Gallium Disrupts Iron Metabolism of Mycobacteria Residing within Human Macrophages

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Received 6 January 2000/Returned for modification 18 February 2000/Accepted 29 June 2000

Mycobacterium tuberculosis and M. avium complex (MAC) enter and multiply within monocytes and macrophages in phagosomes. In vitro growth studies using standard culture media indicate that siderophore-mediated iron (Fe) acquisition plays a critical role in the growth and metabolism of both M. tuberculosis and MAC. However, the applicability of such studies to conditions within the macrophage phagosome is unclear, due in part to the absence of experimental means to inhibit such a process. Based on the ability of gallium (Ga3+) to concentrate within mononuclear phagocytes and on evidence that Ga disrupts cellular Fe-dependent metabolic pathways by substituting for Fe3+ and failing to undergo redox cycling, we hypothesized that Ga could disrupt Fe acquisition and Fe-dependent metabolic pathways of mycobacteria. We found that Ga(NO3)3 and Ga-transferrin produce an Fe-reversible concentration-dependent growth inhibition of M. tuberculosis strains and MAC grown extracellularly and within human macrophages. Ga is bactericidal for M. tuberculosis growing extracellularly and within macrophages. Finally, we provide evidence that exogenously added Fe is acquired by intraphagosomal M. tuberculosis and that Ga inhibits this Fe acquisition. Thus, Ga(NO3)3 disruption of mycobacterial Fe metabolism may serve as an experimental means to study the mechanism of Fe acquisition by intracellular mycobacteria and the role of Fe in intracellular survival. Furthermore, given the inability of biological systems to discriminate between Ga and Fe, this approach could have broad applicability to the study of Fe metabolism of other intracellular pathogens.

Fe is critical for the metabolism and growth of most microorganisms. Limitation of Fe availability is utilized by many animal species, including humans, as a means of host defense (20, 29). Chelation of Fe to proteins such as transferrin markedly decreases its accessibility to pathogenic microbes that grow and replicate extracellularly (3). Beyond this, infection leads to a shift of extracellular Fe from serum to the reticuloendothelial system. Microbial pathogens utilize several distinct means to counteract this strategy and obtain extracellular Fe from the host. Among these is siderophore production (39, 41).

Not all pathogens grow and replicate extracellularly. Mycobacterium tuberculosis and M. avium complex (MAC) are among a number of human intracellular pathogens that enter and multiply within monocytes and macrophages. Fe is necessary for mycobacterial growth in vitro culture media, and siderophore production is felt to be critical in this process (13, 53). Mycobacterium and MAC generally produce two types of siderophores, exochelins (also referred to as water-soluble mycobactins) and mycobactins (1, 13, 24, 25, 49, 53). Exochelins are hydrophilic high-affinity Fe3+ chelators which are secreted (24, 25, 48, 55). Mycobactins are hydrophobic siderophores that are associated with the bacterial cell membrane (24). Mycobacterial Fe acquisition is postulated to involve the acquisition of Fe from host high-affinity Fe-binding molecules such as transferrin by exochelin, followed by transfer of this Fe to mycobactin for subsequent internalization (24, 25). Extracellular transferrin has been shown to traffic to the M. tuberculosis-containing phagosome (10, 50), but there is no conclusive evidence that M. tuberculosis acquires Fe bound to this extraacellular protein during intracellular growth.

Most evidence that mycobacteria residing within human macrophages require a source of Fe has been indirect through studies with other intracellular pathogens in which the host cell Fe pool has been decreased or enhanced through the addition of Fe chelators or Fe supplementation of culture medium, respectively (5, 37). Conclusions drawn from such approaches may be problematic since they mediate their effects through modulations of host cell physiology rather than by directly altering microbial access to Fe. The ability to investigate Fe acquisition mechanism(s) of mycobacteria and other intracellular pathogens residing within macrophages, as well as the role of these processes in the pathogenesis of infection with such organisms, would be greatly facilitated by the development of new strategies to disrupt Fe acquisition by such bacteria.

Gallium (Ga), a group IIIA metal, particularly in the form of Ga nitrate [Ga(NO3)3], is preferentially taken up by phagocytes at sites of inflammation (52) and by certain neoplastic cells, for which it is cytotoxic (22, 31, 32, 42, 47, 51). The biological and therapeutic effects of Ga3+ appear to relate to its ability to substitute for Fe3+ in many biomolecular processes, thereby disrupting them (8, 27). Ga3+, like Fe3+, enters mammalian cells, including macrophages, via both transferrin-dependent and transferrin-independent Fe uptake mechanisms (9, 40). In rapidly dividing tumor cells (as opposed to terminally differentiated cells such as macrophages), Ga interferes with cellular DNA replication via its ability to substitute for Fe in ribonucleotide reductase, resulting in enzyme inactivation due to the fact that Ga, unlike Fe, is unable to undergo redox cycling (8).

Based on (i) the ability of Ga to concentrate within mononuclear phagocytes and (ii) evidence that Ga disrupts Fe-de-
pendent metabolic pathways, we hypothesized that Ga could serve as an experimental tool to disrupt acquisition and utilization of Fe by mycobacteria residing within human macrophages. Here we demonstrate that Ga-containing compounds inhibit the growth of *M. tuberculosis* and MAC regardless of whether they are growing extracellularly or within human macrophages. The mechanism appears to involve disruption of mycobacterial Fe-dependent metabolism. Furthermore, we provide the first definitive evidence for the acquisition of Fe from extracellular transferrin by intraphagosomal mycobacteria and demonstrate that Ga significantly decreases this process.

**MATERIALS AND METHODS**

**Myobacteria.** *M. tuberculosis* Erdman (American Type Culture Collection [ATCC] 35801, a virulent strain) and H37Ra (ATCC 25177, an attenuated strain) were cultivated and harvested to form predominantly single-cell suspensions (45). A multidrug-resistant (MDR) isolate of *M. tuberculosis* (100% resistant to isoniazid and rifampin) was obtained from the State Hygienic Laboratory (University of Iowa, Iowa City, Iowa). The two MAC strains used were ATCC 25291 (MAC 1) and a clinical isolate obtained from the Clinical Microbiology Laboratory of the University of Iowa Hospitals and Clinics (MAC 2).

**Macrophage culture.** Monocyte-derived macrophages (MDMs) and human alveolar macrophages (HAMs) were obtained as described elsewhere (23) from healthy adult volunteers who were purified protein derivative negative and had no history of mycobacterial infection. MDM monolayers (1.0 × 10^5 MDMs/well) were prepared on day 5 and incubated for an additional 7 days at 37°C in RPMI supplemented with 20% autologous serum in order to stabilize the monolayer for subsequent incubation with mycobacteria. HAM monolayers were formed as for MDMs and were used immediately.

**Analysis of extracellular mycobacterial growth.** Mycobacteria (3 × 10^7) were inoculated into BACTEC 12b broth culture bottles in the BACTEC 460TB system (Becton Dickinson Diagnostic Instrument System, Sparks, Md.) from Teflon wells on day 5 and then incubated for an additional 7 days at 37°C in RPMI supplemented with 20% autologous serum in order to stabilize the monolayer for subsequent incubation with mycobacteria. HAM monolayers were formed as for MDMs and were used immediately.

**Intracellular mycobacterial growth.** Mycobacteria (3 × 10^7) were inoculated into BACTEC 12b broth culture bottles in the BACTEC 460TB system (Becton Dickinson Diagnostic Instrument System, Sparks, Md.) from Teflon wells on day 5 and then incubated for an additional 7 days at 37°C in RPMI supplemented with 20% autologous serum in order to stabilize the monolayer for subsequent incubation with mycobacteria. HAM monolayers were formed as for MDMs and were used immediately.

**To determine whether Ga is bactericidal for extracellular *M. tuberculosis*, Erdman strain *M. tuberculosis* (10^5 bacteria in 1 ml of 7H9 medium without added Fe) was placed in Teflon wells overnight at 37°C. Ga(NO_3)_3 (0 to 80 μM) was then added. Teflon wells were vortexed briefly each day; after 72 h the bacteria were resuspended by pipetting, and aliquots were removed to assess bacterial growth by counting the CFU after 2 weeks (reassessed after 5 weeks).

**Analysis of the growth of mycobacteria in macrophages.** MDM or HAM monolayers were incubated with mycobacteria (bacterium/macrophage ratios of 1:3 to 1:5) for 2 h in RPMI containing 10 mM Hepes and 1 mg of human serum albumin (HSA; Calbiochem, La Jolla, Calif.) per ml, followed by washing to remove nonadherent bacteria. Monolayers were then covered with repletion medium (RPMI containing 1% autologous serum and, in the case of HAMs, supplemented with 2,000 U of penicillin per ml). Twenty-four hours later, Ga(NO_3)_3 at various concentrations was added. In some experiments, Ga(NO_3)_3 or Ga-transferrin was added either in combination with or 24 h following the addition of human recombinant gamma interferon (IFN-γ; Genzyme, Cambridge, Mass.) (100 U/ml). In each experiment, mycobacterium-containing macrophage monolayers devoid of inhibitors were included as controls. The supernatant from each well was removed, and cold sterile water (300 μl) was added to the monolayer. After 10 min with periodic agitation, 7H9 culture broth (600 μl) was added to the monolayer, followed by lysis with 0.25% SDS in phosphate-buffered saline (pH 7.4 with saturated aqueous NaHCO_3, and the mixture was incubated overnight at 80°C. The bacterial suspension released from the MDMs was diluted fivefold, and 1 ml was spotted onto a piece of nitrocellulose. The nitrocellulose was air dried in the biosafety cabinet and then wrapped in a plastic sheet. The nitrocellulose was exposed to film at −80°C.

**Intracellular “mixing” experiment.** Uninfected MDM monolayers pulsed with 10 μM [59Fe]citrate for 24 h were lysed as described above and mixed with lysates of *M. tuberculosis*-infected MDMs that had not been pulsed with [59Fe]. *M. tuberculosis* bacilli were then filtered through the centrifuge tube filter as described above, and the amount bacterium-associated [59Fe] was determined.

**To determine whether Ga inhibits mycobacterial growth in macrophages.** To determine the morphology of broth-grown (extracellular) and intracellular *M. tuberculosis* released from MDMs in the presence or absence of Ga(NO_3)_3, scanning electron microscopy (SEM) was performed. H57RA *M. tuberculosis* in 7H9 broth was inoculated in the absence or presence of Ga(NO_3)_3 (250 μM). For 24 h, the medium was washed, and filtered through the centrifuge tube filter as described above. Bacilli on the filter were fixed in 2.5% glutaraldehyde in 0.1% cacoodylate buffer for 1 h at room temperature, postfixed in 1% buffered osmium tetroxide, dehydrated through a graded series of acetone, critical-point dried, and sputter coated with a 60/40 mixture of gold and palladium. Specimens were imaged using a Hitachi S-4000 FESEM.

**Measurement of the growth of Fe and Ga from broth medium by BACTEC culture.** To determine the effect of Ga on the growth of *M. tuberculosis* and MAC, we inoculated 3.0 × 10^7 mycobacteria into BACTEC bottles and monitored bacterial growth over time in the absence or presence of Ga(NO_3)_3. A concentration-dependent growth inhibition of mycobacteria was observed in the presence of Ga(NO_3)_3 (Fig. 1). This growth inhibition was observed regardless of whether we used an attenuated strain of *M. tuberculosis* (bacterium/MDM ratio of 1:1) exactly as described above. Ga(NO_3)_3 (500 μM) was added 24 h later. Bacterial growth was assessed by measuring CFU at 0, 2, 4, 6, 8, and 10 days postaddition of Ga using the combined cell lysates, and supernatants were also assessed as described above.

**Measurement of the acquisition of extracellular Fe by intracellular *M. tuberculosis*.** MDM monolayers, formed in four-well tissue culture plates (2.0 × 10^6 MDMs/well), were incubated with or without (control) H37Ra *M. tuberculosis* for 2 h, washed, and covered with repletion medium. After 48 h, 10 μM [59Fe]citrate (0.1 μCi/ml; Amersham, Arlington Heights, Ill.; 14.2 μCi/mg; NEN Life Science Products, Inc., Boston, Mass.) complexed to transferrin was added in the presence or absence of Ga(NO_3)_3. After 12 and/or 24 h, the monolayer was lysed in 0.1% SDS in the presence of DNase (200 μg/ml), and *M. tuberculosis* bacilli were released. The released bacteria were combined with the supernatant, washed in RPMI containing 0.01% SDS, resuspended, and filtered through a 0.22-μm (pore-sized) Spin-X centrifuge tube filter (Costar Corp., Cambridge, Mass.). The filter was placed in an O-ringed tube, and bacterium-associated [59Fe] was then determined by using a gamma counter. Initial experiments revealed that approximately 86% of [59Fe]was associated with the MDMD lysis; and <1% was associated with a pellet derived from the supernatant devoid of MDMs. Thus, ≥99% of [59Fe] was MDM associated. In certain experiments, M. tuberculosis released from MDMs pulsed with [59Fe] were subjected to autoradiography. In these experiments, the bacterial suspension released from the MDMs was diluted fivefold, and 1 ml was spotted onto a piece of nitrocellulose. The nitrocellulose was air dried in the biosafety cabinet and then wrapped in a plastic sheet. The nitrocellulose was exposed to film at −80°C.

**RESULTS**

**Ga inhibits the growth of mycobacteria in broth culture.** To determine the effect of Ga on the growth of *M. tuberculosis* and MAC, we inoculated 3.0 × 10^7 mycobacteria into BACTEC bottles and monitored bacterial growth over time in the absence or presence of Ga(NO_3)_3. A concentration-dependent growth inhibition of mycobacteria was observed in the presence of Ga(NO_3)_3 (Fig. 1). This growth inhibition was observed regardless of whether we used an attenuated strain of *M. tuberculosis*.
M. tuberculosis (H37Ra), the virulent Erdman strain of M. tuberculosis, MDR M. tuberculosis, or MAC.

Ga and Fe compete for acquisition by mycobacteria. Previous data with both eukaryotic and prokaryotic systems (8, 18, 22, 27, 51) led us to hypothesize that the inhibitory effect of Ga on mycobacterial growth occurs via disruption of bacterial Fe metabolism. Because of the high and variable amount of Fe we measured in commercial BACTEC medium (0.25 to 1.6 mM), we could not test this hypothesis directly in the BACTEC system. Therefore, mycobacteria were cultivated in defined 7H9 medium in which the basal Fe content was controlled. In this defined medium in which the Fe content (2 μM) is closer to that which occurs in vivo (43), much lower concentrations of Ga(NO₃)₃ markedly inhibited mycobacterial growth than those required using the BACTEC system, and the extent of growth inhibition increased over time (Fig. 2A). The 50% inhibitory concentration was approximately 1.25 to 2.5 μM at 72 h of Ga exposure. Importantly, the effect of Ga was prevented by the addition of exogenous Fe in a concentration-dependent manner (Fig. 2B).

These data are consistent with the ability of Ga to inhibit Fe-dependent processes. We next assessed the ability of M. tuberculosis to acquire Ga and Fe. M. tuberculosis was suspended in 7H9 medium without added Fe or in OADC and increasing concentrations of either ⁶⁷Ga or ⁵⁹Fe. Cultures were incubated for defined time periods, following which the amount of bacterium-associated ⁶⁷Ga or ⁵⁹Fe was determined.

FIG. 1. Ga(NO₃)₃ inhibits the growth of M. tuberculosis and MAC in broth culture. A total of 3.0 × 10⁵ mycobacteria (Erdman M. tuberculosis, H37Ra M. tuberculosis, an MDR M. tuberculosis clinical isolate, or two MAC isolates) were inoculated into BACTEC 12B bottles in the presence or absence (control) of the indicated concentrations of Ga(NO₃)₃. In panel A, growth is expressed in Growth Index Units. The results are presented as the mean ± the standard deviation (SD) for triplicate bottles for a representative experiment. In panel B, cumulative data are expressed as the percentage of control (absence of Ga). Each datum point represents the mean ± the SEM of two to seven independent experiments, each of which was done in duplicate or triplicate.

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FIG. 2. Low concentrations of Ga(NO₃)₃ inhibit the growth of M. tuberculosis under physiologic Fe conditions; the inhibition of growth is prevented in the presence of excess Fe. (A) Erdman M. tuberculosis (10⁶/ml) was incubated in 7H9 medium without added OADC and Fe in the presence of the indicated concentrations of Ga(NO₃)₃. At defined time points aliquots of bacterial suspensions were inoculated into duplicate BACTEC 12B bottles, and the subsequent growth index was determined. The cumulative data for the indicated concentrations of Ga(NO₃)₃ at 24, 48, and 72 h are shown and represent the mean ± the SEM of three independent experiments. (B) Erdman M. tuberculosis (10⁶/ml) was incubated in 7H9 medium without added ODAC and Ga, to which was added 10 μM Ga(NO₃)₃ and increasing concentrations of Fe-citrate. At 72 h bacterial suspensions were inoculated into BACTEC 12B bottles, and the subsequent growth index was determined. The results shown (mean ± the SD) are from a representative experiment (n = 2). Fe was also found to reverse the growth-inhibitory effect of Ga(NO₃)₃ on Erdman M. tuberculosis and MAC when the experiments were performed in BACTEC bottles (high-Fe-containing medium) (data not shown).
Mycobacterial acquisition of both Ga and Fe was readily demonstrable. At all time points up to 8 h, the uptake of Fe exceeded that of Ga. At 8 h of incubation with a 16 μM concentration of each metal, 115.8 ± 28.5 fmol of Fe and 3.38 ± 0.41 fmol of Ga were associated with 10^6 bacteria, respectively (n = 3). Thus, the bacteria appear to have a greater capacity for Fe accumulation than for Ga accumulation.

We next assessed the ability of Ga to compete for the acquisition of Fe by *M. tuberculosis* and vice versa. *M. tuberculosis* was suspended in 7H9 medium, to which was added either ^67^Ga or ^59^Fe in the absence or presence of increasing concentrations of either "cold" Fe or Ga. Cultures were incubated for defined time periods following which the amount of bacterium-associated metal was determined. Whereas Ga was highly effective in competing for the acquisition of Fe by *M. tuberculosis*, Fe was relatively ineffective in competing for Ga acquisition (Fig. 3).

Ga compounds inhibit the growth of mycobacteria in macrophages. The critical site of growth of mycobacteria in vivo is within host macrophages. Analogous to the results with the broth culture experiments described above, Ga(NO_3)_3 inhibited mycobacteria growing within both human MDMs and HAMs in a concentration-dependent (Fig. 4) and time-dependent manner. Although mycobacterial growth was inhibited at 24 h (34 ± 5%, n = 8, for the Erdman strain; 50 ± 18%, n = 3, for the H37Ra strain), more-striking inhibition was observed 48 h (77 ± 4% for Erdman and 77 ± 1% for H37Ra) and 72 h (92 ± 3% for Erdman and 83 ± 12% for H37Ra) after the addition of Ga. NaNO_3 (1.5 mM, equal to that present in 500...
mM Ga(NO$_3$)$_3$ had no effect on mycobacterial growth in macrophages (data not shown), indicating that the Ga$^{3+}$ and not the NO$_3^-$ was responsible for the growth inhibitory activity of Ga(NO$_3$)$_3$. *M. tuberculosis*-infected monolayers were lysed over time in culture due to bacterial multiplication. In contrast, Ga(NO$_3$)$_3$-treated macrophage monolayers harboring *M. tuberculosis* remained more intact over the same time period (Fig. 5). In the absence of mycobacteria, Ga(NO$_3$)$_3$ at concentrations of up to 2 mM did not influence the density of the macrophage monolayer for up to 37 days as viewed by inverted phase microscopy.

Recent findings provide evidence that exogenous transferrin is transported to the *M. tuberculosis*-containing phagosome by phagosome–early-endosome fusion (10, 50). Since transferrin is capable of chelating Ga analogous to Fe and is the major physiologic chelate, we assessed the ability of Ga-transferrin to inhibit mycobacterial growth within human macrophages. The Ga-transferrin complex proved to be as effective as Ga(NO$_3$)$_3$ in inhibiting mycobacterial growth in both liquid media and within human macrophages. In macrophages, 72 h of exposure to Ga-transferrin (62.5 μM) inhibited *M. tuberculosis* growth by 50.7 ± 17.3% (mean ± the standard error of the mean [SEM], n = 5), MAC by 32.5 ± 0.2% (mean ± SEM, n = 2), and MDR *M. tuberculosis* by 73.0 ± 9.6% (mean ± SEM, n = 2).

Secretion of IFN-γ is important in regulating the immune response during tuberculosis (12, 21, 28, 38). IFN-γ downregulates macrophage ferritin levels and expression of the transferrin receptor (4). Thus, we reasoned that IFN-γ might reduce the ability of Ga to inhibit the growth of *M. tuberculosis* in macrophages. In agreement with published literature (14), IFN-γ alone did not inhibit growth of *M. tuberculosis* in human macrophages. *M. tuberculosis* growth in IFN-γ-treated MDMs was 90.6 ± 4.7% of control (mean ± SEM, n = 4) at 72 h. Preincubation of macrophages with IFN-γ did not influence the ability of Ga(NO$_3$)$_3$ or Ga-transferrin to inhibit growth of *M. tuberculosis* in these cells. The growth of *M. tuberculosis* in Ga-treated (100 μM) MDMs was reduced to 8.3 ± 3.1% and 13.8 ± 9.1% (mean ± SEM, n = 4) of the non-Ga-treated control cells when the MDMs were or were not exposed to IFN-γ over a range of concentrations (10 to 1,000 U/ml), respectively. Thus, the effects of Ga in the form of Ga(NO$_3$)$_3$ or Ga-transferrin on *M. tuberculosis* growth were not influenced by treatment of MDMs with IFN-γ.

Overall, these data provide evidence that Ga effectively inhibits the growth of two mycobacterial species in both simple and complex broth culture media and in two different types of human macrophages (MDMs and HAMs).

Ga is bactericidal for mycobacteria growing within macrophages. Lack of growth in the BACTEC system may occur as a result of either bacterial survival without multiplication or bacterial death. To distinguish between these possibilities, we evaluated the influence of Ga(NO$_3$)$_3$ on the viability of *M. tuberculosis* in macrophages using a CFU assay. As shown in Table 1, there was a progressive decline in the number of CFUs with Ga treatment over 10 days. The number of CFU in control wells (without Ga) continued to increase over this time.
period. Even by day 2, the CFU in Ga-containing wells fell below the initial amount of bacteria ($4.3 \times 10^4$ in the experiment shown in Table 1) in the macrophages, a finding consistent with bacterial killing. By day 10, the CFU count decreased by nearly 2 logs compared to the initial CFU count. Thus, Ga exposure exerts bactericidal activity against mycobacteria growing within macrophages. CFU studies also revealed that Ga was bactericidal in broth culture. In the presence of 80 $\mu$M Ga(NO$_3$)$_3$ for 72 h, CFU numbers for Erdman *M. tuberculosis* decreased from $(4.5 \pm 0.8) \times 10^6$ to $(1.7 \pm 0.1) \times 10^6$ ($n = 2$).

**Ga disrupts Fe acquisition of intracellular *M. tuberculosis***. There is transport of exogenously added transferrin to the *M. tuberculosis*-containing phagosome of phagosomes of macrophages, suggesting that this may be an important source of Fe for the organism growing intracellularly (10, 50). We hypothesized that Ga would compete with Fe and therefore inhibit Fe uptake by mycobacteria dividing within the phagosome.

In order to test this hypothesis, we developed a method to quantitate the acquisition of extracellular Fe bound to transferrin by *M. tuberculosis* located within the macrophage phagosome. [$^{59}$Fe]transferrin was added to MDM monolayers in the presence or absence of Ga-transferrin 2 days following the addition of H37Ra *M. tuberculosis*. After an additional 12 and 24 h, the monolayers were lysed and *M. tuberculosis* bacilli were released from the phagosome by detergent treatment, washed, and trapped during filtration on a 0.22-$\mu$m (pore-size) filter. [$^{59}$Fe] was detected on the filter from *M. tuberculosis*-infected MDMs prior to filtration contained slightly less than at 12 h after addition (data not shown). Together, these data provide further evidence that the [$^{59}$Fe] associated with the filter from lysed *M. tuberculosis*-infected macrophages was from the bacteria and not from host cell proteins or organelles which had become adherent to the bacteria during the lysis-centrifugation process.

Periods of control experiments were undertaken to examine whether 59Fe complexed to host cell proteins bound nonspecifically to bacteria during the lysis process. Bacteria recovered from MDMs which had not been incubated with [$^{59}$Fe]transferrin or Ga were mixed on the day of harvesting with lysates from uninfected MDMs which had been incubated for 24 h with [$^{59}$Fe]transferrin. Under this condition, the counts detected from isolated bacteria on the filter (89 $\pm$ 43 cpm [mean $\pm$ SEM], $n = 2$) did not differ significantly from the counts on the filter from uninfected MDMs (208 $\pm$ 94 cpm [mean $\pm$ SEM], $n = 7$). Scanning EM of filters containing embedded bacilli revealed intact organisms with no evidence of associated cellular organelles or debris which could contain macrophage-derived Fe-containing molecules (Fig. 6).

### DISCUSSION

Although it has been proposed that Fe metabolism is important in the pathogenesis of infection with intracellular pathogens such as *M. tuberculosis* and MAC, the evidence for this has been for the most part indirect. Cultivation of intracellular pathogens such as mycobacteria in microbiologic media clearly requires the presence of exogenous Fe. Under these conditions, the mycobacteria are felt to acquire Fe through the use of siderophore-based system (1, 24, 25, 49, 53). However, the potential sources of Fe to which mycobacteria have access when residing within a macrophage phagosome are likely to be quite different from those available when they grow extracellularly. Also, whether bacilli acquire Fe while residing in a macrophage phagosome or depend on Fe stores accumulated during their extracellular phase to meet their metabolic needs is unknown. Transferrin has been found to traffic from the extracellular space to the phagosome (10, 50), but it is not known whether Fe initially complexed to the protein also makes the journey or, if it does, whether the organism has the capacity to acquire it.

In order to address such issues, experimental means to modulate intraphagosomal mycobacterial Fe metabolism are needed. Approaches used in studies of extracellular pathogens, such as the use of Fe-depleted growth media or the addition of Fe chelating agents, are suboptimal for the study of intracellular pathogens. Fe-limited culture media may not significantly

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**TABLE 1. Ga(NO$_3$)$_3$ kills *M. tuberculosis* growing within human macrophages**

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<tr>
<th>Test group</th>
<th>Mean CFU ± SD at (days post-Ga):</th>
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<tbody>
<tr>
<td>Control</td>
<td>$(4.3 \pm 0.4) \times 10^4$</td>
</tr>
<tr>
<td>Ga(NO$_3$)$_3$</td>
<td>$(1.2 \pm 0.2) \times 10^4$</td>
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<tr>
<td></td>
<td>$(8.0 \pm 1.7) \times 10^4$</td>
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<td>$(7.3 \pm 1.8) \times 10^4$</td>
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<td>$(6.0 \pm 0.1) \times 10^4$</td>
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<td>$(3.0 \pm 0.0) \times 10^3$</td>
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<td>$(6.3 \pm 1.8) \times 10^2$</td>
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*Data represent the mean ± the SD of duplicate samples from a representative experiment ($n = 3$). Harvested combined supernatants and cell lysates from wells were plated on 7H11 agar in serial dilutions, and CFU were counted after 2 weeks.*
alter intracellular Fe stores in macrophages. Many Fe chelating agents (e.g., deferoxamine) penetrate cells poorly (6) and thus have variable access to intracellular mycobacteria.

Because of its ability to be readily taken up by macrophages and its known ability to compete with Fe in biologic systems, we examined the potential for Ga to be used as a means to modulate the Fe metabolism of mycobacteria (both *M. tuberculosis* and MAC). The incorporation of Ga into specific Fe-dependent enzymes leads to the inactivation of these enzymes because Ga\(^{3+}\), in contrast to Fe\(^{3+}\), is not able to undergo redox cycling (2). The cumulative data reported here demonstrate that Ga inhibits the growth of two mycobacterial species, *M. tuberculosis* and MAC, in media as well as when cultivated within two types of human macrophages in monolayer culture. Both Ga\((NO_3)_3\) and Ga-transferrin exhibit this effect. The inhibitory growth effect of Ga is prevented in a concentration-dependent fashion by excess Fe, implying that Ga acts primarily by interfering with bacterial Fe metabolism. Finally, our data are consistent with the hypothesis that Fe bound to transferrin moves there as well and is subsequently acquired by the organism. Such a process would appear to required the movement of mycobacterial siderophore(s) into the macrophage cytoplasm to capture this Fe. There is recent evidence for the movement of Fe from intracellular Fe pools to the phagosomal membrane around MAC (56). Second, that extracellular Fe is taken up by the macrophage via a transferrin-independent pathway (40) and reaches intraphagosomal mycobacteria via another route.

Our results show that Ga competes with Fe for acquisition by *M. tuberculosis* grown in broth culture as well as within phagosomes in macrophages. Ga is known to be released from transferrin at a higher pH (ca. 6.5) than that necessary for the release of Fe from transferrin (pH 5.5) (34). Since the pH of the mycobacterial phagosome is \(\approx 6.0\), Ga may be particularly effective for competing with Fe in this environment. How Ga interferes with mycobacterial Fe acquisition is only partially addressed by our work. The most straightforward mechanism would involve direct competition with Fe for binding by the mycobacterial siderophores exochelin and/or mycobactin. In this regard, Ga binds to the siderophores of other bacteria (18, 27, 35). Alternatively, Ga that is internalized by the mycobacteria may also disrupt subsequent Fe acquisition through effects on transcriptional regulators involved in the Fe acquisition apparatus. Preliminary data indicate that Ga does not bind to IdeR (I. Smith, O. Olakanmi, B. Britigan, and L. Schlesinger, unpublished observation), which plays a key role in regulating mycobacterial siderophore production (16).

Our data provide evidence that *M. tuberculosis* can take up

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**FIG. 6.** Scanning EM of *M. tuberculosis* bacilli grown extracellularly or within macrophages. H37Ra *M. tuberculosis* grown in 7H9 broth (A), 7H9 broth in the presence of 500 \(\mu\)M Ga\((NO_3)_3\) for 24 h (B), and MDMs for 72 h (C) were harvested and trapped during filtration on a 0.22-\(\mu\)m (pore-size) filter. Fixed bacteria on the filter were then visualized by scanning EM. (D) Bacteria and macrophage lysates were mixed, and washed bacteria were fixed on the filter and then visualized as described above. Magnification, \(\times 40,000\).
greater amounts of Fe than Ga. Ga is highly effective in competing for Fe acquisition by the bacterium; whereas Fe is relatively ineffective in competing for Ga acquisition. The uptake and competition experiments were performed at a time of exposure to Ga (1 to 6 h) and at a concentration below that which inhibits growth. Thus, the effect of Ga cannot be attributed to a general toxic effect on the organism.

The mechanisms involved in the uptake and transport of Fe-containing siderophores across the mycobacterial membrane are not understood and are likely complex. They are thought to involve specific receptors and transport molecules (7, 13, 15, 26). The efficient internalization of Fe$^{3+}$ is thought possibly to be linked to its reduction to the ferrous form either enzymatically via a reductase or nonenzymatically (13). It is thus plausible that the lower capacity for Ga uptake by \textit{M. tuberculosis} (compared to Fe uptake) is related to its inability to be reduced to the divalent state. This would not preclude the possibility that Ga bound to a receptor would effectively inhibit Fe binding and/or uptake. Our results demonstrating the relative inability of Fe to compete for Ga acquisition raises the possibility for a unique binding site for Ga distinct from that utilized by the Fe uptake pathway. The effect(s) of Ga on microbial physiology in general has received minimal attention (19, 27, 35).

Current studies are aimed at determining the intraphagosomal target(s) for Ga in mycobacteria. Ribonucleotide reductase (RR) is a cell cycle regulated, two-subunit, allosteric enzyme that catalyzes the reduction of nucleoside diphosphates to deoxynucleoside diphosphates (44). RR appears to be an important target for Ga, resulting in the inhibition of growth of rapidly dividing eukaryotic cells (8). Terminally differentiated cells such as macrophages have negligible RR activity (33). RR is also critical for DNA synthesis in bacteria (44). Its affinity for Fe differs from that of mammalian cells (17). Preliminary studies demonstrate that Ga is a potent inhibitor of purified \textit{M. tuberculosis} RR activity (H. Rubin, O. Olakanmi, B. Britigan, and L. Schlesinger, unpublished observation). Additional studies are under way to define the extent to which RR and other Fe-dependent mycobacterial enzymes (such as Fe-containing antioxidant enzymes) are modulated during mycobacterial exposure to Ga. In this regard, it is possible that the bactericidal effect of Ga on mycobacteria relates in part to the inactivation of bacterial Fe-centered antioxidant enzymes, leading to greater amounts of reactive oxygen or nitrogen products rather than simply Fe limitation, which typically results in bacteriostasis.

Together these studies demonstrate that intraphagosomal mycobacteria are capable of acquiring Fe bound to extracellular transferrin and that this process is disrupted by extracellular Ga. These data provide further evidence that Fe acquisition and Fe-dependent metabolism are critical to the survival of intracellular mycobacteria. Ga may serve as a novel experimental method to limit mycobacterial Fe acquisition and thereby investigate the mechanism and role of this process in mycobacterial survival and pathogenesis. Given that most biologic systems are unable to distinguish Ga from Fe, this approach may prove to be useful for disrupting the Fe acquisition mechanisms of other intracellular pathogens. Finally, our data also suggest a potential role for Ga in the therapy of mycobacterial infections in humans, where the problem of increasing resistance to antimicrobial agents is occurring (11, 36). Ga(NO$_3$)$_3$ is a drug approved by the U.S. Food and Drug Administration for the treatment of hypercalcemia of malignancy. The concentrations of Ga(NO$_3$)$_3$ which demonstrate antimicrobial activity in our in vitro systems are in the range of those achievable in vivo and found to be safe for human use (2, 51).

**ACKNOWLEDGMENTS**

This work was supported in part by VA Merit Review Grants (B.E.B. and L.S.S.), an AHA Established Investigator Award (B.E.B.), and NIH grants (AI24954 [B.E.B.] and HL51990, AI33004, and AI43870 [L.S.S.]).

We thank Thomas Kaufman for his expert technical assistance (particularly in the development of the bacterial growth assays in macrophages), Lucy DesJardin for advice, and J. Scott Ferguson and Stephen McGowan for assistance with the bronchoalveolar lavage for HAMs. We thank members of the Central Microscopy Research Facility at the University of Iowa and members of the Clinical Microbiology laboratory at the VAMC. Finally, we thank the National Cancer Institute for kindly providing human-injectable Ga(NO$_3$)$_3$.

**REFERENCES**
