Human Granulocytic Ehrlichiosis in Wisconsin and Minnesota: A Frequent Infection with the Potential for Persistence

J. Stephen Dumler and Johan S. Bakken

Department of Pathology, University of Maryland School of Medicine, Baltimore; Section of Infectious Diseases, Duluth Clinic, Duluth, Minnesota

Human granulocytic ehrlichiosis (HGE) is a tickborne illness caused by an agent closely related to *Ehrlichia equi* and *Ehrlichia phagocytophila*. The clinical presentation is nonspecific, and diagnosis is made infrequently. Sixty-six sera were obtained from 54 patients in Minnesota and Wisconsin with undifferentiated fever during the summer and fall of 1993. Serologic reactivity with *E. equi* was observed in 6 (11%), including 4 seroconversions, 1 stable titer, and 1 seroreversion. Of the seropositive patients, HGE agent DNA was detected by polymerase chain reaction in the first serum from 4 of 5 patients and was present in the serum of 1 of 2 untreated patients after 21 days, even when *E. equi* antibodies also were present. HGE is a significant and potentially frequent cause of undifferentiated fever in Wisconsin and Minnesota during seasons with tick activity. The agent may persist in untreated patients for at least 1 month or may be cleared earlier, even if not treated with doxycycline or tetracycline.

Human granulocytic ehrlichiosis (HGE) is a newly described, tickborne, zoonotic infection probably transmitted to humans by the bite of *Ixodes scapularis* ticks [1–4]. The causative agent is nearly identical with *Ehrlichia phagocytophila* and *Ehrlichia equi*, both veterinary granulocytic ehrlichiae, based on serologic reactions and 16S ribosomal RNA gene sequence analysis. The clinical disease caused by infection with the agent of HGE is generally nonspecific and is characterized by fever, headache, myalgias, and malaise, with other systemic findings referable to the gastrointestinal and central nervous system occurring in <50% of patients [2]. Laboratory tests are helpful, since leukopenia, anemia, thrombocytopenia, or elevations in serum hepatic transaminase activities occur in >50% of patients. However, the clinical and laboratory findings are nonspecific and thus the infection is often unrecognized.

The original cases of HGE were described in the regions of Minnesota and Wisconsin that surround Duluth. Thus, we tested serum samples submitted by local physicians from patients within this region who had undiagnosed fevers that occurred during the summer and fall of 1993 for evidence of serologic reactions suggestive of infection with the agent of HGE.

Materials and Methods

Patients and sera. Acute and convalescent sera were collected from patients from Minnesota and Wisconsin in the region surrounding Duluth who presented with an acute febrile illness. Serum samples and peripheral blood smears were submitted by private physicians to the Duluth Clinic for evaluation of serologic responses to the HGE agent. Clinical information usually included the date the sample was obtained and the approximate date of onset of illness. For patients with *E. equi* antibodies detected, follow-up convalescent sera were requested to demonstrate seroconversion. All sera were stored frozen (−70°C) until tested. Wright-stained peripheral blood smears were reviewed for the presence of typical morulae.

*E. equi* indirect fluorescent antibody (IFA) test. The serologic test for antibodies reactive with the agent of HGE used *E. equi* antigen and was done as described [2, 5]. All sera were initially screened at a dilution of 1:80, and sera reactive at that dilution were then serially titrated.

Polymerase chain reaction (PCR) for HGE DNA. At the time of the study, the agent of HGE was not yet cultivable. Thus, PCR was done for patients whose sera contained antibodies reactive with *E. equi* to attempt confirmation of whether these patients were recently or persistently infected. The PCR procedure was modified from those of Chen et al. [1] and Pancioli et al. [4] by extraction of nucleic acids from 0.5 mL of serum, followed by purification with phenol-chloroform, precipitation with 3 M sodium acetate and ethanol, and suspension in water. For maximum sensitivity in serum, the PCR method used two different primer pairs, GE9f/GE10r and Ehr545/ Ehr747, in separate reactions, essentially as described. Two consecutive rounds of amplification were used to detect DNA amplified with GE9f/GE10r. Amplified DNA was detected by electrophoresis through 1.0% agarose gels and staining with ethidium bromide. The approximate molecular size of the amplified DNA was estimated by comparison with a known DNA...
when HGE agent DNA was present in the serum, and 2 patients (table 1). In 4 of these patients, HGE agent DNA was detected in the serum obtained on days 2, 3, 18, and 30 after onset of illness, respectively. Two patients had no antibody detectable at the time serum was available and was tested by PCR for HGE agent DNA. Of these 6 patients, 1 had a late convalescent serum (531 days) submitted, who had an early convalescent-phase serum available for testing, and both patients demonstrated seroconversion. Of the remaining 4 patients with serologic reactions to E. equi, each had early (18–33 days after onset of illness) and late convalescent sera available. Two of these patients had seroconversions, 1 had a stable E. equi titer, and 1, who had a late convalescent serum (531 days) submitted, had a seroreversion.

In 5 of the 6 patients with E. equi serologic reactions, sufficient volume of the initial serum sample submitted for each was available and was tested by PCR for HGE agent DNA (table 1). In 4 of these patients, HGE agent DNA was detected in the serum obtained on days 2, 3, 18, and 30 after onset of illness, respectively. Two patients had no antibody detectable when HGE agent DNA was present in the serum, and 2 patients had significant titers of E. equi antibodies (160 and 320) concurrent with the presence of HGE agent DNA in the serum. Doxycycline therapy was given for only 2 of these 6 patients and was continued for a total of 14 days in both. Of the 4 patients who were not treated, 3 had HGE agent DNA present in serum at the time therapy was initiated. HGE agent DNA was not detected in the serum of only 1 of the patients who were tested by PCR, and this patient was not treated. This patient’s sample was obtained 21 days after the onset of illness and had an E. equi titer of 640. Only 1 patient, who was seronegative in both acute and convalescent sera, had acute-phase serum tested by PCR for HGE agent DNA, which was not detected (data not shown). No other acute-phase sera were tested by PCR.

### Discussion

HGE is increasingly recognized as a zoonotic, tickborne infection with a potential for fatal outcome that occurs in the upper midwest and northeastern United States. Because the agent of HGE is difficult to cultivate and rarely isolated in vitro and the clinical findings associated with infection are nonspecific, the exact prevalence and incidence of infection are unknown. As has probably been true for HGE for many years, undifferentiated fevers that occur in association with tick bites or tick exposures often do not receive a specific or confirmed etiologic diagnosis. For example, serologic studies of patients with a Rocky Mountain spotted fever–like illness in Oklahoma in 1987 revealed an equal incidence of Rocky Mountain spotted fever and mononuclear ehrlichiosis caused by Ehrlichia chaffeensis [6]. In spite of the identification of new etiologic agents as causes of some of the nonspecific febrile illnesses associated with tick exposure, a substantial proportion of patients for whom sera are submitted for rickettsial or ehrlichial serology receive no definite laboratory-confirmed diagnosis. Depending on the clinical presentation and severity, these patients would probably be either treated empirically with ineffective antimicrobial agents or not treated. Given the high prevalence of tickborne infections in these regions, a rational therapeutic choice under the appropriate epidemiologic and clinical circumstances would include the use of doxycycline for treatment of both acute Lyme borreliosis and HGE.

The differential diagnosis of tick-associated undifferentiated fever in the upper Midwest is wide, and during periods with tick activity, Lyme disease is often considered. The current study focuses on a group of patients with fever and no specific diagnosis in a region where and season when tick bites and tickborne infections occur frequently. The samples tested must be considered to be a heterogeneous group of acute- and convalescent-phase sera, and therefore, in this selected population of febrile patients, a conservative approximation of incidence shows that 11% of patients tested had serologic reactions diag-

### Table 1. Day of illness, E. equi indirect fluorescent antibody (IFA) titers, and HGE polymerase chain reaction (PCR) results for 6 patients with HGE and undifferentiated fevers.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Day after onset of illness</th>
<th>E. equi IFA titer</th>
<th>HGE PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>3</td>
<td>&lt;80</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>201</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>18</td>
<td>160</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>418</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>26*</td>
<td>2</td>
<td>&lt;80</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>155</td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>12*</td>
<td>33</td>
<td>≥1280</td>
<td>ND²</td>
</tr>
<tr>
<td></td>
<td>508</td>
<td>&lt;80</td>
<td></td>
</tr>
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<td>4</td>
<td>30</td>
<td>320</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>243</td>
<td>5120</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>640</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>5120</td>
<td></td>
</tr>
</tbody>
</table>

* For HGE agent 16S rRNA gene DNA.

† Treated with doxycycline on day that acute-phase serum was obtained.

² Not done; serum volume insufficient for PCR analysis.

size standard. All PCR reagents and reaction mixtures were prepared in a special "pre-PCR" containment hood, and amplifications and electrophoresis were done in separate laboratories to minimize the possibility of contamination.

### Results

In total, 66 sera were obtained from 54 patients. These included single samples from 43 patients with acute illness, 2 single samples from patients convalescent from their illness, 1 sample for which the date of onset of illness was not provided, and paired sera obtained from 10 patients; 2 of these paired sera had early (18 and 30 days) and late convalescent-phase samples available. Peripheral blood smears were available for review from all but 2 patients; no morulae were observed on any smear.

Of the sera obtained from 54 patients, reactions with E. equi by IFA were detected in 6 (11%). Of these 6 patients (table 1), 2 had both acute (<14 days after onset of illness) and convalescent sera available for testing, and both patients demonstrated seroconversion. Of the remaining 4 patients with serologic reactions to E. equi, each had early (18–33 days after onset of illness) and late convalescent sera available. Two of these patients had seroconversions, 1 had a stable E. equi titer, and 1, who had a late convalescent serum (531 days) submitted, had a seroreversion.

In 5 of the 6 patients with E. equi serologic reactions, sufficient volume of the initial serum sample submitted for each was available and was tested by PCR for HGE agent DNA (table 1). In 4 of these patients, HGE agent DNA was detected in the serum obtained on days 2, 3, 18, and 30 after onset of illness, respectively. Two patients had no antibody detectable when HGE agent DNA was present in the serum, and 2 patients...
nomic or highly suggestive of HGE. Since the majority of these patients had only acute-phase serum tested, the incidence of HGE in this area is likely to be very high if extrapolated to the entire population. This situation is also underscored by the likelihood that the agent of HGE is also transmitted by the same tick species responsible for transmission of *Borrelia burgdorferi*, and as such, a substantial number of patients in this region may be at risk for HGE [2, 4, 7].

*Ehrlichio*ses in humans are recent discoveries and are incompletely investigated [7, 8]. In animals, *ehrlichi*ae often cause persistent infection, accompanied by chrlhemia [9–11]. This strategy enables uninfected ticks to acquire the infectious agent from a single infected host over a very long interval and is probably an important evolutionary adaptation for the *ehrlichi*ae. The fact that some mammals can maintain a persistent infection suggests that *ehrlichi*ae may be competent at persistent infection of humans as well. The demonstration that HGE agent DNA was still present in the serum of patients as late as 21 days after the onset of illness seems to confirm this possibility. In fact, HGE agent DNA may be detected in the serum of patients infected long enough to have developed significant immunity and antibody titers, a phenomenon well recognized to occur with other rickettsial infections and infections by intracellular pathogens [12, 13].

Persistent infection of humans by *ehrlichi*ae has been long suspected. However, to date, persistence of an *Ehrlichia* species in a human patient is documented in only 2 cases [14, 15]. The finding that *ehrlichi*ae persisted in the tissues of 1 patient despite therapy with both doxycycline and chloramphenicol seems to confirm similar observations in dogs with *E. canis* infection [12]. It is interesting that 1 untreated patient studied here who had a significant antibody titer did not have HGE agent DNA present in serum at 21 days after infection. This observation implies that in humans, elimination of the infectious agent may be achieved in association with production of serum antibody and may potentially be the result of effective immune stimulation. This situation is probably the natural and usual outcome of infection in humans, but no studies have been published that establish the absence of the agent or transmissibility from previously infected human patients. The persistence of serum antibody for >36 months in treated patients [16] suggests that prolonged low-level antigenic stimulation may continue to occur after apparently effective therapy and recovery. The mechanisms governing the transition from host tolerance with *ehrlichial* persistence to *ehrlichial* elimination are unknown.

The results of this study indicate that HGE is a relatively frequent cause of undifferentiated fever in the US upper Midwest and that this infection may occur undetected by routine clinical evaluation including peripheral blood smear examination. Although a specific rate of incidence cannot be calculated on the basis of these data, the accrued data obtained from patients infected in counties in northwestern Wisconsin indicate that the annual incidence may exceed 50 cases per 100,000 population [16]. The very frequent occurrence in this study is probably partly biased by collection of samples from patients at a time of peak tick activity; however, HGE in the upper Midwest has been detected in every month of the year except February and September [16]. The high rate of infection in this geographic region ensures that a substantial risk exists for immunocompetent patients as well as for patients with underlying defects in immunity or host defenses that may lead to severe and potentially fatal infections [2, 15, 17].

Accordingly, serology is the most sensitive confirmatory test but is usually not diagnostically useful during the active phase of illness [16], and the presence of serum antibodies is not an indicator of *ehrlichial* clearance. However, we tested all sera submitted to obtain a baseline E. *equi* titer. The use of a specific PCR assay for HGE agent DNA holds promise as a rapid diagnostic tool at a time when specific treatment may be beneficial; however, the test is currently available only as a tool for research. While PCR on serum appears promising in this limited study, retrospective analysis of acute-phase serum from other patients with known HGE has revealed limited sensitivity (unpublished data). Acute-phase whole blood is likely to be a more optimal substrate for HGE PCR.

The persistence of HGE agent DNA for 1 month in the absence of specific therapy may prove to be significant. Importantly, the ability of the host to resolve the infection in the absence of specific antimicrobial therapy is documented. The molecular and cellular mechanisms that allow recovery or persistence need further investigation to aid with effective management and treatment of patients with HGE.

**Acknowledgments**

We acknowledge the technical expertise of Kristin Asanovich and Ellen Trigiani in serologic and molecular assays and of Jenny Krueh and Cindy Wilson-Nordskog in evaluation of peripheral blood smears.

**References**

Late Acquisition of Hyporesponsiveness to Lipopolysaccharide by \textit{Mycobacterium avium}—Infected Human Macrophages in Producing Tumor Necrosis Factor-\(\alpha\) but Not Interleukin-1\(\beta\) and -6

Lanfranco Fattorini, Yan Xiao, Clara M. Ausiello, Francesca Urbani, Andrea laSala, Maurizio Mattei, and Graziella Orefici

To investigate whether infection with \textit{Mycobacterium avium} modifies the cytokine response of human macrophages (M\(\phi\)) to lipopolysaccharide (LPS), the release of interleukin (IL)-1\(\beta\), IL-6, and tumor necrosis factor (TNF)-\(\alpha\) was determined in infected and uninfected M\(\phi\), unstimulated or stimulated with LPS. In unstimulated M\(\phi\), the release of IL-1\(\beta\) and IL-6 increased with the progress of infection while that of TNF-\(\alpha\) progressively decreased. When M\(\phi\) were stimulated with LPS, IL-1\(\beta\) and IL-6 levels were always higher in infected than in uninfected cells, but levels of TNF-\(\alpha\) significantly decreased in infected M\(\phi\). A similar trend was obtained for TNF-\(\alpha\) mRNA expression. Altogether, these results indicate that infected M\(\phi\) react to LPS stimulus with enhanced levels of IL-1\(\beta\) and IL-6 but are unable to restore the production of TNF-\(\alpha\) impaired by the growth of the intracellular mycobacteria.

Organisms belonging to the \textit{Mycobacterium avium} complex (MAC) are relatively avirulent for healthy subjects, although they can cause chronic pulmonary infections in patients with underlying disease [1]. In contrast, MAC is the most common cause of disseminated bacterial infections in patients with AIDS, who show limited inflammatory responses and poorly defined granulomas [1, 2].

MAC isolates from blood of AIDS patients produce almost exclusively smooth transparent (SmT) colonial variants [3], while isolates from non-AIDS patients yield a mixture of SmT, smooth domed—opaque (SmD), and rough colonies [4]. SmT isolates are more resistant to drugs and chemicals and better able to replicate within human macrophages (M\(\phi\)) than are SmD variants [1]; in addition, SmD colonies elicit lower levels of cytokines in human monocytes and M\(\phi\) than do SmD variants [5–7].

The ability of SmT organisms to efficiently grow inside murine M\(\phi\) is associated with a delay in M\(\phi\) secretion of tumor necrosis factor (TNF)-\(\alpha\) [8], a cytokine important for macrophagic aggregation [9] and development of bactericidal granulomas in mycobacterial infections [10]. In human M\(\phi\), the TNF-\(\alpha\) response to MAC is brief [11, 12], but it is not completely understood whether the impairment in TNF-\(\alpha\) production is due to a general blockade of M\(\phi\) or whether they can still be efficiently stimulated by nonmycobacterial stimuli.

In this study, we determined the expression of interleukin (IL)-1\(\beta\), IL-6, and TNF-\(\alpha\) in a model of heavily SmT-infected human M\(\phi\). In addition, we verified whether MAC infection impairs the M\(\phi\) response to a second, different stimulus (lipo-