>
<td>20</td>
<td>7.4</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis.** Data were analyzed using the chi-square test to determine statistical differences between the patient groups and the control group.

**DISCUSSION**

Human granulocytic ehrlichiosis, first described in 1994,\(^1\)\(^,\)\(^2\) is an increasingly reported tick-borne infection. To date, the majority of infections have been identified in the United States. Recently, the etiologic agent of HGE was cultivated\(^22\) and applied as an antigen for an IFA. Thus, most of the studies concerning HGE seroprevalence have been performed using *E. equi* or *E. phagocytophila*, which are genetically and antigenically closely related to the HGE agent *ehrlichiae*.\(^21\) In Europe, only one serologic survey based on the HGE agent–IFA has been conducted\(^17\) and only one PCR-confirmed case has been documented.\(^18\) No data exist from eastern Europe. We used the HGE agent–based IFA and demonstrated serologic evidence for HGE in tick-exposed patients in Bulgaria.

 Serum samples from 200 patients with tick bites, separated into four study groups, were tested serologically for HGE. In 18 (9%) patients, specific antibodies to the HGE agent were found. Seventy healthy blood donors served as a control group and two (2.9%) of their serum samples were also

**TABLE 2**

Incidence of antibodies reactive with *Borrelia burgdorferi* in serum samples containing human granulocytic ehrlichiosis (HGE) agent antibodies

<table>
<thead>
<tr>
<th>Anti-HGE agent-positive serum samples</th>
<th>Immuno-</th>
<th>IgM only</th>
<th>No/IHA* tit</th>
<th>IgG only</th>
<th>No/IHA tit</th>
<th>IgM and IgG</th>
<th>No/IHA tit</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. reactive</td>
<td>3</td>
<td>1/1:80</td>
<td>2/1:160</td>
<td>10</td>
<td>1/1:80</td>
<td>2/1:160</td>
<td>7/1:320</td>
</tr>
<tr>
<td>Anti-<em>B. burgdorferi</em>-positive serum samples</td>
<td>IgM only</td>
<td>IgG only</td>
<td>IgM and IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. reactive</td>
<td>5</td>
<td>2/1:80</td>
<td>3/1:160</td>
<td>14</td>
<td>2/1:80</td>
<td>3/1:160</td>
<td>7/1:320</td>
</tr>
</tbody>
</table>

\* IFA = immunofluorescent antibody assay.
\* *B. burgdorferi* serology performed by flagellin ELISA.
positive. The results show that HGE might be common in Bulgaria. Previous investigations documented a high prevalence of the I. ricinus tick in Bulgaria, more than 90% of the entire tick population.19 Taken together, our data support the possible role of I. ricinus as a vector of HGE in Europe. These data also suggest that HGE should be considered in the differential diagnosis of febrile patients from Bulgaria who have tick bites.

Fourteen (9.7%) of the 145 patients with erythema migrans (patient group 1) were seropositive for HGE. Three of them had IgM antibodies reactive with both the HGE agent and B. burgdorferi, and nine had IgG anti-HGE agent antibodies and either IgM alone or IgM and IgG antibodies to B. burgdorferi. Our results are in agreement with data from other investigators, which showed an incidence of 5.2–17.1% of HGE in Lyme borreliosis patients from both Europe and North America.15–17,24–27 In Europe, the I. ricinus complex also seems to be the common vector for both infections. Coinfection of ticks and humans has been proved.6,12,13,27 It is very plausible that HGE seropositivity of the patients with Lyme borreliosis was due to coinfection and even to concurrent infection, especially in cases with established IgM antibodies to both pathogens.

In addition, we found serologic evidence for HGE in four patients from the other three groups of the patients with tick bites. One of the patients presented with fever and headache, symptoms very suspicious for HGE; two other patients presented only with enlarged lymph nodes, and one other had no clinical signs. Two of these four patients also had antibodies reactive with B. burgdorferi. Since clinical signs of Lyme disease were not clear, we suspected either that the serologic results occurred after coinfection or from cross-reactivity in these cases. False-positive serology for Lyme borreliosis has been previously suspected in patients with HGE.28 Immunoblots have implicated reactions to heat-shock proteins as a potential cause of false-positive Lyme disease serology.29 However, to what extent positive serologic results for HGE and Lyme borreliosis in such cases are due to coinfection or to cross-reactivity remains unknown. In fact, the extent to which clinical manifestations accompany HGE agent or B. burgdorferi infection is also unknown.

In conclusion, we demonstrate that 9% of Bulgarian patients with tick bites had serologic evidence for HGE. Taking into account the established high prevalence of Lyme borreliosis in Bulgaria, the results confirm the hypothesis that HGE is also endemic in areas endemic for Lyme borreliosis. Seropositivity for HGE, especially in patients with Lyme disease, suggests that coinfection with both the HGE agent and B. burgdorferi is not uncommon. Our report further elucidates the geographic distribution of HGE in Europe.

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Authors’ addresses: Iva S. Christova, National Center of Infectious and Parasitic Diseases, 26 Yanko Sakazov Bul., Sofia 1504, Bulgaria. J. Stephen Dumler, Division of Medical Microbiology, Department of Pathology, The Johns Hopkins Medical Institutions, Meyer B1-193, 600 North Wolfe Street, Baltimore, MD 21287.

Reprint requests: J. Stephen Dumler, Division of Medical Microbiology, Department of Pathology, The Johns Hopkins Medical Institutions, Meyer B1-193, 600 North Wolfe Street, Baltimore, MD 21287.

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