Ultrastructural and Antigenic Characterization of a Granulocytic Ehrlichiosis Agent Directly Isolated and Stably Cultivated from a Patient in New York State

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A human granulocytic ehrlichiosis (HGE) agent with 16S rDNA sequence identical to the published sequence of HGE agents was isolated from a patient from New York State by inoculation of the blood leukocyte fraction directly into a human promyelocytic leukemia cell line HL-60. The HGE agent was also isolated from the leukocyte fraction of the blood and bone marrow of a mouse inoculated with the leukocyte fraction of the patient's blood. The isolate has been passaged in tissue culture 30 times over 8 months. Electron microscopy revealed pleomorphic coccobacilli with a thin and highly rippled outer membrane in the clear inclusion matrix. Comparison of IFA reactivity of antisera obtained from a variety of sources with the cell-cultured HGE agent revealed that 3 HGE agent strains (New York isolate, Wisconsin [BDS] isolate, and a tick-derived isolate) are highly cross-reactive and there are diverse antigenic cross-reactivities between HGE agent and *Ehrlichia chaffeensis*.

Two tickborne diseases caused by *Ehrlichia* species have been recognized in humans in the United States: human monocytic ehrlichiosis, caused by *Ehrlichia chaffeensis*, and human granulocytic ehrlichiosis (HGE), caused by an agent closely related to *Ehrlichia equi* or *Ehrlichia phagocytophila* [1]. HGE was first described in 1994 among patients in Minnesota and Wisconsin [1, 2]. In addition to these cases, acquisition of HGE may have occurred in California, Florida, Maryland, Massachusetts, Connecticut, and New York [1–4]. As of 15 August 1995, 29 patients from New York State were considered to have either confirmed (23 patients) or probable (6 patients) cases of HGE, 20 from 1995 and 9 from 1994 [3].

An HGE agent has been isolated from 3 patients from Minnesota and Wisconsin, and 2 of these isolates have been subcultured [5]. Here we describe the direct and indirect isolation and cultivation in a continuous cell line of an HGE agent from a patient residing in New York State. A serologic comparison with HGE agent, Wisconsin isolate, and *E. chaffeensis* was made using the cell-cultured HGE agent, and the isolate was characterized by ultrastructural analyses.

Materials and Methods

Isolation of HGE agent from leukocyte fraction from the blood. A blood specimen was collected in an EDTA tube from a 74-year-old man from Westchester County, New York, with typical clinical signs and laboratory findings of HGE [2, 3]. The 3-mL blood sample was overlaid on Histopaque 1119 (Sigma, St. Louis) and centrifuged at 1500 g for 15 min. The interphase fraction was collected and washed with RPMI 1640 medium (Life Technologies GIBCO BRL, Gaithersburg, MD). One-third of the leukocyte fraction was resuspended in 0.5 mL of RPMI 1640 containing 20% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM Na pyruvate, and 0.1 mM nonessential amino acid mixture and cocultured with 10^6 HL-60 cells (American Type Culture Collection, Rockville, MD) in a well of a 12-well plate at 37°C in 5% CO2 and 95% air. Infectivity of the cells was determined every 2–4 days by Diff-Quik (Baxter Scientific Products, Oceanside, OH) staining and light microscopic examination at ×1000 magnification.

Subculturing of HGE agent in HL-60 cells. Both infected and uninfected HL-60 cells were examined daily under a phase contrast microscope for confluence, general health, and absence of contamination. HL-60 cells were split every 3–5 days to prevent confluence. When 90% of HL-60 cells were infected with the HGE agent, 1 mL of infected cells (~10^9 cells) was inoculated in 5 mL of 5 × 10^6 HL-60 cells in 25-cm² flasks. Viability and infectivity of the cells were examined every 2 days by Diff-Quik staining of an aliquot of cells. The infected cells were frozen in RPMI medium containing 30% FBS and 10% dimethyl sulfoxide at −80°C or continuously passaged at a 1:5 infected-to-uninfected cell ratio.

Mouse inoculation. The leukocyte fraction obtained from ~1 mL of patient blood was suspended in 0.1 mL of RPMI without serum and intraperitoneally inoculated into a female 5-week-old BALB/c mouse (Harlan Sprague-Dawley, Indianapolis). Using the protocol of Tachibana et al. [6], the mouse was inoculated intraperitoneally with 0.16 mg of cyclophosphamide/g of body weight at 0, 4, and 8 days after inoculation of the patient’s cells. The mouse...
was observed daily for clinical signs of infection (ruffled fur, inactivity, weight loss, squinty eyes, and anorexia). At day 10 after inoculation, the mouse was anesthetized with ether, and blood was collected by heart puncture into a heparinized tube. Bone marrow cells were collected from the femur. The bone marrow cells and blood leukocytes were cocultured with HL-60 cells in wells of a 12-well plate.

IFA. IFA antigen slides of the cell-cultured HGE agent and E. chaffeensis were prepared, and the IFA was done as described [7]. Briefly, HL-60 cells infected with the HGE agent (90% infected cells) were suspended in RPMI medium and placed on 12-well slides at 10^4 cells/well. The slides were air-dried and acetone-fixed. Eight-well antigen slides of horse leukocytes infected with HGE agent (BDS strain, Wisconsin isolate) [8] were provided by J. Madigan (University of California, Davis). Serial 2-fold dilutions of test sera in PBS (pH 7.4), starting at a 1:20 dilution, were made in 96-well microtiter plates in a 100-μL volume, and a 10-μL volume of each dilution was applied to a well of antigen-coated IFA slides. The sera examined were the acute and convalescent sera from the HGE patient from whom the HGE agent was isolated, human anti–E. chaffeensis serum (provided by J. R. Harkess and J. Kudlik, Oklahoma State Department of Health, Oklahoma City; the serum was previously characterized by Western immunoblot analysis using purified E. chaffeensis and Ehrlichia canis antigen [9]), dog anti–E. chaffeensis serum, horse anti–E. equi, MRK strain, and horse anti-HGE, BDS strain sera (provided by J. Madigan), dog anti–granulocytic ehrlichia (tick isolate [10]) serum (provided by R. Coughlin, Cambridge Bio-Tech, Worcester, MA), dog anti–Ehrlichia ewingii serum (provided by S. Ewing, Oklahoma State University, Stillwater), rabbit anti–Ehrlichia sennetsu serum, and mouse anti–Ehrlichia muris serum, at 37°C for 1 h. The cells were then incubated with FITC-conjugated goat anti-human, anti-horse, anti-dog, anti-rabbit, or anti-mouse IgG or anti-human IgM (Organon Teknika, West Chester, PA) at a dilution of 1:100. The slides were incubated, washed, and counterstained with Evans blue (Sigma), and a small drop of mounting fluid (70% glycerol in PBS) was placed in each well. Coverslips were added, and the slides were examined with an epifluorescent microscope.

Electron microscopy. Electron microscopy of HGE agent–infected HL-60 cells was done as described [7].

Sequencing of the 16S DNA of the HGE agent. The DNA was extracted from HGE agent–infected HL-60 cells with a tissue kit (QIAamp; Qiagen, Chatsworth, CA). The total 16S rRNA gene was amplified using primers A-17 and 3-17 as described [11]. The amplified DNA fragment was purified by DNA purification system (Promega, Madison, WI) and used as the template for sequencing with a double-stranded DNA cycle sequencing system [11]. The sequencing primers used were primers A-17, 3-17, ER323-341, ER757-775, ER1043-1062, and ER864-846 as described [11] and HGE 2 (5'–TG TAG GT ACC GT CATT AT CT CCT AC-3').

Results

Isolation and cultivation of the HGE agent. An HGE agent was isolated from the patient’s blood in HL-60 cells. Ehrlichial organisms appeared as small cocci and formed multiple distinct clusters that were visible after 1 week of culture. Both the number of organisms and the number of infected cells rapidly increased. By the end of 2 weeks of culture, almost 100% of HL-60 cells were heavily infected (figure 1, inset). Sizes and numbers of morulae were highly variable from cell to cell. The HGE agent was continuously subcultured in HL-60 cells up to 30 times over 7 months.

Electron microscopy of the isolated HGE agent. Electron microscopy revealed pleomorphic coccobacilli loosely packed in several small membrane-lined vacuoles in the cytoplasm of HL-60 cells. Occasionally extracellular ehrlichiae attached to the cell surface were also seen. HGE agents were variable in their cytoplasmic density, size, and shape. Two layers of trilaminar membranes, clumps of ribosomes, and fine DNA strands were clearly visible. The highly rippled thin outer membrane of two leaflets of almost equal thickness was characteristic of ehrlichial organisms (figure 1). Various kinds of small membranous structures were present in ehrlichial inclusions, and the matrix of the inclusion was clear.

Isolation of the HGE agent following mouse inoculation. A mouse inoculated with blood leukocytes of the human patient did not develop any clinical signs during the 10-day infection period. However, after the blood leukocytes or bone marrow cells were cocultured with HL-60 cells for 4 days, the HGE agent was isolated.

Serologic tests and sequence of 16S rDNA. Acute and convalescent (2 weeks apart) sera of the patient had IgG IFA titers of 1:640 and 1:1280, respectively, to the homologous strain of HGE cultured in HL-60 cells. For both sera, IgM IFA titers were similar to IgG IFA titers. IgG IFA titer of the convalescent serum against HGE agent, BDS (Wisconsin) strain, in horse granulocytes was also 1:1280. IFA titers of anti–E. equi serum (1986) against the New York and BDS strains of HGE agent were 1:640 and 1:1280, respectively. Dog anti–granulocytic ehrlichia (tick-derived isolate) serum also reacted with the cell-cultured New York strain. Acute and convalescent sera of the patient had IgG IFA titers of 1:640 and 1:1280, respectively, to E. chaffeensis, but IgM IFA titers of both sera were negative. Horse anti–E. equi serum (1986) also reacted with E. chaffeensis (table 1). Conversely, human anti–E. chaffeensis serum reacted with 2 strains of HGE agent by IgG IFA. Dog anti–E. chaffeensis serum, however, did not react with the HGE agent. Both dog anti–granulocytic ehrlichia (tick-derived isolate) and horse anti–HGE agent (BDS strain) sera did not contain IgG against E. chaffeensis (table 1). Dog anti–E. ewingii serum, rabbit anti–E. sennetsu serum, and mouse anti–E. muris serum did not react with the HGE agent New York strain (table 1).

The 1410-bp sequence of 16S rDNA of the HGE agent did not differ from the sequence of the 16S rRNA gene amplified from the blood of a person with HGE in Wisconsin, including three polymorphic positions found between E. equi, or E. phagocytophilum [1].

Discussion

This report describes the direct isolation and establishment of a stable culture of an HGE agent from an elderly man.
in Westchester County, New York. Although some Ehrlichia risticii strains isolated in different geographic locations vary in 16S rDNA base sequences [11], the New York strain had the identical 16S rDNA sequence as that of Wisconsin and Minnesota strains [1, 2, 5]. We also report the isolation of the HGE agent from a cyclophosphamide-treated mouse after inoculation of the patient blood leukocyte fraction. An HGE agent from a Massachusetts patient's blood has been serially passaged in splenectomized and intact mice [12]. Thus, the mouse isolation may be useful when cell culture isolation is not immediately feasible.

Although the ultrastructure of an HGE agent was seen in the spleen of a patient [1] and in an equine peripheral blood neutrophil [13], a cell-cultured HGE agent has not been exam-

### Table 1. Reaction of patient's and other sera with HGE and Ehrlichia chaffeensis.

<table>
<thead>
<tr>
<th>Sera</th>
<th>HGE* in HL-60</th>
<th>E. chaffeensis in DI82</th>
<th>HGE* in horse leukocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Patient acute serum</td>
<td>1:640</td>
<td>1:320</td>
<td>1:640</td>
</tr>
<tr>
<td>Patient convalescent serum</td>
<td>1:1280</td>
<td>1:1280</td>
<td>1:1280</td>
</tr>
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<td>Dog anti-granulocytic ehrlichia</td>
<td>1:160</td>
<td>ND</td>
<td>&lt;1:20</td>
</tr>
<tr>
<td>Horse anti-HGE*</td>
<td>1:2560</td>
<td>ND</td>
<td>&lt;1:20</td>
</tr>
<tr>
<td>Human anti-E. chaffeensis</td>
<td>1:80</td>
<td>ND</td>
<td>1:5120</td>
</tr>
<tr>
<td>Dog anti-E. chaffeensis</td>
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<td>ND</td>
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</tr>
<tr>
<td>Horse anti-Ehrlichia equi (1995)</td>
<td>1:320</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Horse anti-E. equi (1986)</td>
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<td>ND</td>
<td>1:320</td>
</tr>
<tr>
<td>Dog anti-Ehrlichia ewingii</td>
<td>&lt;20</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rabbit anti-Ehrlichia sennetsu</td>
<td>&lt;20</td>
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<tr>
<td>Mouse anti-Ehrlichia muris</td>
<td>&lt;20</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

**NOTE.** ND, not determined.
* New York isolate.
† BDS strain [8].
ined in this way before. This study suggests that the ultrastructure of the HGE agent differs from that of other human ehrlichiosis agents. Loosely packed inclusions with a small number of ehrlichial organisms is characteristic of the HGE agent, which is similar to that of E. equi and E. phagocytophila inclusions [14]. E. sen netsu is individually and tightly surrounded by the host membrane [15]. Inclusions of E. chaffeensis and E. canis are densely packed with larger numbers of organisms [14, 15]. In contrast to E. canis or E. chaffeensis inclusions [14, 15], densely filamentous ground substance was absent in the matrix of HGE agent inclusions.

IFA testing with a cell-cultured HGE agent as antigen has not been reported before. In this study, using the cell-cultured HGE agent, comparative IFAs were done. The cell-cultured New York strain antigen and the Wisconsin (BDS) strain in horse granulocytes as antigen generated similar titers using 3 different antisera. The IFA using HGE patient sera, horse anti-HGE agent serum (BDS strain), and dog anti-E. chaffeensis serum (BDS strain), and several anti-HGE agent sera, revealed various levels of serologic cross-reactivity between E. chaffeensis and the HGE agent. Based on the serologic studies, either the immune system of the New York HGE patient and the E. equi–infected horse strongly recognized antigens common between E. chaffeensis and HGE agent, or infection with both agents occurred in the human and the horse. The first hypothesis is more likely, since horse infection with E. chaffeensis is not known to occur. Western immunoblot analysis using purified HGE agent and E. chaffeensis antigens in progress to identify the common antigens between HGE agent and E. chaffeensis. The prevalence of the serologic cross-reactivity does not appear to be high among HGE patients. Five (17%) of the first 29 patients diagnosed with HGE in New York State [3] and 3 (8%) of 40 patients from Wisconsin and Minnesota [2] had a positive IFA titer (≥64) to E. chaffeensis. However, the 16S rRNA gene sequences of the HGE agent and E. chaffeensis are distinct, PCR based on divergent base sequences may be a more reliable method than serology to distinguish between them.

Either direct or indirect isolation of HGE agent would provide the definitive diagnosis of HGE. Establishment of a stable HGE culture should provide sufficient HGE antigen to be useful for serodiagnosis and permit opportunities for research at the molecular and cellular levels.

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References