Cutting Edge: Infection by the Agent of Human Granulocytic Ehrlichiosis Prevents the Respiratory Burst by Down-Regulating gp91phox

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The agent of human granulocytic ehrlichiosis (HGE) is an emerging tick-borne pathogen that resides in neutrophils and can be cultured in a promyelocytic (HL-60) cell line. In response to microbes, polymorphonuclear leukocytes normally activate the NADPH oxidase enzyme complex and generate superoxide anion (O$_2^-$). However, HL-60 cells infected with HGE bacteria did not produce O$_2^-$ upon activation with PMA. RT-PCR demonstrated that HGE organisms inhibited mRNA expression of a single component of NADPH oxidase, gp91 phox, and FACS analysis showed that plasma membrane-associated gp91 phox protein was reduced on the infected cells. Infection with HGE organisms also decreased gp91 phox mRNA levels in splenic neutrophils in a murine model of HGE, demonstrating this phenomenon in vivo. Therefore, HGE bacteria repress the respiratory burst by down-regulating gp91 phox, the first direct inhibition of NADPH oxidase by a pathogen. The Journal of Immunology, 2000, 164: 3946–3949.

Human granulocytic ehrlichiosis (HGE) is a newly described tick-borne disease that is caused by an obligate intracellular pathogen with a tropism for neutrophils (1, 2). Infection is often accompanied by fever, myalgia, and leukopenia and can sometimes result in death (1, 2). Morulae containing HGE bacteria can be found within the cytoplasm of bloodstream polymorphonuclear neutrophils (PMNs) during acute disease (1). Bone marrow progenitors (3), HL-60 cells (a promyelocytic tumor cell line), and C3H/HeN mice can become infected with the HGE agent, facilitating the in vitro and in vivo study of this pathogen (4, 5).

Neutrophils are primary effector cells in host defenses (6), and the respiratory burst that is initiated by NADPH oxidase plays a major role in microbial eradication (7). In resting cells, the four components of the inactive oxidase are unassembled: p47phox and p67phox are present in the cytosol and gp91phox and p22phox are in the plasma membrane (8–10). During activation, p47phox and p67phox, along with Rac2, translocate to the plasma membrane, where they associate with flavocytochrome b558, the key membrane-bound component that is composed of gp91 phox and p22 phox (7, 11). Formation of the complex is essential for superoxide anion (O$_2^-$) generation. Defects in oxidase activity, as demonstrated in chronic granulomatous disease, result in increased susceptibility to various infectious agents (12, 13). To survive, the agent of HGE must have evolved strategies to persist in this hostile environment. Indeed, HGE organisms reside in vacuoles that do not fuse with lysosomes, providing insight into one such tactic (14, 15). We now investigate the effect of HGE bacteria on the respiratory burst because of the paradox that this organism preferentially persists within neutrophils.

Materials and Methods

Cultivation of the HGE agent and superoxide release

HL-60 cells were cultured in Dulbecco’s medium with 20% FCS at 37°C in 5% CO$_2$ and infected with the HGE agent (4, 5). At 5 days, >90% of the cells contained morulae. In some assays, HL-60 cells were exposed to heat-killed HGE bacteria for 24 h or to supernatant (10 ml of supernatant from Ehrlichia-infected HL-60 cells) for 5 days. HL-60 cells (2 × 10$^5$/ml) were incubated at 37°C in 5% CO$_2$ with 1 μM retinoic acid and cultured for 6 days for maximum differentiation (3, 16, 17). Superoxide anion was measured in both control and infected (both uninduced and retinoic acid-induced) HL-60 cells. In some assays, cells were also treated with IFN-γ (1000 U/2 × 10$^5$ cells) for 48 h before the assay and then centrifuged at 500 × g for 10 min at 4°C to harvest the cells (18). In all assays, PMA (200 ng/ml) was used as a stimulating agent along with luminol and an enhancer of chemiluminescence, and superoxide anion was expressed in relative luminometer units (RLU). For the studies with HL-60 cells induced with IFN-γ, a Lumat LB 9501 luminometer (Wallac, Gaithersburg, MD) was used, and for the retinoic acid-differentiated HL-60 cells, a TD-20/20 luminometer (Promega, Madison, WI) was used. The RLU for the two machines are different and should not be directly compared.
RT-PCR detection of NADPH oxidase subunits and HGE bacteria in HL-60 cells
cDNA was prepared from 5 μg of total RNA using random primers, and PCR amplification was then performed (19). The reaction mixture contained 5 μl of 10× PCR buffer with MgCl₂, 1 μl of 10 mM dNTP, 4 μl of 20 μM primers, 0.5 μl of Taq polymerase (5 U/μl), and 2 μl of cDNA. For semiquantitative PCR, serial dilutions of the template were used. The primers were gp91phox (403 bp, 5'-GGTGCGCTGGATGGT-3'), 5'-TCTTCTCTCATGGGTGCA-3'), p22phox (325 bp, 5'-GTGTGTTTTGTGCCGCTGGAGT-3'), 5'-TGAGGCCGCTGGTATGGT-3'), p67phox (726 bp, 5'-CGAGGGACACCTGATAGA-3'), 5'-CATGGGAAACATGTTCA-3'), 5'-AGCGGGAAATCGTGCGTG-3'), 7D5 (403 bp, 5'-GCTGTTCAATGCTTGTGGCT-3'), 5'-AGTACCTGCTGAATCT-3'), HGE 16S rRNA (403 bp, 5'-ACCCAGCCAGCACTATGTGT-3'), p47phox (767 bp, 5'-GCTGTTCAATGCTTGTGGCT-3'), 5'-TGAGGCCGCTGGTATGGT-3'), 5'-TGGGCCGCTGGATGTGGT-3'), and β-actin (300 bp, 5'-AGCGGGAAATCTGCTGGT-3'), 5'-CAGGTTACATGTTGGTCG-3').

Flow cytometric analysis of plasma membrane-associated gp91phox protein
Plasma membrane-associated gp91phox protein was determined using mAb 7D5, both control and infected (treated with or without IFN-γ), were resuspended in PBS/1% FCS, and gp91phox protein was detected with mAb 7D5 and a fluorescein-conjugated goat-anti-mouse-IgG Ab (20). HL-60 cells stained with a control IgG1 mAb of the same isotype did not demonstrate binding (data not shown).

Infection of C3H mice with the HGE agent
Six-week-old C3H/HeN mice were housed in filter-framed cages. A volume of 0.1 ml of blood from an Ehrlichia-infected SCID mouse was used to inoculate groups of five C3H mice (4, 21). Mice were sacrificed at 2 and 8 days, and splenic neutrophils were used to examine gp91phox expression. Spleen cells from five mice were pooled and plated in flasks in RPMI with 10% FBS at 37°C, 5% CO₂. Nonadherent cells were removed after 1 h and subjected to negative selection using mouse anti-CD4, anti-CD8a, anti-B220, and anti-Pan-NK cells (PharMingen, San Diego, CA) and goat-anti-mouse-IgG bound to magnetic beads (Perspective Biosystems, Cambridge, MA). A total of 2 × 10⁶ neutrophils were used to isolate RNA that was then reverse transcribed to obtain cDNA. The primers for murine gp91phox were 5'-GTGACCCGAAGCTGATCCA-3' and 5'-TGTAGTACCTGAGGAA-3'.

Results and Discussion
The production of O₂⁻ in HL-60 cells infected with the HGE agent was first examined (Fig. 1). As expected, some O₂⁻ was detected in HL-60 cells activated with PMA (13). In contrast, HL-60 cells infected with Ehrlichia failed to produce O₂⁻ (Fig. 1). As a control, Escherichia coli did not inhibit O₂⁻ production (not shown). Cells were then stimulated with IFN-γ to increase NADPH oxidase activity. IFN-γ induced O₂⁻ levels in uninfected HL-60 cells but not in the Ehrlichia-infected cells (Fig. 1). Similar results were observed with HL-60 cells terminally differentiated into neutrophils with retinoic acid (Fig. 2). These data show that HGE bacteria inhibit the respiratory burst under a variety of conditions.

To explore the mechanism by which O₂⁻ was suppressed, the influence of HGE bacteria on the expression of genes encoding the multicomponent NADPH oxidase complex was examined by RT-PCR (Fig. 3). Similar levels of p22phox, p47phox, and p67phox mRNA were apparent in control and infected cells (Fig. 3a, lanes 1 and 2). In contrast, gp91phox mRNA was not present in HL-60 cells infected with live Ehrlichia. Infection with HGE bacteria also inhibited the expression of gp91phox mRNA in HL-60 cells that were differentiated into neutrophils using retinoic acid (Fig. 3a, lanes 3 and 4). gp91phox mRNA expression was not affected when HL-60 cells were exposed to heat-killed organisms, or incubated with medium from Ehrlichia-infected HL-60 cells (Fig. 3b), demonstrating that dead bacteria or a soluble factor secreted by the HGE agent were not capable of down-regulating gp91phox. Cells were then induced with IFN-γ to determine whether HGE bacteria could alter gp91phox mRNA levels under conditions of maximal stimulation (18). As expected, IFN-γ markedly increased gp91phox mRNA expression.
mRNA expression (Fig. 4a) in uninfected HL-60 cells. Lower levels of gp91phox mRNA were detected in the Ehrlichia-infected cells in response to IFN-γ than in uninfected cells. Serial dilution PCR analysis indicated that gp91phox mRNA was evident in Ehrlichia-infected cells when the cDNA template was used at a 1:4 dilution and in control cells at a 1:32 dilution (not shown). Ehrlichia mRNA was detected in these cells, verifying that the bacteria persisted (Fig. 4b). However, this lower level of gp91phox transcription could not reverse the respiratory burst arrest by Ehrlichia (Fig. 1). Therefore, additional Ehrlichia-induced effects must also contribute to respiratory burst inhibition.

gp91phox protein expression was then examined to determine whether HGE bacteria influenced formation of the NADPH oxidase complex on the plasma membrane. HL-60 cells were analyzed by FACS using mAb 7D5, which recognizes an extracellular epitope of the gp91phox protein (22). mAb 7D5 bound to stimulated HL-60 cells (lane 1), Ehrlichia-infected HL-60 cells (lane 2), and IFN-γ-stimulated Ehrlichia-infected HL-60 cells (lane 4). RT-PCR showing HGE bacterial mRNA (16S rRNA primers) load in unstimulated (lane 1) and IFN-γ-stimulated HL-60 cells (lane 2). One of four experiments with similar results is shown.

Inhibition of gp91phox mRNA expression by the HGE agent was then assessed in a murine model of granulocytic ehrlichiosis (4). As expected, morulae were evident during the first weeks of infection (4) and observed in 12% of the splenic neutrophils on 8 days. At 2 and 8 days, splenic neutrophils were examined for gp91phox expression (Fig. 6). gp91phox mRNA levels were lower in the infected tissue than in uninfected controls. gp91phox mRNA could be detected when the cDNA template was used at a dilution of 1:100 (barely visible) and 1:400 in mice infected with Ehrlichia for 2 and 8 days, respectively, and at a dilution of 1:1600 in uninfected mice. The disproportionate degree of suppression may be due to an Ehrlichia burden in some neutrophils that is too low for direct visualization. These data demonstrate that down-regulation of gp91phox mRNA levels also occurs in vivo.

Diverse pathogens, including Legionella pneumophila, Toxoplasma gondii, Chlamydia, Ehrlichia risticii (which infects macrophages), Entamoeba histolytica, and Leishmania, have been shown to inhibit the respiratory burst; however, the mechanism(s) is (are) not known (23–28). Suppression of NADPH oxidase activity by down-regulating expression of a critical subunit of the enzyme complex by HGE bacteria represents a new mechanism by which microbes circumvent the oxidant-generating respiratory burst. It is intriguing that Ehrlichia targets the gene, gp91phox, which is associated with chronic granulomatous disease (12), and suggests that HGE bacteria induces a transient state in which the host may be more susceptible to secondary infections. Understanding the biological basis of respiratory burst arrest by pathogens should facilitate the development of new strategies to prevent infectious diseases and modify inflammatory responses.

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References

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