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ORIGINAL RESEARCH

Chemoprophylactic Antimicrobial Activity of Gallium Maltolate against Intracellular *Rhodococcus equi*

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ABSTRACT

The objective of this study was to assess the ability of prophylactically administered gallium maltolate (GaM) to inhibit growth or kill *Rhodococcus equi* within murine macrophage-like (J774A.1) cells. Cells were treated with three different doses of GaM or a single dose of maltol (equivalent to the concentration present in the highest dose of GaM studied); they were then infected with virulent *R. equi* and incubated. Quantitative cultures of *R. equi* from lysed macrophages were obtained at 0, 24, 48, and 72 hours. All GaM dosages resulted in significant reductions of intracellular *R. equi* compared with untreated control cells, and a dose–response effect was evident. Maltol treatment alone had no significant effect on intracellular *R. equi* concentrations compared with untreated control cells. Results suggest the potential usefulness of gallium maltolate for the prevention or control of *R. equi* infections.

Keywords: *Rhodococcus equi*; Gallium; Chemoprophylaxis; Antimicrobial; Intracellular bacteria

INTRODUCTION

Rhodococcus equi is an opportunistic bacterium that survives and replicates in macrophages. It causes severe disease, principally pneumonia characterized by pyogranulomatous lesions, in foals[1] and immunodeficient people.[2] Most foals likely become infected within the first few days of life,[3] when they may have immature or ineffective innate immune responses.[4-7] Effective vaccines are not available, and *R. equi* hyperimmune plasma, the only prophylactic strategy proven to significantly reduce the incidence and severity of this disease,[8-10] has important limitations.[1]

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Ferric iron (Fe^{3+}) is essential for the growth of most microorganisms, including *R. equi*,[11] because of its requirement in various metabolic and DNA-synthetic pathways.[12] Ferric iron sequestration by the host's iron transport proteins, primarily transferrin, is an innate defense mechanism to limit the availability of iron to pathogenic microbes. *Rhodococcus equi*, however, can use transferrin-bound iron, thereby circumventing this defense mechanism.[11]

Gallium (Ga) is a trivalent semimetallic element that mimics Fe^{3+}: it binds to ferric sites on transferrin, is acquired from transferrin by certain bacteria, and is preferentially taken up by mononuclear phagocytes at sites of inflammation.[12,13] Unlike Fe^{3+}, however, Ga is not reducible to a divalent form under physiologic conditions. This precludes its participation in crucial bacterial iron-dependent DNA-synthetic pathways, thereby accounting for gallium's antimicrobial activity. Experimentally, Ga inhibits various microorganisms, including *R. equi*, by exploiting their iron dependency.[12-15] Gallium (as gallium nitrate) is bactericidal for extracellular *R. equi* because of interference with bacterial iron utilization.[14]

Gallium maltolate (GaM), a coordination complex of gallium and maltol, provides high Ga bioavailability after oral administration in humans and a variety of animal species, including newborn foals,[16] and it has not been associated with significant toxicity or gastrointestinal irritation.[17] Prophylactic intragastric administration of GaM to experimentally infected mice reduced their *R. equi* tissue burdens.[14] The purpose of this study was to assess the ability of prophylactically administered GaM to inhibit growth or kill virulent *R. equi* within macrophages.

MATERIALS AND METHODS

Bacteria

Virulent *R. equi* (ATCC 33701) were grown in *R. equi* minimal medium[11] for 24 hours at 37°C with rotation (80 rpm), centrifuged at 1,500*g*, washed with RPMI-1640 (Invitrogen Corp., San Diego, CA), suspended in aliquots of RPMI-1640 with 15% glycerol, and stored at −80°C. For each experiment, bacteria were thawed at 37°C, centrifuged at 2,000*g* for 10 minutes, and washed twice with Ca^{++}- and Mg^{++}-free phosphate-buffered

saline (PBS-free) (Invitrogen Corp., San Diego, CA). Bacterial pellets were resuspended in a volume of complete Dulbecco's modified Eagle's medium (CDMEM) (Invitrogen Corp., San Diego, CA), plus 10% fresh-frozen and filter-sterilized equine serum as a source of complement, *R. equi* antibody, and transferrin (CDMEM-EqFF) equal to that of the original aliquots. After 1 hour incubation at 37°C with rotation for opsonization, bacterial concentrations were adjusted spectrophotometrically at 600 nm to approximately 1×10^6/ml. Bacterial concentrations were assessed by duplicate plate counts of 10-fold serial dilutions on trypticase soy agar with 10% sheep red blood cells (Becton-Dickinson Microbiology Systems, Cockeysville, MD) and recorded as colony-forming units (CFU)/ml.

Macrophages

Murine macrophage-like J774A.1 cells were propagated in 75 cm^2 tissue culture flasks (BD Falcon, Bedford, MA) with CDMEM + 200 mM glutamine, 0.075% $NaHCO_3$, 1% essential amino acids, and 10% equine serum (Invitrogen Corp., San Diego, CA) (CDMEM-Eq) at 37°C in 5% CO_2. Cells were washed once with PBS-free to remove and discard unattached cells. Remaining cells were detached by incubating in PBS-free for 30 minutes at 37°C in 5% CO_2, and scraping. Cell suspensions were transferred to tubes and centrifuged 10 minutes at 1,100*g*. Supernatants were discarded, cells resuspended in CDMEM-EqFF, and cell counts and viability determined by hemacytometer and trypan blue dye-exclusion, respectively. Concentrations of viable J774A.1 cells were adjusted to approximately 1×10^5 cells/ml with CDMEM-Eq. One milliliter cell suspension was seeded into each well of 24-well poly-D-lysine plates (BD Biosciences, Bedford, MA), in triplicate, for each of five GaM and three maltol treatment experiments and for four incubation times (ie, 0, 24, 48, and 72 hours).

Treatment and Infection

Gallium maltolate treatment groups were: (1) cells only; and (2) cells + 10, 50, or 75 μM GaM (Norac Inc., Azusa, CA). Maltol groups were: (1) cells only; and (2) cells + 225 μM maltol (Sigma Chemical Co., St. Louis, MO) (a concentration equivalent to that contained in 75 μM GaM). Cells were incubated overnight at 37°C in 5% CO_2, and *R. equi* were then added to each well at a multiplicity of infection of 1 (ie, 1 bacterium per macrophage). The cells were continuously exposed to their assigned concentrations of GaM or maltol before and after infection with *R. equi*. Cells were incubated 1 hour at 37°C to permit bacterial phagocytosis, washed with CDMEM-EqFF to remove unattached bacteria, and re-suspended in CDMEM-EqFF containing 10 μM gentamicin to control remaining extracellular bacteria, as previously described.[18]

Bacterial Quantification

At 0, 24, 48, and 72 hours postinfection, medium was removed, centrifuged at 2,000*g* for 10 minutes, and the supernatant discarded. Adherent cells were lysed by adding 500 μl 0.05% Tween 20 in ddH_2O (Sigma Chemical Co., St. Louis, MO) to each well and pipetted vigorously. Cell lysates were combined and vortexed with pelleted nonadherent cells for 1 minute to complete lysing, and sonicated (Ultrasonic Cleaner) (American Scientific Products, McGaw Park, IL) 5 minutes to minimize clumping of *R. equi* (S. Giguere, University of Florida, personal communication). Lysate bacterial concentrations were assessed by duplicate plate counts of 10-fold serial dilutions on trypticase soy agar with 10% sheep red blood cells, and recorded as CFU/ml.

Data Analysis

The outcome of interest was the extent to which GaM influenced intracellular concentrations of *R. equi* over time, relative to the initial (baseline) concentration. Thus, the dependent variable in analyses was the ratio of concentration of *R. equi* at a given experiment, time, and treatment relative to the corresponding initial concentration, indicating the change in concentration over time relative to baseline: ratio values significantly less than 1 indicated reduced concentrations of *R. equi* relative to time 0, and ratio values significantly greater than 1 indicated increased concentrations of *R. equi* relative to time 0. Because the ratio data were not normally distributed, ratios were transformed logarithmically (base 10). Linear mixed-effects models were used for analysis,[19] where experiment was modeled as a random effect, and time, treatment, and their interaction term were modeled as fixed effects. Post hoc testing was performed using the method of Sidak.[20] A significance level of $P \leq .05$ was used.

RESULTS

On the basis of microscopic assessments, there were no apparent differences in J774A.1 cell morphology or growth characteristics within groups and among groups at any time. The concentration ratios of *R. equi* varied significantly as a function of time for all groups, including controls (Fig. 1). By 48 hours, all three GaM treatment groups were significantly different from untreated controls, but not from each other. Ratios were inversely proportional to treatment dose at 48 hours and 72 hours (Fig. 1), although the differences were not significant. The untreated controls had significantly higher concentrations of *R. equi* after 24 hours relative to time 0, and this difference was maintained through 72 hours. For the GaM-treated groups, there appeared to be some increase in *R. equi* relative to baseline after 24 hours, although this increase was only significant for the 10-μM dose. Concentrations of *R. equi* then began to decrease among GaM-treated cells, and were significantly different from baseline for the 50-μM and 75-μM doses but not for the 10-μM dose; ratios for all three GaM treatment groups were significantly lower at 72 hours than at 24 hours. There was no significant ($P \geq .373$) difference in *R. equi* concentrations

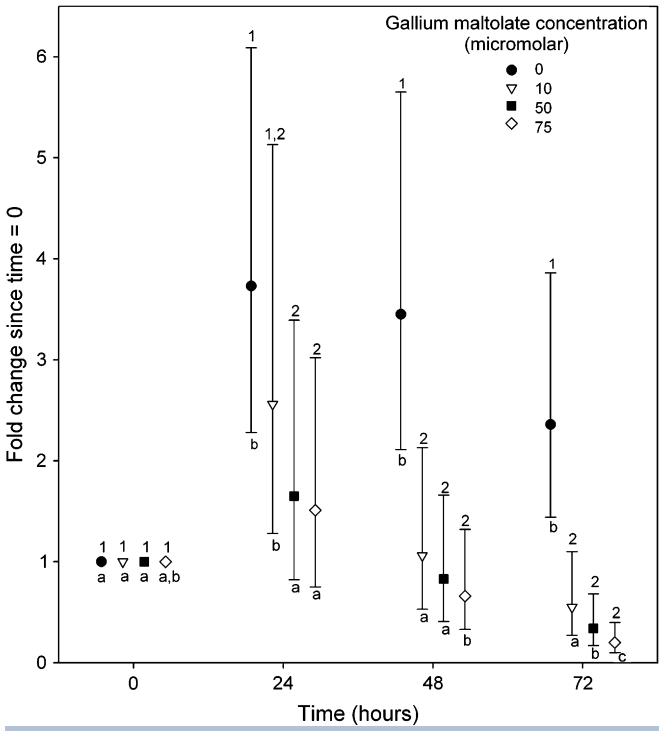

Figure 1. Mean ratios of *R. equi* within J774A.1 macrophage cells after 0, 24, 48, and 72 hours' exposure to gallium maltolate (GaM), to concentrations at 0 hours ("fold change"). Error bars indicate 95% confidence intervals. Values with different numbers above the upper confidence bounds represent significant ($P < .05$) differences among GaM dosages at given time periods. Values with different letters below the lower confidence bounds represent significant ($P < .05$) differences among times at given GaM dosages.

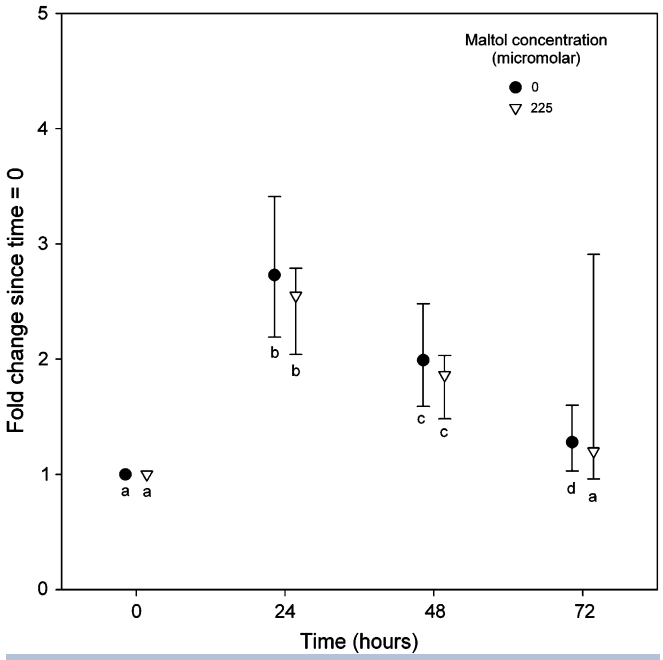

Figure 2. Mean ratios of *R. equi* within J774A.1 macrophage cells after 0, 24, 48, and 72 hours' exposure to maltol, to concentrations at 0 hours ("fold change"). Error bars indicate 95% confidence intervals. There were no significant effects of maltol treatment at any time. For a given maltol dose, values with different letters below the lower bound of the 95% confidence interval represent significant ($P < .05$) differences among times.

between maltol-treated and untreated control cells at any sampling time, indicating that the GaM-elicited response was not attributable to maltol alone (Fig. 2).

DISCUSSION

Rhodococcus equi is a facultative intracellular pathogen whose virulence appears to be related to its ability to survive in macrophages by inhibiting events associated with phagolysosome acidification or other late endosomal reactions.[21] Thus, antimicrobial strategies to prevent or control *R. equi* infections must be able to target this pathogen intracellularly. These data provide the first evidence that GaM significantly reduces the intracellular concentration of a pathogen, viz., *R. equi* (Fig. 1) and that this negative impact on intracellular growth and viability of *R. equi* is not attributable to maltol (Fig. 2). In addition, they further substantiate a potential role for GaM-prophylaxis in the control of *R. equi* foal pneumonia.

On the basis that: (1) prophylactic administration of gallium maltolate to newborn foals has been proposed as a potential strategy for the control of *R. equi* pneumonia[22]; (2) foal tissues would be continuously exposed to transferrin-bound GaM throughout the proposed period of chemoprophylaxis; and (3) transferrin-bound gallium is preferentially taken up by activated phagocytic cells and accumulates at sites of infection and granulomatous lesions,[12,23] macrophages in this study were continuously exposed to GaM or maltol before and after infection with *R. equi*.

The effectiveness of gallium to control *R. equi* is dependent on its concentration in infected tissues, principally macrophages (the target cell for *R. equi*),[18] rather than in blood serum, or in the case of this study, tissue culture media. In a previous study involving prophylactic GaM treatment of mice experimentally infected with *R. equi*, there was a reduction in tissue concentrations of *R. equi* compared with untreated controls.[14] This reduction was not statistically significant; however, extrapolation of serum Ga concentration and tissue *R. equi* concentration data indicated that a serum Ga concentration of 700 ng/ml would reduce *R. equi* tissue burdens by approximately 90%.[14] The lowest GaM concentration used in the current study (ie, 10 μM) contained 697 ng/ml elemental Ga. This concentration of GaM killed significant numbers of intracellular *R. equi*, with even greater numbers of bacteria being killed at higher GaM concentrations. This is of major clinical importance, because relatively low Ga serum

concentrations can result in high concentrations within infected tissues (well known from ^{67}Ga scans used to diagnose sites of infection[12]). A recent study by these investigators confirmed that Ga serum concentrations of 700 ng/ml are readily and safely achievable in neonatal foals after enteral administration of GaM.[16]

Murine macrophage-like J774A.1 cells were used in this study on the basis that virulent *R. equi* (ATCC 33701) survive and multiply in those cells[18] in a manner similar to that observed in murine peritoneal macrophages and equine alveolar macrophages.[18] Some growth of *R. equi* within the J774A.1 cells was therefore anticipated, and we hypothesized that GaM would negatively influence that growth. As illustrated, *R. equi* concentrations in control cells in the GaM study (Fig. 1) and both control and treated cells in the maltol study (Fig. 2) exhibited similar temporal patterns: an increase over baseline that was greatest at 24 hours, with gradual reductions at 48 and 72 hours. As hypothesized, the GaM had a significantly negative impact on the intracellular growth and viability of *R. equi* across times (Fig. 1), and this negative influence was not attributable to maltol (Fig. 2). Because virulent *R. equi* can destroy infected macrophages,[24] the possibility that some extracellular bacteria released from extirpated cells may have been killed by gentamicin or GaM in the media cannot be ruled out. This may account for the slight reduction in *R. equi* concentrations in both sets of control cells and maltol-treated cells after 24 hours, and reductions of similar magnitude in GaM-treated cells (Figs. 1 and 2). Alternatively, sonication of cell lysates may not have dissipated all of the clumped *R. equi*. In that case, each clump of *R. equi* would have produced a single colony when quantitatively cultured, thereby artificially lowering bacterial counts as previously described.[18]

On the basis of pharmacokinetic studies in foals[16] and in vivo studies in mice,[14] daily enteral administration of GaM for the first weeks of a foal's life may provide adequate protection against early infection by preventing invading *R. equi* from growing to overwhelming numbers before innate immune responses sufficiently mature or specific adaptive immunity can be established to control infections. This, in turn, could substantially reduce the incidence of disease on farms on which *R. equi* foal pneumonia is endemic. In addition, GaM used alone or in conjunction with standard antibiotic protocols may be effective for the treatment of established infections.

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