
Primary Bone Marrow Progenitors of Both Granulocytic and Monocytic Lineages Are Susceptible to Infection with the Agent of Human Granulocytic Ehrlichiosis

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Human granulocytic ehrlichiosis (HGE) is an emerging tickborne infection resulting in an acute febrile illness associated with cytopenias and characteristic intracellular organisms within peripheral blood granulocytes. The etiologic agent of HGE has recently been isolated and cultivated in the HL-60 promyelocytic leukemia cell line, but the spectrum of host cells that it naturally infects remains unknown. To determine if normal hematopoietic progenitors could be targets of infection, CD34+ primary human bone marrow cells, stimulated to differentiate along myelomonocytic lineages, were incubated with the HGE agent. Immature marrow progenitors and, remarkably, not only granulocytic but also CD14+ monocytic cells from these cultures supported replication of the HGE agent, suggesting that all are potential targets of infection in vivo. Infection of bone marrow progenitors may contribute to the hematologic manifestations of HGE. Furthermore, the ability of the agent to interact with monocytes has significant implications regarding disease pathogenesis and host response.

Human granulocytic ehrlichiosis (HGE) is a tickborne infection first described in 1994 [1]. This disease has tentatively been given the name of HGE because colonies of intracellular bacteria (morulae) are often seen within peripheral blood granulocytes. This feature helps to clinically distinguish HGE from ehrlichiosis caused by Ehrlichia chaffeensis, in which infection of peripheral blood monocytes is observed [2]. HGE is caused by an obligate intracellular gram-negative bacterium, which is genetically closely related, if not identical, to the ehrlichiae that infect horses and ruminants, Ehrlichia equi and Ehrlichia phagocytophila, both of which also infect peripheral blood granulocytes [3–5].

The clinical manifestations of HGE include fever, myalgias, and headache. Infection may be severe, with renal, pulmonary, and neurologic complications, and deaths have been reported in up to 5% of cases [6]. Most patients have elevated hepatic transaminase levels and significant hematologic abnormalities, including thrombocytopenia and leukopenia [1, 6].

The spectrum of host cells and tissues infected with the HGE agent remains unknown. So far, mature granulocytes are the
only cells found to be infected in patients. The underlying pathogenic process(es) responsible for the diverse clinical manifestations and the cytopenias have yet to be defined. Our laboratory recently isolated the etiologic agent of HGE in the human promyelocytic leukemia line HL-60 [5]. The short life span of peripheral blood granulocytes and the susceptibility of the promyelocytic HL-60 cell line to infection suggested that normal hematopoietic progenitors might be targets of HGE infection. To evaluate this hypothesis, we inoculated CD34+ human marrow progenitors (stimulated to differentiate along myelomonocytic lineages) with the agent of HGE.

Material and Methods

Isolation and cultivation of the HGE agent. We used 2 midwestern isolates made by our laboratory from the blood of patients with acute ehrlichiosis, which were confirmed to be HGE isolates by genospecies-specific polymerase chain reaction, 16S rDNA sequencing, and serologic testing against E. equi and HGE antigens. The HGE isolates were propagated continuously in the human leukemia cell line HL-60 (CL240; American Type Culture Collection, Rockville, MD) as previously described [5]. Cells were maintained between 2 × 10^6 and 1 × 10^6 cells/mL in RPMI 1640 (Celox Laboratories, Hopkins, MN) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine at 37°C and 5% CO2. Infection was monitored by use of Giemsa-stained cytocentrifuged preparations (see below).

Isolation and cultivation of bone marrow progenitors. Bone marrow from the posterior iliac crest of healthy volunteer donors was used. Bone marrow mononuclear cells were obtained by ficoll-hypaque (Sigma Diagnostics, St. Louis) density gradient centrifugation and enriched for CD34+ cells, using an avidin-biotin column (Cellpro, Bothell, WA) as recommended by the manufacturer. Resultant cells were stained with CD34-biotin (Cellpro) and phycocerythrin-conjugated HLA-DR (Becton Dickinson, Mountain View, CA) for multicolor sorting, as previously described [7]. A population that was positive for CD34 and HLA-DR (CD34+HLA-DR+) and known to contain committed progenitors of multiple lineages [8] was selected for study. CD34+HLA-DR+ cells (10^5) were seeded into each well of a 6-well tissue culture plate in 3 mL of Iscove’s modified Dulbecco medium (GIBCO BRL, Grand Island, NY) supplemented with 12.5% fetal calf serum (HyClone), 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), and 2 mM l-glutamine. To stimulate differentiation toward granulocytes and monocytes [8], 10^-6 M hydrocortisone (Abbott, Abbott Park, IL), 5 ng/mL interleukin-3 (R&D Systems, Minneapolis) and 10 ng/mL stem cell factor (provided by Amgen, Thousand Oaks, CA) were added. Cells were maintained at 37°C in 5% CO2.

Phenotype analysis. Cell surface antigens were determined by direct staining of cells, using monoclonal antibodies (0.15 μg per 2 × 10^6 cells) against CD34, CD15, and CD14 (Becton Dickinson). Antigens were analyzed by flow cytometry (FACStar® Plus; Becton Dickinson), using a computer (Consort 32, model 340; Hewlett Packard, Palo Alto, CA), and isotype-matched antibodies were used for controls.

Infection with the HGE agent. Both early (passage 7) and laboratory-adapted (passage >40) HGE isolates were used. Each experiment was repeated at least twice to confirm results. At 1, 4, 5, and 7 days after initiation into culture, marrow progenitors were infected by inoculation with either 10^5–10^6 heavily infected HL-60 cells (near lysis) or cell-free bacterial preparations. To prepare cell-free bacterial suspensions, we centrifuged cultures containing 1–2 × 10^6 HL-60 cells (>99% infected) at 125 g for 10 min to pellet cells. The resulting supernatant was removed and centrifuged at 3000 g for 15 min to produce a bacterial pellet, which was used to inoculate marrow progenitors harvested from culture. The bacterial preparations, which were examined by microscopy, were free from visible contamination by intact cells. In all, samples from 6 marrow donors were infected with HGE. Uninfected control cells were prepared and studied in parallel. Samples were examined 3, 5, 7, and 10 days after inoculation with HGE.

Microscopy. Cytospin slides were stained with Giemsa and observed under oil immersion (×1000) for the presence of intracellular bacterial inclusions (morulae). To characterize phenotypic differentiation, myeloperoxidase and nonspecific esterase staining were done as described [9, 10]. To confirm infection of marrow-derived monocytes, cells were double-labeled for both HGE antigens and the monocytic-specific marker, CD14. For the detection of HGE antigens, serum from a patient who had recovered from culture-proven HGE (HGE IFA titer, 1:5120) was diluted 1:500 in TRIS-buffered saline with 3% bovine serum albumin and detected by secondary labeling with rhodamine-isothiocyanate (FITC)–conjugated anti-CD14 antibody (Leu-M3, Becton Dickinson, San Jose, CA) followed by amplification using secondary labeling with FITC-conjugated anti-mouse Ig (Organon Teknika, West Chester, PA). At the same time, to identify monocytes, direct labeling with fluorescein isothiocyanate (FITC)–conjugated anti-CD14 antibody (Leu-M3, Becton Dickinson, San Jose, CA) was followed by amplification using secondary labeling with FITC-conjugated anti-mouse Ig (Organon Teknika) was done. Negative controls included uninfected marrow and HL-60 cells for HGE and CD14 antigens, respectively. Peripheral blood monocytes purified by magnetic bead selection (Miltenyi Biotec, Auburn, CA) were used as a positive control for the detection of CD14.

Results

Human bone marrow progenitors from all 6 donors became infected with the HGE agent regardless of whether infected cells or cell-free bacterial preparations were used as the inoculum. The degree of infection and the rapidity of its progression were dependent on the time that cells were maintained in culture before being inoculated with HGE. Cells infected 1 day after culture initiation were only 5% infected 10 days later, whereas cells infected at 4 and 5 days of culture were 80%–90% infected 3 and 5 days later, respectively, and were completely lysed by bacteria by 7 days. Cells became less susceptible after having been cultured for 7 days before inoculation; only 30% contained morulae 7 days later.

Initially, all cells were of similar appearance, with a high nuclear-to-cytoplasmic ratio, dense blue cytoplasm, and absent granules. After 3–5 days in culture, cells showed differentiation of marrow progenitors, with increasing granularity, decreased nuclear size, fewer nucleoli, and the appearance of granulocytic and monocytic precursors. By days 7–10, mono-
cytes, metamyelocytes, and granulocyte band forms were visible. At the time of greatest susceptibility to infection (4 days), >70% of cells had lost expression of the primitive marker, CD34. Six percent to 9% of cells were CD14+, and 40%–60% were CD15+CD14−, demonstrating monocytic and granulocytic differentiation, respectively (table 1) [8]. The time course of differentiation was not altered by infection with HGE.

Remarkably, cells morphologically characteristic of monocytes and granulocytes were equally infected by the HGE agent (figure 1A). Myeloperoxidase staining was done as a marker for granulocytic differentiation [9], and revealed that both peroxidase-positive and -negative cells were infected (figure 1B).

Nonspecific esterase, a marker of monocyte differentiation [10], stained 30%–40% of cells examined 10 days after culture initiation, but the stain was too intense for photography of morulae within stained cells. Immunofluorescence demonstrated that 30% of cells were positive for the monocyte marker CD14 after 10 days in culture, and double immunofluorescence for CD14 and the HGE agent revealed that 90% of these CD14+ cells were infected with HGE (figure 1C).

**Discussion**

Normal CD34+HLA-DR+ primary human bone marrow progenitors, which share with susceptible HL-60 cells [11] the

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**Figure 1.** Photomicrographs of bone marrow progenitors infected with agent of human granulocytic ehrlichiosis (HGE). **A,** Bone marrow progenitors shown 5 days after inoculation with HGE agent, demonstrating infection of undifferentiated (u), granulocytic (g), and monocytic (m) cells (Giemsa stain; ×4350). **B,** Myeloperoxidase stain demonstrating infection of both peroxidase-positive (blue) and -negative cells (×4350). **C,** Dual immunofluorescence showing HGE infection (rhodamine) of CD14+ (fluorescein) and CD14− cells (×4350).
Table 1. Bone marrow progenitor cell surface markers and susceptibility to infection with human granulocytic ehrlichiosis (HGE).

<table>
<thead>
<tr>
<th>No. of days in culture</th>
<th>Cells infected 10 days after HGE inoculation</th>
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<tr>
<td></td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGE inoculation</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&gt;99</td>
</tr>
<tr>
<td>4</td>
<td>22.5–30.0</td>
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<tr>
<td>7</td>
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NOTE. Data are %, based on analysis of 2 donors on indicated day of inoculation with HGE agent.

ability to differentiate along both granulocytic and monocytic lines, are susceptible to infection with the agent of HGE. To our knowledge, this is the first report of cultivation of the HGE agent in primary cells.

The peak period of susceptibility to infection was 4–5 days after initiation into the culture system, a time at which there are few fully mature cells but clear evidence, by morphology and cell marker studies, of differentiation along both lineages. Cells infected at earlier and later time points were less susceptible, suggesting targeting of the HGE agent to committed myelomonocytic progenitors or alterations in susceptibility to HGE related to factors such as cell proliferation or metabolic activity.

There are no published studies detailing bone marrow findings in patients with HGE. *E. chaffeensis* morulae have been observed in bone marrow mononuclear cells in association with progressive pancytopenia [12, 13] in human monocytic ehrlichiosis, demonstrating that direct infection of hematopoietic cells can occur and that such infection or alterations in the bone marrow microenvironment resulting from it, may contribute to myelosuppression in some cases.

To our surprise, we found that bone marrow cells not only of granulocytic but also of clearly monocytic lineage were susceptible to infection with HGE. There have been no reports of infection of monocytic cells in patients with HGE, although *E. phagocytophila* has been observed in monocytes of experimentally infected sheep [3]. While bone marrow monocytes are susceptible to infection with HGE in vitro, they may not provide the appropriate environment for continued growth of the organism in vivo, or they may be able to kill the organism once activated, as has been demonstrated with *Ehrlichia risticii* [4]. Alternatively, some monocytes or their precursors (or both) may become infected, but as seen with *E. chaffeensis* and other monocytic ehrlichioiises, are rarely present in the circulation [2, 4]. Infected monocytes may reside predominantly in tissues or bone marrow, areas not well studied in HGE. In any case, the HGE agent clearly is not restricted to replicating in cells of granulocytic lineage.

Conversely, it is of interest that *E. chaffeensis* has been found occasionally in granulocytes [2]. It is unknown what determines the relative specificity of different species of ehrlichiae for their host cells. Our observations raise the possibility that there may be receptor(s) or pathway(s) of entry for the HGE agent common to granulocytic and monocytic cells but that survival and proliferation of the organism may be dependent on an appropriate intracellular milieu. Furthermore, while clinically useful, the designation of this new agent as “granulocytic ehrlichiosis” may be biologically imprecise. Studies of bone marrow from patients infected with HGE will be of interest to determine whether these in vitro observations parallel events during natural infection.

Even short-lived infection of monocytic cells could contribute to the clinical manifestations observed in HGE. For example, the release of cytokines by monocytes could result in fever, myalgias, and hepatic dysfunction [14] and could act on the bone marrow to down-regulate hematopoiesis [15]. Circulating monocytes could participate in disseminating or eliminating infection. Tissue macrophages and related skin Langerhans cells could be important during infection either as targets of initial infection or as effectors in the early host response, as observed with other tickborne infections [16].

The susceptibility of the HL-60 promyelocytic leukemia cell line to infection with the HGE agent suggested that myelomonocytic precursors in the bone marrow might be targets of natural infection. The ability to infect primary CD34<sup>+</sup> HLA-DR<sup>+</sup> human bone marrow progenitors provides support for this hypothesis. The finding that cultured normal hematopoietic cells, both granulocytic and monocytic, are susceptible to HGE infection provides new opportunities for studying the biology of this agent and its interactions with pathogenically relevant nontransformed target cells.

Acknowledgments

We thank Richard Brunning and the University of Minnesota Hematopathology Laboratory for performing and reviewing histochenal stains, Valerie McCullar and Brad Anderson for their technical support, and Mary Hayes for her assistance with manuscript preparation.

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