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Persistent *Mycobacterium bovis*-BCG is resistant to glutathione induced reductive stress killing

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ABSTRACT

This study focuses on the redox stress response in mycobacteria elicited by a host-derived, thiol-based detoxification molecule, glutathione (GSH). Although the growth and viability of *Mycobacterium bovis*-BCG (BCG) was hampered by exposure to 8 mM GSH, oxygen depleted, persistent BCG (NRP BCG) resisted GSH-mediated killing. Fast growing mycobacteria also resisted GSH-mediated killing. To determine the mechanisms behind these observations, we evaluated the levels of intracellular ATP in both BCG and NRP BCG exposed to 8 mM GSH. Intracellular ATP levels increased from 0.13 to 2.3 μM in BCG upon exposure to GSH. The levels of ATP remained low and unchanged when NRP BCG was exposed to GSH. Using both HPLC and a cell-based thiol detection assay, it was determined that GSH stimulates the production of mycothiol (MSH) by BCG approximately 5.7 fold. The levels of MSH did not change upon exposure of NRP BCG to GSH. MSH is an alternative, thiol-based detoxification molecule employed by mycobacteria. Changes in the cytoplasmic concentrations of this molecule are suggestive of redox imbalances. Together, GSH and MSH may introduce excess reducing equivalents into the mycobacterial cytoplasm; leading to reductive stress. The modulation of NAD^+ levels through alterations in ATP metabolism can enhance the cells ability to bind excess reducing equivalents and serve as a mechanism to restore the cellular redox balance when cells experience reductive stress. These data suggest that killing of BCG by GSH may result from reductive stress that cannot be controlled. NRP BCG appears to be resistant to GSH-induced reductive stress.

1. Introduction

The World Health Organization (WHO) recently reported that approximately one-third of the world's population are thought to be infected with *Mycobacterium tuberculosis*. At least half of those infected individuals are infected with drug/multi-drug resistant strains. Although the global incidence of tuberculosis has been slowly declining since 2003, approximately nine million individuals contracted the disease with mortality rates approaching 20% in 2013 [37].

Upon inhalation, *M. tuberculosis* is engulfed by alveolar macrophages and other phagocytic cells, initiating the immune response infection. The infected phagocytic cells generate antimicrobials including reactive oxygen and reactive nitrogen intermediates (ROIs and RNIs, respectively) to kill the invading mycobacteria. Mycobacteria have the ability to evade many of the antimicrobial effects elicited by the effector molecules produced by the phagocytic cells, enabling them to hide and persist within the host in a state of non-replicative persistence (NRP; [15]).

The host phagocytes that produce RNIs and ROIs in response to infection also produce a thiol-based detoxification molecule, glutathione (GSH; [25]). GSH is a tripeptide consisting of the amino acids, γ-glutamate, cysteine, and glycine. The tripeptide protects host tissues from the damage associated with exposure to the oxidants by ensuring that the reducing environments within the host cell cytoplasms are maintained [9]. GSH is a reduced molecule that is converted to glutathione thiodisulphide (GSSG) during oxidative stress while donating electrons to protein thiodisulphides arising from the stress. GSSG can be converted back to GSH by the NADPH requiring enzyme, GSH reductase. The ratio of GSH:GSSG within a cell can be used as an indicator of redox stress. Typically, cells at redox homeostasis will have approximately 90% GSH and 10% GSSG. Intracellular concentrations of GSH can range between 0.1 and 15 mM [13].

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The Connell laboratory has demonstrated that 4 mM GSH is bacteriostatic to *M.bovis*-BCG (BCG) after three days of exposure [11] BCG is a biosafety-level two model organism for the study of *M. tuberculosis*. BCG has 99.95% sequence identity to *M. tuberculosis* but contains three large genomic deletions that impact virulence [18]. In a follow-up study, Connell's lab demonstrated that GSH has significant roles in controlling early mycobacterial growth in both murine and human macrophages [33]. Similar phenomena were observed with *M. tuberculosis* H37Rv upon exposure to GSH *in vitro* and in early murine macrophage infection models [34]. Connell hypothesized that the mechanism behind GSH mycobacterial growth inhibition was due to an alteration in the redox balance within the cytoplasms of the exposed organisms [11,33,34].

Mycobacteria employ two thiol-based detoxification molecules of their own to combat redox stress – mycothiol (MSH) and ergothioneine [6]. MSH is composed of a central, reactive cysteine residue flanked by the saccharides inositol and N-glucosamine [20]. MSH functions similarly as GSH in maintaining mycobacterial disulphide homeostasis. MSH is the reduced form of the molecule and mycothione (MSSM) is the oxidized form. Mycothiol disulphide reductase, using NADPH as a co-factor, is responsible for converting MSSM back to MSH to maintain the reducing environment in the mycobacterial cytoplasm [22,31] demonstrated that the basal ratio of MSH:MSSM in redox balanced BCG was 50:1. Very little is known about ergothioneine. Currently, it is thought that ergothioneine shares overlapping functions with MSH [6].

Mycobacteria possess several additional enzymes that can interact with MSH to resist the damaging effects of redox stress. MSH can detoxify alkylating agents, electrophiles, and antibiotics by forming MSH *S*-conjugates. These conjugates can be cleaved by the amidase, Mca, to produce modified and inactive toxins linked to AcCys [24,29]. Additionally, the mycobacterial genome also encodes three thioredoxins and a single copy of thioredoxin reductase belonging to the thioredoxin group of thiol-disulphide oxidoreductases [5]. The thioredoxins contain conserved CXXC catalytic motifs that enable the transfer electrons from NADPH via the thioredoxin reductase to oxidized substrates such as MSSM and protein disulphides [14]. Van Lear and colleagues [32] recently identified another oxidoreductase with a CGYC motif, mycoredoxin-1, that can also reduce *S*-mycothiolated mixed disulphides. Thus, mycobacteria possess at least four alternative, yet complementary pathways that each utilize MSH to combat redox stress.

As described above, *M. tuberculosis* enters the NRP state in order to enhance its survival in the toxic environment within the host. The NRP state is characterized by a change in energy metabolism [26], a decrease in the production of ATP [17] and a strong induction of respiratory nitrate reductase activity [28]. There are two *in vitro* models to study mycobacteria in the NRP state – the oxygen depletion model (Wayne model [36]); and the nutrient starvation model (Loebel model; [3]). Thus far, there have been no studies described in the literature that describe the impact of GSH on persistent mycobacteria. Here, we describe a study to elucidate the mechanism responsible for GSH killing of BCG grown in culture. Additionally, we demonstrate the impact of GSH on BCG that has entered the persistent state using the Wayne oxygen depletion model. The results from our study indicate that exposure to GSH induces a state of reductive stress in the cytoplasm of BCG leading to cell death. NRP BCG appear to be resistant to the toxic effects of GSH.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

BCG Pasteur strain (ATCC), *Mycobacterium phlei* (Carolina Biological Supply Company), *Mycobacterium marinum* (kindly supplied by ND Connell), and *Mycobacterium smegmatis* (Carolina) were all grown in Middlebrook medium [per liter: (NH4)SO4, 0.5 g; L-glutamic acid, 0.5 g; sodium citrate, 0.1 g; pyridoxine, 0.001 g; biotin, 0.0005 g; Na2HPO4, 2.5 g; KH2PO4, 1.0 g; ferric ammonium citrate, 0.04 g; MgSO4, 0.05 g; CaCl2, 0.0005 g; ZnSO4, 0.001 g; CuSO4, 0.001 g; Sigma-Aldrich]. Middlebrook 7H9 (liquid) and 7H11 (1.5% agar) media were supplemented with glycerol (0.5%, vol/vol, Sigma-Aldrich) and ADC (0.5% bovine serum albumin, fraction V, 0.2% dextrose, 0.85% NaCl; all from Sigma-Aldrich). All liquid cultures were also supplemented with 0.05% Tween 80 to prevent clumping (Sigma-Aldrich).

To generate NRP BCG, we followed the protocol developed by Hu and colleagues [16]. This protocol is a modification of the protocol originally developed by Ref. [36]. Basically, 10 ml late mid-logarithmic phase cultures of BCG (Optical Density at 600 nm [OD_{600}] = 0.7–0.8) were inoculated in Middlebrook 7H9 media into Nunc 25 ml culture tubes (Fisher Scientific) and incubated at 37 °C without agitation for 100 days. Most of the bacteria in the tubes had clumped and settled to the bottom. Oxygen depletion was confirmed using a GasPack EZ Anaerobe Sachet Indicator (BD Diagnostics) inside control Nunc tubes. Transition to the persistent state was confirmed by a reduction of intracellular ATP concentration after the 100 day time period (data not shown).

When appropriate, GSH (Sigma-Aldrich) was added to the bacterial cultures at concentrations of 4 mM and/or 8 mM. The half-life of GSH can be approximately 7 h; depending upon the culture conditions [23]. As such, GSH was replaced daily at the appropriate concentrations (4 or 8 mM) for studies requiring multiple days. Ampicillin was added to all cultures at 20 μg/ml to maintain sterility. Mycobacteria are intrinsically resistant to ampicillin [4]. Fresh preparations of all reagents were used in all plates and liquid media.

2.2. Growth and viability studies

Mid-logarithmic (OD_{600} = 0.4–0.7; OD = optical density) BCG, *M. phlei, M. marinum,* or *M. smegmatis* were all diluted to OD_{600} = 0.1 and inoculated into Middlebrook 7H9 medium to a final volume of 5 ml in 15 ml conical vials (Fisher Scientific). Following inoculation, 20 μg/ml ampicillin and the appropriate concentrations of GSH were added to the cultures (0, 4, or 8 mM). For the fast growing mycobacterial cultures of *M. phlei, M. marinum,* and *M. smegmatis* ODs were taken every 3 h for 24–30 h to evaluate growth and samples were removed at each time point for plating onto Middlebrook 7H11 plates lacking the thiols to evaluate viability. For the BCG samples, ODs were taken daily over four consecutive days. Samples were also removed at each time point to evaluate viability. All liquid cultures were incubated in a rotating incubator at 37 °C between sample removal.

The NRP BCG samples were also diluted to an OD_{600} = 0.1 in 8 ml of Middlebrook 7H9 media after dispersal of mycobacterial clumps using 0.4 μm filter units (Fisher Scientific). Sterile 3 mm glass beads (Fisher Scientific) were added to each NRP culture vial to the total volume permitted in the vial (15 ml) to displace any oxygen that might enter the tubes during the inoculation and sample removal process. NRP BCG samples were incubated at 37 °C without agitation between sample removal.