Monocytic Differentiation Inhibits Infection and Granulocytic Differentiation Potentiates Infection by the Agent of Human Granulocytic Ehrlichiosis

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Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne infection with a specific tropism for granulocytes. We previously isolated and cultivated the HGE agent in the promyelocytic leukemia cell line HL-60 and have also demonstrated the susceptibility of both granulocytic and monocytic human marrow progenitors. Circulating monocytes have not been observed to be infected, suggesting that cell susceptibility may be differentiation specific. To evaluate this hypothesis, HL-60 cells were differentiated towards granulocytes (with dimethyl sulfoxide or all-trans retinoic acid) or toward monocytes-macrophages (with 12-O-tetradecanoylphorbol-13-acetate [TPA], gamma interferon, or 1,25-dihydroxyvitamin D3) and then challenged with HGE. HGE binding, internalization, and proliferation were compared in differentiated and untreated control HL-60 cells by immunofluorescence, electron microscopy, and Giemsa staining. Granulocytic differentiation resulted in a doubling of HGE binding and enhanced infection consistent with the agent's clinical tropism for neutrophils. Granulocytic cells were unable to kill internalized ehrlichiae even after activation induced by N-formyl-Met-Leu-Phe alone or together with tumor necrosis factor alpha. In contrast, monocyte-macrophage differentiation with TPA resulted in complete resistance to infection through at least two distinct mechanisms: (i) reduction in binding and uptake and (ii) killing of any internalized organisms. Diminished binding in TPA-treated cells correlated with their reduced expression of sialyl Lewis x (CD15s), a putative cellular receptor component for HGE. The degree of monocytic differentiation and activation induced (i.e., TPA > gamma interferon > vitamin D3) correlated with resistance to HGE. Thus, HL-60 cells exhibit a striking differentiation-specific susceptibility to HGE. Differentiation-induced changes in bacterial adhesion and killing capacity underlie the tropism of HGE for granulocytic HL-60 cells and, conversely, the resistance of activated macrophages to infection.

The ehrlichiae are obligate intracellular tick-borne pathogens with specific cellular tropisms. Two distinct ehrlichial species have recently emerged as important human pathogens: Ehrlichia chaffeensis, which infects monocytes (17), and the agent of human granulocytic ehrlichiosis (HGE), which is unique for its preferential growth within granulocytes (2, 7). HGE causes an acute febrile illness associated with cytopenias and the presence of bacterial inclusions (morulae) within peripheral blood neutrophils. Infection can be complicated by renal, pulmonary, and neurologic manifestations (1).

We previously isolated and cultivated the etiologic agent of HGE by using the human promyelocytic leukemia cell line HL-60 (9). The short life span of peripheral blood granulocytes and the susceptibility of HL-60 cells to infection also led us to investigate whether human bone marrow progenitors might be targets of HGE infection. Indeed, we found that both granulocytic and monocytic marrow progenitors were susceptible to infection, suggesting the presence of a common receptor and/or pathway of entry in the two cell types (14). However, peripheral blood monocytes have not been observed to be infected in vivo, raising the possibility that the susceptibility of monocytic cells to HGE is determined by their state of differentiation or activation.

Like marrow progenitors, HL-60 cells have the potential to differentiate along both granulocytic and monocytic pathways. HL-60 cells incubated in the presence of dimethyl sulfoxide (DMSO) undergo progressive and terminal differentiation toward mature granulocytes (5, 8, 20). Monocytic differentiation of HL-60 cells can be induced by using a variety of agents (3, 15, 18, 21). For example, the protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent inducer of terminal macrophage differentiation (3, 21). Therefore, we performed detailed studies of the effects of HL-60 cell differentiation upon cellular susceptibility to infection with the agent of HGE.

MATERIALS AND METHODS

Cultivation of the HGE agent. We used two 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 PCR, 16S rDNA sequencing, and serologic testing against Ehrlichia equi and HGE antigens. The HGE isolates were propagated continuously in the HL-60 cell line (CL240; American Type Culture Collection) in RPMI 1640 medium (CellGro Laboratories, Hopkins, Minn.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah) and 2 mM L-glutamine as previously described (9). Infection was monitored by examining cytospin preparations for the presence of intracellular bacteria by both immunofluorescence (see below) and Giemsa staining.

Differentiation of HL-60 cells. To induce granulocytic differentiation, uninfected HL-60 cells (5 × 10⁶ cells/ml) were incubated for 6 days with 1.25% DMSO (Sigma, St. Louis, Mo.) or 1 μM all-trans retinoic acid (RA; Sigma). RA was freshly made from a 1 mM stock solution prepared in 95% (vol/vol) ethanol and stored at −80°C (final ethanol concentration, 0.01%). Ethanol (0.01%) alone had no effect on growth or differentiation of HL-60 cells.

TPA (Sigma) was prepared as a 1.6 × 10⁻⁴ M stock in DMSO and stored at −80°C for ≤6 months. To induce monocytic differentiation, HL-60 cells were incubated in 25-cm² tissue culture flasks with TPA (1.6 × 10⁻⁴ to 1.6 × 10⁻¹⁵ M) freshly prepared from the stock solution in growth medium (final DMSO con-
Infection of HL-60 cells. Prior to experiments, cells were washed free from any inducing agent with fresh RPMI 1640 or PBS. Both early-passage (<7) and laboratory-adapted (passage >40) HGE isolates were used, and each experiment was repeated at least three times to confirm results. Cell-free bacterial suspensions were prepared from cultures containing 108 HL-60 cells/ml (>99% infected), passaged through a 27-gauge needle three times to aid in lysis, and then centrifuged at 125,000 x g for 10 min to pellet cell debris. The resulting supernatant was challenged at 2 x 105 cells/ml for 15 min at 37°C to produce a bacterial inoculum. Each sample (1 ml) was immediately incubated with fresh RPMI 1640 or PBS. Both early-passage (<7) and laboratory-adapted (passage >40) HL-60 cells were retained for study ranging from 1 h to 6 days. The resulting adherent cells were harvested for study by vigorous pipetting and/or gentle scraping.

Recombinant gamma interferon (rIFN-γ; 105 U/ml stock solution; Genentech, San Francisco, Calif.) was prepared in Dulbecco's phosphate-buffered saline (PBS) (without Ca or Mg) and stored at 4°C. HL-60 cells (3 x 105/ml) were exposed to concentrations ranging from 200 to 2,000 U/ml in growth medium in 24-well tissue culture plates for 4 to 6 days. 1,25-Dihydroxyvitamin D3 were exposed to concentrations ranging from 200 to 2,000 U/ml in growth medium in 24-well tissue culture plates for 4 to 6 days. The resulting adherent cells were harvested for study by vigorous pipetting and/or gentle scraping.

RESULTS

Granulocytic differentiation enhances susceptibility to infection with the HGE agent. After 6 days of exposure to 1.25% DMSO, 40 to 50% of HL-60 cells demonstrated granulocytic morphology and were myeloperoxidase positive. Greater than 90% of DMSO-treated cells reduced NBT (compared with <5% of untreated controls), indicating that functional differentiation had also taken place. In repeated experiments, DMSO-treated cells were 2 to 5 times more susceptible to HGE infection than untreated controls (compare Fig. 1E and D). The mean proportions of cells infected at 48 h were 70% DMSO-treated cells and 29% untreated HL-60 controls (P < 0.001) (Table 1). Infection also progressed more rapidly in DMSO-differentiated cells; DMSO-treated cells reached terminal stages of infection 3 to 4 days after inoculation, compared with 7 days for controls. Treatment with RA (1 μM), which also induced granulocytic differentiation of HL-60 cells, similarly resulted in enhanced susceptibility to HGE infection, although its effect was less pronounced (Table 1). DMSO treatment was therefore used to induce granulocytic differentiation in subsequent studies.

Stimulation of DMSO-treated HL-60 cells with G-CSF, TNF-α, FMLP, or the combination of TNF-α and then FMLP immediately after HGE inoculation did not prevent the development of infection. Furthermore, 48 h after infection was established, such stimulation did not induce killing of the HGE. Despite their inability to kill HGE, infected DMSO-treated cells continued to reduce NBT normally.

Monocytic differentiation results in resistance to HGE infection. Greater than 95% of cells treated with TPA became clumped, adherent, and strongly nonspecific esterase positive within 24 h. Forty percent of TPA-treated cells reduced NBT. In contrast to the enhancement of infection observed following granulocytic differentiation on DMSO or RA, as little as a 1-h exposure to TPA (>1 nm) rendered HL-60 cells resistant to HGE infection. When examined 3 days following inoculation, none of the TPA-treated HL-60 cells contained bacterial colonies (Fig. 1F), compared with 29% of untreated controls. Seven days following inoculation, TPA-treated cells remained completely uninfected whereas >99% of untreated HL-60 cells were infected. Occasionally, some TPA-treated cells (i.e., 1 to 5%) contained solitary intracellular bacilli 24 to 48 h after inoculation but never developed classic membrane-enveloped ehrlichial colonies (morulae), suggesting that bacterial replication did not take place. Treatment of HL-60 cells with rIFN-γ in concentrations of ≥1,000 U/ml also resulted in differentiation along the monocytic pathway. However, rIFN-γ-treated cells, unlike those treated with TPA, continued to proliferate and generally remained nondifferentiated. In addition, only 60% demonstrated monocytic morphology, indicating that many cells still retained undifferentiated features. rIFN-γ-treated cells demonstrated intermediate susceptibility to HGE: up to 23% of cells supported ehrlichial growth initially (48 h) but the infection diminished over time, with fewer than 10% of cells observed to be infected at 7 days postinoculation. Interestingly, although treatment with VD3 in concentrations as high as 1 μM resulted in some features of monocytic differentiation such as adherence (20 to 40%) and NBT reduction (65%), VD3-treated cells remained susceptible to HGE (Table 1).

Susceptibility to infection in differentiated cells correlates with bacterial adhesion and invasion. To explore potential mechanisms for the differentiation-specific susceptibility to HGE, bacterial adherence to differentiated and untreated control HL-60 cells was assayed (four independent experiments). DMSO-treated granulocytic cells bound a mean of 8.6 ± 3.2
bacteria/cell, compared with 4.2 ± 1.4 bacteria/cell bound by untreated controls ($P = 0.07$). Approximately 20% of DMSO-treated cells were “hyperbinders,” binding >20 bacteria/cell, while such cells were rarely noted in untreated controls (Fig. 1A and B). In contrast, TPA-treated monocytic HL-60 cells exhibited a twofold reduction in binding compared with controls (mean of 2.0 ± 0.8 bacteria/cell; $P = 0.04$). Many TPA-treated cells exhibited no bacterial binding (Fig. 1C). HGE adhesion to rIFN-γ- and VD₃-treated cells did not differ significantly from that observed in control HL-60 cells (means of 3.0 ± 2.9 and 3.4 ± 2.6 bacteria/cell, respectively).

Electron microscopy revealed that DMSO-treated cells rapidly ingested ehrlichiae; many cells contained bacterial inclusions as early as 20 min postinoculation (Fig. 2B). In contrast, untreated HL-60 cells rarely exhibited internalized organisms until 4 to 6 h following inoculation (Fig. 2A). By 24 h, both DMSO-treated and untreated HL-60 cells demonstrated typical bacterial replication within endosomes. Again, DMSO-treated cells became more heavily infected than controls (not shown).

Bacterial binding to differentiated cells correlates with their surface CD15s expression. Recently we demonstrated that the leukocyte cell surface carbohydrate CD15s (sialyl Lewis x) plays an important role in HGE binding to and infection of susceptible cells (10). To evaluate whether differentiation-specific changes in bacterial binding correlated with cellular CD15s expression, we treated HL-60 cells with DMSO or TPA for 6 days and compared CD15s expression and bacterial adherence with results for uninduced controls. DMSO-treated cells expressed CD15s at levels similar to those for untreated control HL-60 cells (3.0 ± 2.9 bacteria/cell), while such cells were rarely noted in untreated controls (Fig. 1A and B). In contrast, TPA-treated monocytic HL-60 cells exhibited a twofold reduction in binding compared with controls (mean of 2.0 ± 0.8 bacteria/cell; $P = 0.04$). Many TPA-treated cells exhibited no bacterial binding (Fig. 1C). HGE adhesion to rIFN-γ- and VD₃-treated cells did not differ significantly from that observed in control HL-60 cells (means of 3.0 ± 2.9 and 3.4 ± 2.6 bacteria/cell, respectively).

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**TABLE 1. Binding and infection of untreated and differentiated HL-60 cells following HGE inoculation**

<table>
<thead>
<tr>
<th>Inducing agent</th>
<th>Bacteria bound/cell</th>
<th>Cells infected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.2 ± 1.4</td>
<td>29</td>
</tr>
<tr>
<td>Granulocytic inducers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>8.6 ± 3.2</td>
<td>70d</td>
</tr>
<tr>
<td>RA</td>
<td>NDc</td>
<td>38</td>
</tr>
<tr>
<td>Monocytic inducers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>2.0 ± 0.8d</td>
<td>0f</td>
</tr>
<tr>
<td>γ-IFN</td>
<td>3.0 ± 2.9</td>
<td>10f</td>
</tr>
<tr>
<td>VD₃</td>
<td>3.4 ± 2.6</td>
<td>21f</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation (four samples per group).

$^a$ Mean percentage of cells infected (four samples per group).

$^b$ $P = 0.07$ compared with untreated controls, Student’s $t$ test.

$^c$ $P < 0.05$ compared with untreated controls, Student’s $t$ test.

$^d$ ND, not done.

$^e$ $P = 0.04$ compared with untreated controls, Student’s $t$ test.

$^f$ $P = 0.04$ compared with other monocytic inducers and control uninduced cells.
HL-60 cells but with a greater degree of heterogeneity. Cells with the highest amount of expression clearly bound the most bacteria (Fig. 1B). In contrast, the low-binding TPA-treated cells were devoid of CD15s expression (Fig. 1C). In other experiments, we exposed HL-60 cells to TPA for various periods of time from 1 h through 6 days and evaluated CD15s expression and bacterial binding. Both CD15s expression and bacterial adhesion diminished gradually over time (Fig. 3). Both rIFN-γ-treated and VD3-treated cells continued to express CD15s and bound HGE normally (see above).

**Monocytic differentiation results in killing of the HGE agent.** Although diminished in number, those bacteria which bound to TPA-treated cells were rapidly internalized (<2 h), as observed both by indirect immunofluorescence and transmission electron microscopy. However, unlike susceptible cells, any organisms which entered TPA-treated cells were noted to undergo degradation within 2 to 4 h (Fig. 2C). No microscopic evidence of infection remained by 24 h. Furthermore, the addition of TPA to undifferentiated HL-60 cells which already contained well-established infection (i.e., 60% of cells exhibiting morulae) led to the prompt inhibition of further bacterial replication and resulted in the eradication of existing infection within 72 h. As a control to exclude direct toxicity of TPA for the HGE agent, bacteria were incubated with 10⁻⁷ M TPA for 30 min. These bacteria remained viable and fully capable of establishing infection in susceptible cells.

**DISCUSSION**

We have demonstrated differentiation-specific susceptibility of HL-60 cells to infection with the agent of HGE—remarkable in that it parallels closely the clinical tropism of this unique organism. Differentiation of HL-60 cells toward granulocytes enhances susceptibility to infection, consistent with the observed tropism of the HGE agent for neutrophils. Granulocytic HL-60 cells exhibit increased binding and uptake of the organism, which they are then unable to kill. Enhanced adhesion and invasion alone may account for increased susceptibility of these cells to infection. It is also possible that additional factors such as alterations in the intracellular environment or available nutrients render granulocytic cells more supportive of ehrlichial growth.

Similar to mature granulocytes, DMSO-treated HL-60 cells have been shown to become chemotactic, undergo degranulation, produce oxidative products in response to stimulation, ingest latex beads, and kill *Staphylococcus aureus* (5, 8, 20) and therefore should be well armed to kill the HGE agent. The DMSO-treated cells used in these experiments were indeed able to reduce NBT (both when infected and when uninfected). However, even when stimulated with G-CSF or TNF and/or FMLP, DMSO-treated cells were unable to kill the organism, suggesting that the HGE agent inhibits, or is resistant to, the cell's microbicidal armamentarium. Neutrophils from sheep experimentally infected with *Ehrlichia phagocytophila* (genetically closely related, if not identical, to HGE) have been reported to have diminished phagocytosis and killing of *S. aureus*, suggesting that ehrlichial infection may globally impair neutrophil function (25).

Treatment of HL-60 cells with RA also enhanced HGE infection, confirming that increased susceptibility occurred in-
FIG. 3. CD15s expression and binding of the agent of HGE relative to time of TPA addition. CD15s expression is graded as follows: 4+, brilliant; 3+, bright; 2+, moderate; 1+, faint; and 0, none. The data represent mean numbers of bacteria adherent per cell as determined by counting >200 cells.

dependently of the agent used to induce granulocytic differentiation. Recently, it was reported that diagnostic culture of granulocytic ehrlichiae from both equine and human sources could be enhanced by the addition of RA to HL-60 cells in a culture system that used PCR as a means of detection (11).

Monocyte-macrophage differentiation, most strikingly that induced by TPA, resulted in restriction of infection through at least two mechanisms: (i) diminished bacterial binding and entry and (ii) growth inhibition and subsequent killing of internalized organisms. Recently we have demonstrated that CD15s, a carbohydrate expressed on the surface of leukocytes that normally functions as a ligand for endothelial selectins, also is critical for HGE binding and subsequent infection of leukocytes (10). Bacterial adhesion in TPA-treated cells correlated with their degree of cell surface CD15s expression. Thus, the observed twofold reduction in HGE binding to TPA-differentiated cells may well be related to the modification or down regulation of CD15s. Diminished binding alone, however, was not the sole mechanism of TPA-induced resistance to HGE infection. First, as little as 1 h of exposure to TPA, which did not immediately alter either cell surface CD15s expression or HGE binding, rendered cells resistant to infection. Furthermore, even after HGE infection of uninduced HL-60 cells was well established, the addition of TPA resulted in inhibition of further bacterial replication and the eradication of existing infection. Similarly, IFN-γ treatment did not reduce binding of the HGE agent or significantly alter CD15s expression, but it did substantially inhibit subsequent infection. Taken together, these findings suggest that in monocyte-differentiated cells, regulation of HGE binding and uptake may be dissociated from the cell’s bacteriostatic and bactericidal properties. For the complete prevention of HGE infection, diminished binding and uptake and the ability to kill HGE all appear to be required.

Both granulocytic and monocytic differentiated HL-60 cells possess potent antimicrobial defenses similar to those of peripheral blood granulocytes and monocytes. Both express Fc receptors and are capable of phagocytosis, degranulation, and oxidative killing (3, 5, 8, 15, 20, 21). What accounts for the ability of monocytes, but not granulocytes, to kill the HGE agent therefore remains enigmatic. TPA-induced phosphorylation of surface receptors, such as transferrin or other important signalling proteins, could have profound effects on the growth of an intracellular organism such as HGE (23, 24).

In contrast to the inhibition of HGE growth by both TPA and IFN-γ, VD3 in concentrations as high as 10−6 M did not result in resistance to infection. Treatment of HL-60 cells with VD3 induces many features of monocytic differentiation including phagocytosis, α-naphthyl acetate esterase activity, and the release of superoxide anions (15, 18). However VD3-treated cells continue to express granulocyte-specific markers such as CD44, FMC10, and FMC12, demonstrate less adherence, and continue to proliferate (4, 18). Thus, VD3 treatment results in a less differentiated phenotype than TPA treatment—a feature that has been attributed to the induction of distinct protein kinase C signal pathways (22).

The greater degree of terminal macrophage differentiation achieved with TPA was accompanied by the highest degree of resistance to HGE infection. Similarly, marrow-derived monocytic cells support the growth of the HGE agent (14) but peripheral blood monocytes have not been observed to be infected in vivo. Our observations suggest that through differentiation and/or activation, permissive monocytes can acquire the ability to kill the organism. Activated monocytes are therefore likely to be important effectors in the host response to HGE.

The observed differentiation-specific susceptibility of HL-60 cells to infection by the HGE agent is remarkable in that it closely parallels events in natural infection. In addition, differentiation of HL-60 cells provides a model for further studies to define critical cellular and molecular determinants underlying the remarkably evolved and restricted tropism of this organism. A greater understanding of how, on the other hand, HGE is able to survive within the hostile environment of the granulocyte while, on the other hand, it can be killed by differentiated monocytic cells may help shed new light both on phagocyte function and on the mechanisms that intracellular pathogens adopt to evade host defenses.

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