

Glutathione and Nitrosoglutathione in Macrophage Defense against *Mycobacterium tuberculosis*

Vishwanath Venketaraman,^{1,2,3,4*} Yaswant K. Dayaram,^{2,3,4} Meliza T. Talaue,^{2,3,4}
and Nancy D. Connell^{1,2,3,4}

Department of Medicine,¹ Department of Microbiology and Molecular Genetics,² New Jersey Medical School National Tuberculosis Center,³ and Center for Emerging and Re-emerging Pathogens,⁴ UMDNJ-New Jersey Medical School, Newark, New Jersey

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We demonstrate that *Mycobacterium tuberculosis* grown in vitro is sensitive to glutathione and its derivative S-nitrosoglutathione. Furthermore, our infection studies with J774.1 macrophages indicate that glutathione is essential for the control of the intracellular growth of *M. tuberculosis*. This study indicates the important role of glutathione in the control of macrophages by *M. tuberculosis*.

Tuberculosis is one of the most prevalent infectious diseases in the world (3). The situation is exacerbated by the emergence of multidrug-resistant strains and an ever-growing number of highly susceptible immunocompromised individuals arising from the AIDS pandemic (3). The production of reactive nitrogen intermediates by murine macrophages is considered to be a relatively effective host defense mechanism against *Mycobacterium tuberculosis* (1, 4, 7, 8, 9, 13, 16).

In this study, we show that a virulent laboratory strain of *M. tuberculosis*, H37Rv, grown in vitro is sensitive to glutathione (GSH) and nitrosoglutathione (GSNO) at physiological concentrations. While GSH at a 5 mM concentration is bacteriostatic to H37Rv (Fig. 1; Table 1), GSNO at a 5 mM concentration is bactericidal (Fig. 1; Table 1), as was observed by determining optical density (OD) and numbers of CFU. H37Rv was grown in 7H9 containing albumin dextrose complex (ADC) and Tween 80 to mid-log phase and then diluted to an OD of approximately 0.1. The bacterial suspensions were then treated with 5 mM GSH (Sigma) or 5 mM GSNO (Sigma) or left untreated. The OD was measured every day for 5 days (Fig. 1). Each day, an aliquot was taken from every sample, diluted 100-fold, and plated on 7H11 plates containing ADC for determination of numbers of CFU (Table 1). All experiments were performed three times in triplicate.

The mechanism of action of the antimycobacterial activity of GSH is not certain. One possibility is that the presence of a high concentration of GSH may result in an imbalance in a bacterium containing an alternative thiol for regulating reduction or oxidation activity (i.e., mycothiol). We also consider the hypothesis of Spallholz in this connection (14). According to Spallholz, "GSH is structurally similar to the precursor of the antibiotics produced in fungi in the genera *Penicillium* and *Cephalosporium*." Its potential conversion to the penicillin-like derivative glutacillin, a β -lactam form of GSH, raises the intriguing question of whether GSH was once a universal penem-like precursor of antibiotics in cells of many life forms (14).

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, UMDNJ-ICPH, 225 Warren St., Newark, NJ 07103. Phone: (973) 972-4483, ext. 28963. Fax: (973) 972-8981. E-mail: venketvi@umdnj.edu.

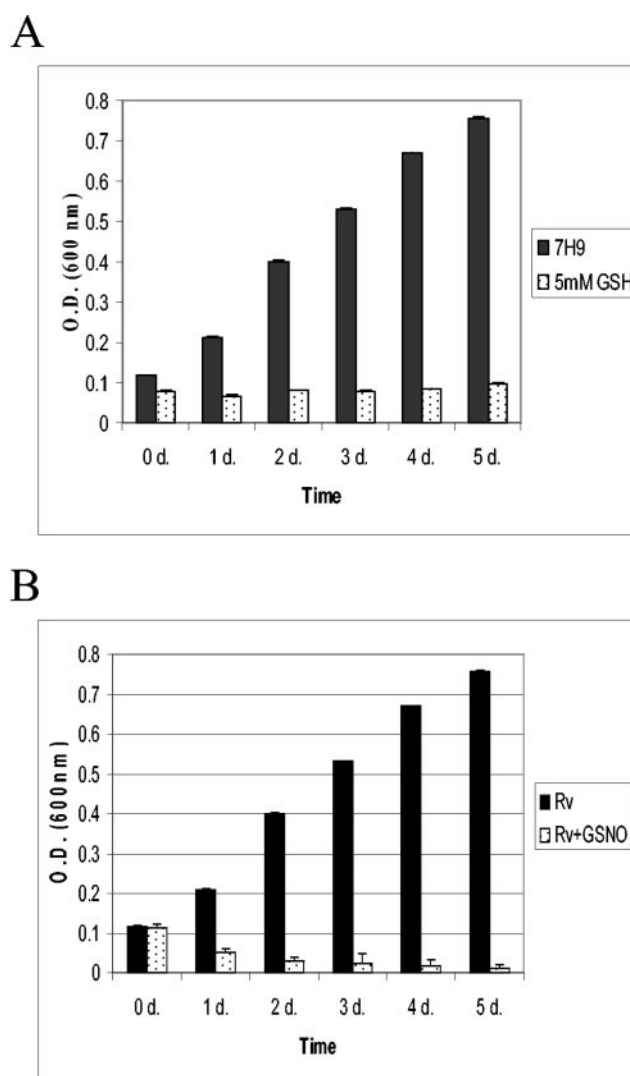


FIG. 1. Growth of *M. tuberculosis* in the presence and absence of GSH (A) and GSNO (B) as determined by measuring OD. The H37Rv (Rv) cell suspension was treated with 5 mM GSH or 5 mM GSNO or left untreated. The OD was measured every day for 5 days (d.). (A). All experiments were repeated three times in triplicate.

TABLE 1. Growth of H37Rv in 7 Hg medium

H37Rv treatment	Mean no. of CFU ^a ± SD on:					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
None	$2.8 \times 10^4 \pm 8.81 \times 10^2$	$6.4 \times 10^4 \pm 4.1 \times 10^3$	$3.3 \times 10^5 \pm 1.8 \times 10^4$	$7.3 \times 10^5 \pm 1.2 \times 10^4$	$7.2 \times 10^6 \pm 1.6 \times 10^5$	$9.8 \times 10^7 \pm 2.0 \times 10^5$
5 mM GSH	$2.6 \times 10^4 \pm 1.2 \times 10^3$	$2.1 \times 10^4 \pm 1.2 \times 10^3$	$2.4 \times 10^{4*} \pm 5.5 \times 10^2$	$2.4 \times 10^{4*} \pm 2.0 \times 10$	$2.4 \times 10^{4*} \pm 6.2 \times 10^2$	$2.7 \times 10^{4*} \pm 6.2 \times 10^2$
5 mM GSNO	$2.6 \times 10^{4*} \pm 1.8 \times 10^4$	$8.6 \times 10^{3*} \pm 8.8 \times 10^2$	$3.5 \times 10^{3*} \pm 3.1 \times 10^2$	$3.2 \times 10^{3*} \pm 1.6 \times 10^3$	$3.0 \times 10^{3*} \pm 6.2 \times 10^2$	$2.6 \times 10^{3*} \pm 2.3 \times 10^2$

^a Asterisks indicate statistical significance.

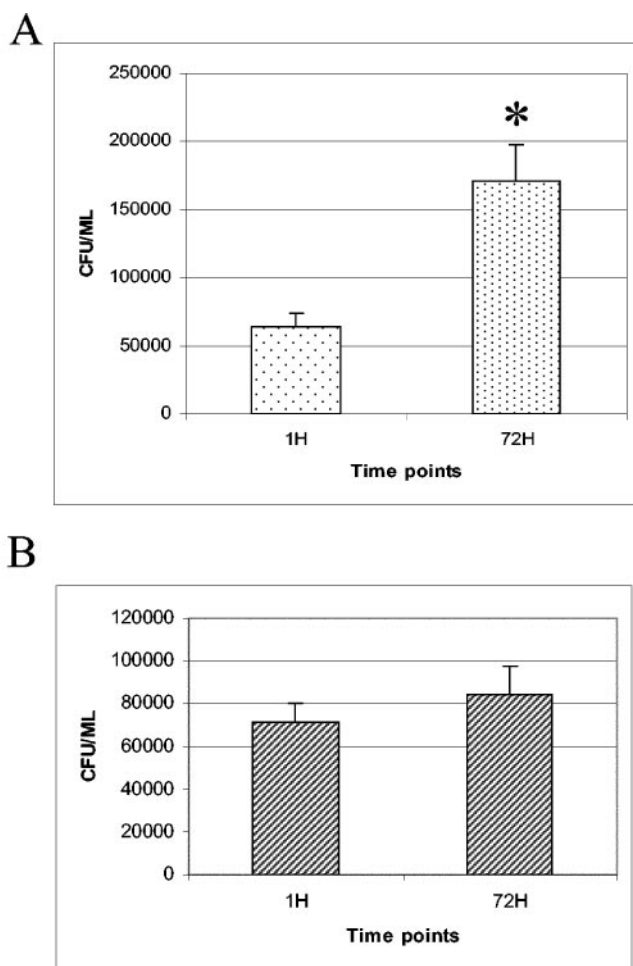


FIG. 2. Growth of H37Rv in untreated (A) and IFN-γ-LPS-treated (B) J774.1 cells. Experiments with H37Rv-infected macrophages, maintained in the absence (A) and presence (B) of IFN-γ plus LPS, were terminated at 1 and 72 h to determine the growth of H37Rv inside J774.1 cells. * denotes a statistically significant increase in the number of CFU between 1 and 72 h ($P < 0.0006$). Data are averages of results from six different experiments performed in triplicate.

GSH is a precursor for GSNO, and GSNO may represent one of the most important active forms of nitric oxide (NO) as an antimicrobial agent (6, 10, 11, 12).

Based on the results of our in vitro studies, we hypothesized that the control of the growth of H37Rv in murine macrophages is mediated in part by GSNO and GSH, generated by macrophages during oxidative or nitrosative stress. We tested this hypothesis by performing in vitro infection studies with a murine macrophage cell line, J774.1. J774.1 macrophages were maintained in vitro and infected with H37Rv, according to methods described by Venketaraman et al. (15). J774.1 cells were stimulated for GSNO synthesis as follows. Gamma interferon (IFN-γ) and lipopolysaccharide (LPS) stimulation result in NO production by macrophages (5) and subsequently in the formation of GSNO. Infected macrophages were therefore maintained in tissue culture medium with and without IFN-γ (100 U/ml) and LPS (1 μg/ml). Infected-macrophage cultures were terminated at 1 and 72 h to allow study of the intracellular viability of H37Rv.

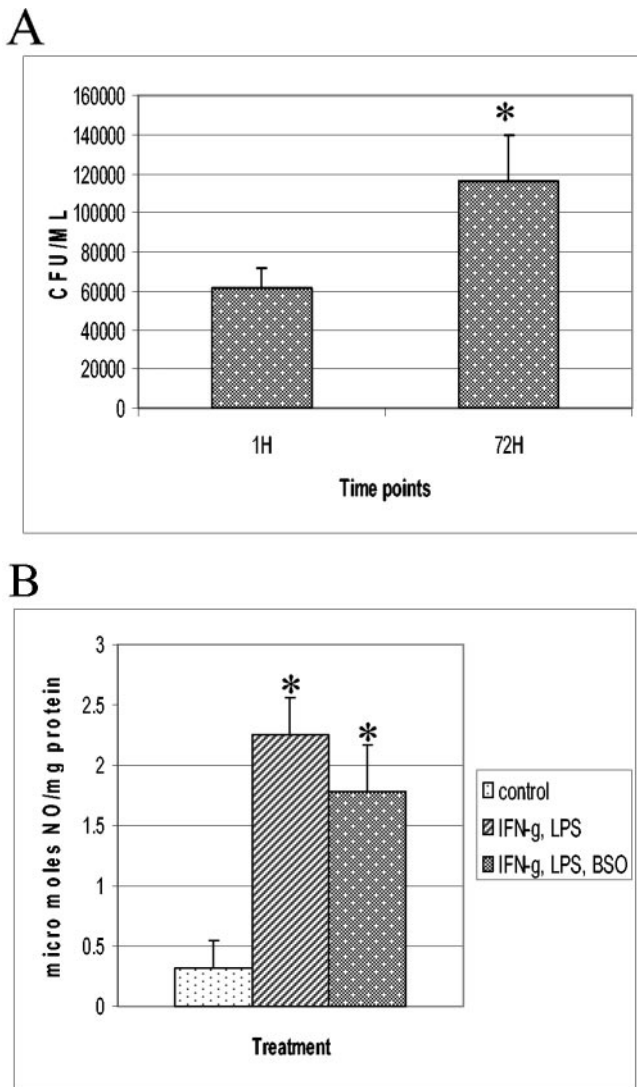


FIG. 3. (A) Growth of H37Rv in IFN- γ -LPS-BSO-treated J774.1 cells. Experiments with H37Rv-infected macrophages treated with IFN- γ plus LPS and BSO were terminated at 1 and 72 h to determine the levels of growth of H37Rv inside J774.1 cells. Statistical significance was calculated with the Statview program. * represents a statistically significant increase in the number of CFU between 1 and 72 h ($P < 0.0083$). Data are averages of results from from six different experiments performed in triplicate. (B) NO estimation in J774.1 cells. Nitrite levels in macrophage supernatants were determined spectrophotometrically by a Greiss reaction. Data are averages of results from five different experiments. * indicates a statistically significant increase in nitrite levels between control and IFN- γ -LPS- or IFN- γ -LPS-BSO-treated macrophages. For values for the control versus those after IFN- γ -LPS treatment, P was <0.0001 . For values for the control versus those after IFN- γ -LPS-BSO treatment, P was <0.0030 .

To prove that the macrophage killing of H37Rv is mediated by GSH or GSNO, macrophages were treated with IFN- γ (100 U/ml), LPS (1 μ g/ml), and buthionine sulfoximine (BSO; 500 μ M), and the growth of H37Rv inside IFN- γ -, LPS-, and BSO-treated macrophages was compared with the growth of H37Rv inside unstimulated and IFN- γ - and LPS-treated macrophages. BSO specifically inhibits the activity of a γ -glutamyl-cysteinyl synthetase enzyme that catalyzes the first-step reaction in the

synthesis of GSH. The intracellular viability of H37Rv was determined by plating the macrophage lysates on 7H11 enriched with ADC to count mycobacterial colonies.

Figure 2 shows the results of these experiments, which are presented as averages of results of six different experiments performed in triplicate. We observed significant growth of *M. tu-*

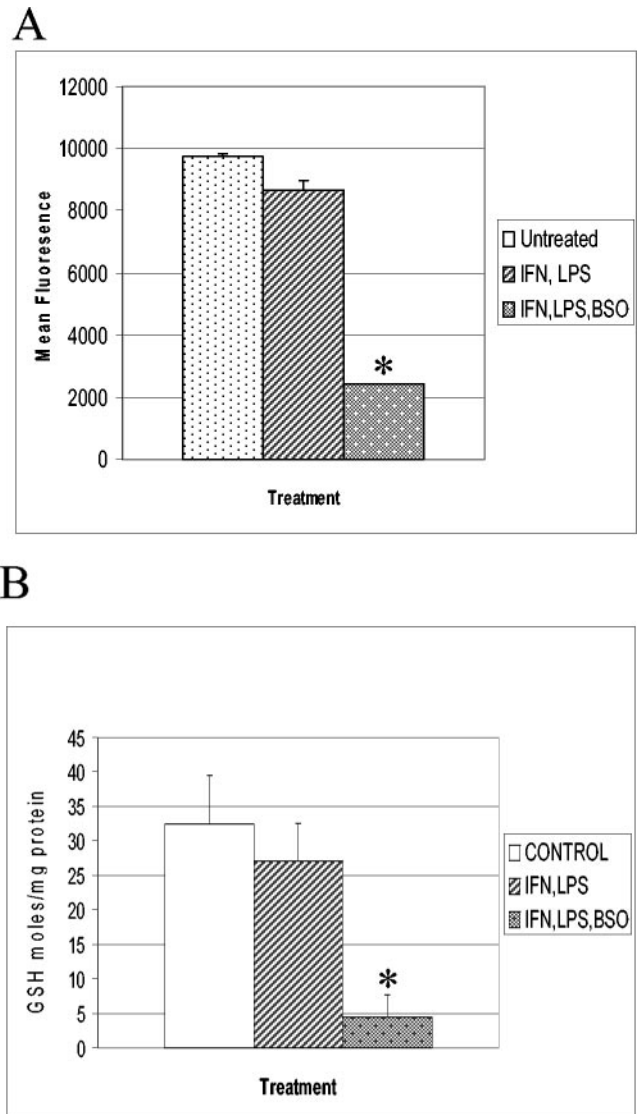


FIG. 4. Estimation of GSH levels in J774.1 cells by flow cytometry (A) and spectrophotometry (B). (A) GSH was quantitated in J774.1 cells by staining with monochlorobimane. * signifies a statistically significant decrease in the number of fluorescent cells in IFN- γ -LPS-BSO-treated macrophages compared to that for control or IFN- γ -LPS-treated macrophages. For values for the control versus those after IFN- γ -LPS-BSO treatment, P was <0.0001 . For values after IFN- γ -LPS treatment versus those after IFN- γ -LPS-BSO treatment, P was <0.0026 . Data are averages of results from three different experiments. (B) GSH was also assayed by spectrophotometry. Data are averages from three different experiments. * designates a statistically significant decrease in intracellular GSH levels in IFN- γ -LPS-BSO-treated macrophages compared to those in control macrophages or IFN- γ -LPS-treated macrophages. For values for the control versus those after IFN- γ -LPS-BSO treatment, P was <0.0024 . For values after IFN- γ -LPS treatment versus IFN- γ -LPS-BSO treatment, P was <0.0028 .

berculosis inside unstimulated J774.1 macrophages between 1 and 72 h (Fig. 2A). Stimulation of J774.1 cells with IFN- γ -LPS resulted in a static effect on the growth of intracellular *M. tuberculosis* (Fig. 2B). Addition of BSO (500 μ M) to IFN- γ - and LPS-treated J774.1 cells resulted in significant growth of H37Rv, similar to what occurred with untreated macrophages and elimination of the static effect brought about by IFN- γ -LPS treatment (Fig. 3A). These findings indicate that intracellular GSH and GSNO play a significant role in the killing of intracellular H37Rv in J774.1 cells.

IFN- γ -LPS treatment is likely to induce several antimicrobial mechanisms within macrophages. In order to demonstrate that GSH and GSNO contribute to a great extent in the growth inhibition of H37Rv inside IFN- γ - and LPS-treated macrophages, we measured nitrite and GSH levels in macrophages treated with IFN- γ -LPS and IFN- γ -LPS-BSO.

Nitrite was detected spectrophotometrically by a Greiss reaction (15). Stimulation of J774.1 macrophages with IFN- γ -LPS resulted in a significant fourfold increase in NO generation (Fig. 3B) compared to NO generation in unstimulated macrophages. Treatment of J774.1 cells with IFN- γ -LPS-BSO also resulted in a significant and almost fourfold increase in NO generation (Fig. 3B) compared to NO generation in unstimulated macrophages.

If NO is the primary species responsible for controlling mycobacterial growth in murine macrophages, then we should observe the inhibition of growth of *M. tuberculosis* in IFN- γ -, LPS-, and BSO-treated macrophages. However, we observed a significant growth of intracellular *M. tuberculosis* (Fig. 3A).

To prove that GSH and GSNO contribute to a great extent in the inhibition of the growth of H37Rv inside IFN- γ -LPS-treated macrophages, we measured GSH in macrophages under different treatments. GSH was assayed by two methods: spectrophotometry (15) and fluorescent detection of monochlorobimane (MCB) staining. J774.1 cells were cultured in 5-ml tissue culture flasks for 24 h at 37°C in the presence or absence of IFN- γ -LPS or IFN- γ -LPS-BSO. Macrophages were stained with MCB (60 μ M) and incubated at 37°C for 30 min. MCB reacts with intracellular GSH to form glutathione-S bimane, a fluorescent adduct retained by the cells and detectable by fluorescence-activated cell sorting using a 351 nm excitation (2). Our results show similar trends by both techniques. As shown in Fig. 4, maximum levels of GSH were observed in untreated macrophages. Treatment of J774.1 cells

with IFN- γ , LPS, and BSO caused a significant decrease in intracellular GSH levels (Fig. 4), possibly leading to an inhibition of GSNO formation, and hence we observed a significant increase in the intracellular growth of H37Rv.

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