The Agent of Human Granulocytic Ehrlichiosis Induces the Production of Myelosuppressing Chemokines without Induction of Prolinflammatory Cytokines

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Infection by human granulocytic ehrlichiosis (HGE) is characterized clinically by cytopenias out of proportion to the number of cells seen to be infected directly. To study the pathogenic role of inflammatory mediators in HGE infection, cytokine production by untreated and dimethyl sulfoxide–treated HL-60 cells, which demonstrate enhanced infection because of granulocytic differentiation, and by normal bone marrow cells was measured using modified sandwich ELISA assays on samples obtained sequentially after inoculation with the HGE agent. All infected cells produced physiological concentrations of CC (monocyte chemotactic protein–1, macrophage inflammatory protein–1α and −β, and RANTES) and CXC (interleukin [IL]–8) chemokines in amounts significantly greater than those produced by uninfected controls. In contrast, infected cells did not secrete the classic proinflammatory cytokines IL-1, IL-6, or tumor necrosis factor–α. The striking production of chemokines, powerful leukocyte chemoattractants capable of suppressing hematopoesis, by susceptible target cells, is likely to be of pathogenic importance both in the observed cytopenias and in mediation of inflammation and host defenses during infection.

The chemokines have recently become a focus of investigation as important mediators of leukocyte activation and chemotaxis in a number of infectious and inflammatory conditions [1–4]. These structurally related proteins are predominantly secreted by inflammatory cells and share the ability to activate and direct the migration of specific leukocyte types [1]. In addition to these properties, several of the chemokines, including interleukin (IL)–8, macrophage inflammatory protein (MIP)–1α, and monocyte chemoattractant protein (MCP)–1, have been shown to be potent inhibitors of stem-cell proliferation both in vitro and in vivo [5–7]. Chemokines are not expressed in resting cells but are up-regulated on activation by a number of stimuli, including the proinflammatory cytokines IL-1, tumor necrosis factor (TNF)–α, and interferon-γ, as well as by reactive oxygen intermediates, lipopolysaccharide (LPS), and other bacterial products [1]. Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne infection endemic to the upper midwestern and northeastern United States [8]. HGE is caused by an obligate intracellular gram-negative bacterium unique in its ability to replicate within granulocytes [9]. Clinically, HGE is an acute febrile illness that, if left untreated, may result in renal, pulmonary, and neurological complications. A prominent clinical feature of HGE infection is the reduction in the number of circulating blood cells, most commonly manifesting as thrombocytopenia (observed in >90% of clinical cases), leukopenia, and normocytic anemia [8]. The etiology of these hematologic abnormalities is unknown. We recently demonstrated that bone marrow progenitors of both granulocytic and monocytic lineage are susceptible to infection by HGE in vitro, which suggests that the bone marrow may be a likely target of infection [10]; however, <10% of granulocytes are seen to be directly infected during the course of HGE infection [8], and there are no reports of overwhelming bone marrow infection. The broad constellation of clinical symptoms seen combined with cytopenias, out of proportion to the number of cells seen to be directly infected, suggests that the stimulation of cytokine production may have an important pathogenic role in this infection.

Our laboratory isolated and cultivated the causative agent of HGE in the human promyelocytic leukemia cells line, HL-60[11]. Furthermore, we have demonstrated that, when induced to undergo differentiation along a granulocytic pathway using
Figure 1. Chemokine production in untreated and dimethyl sulfoxide (DMSO)-treated HL-60 cells over time (human granulocyte ehrlichiosis [HGE] infected [broken line] vs. HGE uninfected [solid line]). Data are mean ± SE of 3 independent experiments. MIP-1α, macrophage inflammatory protein–1α; MCP-1, monocyte chemotactic protein–1; IL-8, interleukin-8. *P < 0.05 for comparison of infected and uninfected samples at same time point, using the Student’s t test.

dimethyl sulfoxide (DMSO), HL-60 cells exhibit a marked increase in susceptibility, in keeping with the clinical tropism of HGE for neutrophils [12]. In the present study, we investigate whether HGE infection of both untreated and differentiated HL-60 cells, as well as bone marrow cells enriched for progenitors, results in the production of proinflammatory cytokines, and we focus particularly on those chemokines—IL-8, MIP-1α, and MCP-1—that have been associated with myelosuppression.

Materials and Methods

Cell culture. HL-60 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Celox Laboratories, Hopkins, MN) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). To induce granulocytic differentiation, uninfected HL-60 cells (5 × 10⁴ cells/mL) were incubated for 5 days with 1.25% DMSO (Sigma, St. Louis). Cells were washed free of DMSO, resuspended in fresh medium, and cultured free of the inducing agent for 24 h prior to use. Bone marrow was obtained from the posterior iliac crest of a normal donor. Bone marrow mononuclear cells were obtained by ficoll-hypaque density gradient centrifugation (Sigma). A population that was both CD34 and HLA-DR positive (CD34⁺/HLA-DR⁺) was selected as described elsewhere [10] and cultured at a concentration of 10⁷ cells/mL in a 25 cm² flask in Iscove’s modified Dulbecco’s medium (IMDM; Gibco BRL, Grand Island, NY) supplemented with 12.5% fetal calf serum, 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), and 2 mM L-glutamine. To stimulate differentiation toward granulocytes and monocytes [10], 10⁻⁶ M hydrocortisone (Abbott Laboratories, North Chicago, IL), 5 ng/mL IL-3 (R&D Systems, Minneapolis), and 10 ng/mL stem-cell factor (kindly provided by Amgen Inc., Thousand Oaks, CA) were added. Cells were maintained at 37°C and 5% CO₂ for 5 days and then were washed free from all additives and cultured in IMDM with serum for 24 h prior to their being used in experiments.
and bacteria were initially coincubated at room temperature in a 100-μL volume for 15 min and then brought up in 10 mL of medium at 37°C (final concentration of cells 5 × 10^7/mL). Samples (1 mL) were taken immediately and placed on ice. Supernatants were collected after centrifugation at 13,000 g for 15 min at 4°C. Aliquots were immediately frozen and stored at −70°C until cytokine assays were performed. The remaining cells were cultured in 25-cm² flasks. Additional samples were taken at 6, 12, 24, 48, and, in some cases, 72 h and processed as above. Viable cells were counted daily using trypan blue exclusion. All experiments included uninfected HL-60 cells as controls, which were handled identically except that medium was used in place of the bacterial inoculum. In some experiments, the bacterial inoculum was inactivated by heating to 60°C for 10 min, with or without subsequent filtration through a 0.22-μm low–protein binding syringe tip filter. In other experiments, LPS (1 μg/mL; Sigma) was used, as a positive control, to stimulate HL-60 cells.

**Cytokine assays.** MIP-1α, MIP-1β, MCP-1, RANTES, IL-1, IL-6, IL-8, and TNF-α levels in culture supernatants were measured using a modified sandwich ELISA, as described elsewhere [13]. Appropriate recombinant human, monoclonal mouse anti-human, and polyclonal goat anti-human antibodies were purchased from R&D Systems and polyclonal rabbit anti-human antibodies from Genzyme (Cambridge, MA). In brief, purified antibodies were respectively diluted in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) at a concentration of 1–2 μg/mL and coated overnight at 4°C onto 96-well ElisaPlates (Corning, Cambridge, MA). The wells were washed and then blocked with a solution containing 1% bovine serum albumin in PBS for 1 h at 37°C, and 50 μL each of samples or standard chemokines was added in triplicate for 2 h at 37°C. The wells were then washed with PBS/Tween 20 (0.05%) and incubated with goat anti-human polyclonal cytokine/chemokine secondary antibodies (1–2 μg/mL) for 1.5 h and washed again, followed by donkey anti-goat IgG–horse radish peroxidase conjugate (1:10,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for an additional 1 h at 37°C. After an intensive wash, K blue substrate (Neogen Corp., Lexington, KY) was added for 10–20 min at room temperature, and the reaction was then stopped by the addition of 100 μL of 1 M H₂SO₄. Optical density was read at 450 nm and compared with standard values for quantification. Sensitivities of the ELISA were as follows: MIP-1β, 7.8 pg/mL; IL-8, 10 pg/mL; MIP-1α, RANTES, and MCP-1, 15.6 pg/mL; IL-6 and TNF-α, 20 pg/mL; and IL-1β, 50 pg/mL. All chemokine and cytokine antibodies were uncontaminated by endotoxin (<0.1 ng/μg).

**Limulus assays and polymixin B.** Infected cell samples were tested for the presence of endotoxin by the amoebocyte limulus assay (sensitivity 0.03 EU), performed as per the manufacturer’s directions (Associates of Cape Cod, Falmouth, MA). As another control for the presence of endotoxin, polymixin B (10 μg/mL; Sigma) was added to some infected cultures and controls during the incubation step.

**Statistics.** Cytokine levels are expressed as the mean ± SE of results obtained from triplicate assays of 3–5 individual experiments and are corrected to represent 10^6 cells. Data were entered into a Microsoft Excel database and groups compared using the Student’s t test with α = .05.

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**Figure 2.** Chemokine production by human bone marrow cells enriched for progenitors over time (human granulocyte ehrlichiosis [HGE] infected [broken line] vs. HGE uninfected [solid line]). Data are mean ± SE of 3 independent experiments. MIP-1α, macrophage inflammatory protein–1α; MCP-1, monocyte chemotactic protein–1; IL-8, interleukin-8. *P < .05 for comparison of infected and uninfected samples at same time point, using the Student’s t test.

**HGE preparation.** We used 2 midwestern isolates (HGE 2 and 6) made by our laboratory from the blood of patients with acute ehrlichiosis, confirmed to be HGE by genospecies-specific polymerase chain reaction, 16S rDNA sequencing, and serologic testing against *Ehrlichia equi* and HGE antigens. The HGE isolates were propagated continuously in HL-60 cells as described elsewhere [11]. Infection was monitored by examining cytocentrifuged preparations for the presence of intracellular bacteria, by Giemsa staining. Both early (<7 passages) and laboratory-adapted (>40 passages) HGE isolates were used to prepare cell-free bacterial suspensions, as described elsewhere [10]. Resulting bacterial pellets were used to inoculate DMSO-differentiated (granulocytic) or untreated HL-60 cells or marrow cells (inoculum of ~10 organisms/cell). Cells were incubated in 100-μL volumes for 15 min and then brought up in 10 mL of medium at 37°C (final concentration of cells 5 × 10⁷/mL). Samples (1 mL) were taken immediately and placed on ice. Supernatants were collected after centrifugation at 13,000 g for 15 min at 4°C. Aliquots were immediately frozen and stored at −70°C until cytokine assays were performed. The remaining cells were cultured in 25-cm² flasks. Additional samples were taken at 6, 12, 24, 48, and, in some cases, 72 h and processed as above. Viable cells were counted daily using trypan blue exclusion. All experiments included uninfected HL-60 cells as controls, which were handled identically except that medium was used in place of the bacterial inoculum. In some experiments, the bacterial inoculum was inactivated by heating to 60°C for 10 min, with or without subsequent filtration through a 0.22-μm low–protein binding syringe tip filter. In other experiments, LPS (1 μg/mL; Sigma) was used, as a positive control, to stimulate HL-60 cells.

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**Results**

**HGE infection does not stimulate production of proinflammatory cytokines in vitro.** No measurable IL-1, IL-6, or TNF-α production was detected in culture supernatants from HGE-infected uninduced and differentiated HL-60 cells or cells enriched for marrow progenitors at any of the time points assayed (data not shown).

**HGE infection of HL-60 cells results in the production of both CC and CXC chemokines.** Production of significant levels of CC and CXC chemokines was stimulated by HGE infection in both uninduced and DMSO-stimulated HL-60 cells, when compared with uninfected control samples (table 1). Nanogram amounts of IL-8, MIP-1α, MIP-1β, MCP-1, and RANTES were secreted beginning 12 h following inoculation with HGE and remained measurable through 72 h. By 24 h following inoculation with HGE, IL-8, MIP-1β, and MCP-1, secretion by uninduced and DMSO-treated HL-60 cells was significantly greater than secretion by uninfected controls ($P < .05$). By 48 h, production of all measured chemokines by infected cells was significantly greater than in uninfected controls ($P < .05$; figure 1). The time course of chemokine secretion correlated closely with the presence of ehrlichial inclusions within infected cells, a pattern reflecting increasing bacterial burden. This pattern differed from that induced by LPS stimulation of cells, in which peak secretion occurred at 24 h. Of note, DMSO-treated granulocytic cells, which we have previously demonstrated to have enhanced susceptibility to HGE infection, secreted proportionately greater amounts of chemokines when compared with uninduced promyelocytic HL-60 cells ($P < .05$ for MIP-1β and RANTES at 48 h).

**Bone marrow cells also produce chemokines when infected with HGE.** Marrow progenitors are likely to be natural targets of HGE infection. Furthermore, regulation of the bone marrow microenvironment is essential to normal hematopoiesis. Therefore, we studied chemokine production by cultured human marrow cells, enriched for progenitors, following inoculation with the HGE agent. Similar to HL-60 cells, we found that HGE infection of these cells resulted in the production of significant levels of IL-8, MIP-1α, MIP-1β, MCP-1, and RANTES when compared with uninfected control cells (figure 2).

**Heat inactivation of the inoculum does not abrogate chemokine secretion.** To determine whether viable bacteria were necessary to induce chemokine production by susceptible cells, ehrlichial isolates were heat-inactivated prior to inoculation. Although no detectable infection of cells resulted following inoculation with heat-treated HGE, measurable chemokine production nonetheless still occurred. The time course of production was shifted and peaked earlier (24–48 h), which was similar to that seen following LPS stimulation of HL-60 cells (figure 3). When ehrlichial preparations were filtered through a 0.22-μm filter to remove intact organisms and the filtrate was then used to inoculate cells, however, no chemokine production was detected.

**Classic endotoxin is not responsible for HGE-induced chemokine secretion.** In addition to the proinflammatory cytokines, which were not detected (above), LPS classically induces the production of chemokines by inflammatory cells. To exclude the possibility that endotoxin, either from HGE or due to contamination of cultures or assays, was responsible for the observed chemokine secretion, limulus assays were performed at multiple time points on the supernatants obtained from 3 independent experiments. No endotoxin was detected at any time point. LPS (1 μg/ml) did stimulate HL-60 cells to secrete chemokines, as expected, and provided a positive control for the assay. Finally, the addition of the LPS inhibitor polymixin B to the culture system did not reduce the production of che-
mokines stimulated by HGE-infected cells but did abrogate the LPS response, as expected.

Discussion

These studies demonstrate that HGE infection of susceptible target cells, including normal marrow cells enriched for progenitors, results in the striking production of both CC and CXC chemokines. In contrast, infected cells did not induce the secretion of the classic proinflammatory cytokines, IL-1, IL-6, or TNF-α. Our findings have important implications for furthering the understanding of the pathogenesis of human infection with this unique intracellular organism.

A predominant feature of infection with the HGE agent is the development of cytopenias that are out of proportion to the number of infected cells [8]. MIP-1α, MCP-1, and IL-8 at 25–100 ng/mL, amounts similar to those we found in preparations from HGE-infected cells, have been shown to be capable of suppressing the proliferation of bone marrow progenitors and consequent hematopoiesis both in vitro and in vivo [6]. Furthermore, even lower amounts of these potent proteins (e.g., MIP-1α and IL-8, each at 0.1 ng/mL) have been shown to act synergistically to down-regulate hematopoiesis dramatically [7]. Thus, even very low levels of these active chemokines, secreted during the course of natural infection, could act on the bone marrow to impact hematopoiesis profoundly. It is, therefore, particularly relevant that normal bone marrow cells were induced to produce MIP-α, MIP-1β, and MCP-1. Although the chemokines have recently been implicated in the pathogenesis of a number of infections as diverse as human immunodeficiency virus infection, Lyme disease, and malaria [4, 14, 15], we present the first evidence suggesting that the induction of these peptides may contribute to cytopenias associated with infection. Because myelosuppression is a feature of many acute infectious diseases, our findings have broader implications for the understanding of pathogenic mechanisms underlying infection-mediated cytopenias.

The clinical spectrum of HGE is protean. The nonspecific constitutional symptoms observed suggest that a proinflammatory response is induced by the agent. The chemokines, particularly MIP-1α and IL-8, have been demonstrated to be important mediators of inflammation through the recruitment and activation of leukocytes in many tissues [1]. In light of the fact that the classic proinflammatory cytokines were not secreted, chemokines may be in part responsible for some of the more severe manifestations of HGE infection.

IL-8 could potentially play another interesting role during HGE infection: the chemotactic recruitment of uninfected neutrophils to sites of other infected cells, thereby facilitating the direct cell-cell spread of ehrlichia. This would represent a remarkable adaptation by this organism, which is specialized to infect neutrophils. It may seem equally remarkable that an IL-8 response would not also induce neutrophils to kill ehrlichia, because the role of IL-8 in enhancing phagocytosis, superoxide, and granule release by neutrophils is well described [16]. This observation belies the extraordinary ability of this organism to survive and proliferate within highly armed phagocytes.

The high level of expression of IL-8 and MCP-1 by epithelial cells has led to the suggestion that they might be key factors in attracting mediators of host defense against invading pathogens at epithelial surfaces, the first point of contact of HGE with the host following a tick bite. Thus, HGE-induced MIP-1α, MCP-1, and RANTES could attract cells important in the early host response to this bacterium.

Viable ehrlichia were not required to generate chemokine secretion. Heat treatment did result in earlier secretion at reduced levels, compared with chemokine secretion by cells infected with viable and replicating organisms. Given the asynchronous nature of HGE infection in cell culture, the prolonged chemokine secretion observed with viable organisms may be a result of the interactions between recently released bacteria and any uninfected cells remaining in the population. It is also possible, as has been reported for Mycobacterium tuberculosis [17], that intracellular growth is a more potent stimulus for chemokine secretion than extracellular organisms.

The bacterial component responsible for inducing chemokine secretion in susceptible cells remains unclear. Although the outer membranes of rickettsiae, such as Rickettsia rickettsiae, have been shown to contain LPS, ehrlichial species have not [18]. Classic LPS was not responsible for the chemokine secretion observed because of HGE, as evidenced by the failure to detect endotoxin in the limulus assay and the inability of polymixin B to inhibit chemokine secretion. Nor was chemokine production the result of known proinflammatory molecules such as TNF-α or IL-1, because they were not detected. It remains possible that other unmeasured cytokines may be at play. Because intact ehrlichia were required to induce the chemokine response, however, it is unlikely that a soluble mediator produced by the bacteria or by the cells was responsible. Rather, the evidence supports the hypothesis that a heat-stable bacterial

### Table 1. Mean chemokine level 48 h after inoculation with human granulocytic ehrlichiosis.

<table>
<thead>
<tr>
<th>Cell type, infection status</th>
<th>MIP-1α</th>
<th>MIP-1β</th>
<th>MCP-1</th>
<th>RANTES</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated HL-60 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>8.74</td>
<td>11.68</td>
<td>8.50</td>
<td>3.58</td>
<td>2.70</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.80</td>
<td>1.51</td>
<td>0.08</td>
<td>0.70</td>
<td>0.31</td>
</tr>
<tr>
<td>DMSO-treated cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>16.55</td>
<td>61.54</td>
<td>50.29</td>
<td>28.4</td>
<td>39.11</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.22</td>
<td>2.51</td>
<td>3.95</td>
<td>5.04</td>
<td>0.31</td>
</tr>
<tr>
<td>Bone marrow cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>21.71</td>
<td>22.77</td>
<td>26.79</td>
<td>14.46</td>
<td>27.33</td>
</tr>
<tr>
<td>Uninfected</td>
<td>1.19</td>
<td>3.79</td>
<td>2.17</td>
<td>2.42</td>
<td>8.65</td>
</tr>
</tbody>
</table>

NOTE: Values are ng/10⁶ cells. MIP-1α, -1β, macrophage inflammatory protein-1α, -1β; MCP-1, monocyte chemotactic protein-1; IL-8, interleukin-8; DMSO, dimethyl sulfoxide.

* P < .05, Student’s t test, infected cells vs. their respective uninfected controls.
component different from classic LPS induces susceptible cells to produce chemokines.

Chemokine secretion may occur through the ehrlichial activation of the nuclear factor-κB (NF-κB) pathway, offering a parallel to events induced by the outer membrane protein A of Borrelia burgdorferi [19, 20]. In addition, we have recently shown that HGE engages sialyl Lewis x modified cell surface molecules [21] and that one, P-selectin glycoprotein ligand-1 (PSGL-1), can serve as an HGE receptor [22]. Binding of ligands to PSGL-1 has been reported to induce cell signaling, including chemokine release and NF-κB translocation [23]. Further studies are required to elucidate the mechanism of chemokine secretion by HGE-infected HL-60 cells and neutrophils and the bacterial components responsible.

In summary, we have described the dramatic production of chemokines in vitro by HGE-infected cells, including normal human marrow cells enriched for progenitors. Animal and clinical studies will be helpful in confirming the in vivo importance of these observations. In the interim, however, our findings suggest that ehrlichial-induced chemokines may play an important role in the pathogenesis of HGE and in the host’s response to this infection.

References