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Cutting Edge: Infection by the Agent of Human Granulocytic Ehrlichiosis Prevents the Respiratory Burst by Down-Regulating *gp91^{phox}*[1]

Rila Banerjee,* Juan Anguita,* Dirk Roos,† and Erol Fikrig[2]*

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)[3] 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: *p47^{phox}* and *p67^{phox}* are present in the cytosol and *gp91^{phox}* and *p22^{phox}* are in the plasma membrane (8–10). During activation, *p47^{phox}* and *p67^{phox}*, 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 $\times$ 10^5 cells) for 48 h before the assay and then centrifuged at 500 $\times$ *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.

*Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520; and †Central Laboratory of the Netherlands Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

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[2] Address correspondence and reprint requests to Dr. Erol Fikrig, 608 Laboratory of Clinical Investigation, Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208031, New Haven, CT 06520-8031. E-mail address: erol.fikrig@yale.edu

[3] Abbreviations used in this paper: HGE, human granulocytic ehrlichiosis; PMN, polymorphonuclear neutrophil; RLU, relative luminometer unit.

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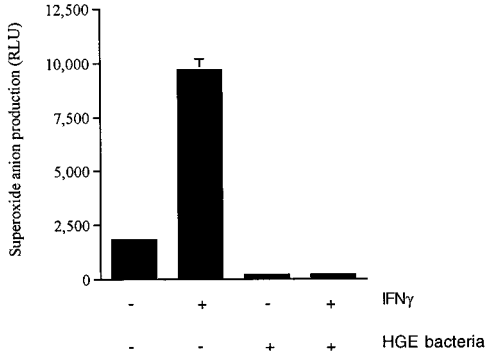

FIGURE 1. Superoxide anion formation in uninfected and *Ehrlichia*-infected HL-60 cells using a chemiluminescence assay. PMA was used as an activating agent in all assays. Data are presented in RLU. Results are the mean ± SDs of three experiments.

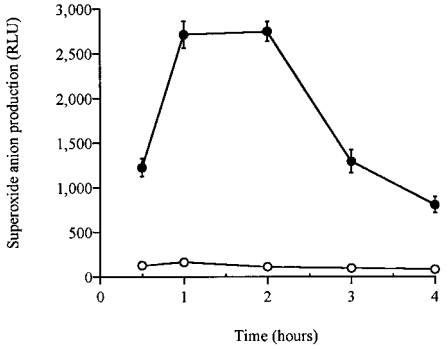

FIGURE 2. Time-course of superoxide anion formation in uninfected and *Ehrlichia*-infected HL-60 cells differentiated to neutrophils using retinoic acid. Cells were incubated with PMA for different time periods (0.5, 1, 2, 3, and 4 h), and O_2^- was expressed in RLU. Results are the mean ± SDs of three studies. ○, Infected cells; ●, uninfected cells.

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 $MgCl_2$, 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 *gp91^{phox}* (403 bp, 5′-GCTGTTCAATGCTTGTGGCT-3′, 5′-TCTCCTCATCATGGTGCACA-3′), *p22^{phox}* (325 bp, 5′-GT TTGTTTT GTGCCTGCTGGAGT-3′, 5′-TGGGCGGCTGCTTGATGGT-3′), *p67^{phox}* (726 bp, 5′-CGAGGGAACCAGCTGATAGA-3′, 5′-CATGGGAACACT GAGCTTCA-3′), *p47^{phox}* (767 bp, 5′-ACCCAGCCAGCACTATGTGT-3′, 5′-AGTAGCCTGTGACGTCGTCT-3′), *HGE 16S rRNA* (4) (250 bp, 5′-TG TAGGCGGTTCGGTAAGTTAAAG-3′, 5′-GCACTCATCGTTTACAGCG TG-3′), and β-actin (300 bp, 5′-AGCGGGAAATCGTGCGTG-3′, 5′-CAG GGTACATGGTGGTGCC-3′).

Flow cytometric analysis of plasma membrane-associated gp91^{phox} protein

Plasma membrane-associated gp91^{phox} protein was determined using mAb 7D5. HL-60 cells (10^7/ml), both control and infected (treated with or without IFN-γ), were resuspended in PBS/1% FCS, and gp91^{phox} 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 as mAb 7D5 were used for comparison and 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 *gp91^{phox}* expression. Spleen cells from five mice were pooled and plated in flasks in RPMI with 10% FBS at 37°C, 5% CO_2. 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 2×10^6 neutrophils were used to isolate RNA that was then reverse transcribed to obtain cDNA. The primers for murine *gp91^{phox}* were 5′-GTCAAGTGCCCCAAGGTATCCA-3′ and 5′-TTGTAGCTGA GGAAGTTGGC-3′.

Results and Discussion

The production of O_2^- in HL-60 cells infected with the HGE agent was first examined (Fig. 1). As expected, some O_2^- was detected in HL-60 cells activated with PMA (13). In contrast, HL-60 cells infected with *Ehrlichia* failed to produce O_2^- (Fig. 1). As a control, *Escherichia coli* did not inhibit O_2^- production (not shown). Cells were then stimulated with IFN-γ to increase NADPH oxidase activity. IFN-γ induced O_2^- 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 O_2^- 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 *p22^{phox}*, *p47^{phox}*, and *p67^{phox}* mRNA were apparent in control and infected cells (Fig. 3*a*, *lanes 1* and *2*). In contrast, *gp91^{phox}* mRNA was not present in HL-60 cells infected with live *Ehrlichia*. Infection with HGE bacteria also inhibited the expression of *gp91^{phox}* mRNA in HL-60 cells that were differentiated into neutrophils using retinoic acid (Fig. 3*a*, *lanes 3* and *4*). *gp91^{phox}* 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. 3*b*), demonstrating that dead bacteria or a soluble factor secreted by the HGE agent were not capable of down-regulating *gp91^{phox}*. Cells were then induced with IFN-γ to determine whether HGE bacteria could alter *gp91^{phox}* mRNA levels under conditions of maximal stimulation (18). As expected, IFN-γ markedly increased *gp91^{phox}*

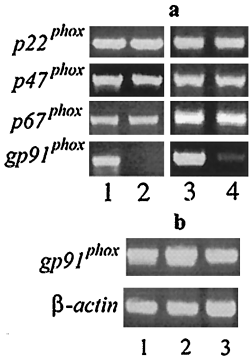

FIGURE 3. Effect of infection with the HGE agent on the expression of mRNA for NADPH oxidase components. *a*, RT-PCR using primers for *p22^{phox}*, *p47^{phox}*, *p67^{phox}*, and *gp91^{phox}* with uninfected HL-60 cells (*lane 1*), HGE bacteria-infected HL-60 cells (*lane 2*), uninfected retinoic acid-differentiated HL-60 cells (*lane 3*), and HGE bacteria-infected retinoic acid-differentiated HL-60 cells (*lane 4*). *b*, Expression of *gp91^{phox}* mRNA in HL-60 cells (*lane 1*), HL-60 cells treated with heat-killed HGE organisms (*lane 2*), and HL-60 cells grown in medium from *Ehrlichia*-infected HL-60 cells (*lane 3*). β-actin levels were measured as a control. One of five experiments with similar results is shown.

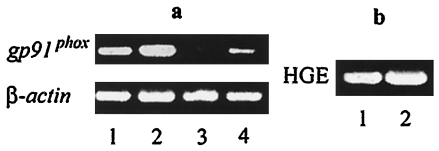

FIGURE 4. Effect of IFN-γ on the expression of *gp91^{phox}* mRNA in uninfected and Ehrlichia-infected HL-60 cells. *a,* RT-PCR analysis of *gp91^{phox}* mRNA expression in HL-60 cells (lane 1), IFN-γ-stimulated HL-60 cells (*lane 2*), *Ehrlichia*-infected HL-60 cells (*lane 3*), and IFN-γ-stimulated *Ehrlichia*-infected HL-60 cells (*lane 4*). *b,* 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.

mRNA expression (Fig. 4*a*) in uninfected HL-60 cells. Lower levels of *gp91^{phox}* mRNA were detected in the *Ehrlichia*-infected cells in response to IFN-γ than in uninfected cells. Serial dilution PCR analysis indicated that *gp91^{phox}* 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. 4*b*). However, this lower level of *gp91^{phox}* transcription could not reverse the respiratory burst arrest by *Ehrlichia* (Fig. 1). Therefore, additional *Ehrlichia*-induced effects must also contribute to respiratory burst inhibition.

gp91^{phox} 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 extracytoplasmic epitope of the gp91^{phox} protein (22). mAb 7D5 bound to uninfected but not to *Ehrlichia*-infected HL-60 cells (Fig. 5). HL-60 cells further stimulated with IFN-γ demonstrated a large increase in mAb 7D5 binding, and only very weak mAb 7D5 reactivity was observed in *Ehrlichia*-infected, IFN-γ-induced cells (Fig. 5). Therefore, infection with the HGE agent reduced gp91^{phox} protein on the plasma membrane.

Inhibition of *gp91^{phox}* 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

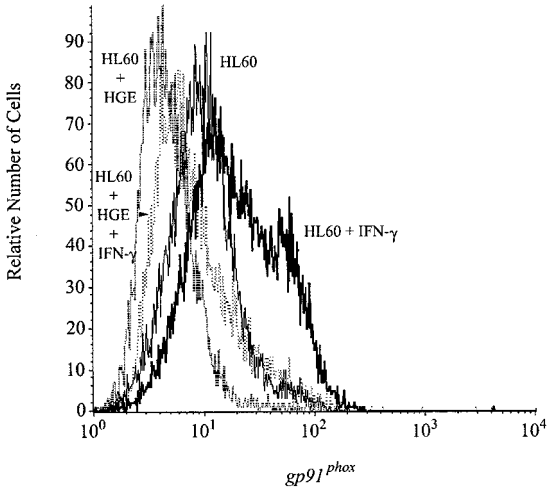

FIGURE 5. Flow cytometric analysis of plasma membrane associated *gp91^{phox}* protein expression in control and HGE-infected HL-60 cells. HL-60 cells (control or infected) were treated with or without IFN-γ and then probed with mAb 7D5. One of four experiments with similar results is shown.

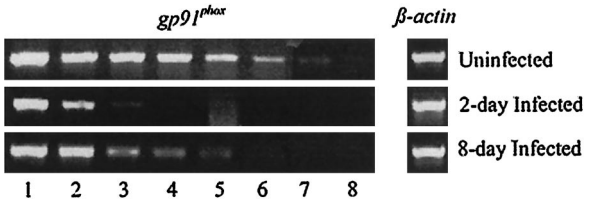

FIGURE 6. The influence on HGE bacteria on *gp91^{phox}* mRNA expression in vivo. RT-PCR analysis of expression of *gp91^{phox}* mRNA in the splenic neutrophils of groups of five uninfected C3H mice, and mice infected with Ehrlichia for 2 days and 8 days are shown. β-actin mRNA levels were measured as a control. cDNAs from the neutrophils were serially diluted, and PCR was performed. Dilutions: *lane 1,* 1:1; *lane 2,* 1:10; *lane 3,* 1:100; *lane 4,* 1:200; *lane 5,* 1:400; *lane 6,* 1:800; *lane 7,* 1:1600; and *lane 8,* 1:3200.

days. At 2 and 8 days, splenic neutrophils were examined for *gp91^{phox}* expression (Fig. 6). *gp91^{phox}* mRNA levels were lower in the infected tissue than in uninfected controls. *gp91^{phox}* 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 *gp91^{phox}* 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, *gp91^{phox}*, 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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