Killing of Virulent *Mycobacterium tuberculosis* by Reactive Nitrogen Intermediates Produced by Activated Murine Macrophages

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Summary

Tuberculosis remains one of the major infectious causes of morbidity and mortality in the world, yet the mechanisms by which macrophages defend against *Mycobacterium tuberculosis* have remained obscure. Results from this study show that murine macrophages, activated by interferon γ, and lipopolysaccharide or tumor necrosis factor α, both growth inhibit and kill *M. tuberculosis*. This antimycobacterial effect, demonstrable both in murine macrophage cell lines and in peritoneal macrophages of BALB/c mice, is independent of the macrophage capacity to generate reactive oxygen intermediates (ROI). Both the ROI-deficient murine macrophage cell line D9, and its ROI-generating, parental line J774.16, expressed comparable antimycobacterial activity upon activation. In addition, the oxygen radical scavengers superoxide dismutase (SOD), catalase, mannitol, and diazabicyclooctane had no effect on the antimycobacterial activity of macrophages. These findings, together with the results showing the relative resistance of *M. tuberculosis* to enzymatically generated H_2O_2, suggest that ROI are unlikely to be significantly involved in killing *M. tuberculosis*. In contrast, the antimycobacterial activity of these macrophages strongly correlates with the induction of the L-arginine-dependent generation of reactive nitrogen intermediates (RNI). The effector molecule(s) that could participate in mediating this antimycobacterial function are toxic RNI, including NO, NO_2, and HNO_2, as demonstrated by the micobacteriocidal effect of acidified NO_2. The oxygen radical scavenger SOD adventitiously perturbs RNI production, and cannot be used to discriminate between cytocidal mechanisms involving ROI and RNI. Overall, our results provide support for the view that the L-arginine-dependent production of RNI is the principal effector mechanism in activated murine macrophages responsible for killing and growth inhibiting virulent *M. tuberculosis*.

Tuberculosis remains the single greatest infectious cause of death in the world today. It is estimated that there are 1.75 billion persons infected with the tubercle bacillus worldwide, with 8 million new cases and 3 million deaths per year (1). Recently, there has been a dramatic increase in the number of persons with disease caused by *Mycobacterium tuberculosis*, and the portentous emergence of multiply resistant organisms, primarily as a result of the HIV epidemic and inadequate control measures (2).

Although *M. tuberculosis* was discovered by Koch (3) over a century ago, the mechanisms of killing and resistance to this facultative intracellular pathogen that can replicate and persist inside mononuclear phagocytes remain unclear. Since the initial report of the killing of *Mycobacterium microti* by immunologically activated macrophages (4), research efforts to understand antimycobacterial mechanisms have focused on the toxic effect of reactive oxygen intermediates (ROI)[1] generated by phagocytes during the respiratory burst. Despite such efforts, the role of ROI as effector molecules mediating antimycobacterial activity remains controversial. Studies on the antimicrobial capability of respiratory burst–deficient macrophages from chronic granulomatous disease patients suggested the existence of "oxygen-independent cytotoxic mechanisms" (5, 6). Such oxygen-independent mechanisms have been reported to participate in the antimicrobial activity of

[1] *Abbreviations used in this paper:* BCG, *Bacillus Calmette-Guerin*; DABCO, diazabicyclooctane; HNO_2, nitrous acid; MOI, multiplicity of infection; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NO_2, nitrogen dioxide; NO_2^-, nitrite; O_2^-, superoxide anion; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; SOD, superoxide dismutase.

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macrophages against *Toxoplasma gondii* (5), *Chlamydia psittaci* (6), *Bacillus Calmette-Guerin* (BCG) (7), *Leishmania donovani* (8), and *Schistosoma mansoni* (9).

Recently, macrophage-mediated cytotoxicity effective against tumor cells (10–12) has been linked to a metabolic pathway that involves oxidation of the guanidino nitrogen of L-arginine by the enzyme nitric oxide (NO) synthase to generate toxic reactive nitrogen intermediates (RNI): NO, nitrogen dioxide (NO_2), and nitrite (NO_2^-) (for review, see references 13 and 14). The effector molecule(s) responsible for this antitumor activity has been identified as NO/NO_2 (11, 12). Subsequently, RNI have been implicated in the antimicrobial effect of activated macrophages against a wide variety of pathogens, including *Cryptococcus neoformans*, *L. major*, *T. gondii*, *S. mansoni*, and *M. leprae* (for review, see reference 13). In this study, we examine the antimycobacterial effect of the L-arginine-dependent cytotoxic activity of murine macrophages.

Materials and Methods

Animals. 8-wk-old BALB/c female mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell Cultures. The murine macrophage cell lines (J774.16 and D9) were passaged in antibiotics-free DME as previously described (15). Cells (1.5×10^5) in 200 μl DME were allowed to adhere in wells of flat-bottomed, 96-well tissue culture plates (Fisher Scientific Co., Pittsburgh, PA) for studies of their antimycobacterial activity.

Primary mouse peritoneal macrophages were harvested from mice that had been injected intraperitoneally with thioglycollate 4–6 d earlier. Cells (2×10^5) in 200 μl DME were allowed to adhere in wells of flat-bottomed, 96-well tissue culture plates for 6 h, and nonadherent cells were washed off before assessing the antimycobacterial activity of these peritoneal macrophages.

Organisms. The virulent *M. tuberculosis* (Erdman strain) was obtained from Dr. Frank Collins (Trudeau Institute, Saranac Lake, New York). All liquid cultures were carried at 37°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with oleic acid–albumin-dextrose complex (OADC) (Difco Laboratories), 0.05% Tween 80, and cyclohexamide (0.1 mg/ml). Determination of colony-forming units (CFU) was done on similarly supplemented Middlebrook 7H10 agar (Difco Laboratories) plates. All experiments were performed with fresh, day 7 cultures of third passage. *L. donovani*, an isolate from a patient with visceral leishmaniasis (obtained from Dr. Murray Wittner, Albert Einstein College of Medicine), was cultured in Liver Infusion Tryptose medium at 25°C.

Reagents. N^G-Monomethyl-L-arginine (NNMA) was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Recombinant murine IFN-γ and TNF-α were obtained from Genzyme (Boston, MA). *Escherichia coli* LPS, L-arginine, sulfanilamide, *N*-1-naphthylethylenediamine, scopoletin, horseradish peroxidase, catalase, hydrogen peroxide, superoxide dismutase (SOD), mannitol, diazabicyclooctane (DABCO), saponin, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphoric acid (85%) was obtained from Fisher Scientific Co. [5,6-^{3}H]Uracil (sp act, 34 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

Measurement of Endotoxin. Media, sera, and reagents used in macrophage cultures were assayed for endotoxin using the quantitative *Limulus amoebocyte* assay (Whittaker M.A. Bioproducts, Walkersville, MD). The amount of endotoxin in these materials was found to be consistently <0.1 ng/ml.

Assessment of Antimycobacterial Activity of Macrophages. The antimycobacterial activity of cytokine-activated macrophages was assessed by metabolic labeling of *M. tuberculosis* with [^{3}H]uracil as previously described with modification (16). It is known that 80% of [^{3}H]uracil is incorporated into the RNA of mycobacteria, and the remainder into DNA (16). Day 7 cultures (third passage) of *M. tuberculosis* (Erdman strain) were used to infect macrophages at a multiplicity of infection (MOI) of 1–10:1. Based on preliminary experiments, a MOI of 5–10:1 was chosen for experiments using differential labeling of RNA, and an MOI of 1–2:1 was selected for experiments using CFU, to assess the antimycobacterial effect of macrophages. The reason for choosing a higher MOI (5–10:1) in the RNA labeling experiments was simply to achieve high enough [^{3}H]uracil incorporation (>12,000 cpm) by the tubercle bacilli above background of cultures without *M. tuberculosis* to allow meaningful data analysis. The data for determination of this MOI (5–10:1) were based on standardization experiments using MOIs of 40:1, 20:1, 10:1, 5:1, 2.5:1, 1.25:1, 1:1, and 0.5:1. For experiments using CFU to assess the antimycobacterial function of RNI-generating macrophages, the MOI of 1–2:1 chosen has also been derived from preliminary experiments that indicated optimal reduction in CFU, or increase in killing at lower MOI. Macrophages were primed with IFN-γ (250–500 U/ml) for 12–16 h at 37°C in 5% CO_2. Medium was removed from wells and LPS (1–3 μg/ml) or TNF-α (4,000 U/ml), and mycobacteria were added back to the primed macrophages in a final volume of 200 μl of DME. Control cultures received no mycobacteria. After 4–6 h of incubation at 37°C in 5% CO_2, cultures were washed three times to remove extracellular organisms. In all experiments using CFU as an index for cytotoxicity and growth inhibition, the number of these extracellular tubercle bacilli of each individual culture was ascertained by counting the number of CFU in the supernatants used to wash cultures by direct plating on 7H10 agar. Intracellular organisms were then determined by subtracting the number of extracellular CFU of each individual culture from the number of input bacilli CFU determined again by plating. These estimates were essentially confirmed by electron microscopic examinations of cultured macrophages infected with *M. tuberculosis*, which demonstrated that virtually all organisms remaining after washing cultures at 6 h postinfection are intracellular, not extracellular or pericellular. At 24 h postinfection, the cultures were pulsed with 2.5 μCi of [5,6-^{3}H]uracil for 16–24 h, lysed, and RNAs were TCA precipitated onto GF/C glass fiber filters (Whatman Inc., Clifton, NJ), and counted as previously described (17). This time for pulse was chosen on the basis of preliminary experiments using BCG that indicated that suppression of [^{3}H]uracil incorporation into RNA of the bacilli was observed after 6–9 h of exposure of organisms to RNI-generating macrophages, and peaked at 24 h. In experiments using virulent *M. tuberculosis*, the degree of suppression of [^{3}H]uracil incorporation by the tubercle bacilli seen after 24 h of exposure of the organisms to activated macrophages (93.7% ± 10%, n = 18) was similar to that seen after 48 h of exposure (97.0% ± 3.1%, n = 6). RNA synthesis by mycobacteria was measured as [^{3}H]uracil incorporation by cultures with organisms minus that by control cultures (dcpm). The inhibitory effect of activated macrophages on mycobacteria was measured as percent suppression of [^{3}H]uracil incorporation, expressed as: $100\times [1-(\text{dcpm}_{\text{stimulated macrophages}})/(\text{dcpm}_{\text{unstimulated macrophages}})]$. In experiments designed to examine the effect of ROI scavengers on the antimycobacterial function of activated macrophages, SOD, catalase, mannitol, and DABCO were added to cultures 4 h before infection at final concentrations of 2.5 mg/ml, 1.0 mg/ml, 50 mM, and 1 mM, respectively. Whenever parallel cultures were set up to assess the antimycobacterial effect

of activated macrophages by CFU, no [^{3}H]uracil was added, and macrophages were lysed with 0.1% saponin in distilled water at the end of experiments and plated onto 7H10 agar plates at serial dilutions.

Measurement of NO_2^-, Superoxide Anion (O_2^-), and Hydrogen Peroxide (H_2O_2). Culture supernatants were allowed to react with the Griess reagent, and the NO_2^- content was quantitated by measuring the absorption at 540 nm as described previously (18). Supernatants from mycobacteria-infected cultures were filter-sterilized before measurement. O_2^- generated by IFN-γ- and LPS-activated D9 and J774.16 cells was measured by the SOD-inhibitable cytochrome *c* reduction assay using a double-beam spectrophotometer as described previously (17). H_2O_2, generated by the glucose/glucose oxidase system, was quantitated by monitoring the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin (19).

Assessment of Antimycobacterial Effect of Enzymatically Generated H_2O_2. 2–10 × 10^6 cells of *M. tuberculosis* or *L. donovani* in 100 μl of Krebs-Ringer phosphate glucose solution (pH 7.4) were incubated with the H_2O_2-generating system (glucose/glucose oxidase) for 120 min at 37°C and 25°C, respectively. Control cultures contained no glucose oxidase. Organisms were then washed, resuspended in medium, and incubated overnight at the appropriate temperature. They were then pulsed-labeled with 2.5 μCi of [^{3}H]uracil for 18–24 h, and TCA precipitated as previously described (17). The inhibitory effect of ROI was measured by the decrease in [^{3}H]uracil incorporation in cultures containing enzymes compared with that of controls.

The Antimycobacterial Effect of Acidified NO_2^-. Approximately 2 × 10^6 cells of *M. tuberculosis* were incubated at 37°C for 24 h in 500 μl of 7H9 medium containing different concentrations of sodium nitrite (range, 0.25–10 mM) titrated to different pH (range, 4.5–6.5). NO_2^- was not added to control samples. Organisms were then washed, resuspended in fresh medium, pulsed with 2.5 μCi of [^{3}H]uracil for 18–24 h, and TCA precipitated as described previously (17). The antimycobacterial effect of NO_2^- was determined by both the decrease of [^{3}H]uracil incorporation and CFU in NO_2^--containing samples compared with controls.

Results

Production of ROI and RNI by J774.16 and D9 Cells. Evidence that ROI may not play a significant role in the antimycobacterial activity of murine macrophages has been derived largely from experiments relying on scavengers of ROI (7, 20). This approach obviously suffers from the drawback that scavengers may have nonspecific effects. We chose to pursue an alternative, somatic cell genetic approach by comparing the antimycobacterial activity of an ROI-producing murine macrophage cell line J774.16, with that of a mutant derived from it, clone D9, which is deficient in ROI production (15). Characterization of the IFN-γ- and LPS-induced production of ROI by these cells indicates that the parental clone J774.16 produces O_2^- efficiently (~4.5 nmol/10^6 cells/10 min), while the mutant clone D9 produces only a low level of superoxide anion (~1 nmol/10^6 cells/10 min) (data not shown). In contrast to the disparity in their ability to produce ROI, both J774.16 and D9 macrophages, after activation with IFN-γ and LPS, generate RNI efficiently, as measured by NO_2^- production (Fig. 1). In addition, the capacity for NO_2^- production by these two cell lines at 24 h

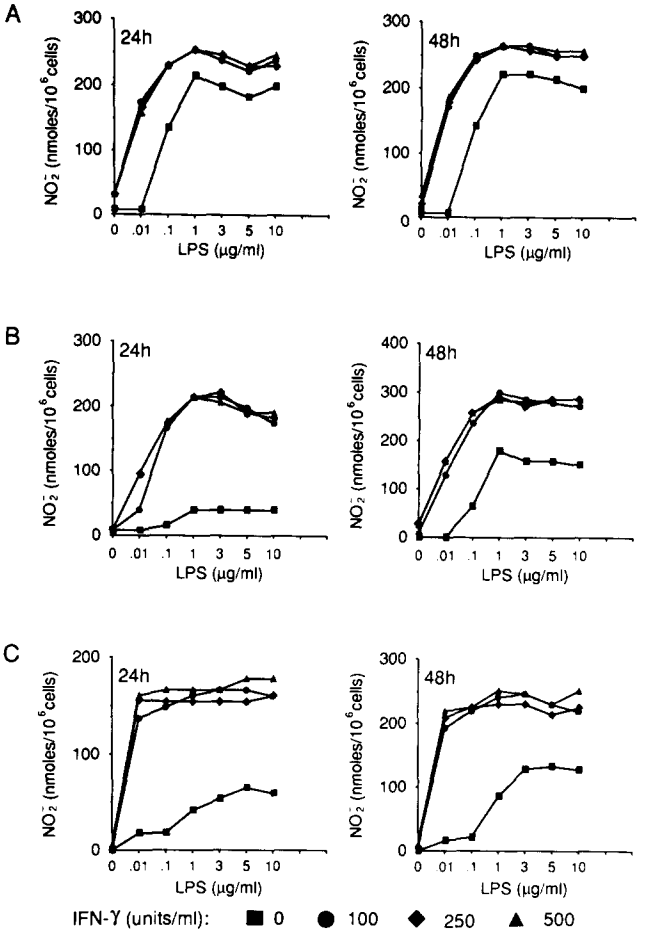

Figure 1. IFN-γ and LPS activate NO_2^- production by D9 (*A*), J774.16 (*B*), and BALB/c peritoneal macrophages (*C*) synergistically. Macrophages (1.5 × 10^5 cells per well in 96-well tissue culture plates) were primed with IFN-γ (500 U/ml) for 12–16 h. Supernatants were then removed from wells and replaced with culture medium containing LPS (3 μg/ml). 24 and 48 h later, supernatants were removed and assayed for their NO_2^- content. Data shown are representative of two experiments. Each point represents the mean of triplicate cultures, whose NO_2^- content was quantitated individually using the Griess reagent. The SDs of all data shown were <5% of the means.

and 48 h is comparable (Fig. 1). From these preliminary titration studies, the IFN-γ and LPS concentrations chosen for activating macrophages in subsequent experiments were 250–500 U/ml and 1–3 μg/ml, respectively.

Inhibition of NO Production by NMMA. NMMA, an analogue of L-arginine, has been shown effectively to block macrophage NO_2^- production (10), through competitive inhibition of NO synthases (21). We confirmed (Fig. 2) that NMMA effectively inhibits NO_2^- production by both J774.16 and D9 cells in a dose-dependent fashion, reaching 70–80% inhibition when used at a final concentration of 300–500 μM. This inhibitory effect of NMMA could be reversed by supplementing the culture with L-arginine, the substrate for NO synthase (data not shown). Based on these results, the concentrations of NMMA and L-arginine used

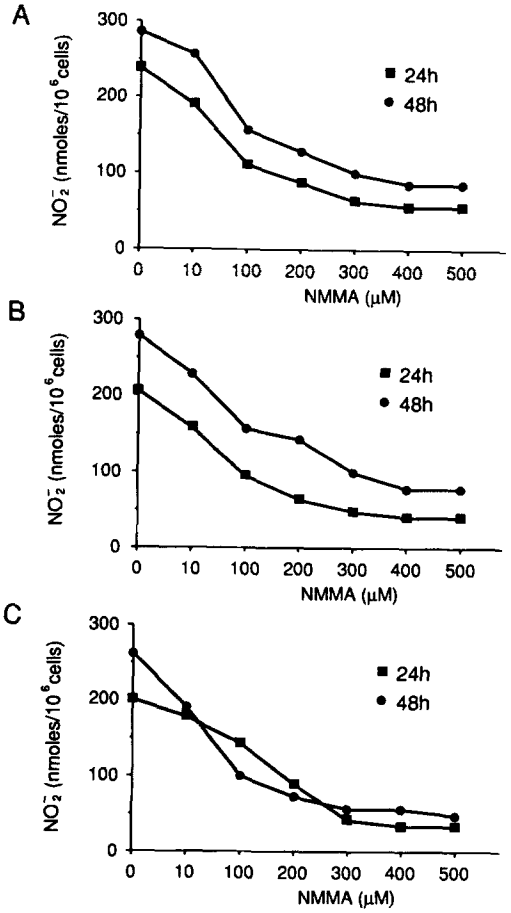

Figure 2. NMMA (250 μM) effectively inhibits NO_2^- production by IFN-γ- and LPS-activated D9 (*A*), J774.16 (*B*), and BALB/c peritoneal macrophages (*C*). Macrophages were stimulated with IFN-γ (500 U/ml) and LPS (3 μg/ml) as described in the legend to Fig. 1. Data shown are representative of two experiments. Each point represents the mean of triplicate cultures, whose NO_2^- content was measured individually by the Griess reagent 24–48 h after the addition of LPS. The SDs of all data shown were <5% of the means.

in subsequent experiments were 250–500 μM and 10 mM, respectively.

Effects of NO-producing Macrophages on [³H]Uracil Incorporation by M. tuberculosis (Erdman). Both D9 and J774.16 cells possess effective antimycobacterial activity upon activation with IFN-γ and LPS, inhibiting >90% of [³H]uracil incorporation by *M. tuberculosis* (Table 1). This antimycobacterial activity was diminished by NMMA, and correlated well with the amount of NO_2^- produced by the activated macrophages (Table 1), suggesting that the antimycobacterial effect of these macrophages was due to RNI. This was supported further by showing that the antimycobacterial effect of NMMA-treated D9 and J774.16 cells could be restored by addition of excess L-arginine, with concomitant restoration of NO_2^- production (Table 1). Several lines of evidence support the view that the [³H]uracil incorporation by *M. tuberculosis* reflects that of intracellular organisms: (*a*) extracellular organisms were washed off carefully 4–6 h postinfection in all experiments; (*b*) electron microscopic evaluation of *M. tuberculosis*–infected macrophage cultures indicated that virtually all organisms remaining after washing cultures at 6 h postinfection were intracellular, and not pericellular or extracellular; and (*c*) we had monitored TCA-precipitable counts of supernatants removed at the end-point of experiments before lysing cultures. No alteration in the percent suppression of [³H]uracil incorporation by *M. tuberculosis* in cytokine-activated macrophage cultures was observed even when the

Table 1. *Reactive Nitrogen Intermediate-generating D9, J774.16, and BALB/c Peritoneal Macrophages Are Inhibitory to M. tuberculosis*

Mϕ	NO_2^-	NMMA	L-Arginine	−IFN-γ/LPS +TB	−IFN-γ/LPS −TB	+IFn-γ/LPS +TB	+IFn-γ/LPS −TB	−IFn-γ/LPS	+IFN-γ/LPS	Suppression of [³H]uracil incorporation
	nmol/10^6 cells			*cpm ± SD*				*dcpm*		*%*
D9	325.3	−	−	18,898 ± 3,836	3,427 ± 243	3,770 ± 829	2,359 ± 310	15,471	1,411	90.9
	63.5	+	−	34,505 ± 3,040	3,521 ± 1,071	29,037 ± 4,007	7,161 ± 2,538	30,984	21,876	32.6
	174.6	+	+	27,612 ± 3,302	5,299 ± 2,152	5,900 ± 169	2,660 ± 1,457	22,312	3,240	85.5
J774.16	365.0	−	−	24,778 ± 2,830	6,236 ± 1,578	6,374 ± 1,703	4,581 ± 1,400	18,541	1,793	90.3
	79.4	+	−	42,202 ± 3,636	4,527 ± 519	34,199 ± 1,919	7,165 ± 136	37,675	27,034	28.2
	174.6	+	+	46,398 ± 5,200	5,168 ± 1,548	6,347 ± 1,740	2,621 ± 1,479	41,230	3,726	91.0
BALB/c	214.3	−	−	28,086 ± 1,403	1,838 ± 210	4,390 ± 430	5,016 ± 852	26,248	−626	102.4
	35.7	+	−	33,009 ± 403	1,032 ± 365	24,431 ± 1,443	5,398 ± 252	32,977	19,033	40.5
	191.4	+	+	30,823 ± 3,969	2,594 ± 539	3,493 ± 154	4,763 ± 752	28,229	−1,270	104.5

Data shown are representative of three experiments. All data were derived from triplicate cultures. Macrophages were infected at an MOI of 5–10:1. Infected cultures were pulsed 24 h postinfection, and harvested 16–24 h later. The antimycobacterial effect of these macrophages was assessed by the inhibition of [³H]uracil incorporation by *M. tuberculosis* (see Methods and Materials for details). The degree of inhibition by the IFN-γ- and LPS-activated macrophages strongly correlates with the amount of NO_2^- produced by these cells. Derivation of dcpm and percent suppression of [³H]uracil incorporation are detailed in Materials and Methods.

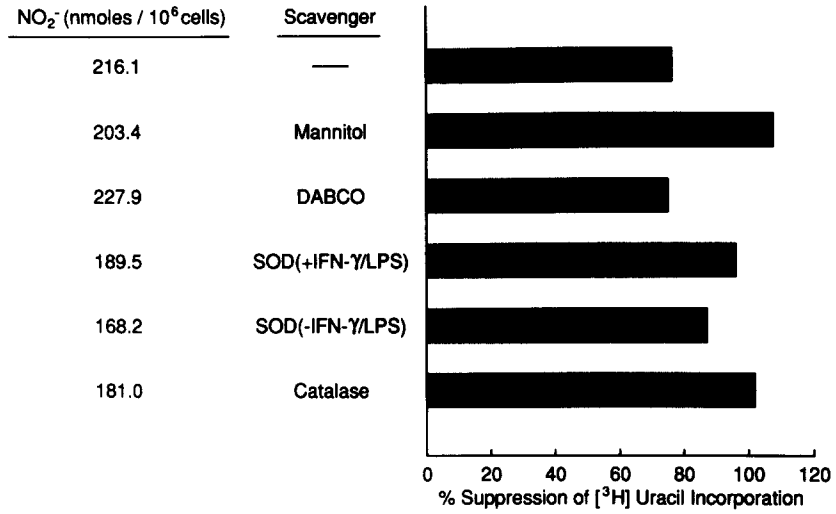

Figure 3. Effects of ROI scavengers on the antimycobacterial activity of IFN-γ- and LPS-activated D9 cells. Experiments were set up as described in the legend to Table 1, except that the various ROI scavengers were added to macrophage cultures 4 h before infection with *M. tuberculosis*. The time of pulsing and harvesting of cultures was as outlined in Materials and Methods. Macrophages were infected at a MOI of 5–10:1. The amount of [^{3}H]uracil incorporated by infected and uninfected cultures was in the same range as that shown in Table 1. SOD, catalase, mannitol, and DABCO had no effect on the antimycobacterial effect of cytokine-activated D9 macrophages, suggesting that O_2^-, H_2O_2, OH·, and $^1O_2$ do not play a significant role in defense against *M. tuberculosis*. SOD resulted in NO_2^- production by unstimulated D9 cells, rendering them inhibitory to *M. tuberculosis*. Consequently, the percent suppression of [^{3}H]uracil incorporation of SOD-treated cultures, both stimulated and unstimulated, was measured against the [^{3}H]uracil uptake of unstimulated cultures containing no scavengers. Experiments were performed twice with similar results. Data shown were derived from triplicate samples. Similar results were also obtained using J774.16 macrophages (data not shown).

supernatant-associated counts were taken into consideration (i.e., correcting the cpm for cell-associated counts) during data analysis (data not shown). Overall, we infer that RNI rather than ROI contribute significantly to the antimycobacterial activity of these macrophages because: (*a*) there was no correlation between the capacity to generate ROI and the antimycobacterial effect of these murine macrophage cell lines; and (*b*) in contrast, the ability of D9 and J774.16 to inhibit mycobacteria strongly correlated with RNI production (Table 1).

Effect of ROI Scavengers on the Antimycobacterial Effect of Macrophages. Although the D9 cell line produced significantly less ROI than J774.16, the possibility that the antimycobacterial activity of the mutant was due to the small amount of toxic oxygen intermediates generated could not be excluded. Consequently, we examined the effect of ROI scavengers on the antimycobacterial effect of D9 cells. Superoxide dismutase, catalase, mannitol, and DABCO were used as scavengers for O_2^-, H_2O_2, hydroxyl radicals (OH·), and singlet oxygen ($^1O_2$), respectively. These scavengers, at the concentrations used, did not affect the viability of macrophages (as assessed by trypan blue exclusion), and had no effect on [^{3}H]uracil incorporation by *M. tuberculosis* (data not shown). SOD, catalase, mannitol, and DABCO had no effect on the antimycobacterial activity of cytokine-activated D9 cells, suggesting that O_2^-, H_2O_2, OH·, and $^1O_2$ do not play a significant role in killing *M. tuberculosis*. Interestingly, treatment with SOD resulted in NO_2^- production by D9 cells that had not been activated with cytokines, rendering these macrophages inhibitory to mycobacteria (Fig. 3). Mannitol, catalase, and DABCO did not affect NO_2^- production. These data indicate that: (*a*) the antimycobacterial activity of D9 macrophages is clearly related to the amount of NO_2^- produced; (*b*) scavengers of ROI did not affect the antimycobacterial activity of RNI-generating macrophages, suggesting that oxygen radicals probably do not play a significant role in the ability of these macrophages to inhibit *M. tuberculosis*; and (*c*) the use of SOD to discriminate between the ROI- and RNI-dependent antimicrobial mechanisms of mononuclear phagocytes could lead to misinterpretation of results because this ROI scavenger renders unstimulated macrophages capable of producing NO_2^-, and therefore, antimycobacterial. The effect of SOD on NO_2^- production by macrophages was dose dependent, and abolished by boiling the native enzyme (Fig. 4).

Effect of H_2O_2 on M. tuberculosis. While it has been

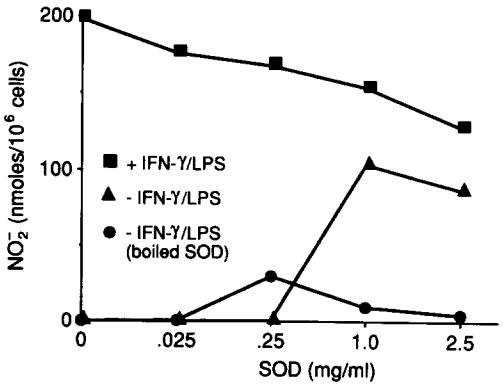

Figure 4. The ROI scavenger SOD perturbs NO_2^- production by D9 cells. Experiments were set up as described in the legend to Fig. 1, except that the various ROI scavengers were added to macrophage cultures 4 h before the addition of LPS. Culture supernatant was assayed for its NO_2^- content 48 h after addition of LPS. Each point represents the mean of triplicate samples, the SD of which is <5%. Results shown are representative of two experiments. SOD treatment of D9 macrophages that were not activated with IFN-γ and LPS resulted in NO_2^- production by these cells, rendering them effectively antimycobacterial (Fig. 3). This effect of SOD is dose dependent, and is abrogated by boiling the enzyme. Similar results were obtained with J774.16 macrophages (data not shown).

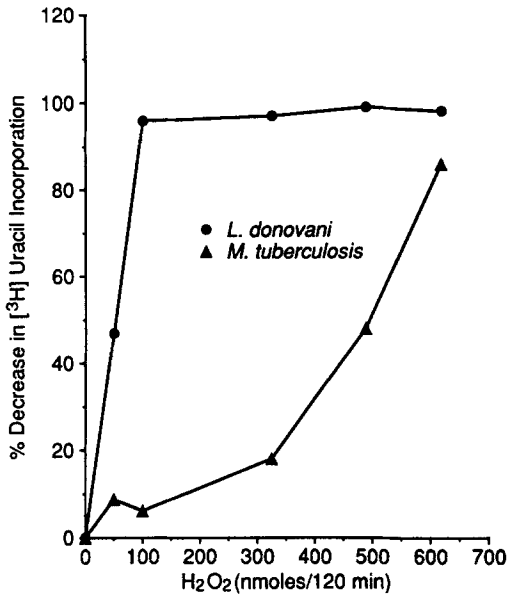

Figure 5. *M. tuberculosis*, is relatively resistant to H_2O_2 in a cell-free system. Hydrogen peroxide, enzymatically generated by glucose/glucose oxidase, effectively inhibited [^{3}H]uracil incorporation by *L. donovani* relative to *M. tuberculosis*. Hydrogen peroxide was quantitated by the horseradish peroxidase–catalyzed oxidation of scopoletin (29). Results shown are representative of four experiments. Each point is shown as the mean of triplicate samples.

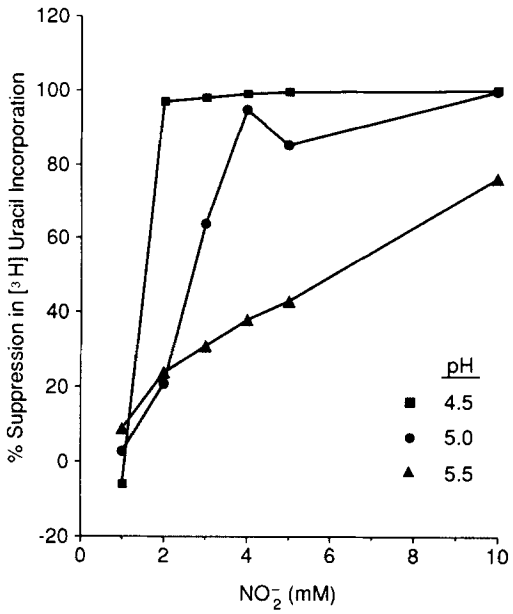

Figure 6. NO_2^- at acidic pH effectively inhibits *M. tuberculosis*. NO_2^--containing 7H9 culture medium inhibits mycobacterial [^{3}H]uracil incorporation, as assessed by [^{3}H]uracil incorporation, in a dose- and pH-dependent fashion. *M. tuberculosis* was exposed to NO_2^--containing medium for 24 h (see Materials and Methods for detail). At pH 6.0 or above, NO_2^- does not inhibit *M. tuberculosis* at concentrations up to 10 mM (data not shown). The experiments were performed three times with similar results. Each point represents the results derived from triplicate samples.

demonstrated that *M. tuberculosis* is resistant to O_2^- (22), the sensitivity of the tubercle bacillus to H_2O_2 remains controversial (23). Consequently, we tested the susceptibility of *M. tuberculosis* to hydrogen peroxide generated by glucose/glucose oxidase in a cell free system. In these experiments, *L. donovani*, the etiologic agent of visceral leishmaniasis, was used as control. The amount of H_2O_2 generated was quantitated in parallel samples without cells under the same experimental conditions. *M. tuberculosis* was significantly more resistant to H_2O_2 compared with *L. donovani* over a wide dose range tested (Fig. 5). This relative resistance of *M. tuberculosis* to H_2O_2, together with the data demonstrating that SOD, catalase, mannitol, and DABCO did not affect the inhibitory effect of activated D9 cells on *M. tuberculosis*, strongly suggests that ROI do not play a significant role in the antimycobacterial activity of macrophages.

Acidified NO_2^- Is Mycobacteriocidal. Recent reports have shown that NO_2^- is tumoristatic at low pH, apparently due to the formation of nitrous acid (pK_a = 3.4), which then dismutates to generate NO and NO_2 (12, 24). Therefore, to examine directly the antimycobacterial effect of RNI, we tested the effect of NO_2^- on *M. tuberculosis* at various pHs. Results in Fig. 6 show that NO_2^- becomes inhibitory to mycobacteria upon acidification. While no antimycobacterial effect of NO_2^- was detected at pH 6.0 and 6.5, even when NO_2^- was used at a final concentration of 10 mM (data not shown), treatment of the bacilli with 2 mM of NO_2^- for 24 h at pH 4.5 inhibited >95% of [^{3}H]uracil incorporation. In fact, acidified NO_2^- is mycobacteriocidal (Table 2), achieving complete killing at 5 mM nitrite at pH 4.5. These results formally establish that RNI are mycobacteriocidal.

Effects of RNI-generating Primary Murine Macrophages on M. tuberculosis. To establish whether the findings in murine macrophage cell lines can be extended to primary murine mononuclear phagocytes, we studied the antimycobacterial effect of IFN-γ- and LPS-activated peritoneal macrophages

Table 2. *Reactive Nitrogen Intermediate(s) Generated by Acidfied NO_2^- Are Mycobacteriocidal*

	CFU (±SD)	
$NaNO_2$	pH 4.5	pH 7.0
mM	$\times 10^{-4}$	
0	590 ± 65	450 ± 100
1	45 ± 5	420 ± 120
2	0.16 ± 0.02	370 ± 38
5	0	580 ± 120
10	0	600 ± 60

Assessment of the effect of acidified nitrite on *M. tuberculosis* by CFU indicates that this RNI-generating system kills *M. tuberculosis* efficiently. The mycobacterial effect is dose and pH dependent. Results shown are representative of three experiments. Data are shown as means of triplicate samples and SDs.

of BALB/c mice. Optimal experimental conditions for RNI production by these cells were found to be similar to those established for J774.16 and D9 macrophages (Fig. 1). Results in Table 1 indicate that the antimycobacterial activity of primary murine peritoneal macrophages is comparable with that of the macrophage cell lines, and again, strongly correlated with the amount of RNI generated.

TNF-α Can Substitute for LPS in the Activation of the Antimycobacterial Effect of the L-arginine-dependent Cytotoxic Mechanism. In our system used to study the antimycobacterial effect of macrophages, IFN-γ alone was insufficient for optimal generation of RNI (Fig. 1). However, both *M. tuberculosis* and LPS stimulated NO_2^- production by IFN-γ-primed macrophages synergistically in a dose-dependent fashion (data not shown and Fig. 1). Indeed, BCG has been found to be effective in stimulating NO_2^- production by macrophages both in vitro and in vivo (25, 26). Since TNF has been shown to act synergistically with IFN-γ in stimulating the production of RNI by murine peritoneal macrophages (27), and to enhance macrophage antimycobacterial activity against BCG and *M. avium* when used in conjunction with IFN-γ (28, 29), we investigated whether TNF-α could substitute for LPS in activating the antimycobacterial activity of D9, J774.16, and BALB/c peritoneal macrophages. The kinetics of NO_2^- production by these macrophages, and the optimal dose of IFN-γ, TNF-α, NMMA, and L-arginine used in these experiments, were determined by preliminary studies (data not shown). The antimycobacterial effect of J774.16, D9, and BALB/c peritoneal macrophages is entirely preserved when TNF-α is used in place of LPS together with IFN-γ as activating agents, inhibiting >90% of [^{3}H]uracil incorporation (Fig. 7). Since *M. tuberculosis* and its cell wall–associated glycolipid, lipoarabinomannan (LAM), can cause the release of TNF from human blood monocytes and mouse peritoneal macrophages (30), the synergistic effect of this cytokine and INF-γ in inducing antimycobacterial activity of macrophages in vitro is likely to have physiological relevance in vivo.

RNI-producing Macrophages Are Cytocidal for Virulent M. tuberculosis. Measurement of inhibition of [^{3}H]uracil incorporation by microbes offers a rapid assessment of antimicrobial activity of infected macrophages (16), and is, in fact, indispensable for experiments involving *M. tuberculosis*, a slow-growing biohazard class 3 pathogen that forms colonies only after 4 wk on agar plates. However, this assay formally cannot distinguish between growth inhibition and killing of microbes. Therefore, we examined whether RNI-generating macrophages were mycobacteriocidal by direct assessment of *M. tuberculosis* CFU. In these experiments, the number of intra- and extracellular bacilli was determined as described in Materials and Methods. Since the number of intracellular bacilli and of macrophages per culture was ~1–2 × 10^5 CFU and 1.5 × 10^5, respectively, the average number of bacilli per macrophage 4–6 h postinfection, in these experiments, was ~0.7–1.3. Light microscopy examination of acid-fast–stained infected cultures, as well as electron microscopic examination of these cultures, showed consistently that ~30–40% of the macrophages were infected, generally with more than one acid-fast bacillus per cell. Evaluation by trypan blue exclusion indicated that viability of macrophages in *M. tuberculosis*–infected cultures was in the range of 85–95%, and that for uninfected cultures, 90–99%. The presence of NMMA and L-arginine did not affect the viability of these macrophages. RNI-generating D9 and J774.16 cells reduced the input CFU by 73% and 72% at 48 h postinfection, respectively, compared with unstimulated controls that did not generate significant amounts of NO_2^- (Table 3). More important, when stimulated to generate RNI, these cells actually decreased the input intracellular *M. tuberculosis* CFU by 42% and 55%, respectively, demonstrating that RNI generating macrophages were mycobacteriocidal (Table 3). Again, the ability of these macrophages to kill *M. tuberculosis* strongly correlated with the amount of NO_2^- generated. The mycobacteriocidal effect of the macrophage cell lines was found to be as effectively exhibited by peritoneal macrophages obtained from BALB/c mice stimulated to generate RNI (Table 4). These primary cells reduced the input *M. tuberculosis* CFU by 58% and 56% at 48 and 96 h postinfection, respectively. These data, together with those demonstrating comparable inhibitory capacity of cytokine-stimulated D9, J774.16, and primary BALB/c macrophages on [^{3}H]uracil incorporation by the tubercle bacillus, indicated that all phagocytes tested were equally effective in killing or growth inhibiting virulent *M. tuberculosis* when activated to produce RNI.

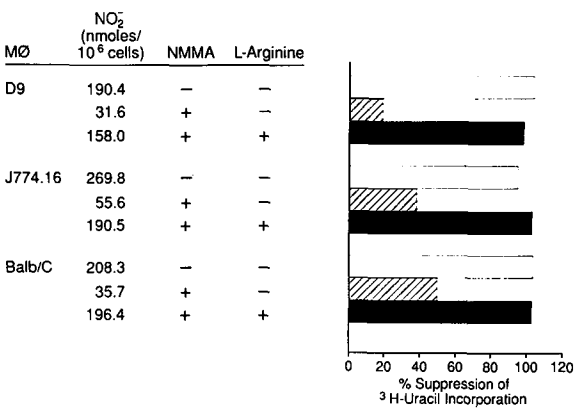

Figure 7. IFN-γ- and TNF-α-activated D9, J774.16, and BALB/c peritoneal macrophages are inhibitory to *M. tuberculosis*. The experiment was performed as described in the legend to Table 1, using TNF-α instead of LPS. Data shown are representative of two experiments. All data were derived from triplicate cultures. The amount of [^{3}H]uracil incorporation by infected cultures was in the same range as that shown in Table 1.

Discussion

Although it is generally accepted that resistance to tuberculosis is dependent upon cell-mediated immunity, the mechanisms by which *M. tuberculosis* is killed or growth inhibited are not fully understood. While it has long been reported that the in vitro killing of *M. microti* by murine macrophages could be inhibited by catalase, the scavenger for H_2O_2 (4), the question as to whether ROI produced by activated macrophages play a significant protective role against *M. tuberculosis* remains unsettled. The recent findings that biosynthesis

Table 3. *Reactive Nitrogen Intermediate-generating D9 and J774.16 Macrophages Are Mycobacteriocidal*

			−IFN-γ/LPS					+IFN-γ/LPS				
			CFU		Decrease	Decrease in CFU		CFU		Decrease	Decrease in CFU	
Mϕ	NMMA	L-Arginine	0 h	48 h	in CFU	(mean ± SD)	NO_2^-	0 h	48 h	in CFU	(mean ± SD)	NO_2^-
			× 10^5			%	nmol	× 10^5		%	%	nmol
D9			–	–	–			1.22	0.56	54.1		
	–	–	1.12	2.4	−114.3	−122.1 ± 11.1	3.2	1.03	0.65	36.9	41.4 ± 11.1	190.6
			1.00	2.3	−130.0			1.02	0.68	33.3		
			1.03	2.0	−94.2			0.94	0.81	13.8		
	+	–	1.08	2.0	−85.2	−95.8 ± 11.4	3.2	0.94	0.78	17.0	15.9 ± 1.3	55.6
			1.01	2.1	−107.9			0.94	0.78	17.0		
			1.00	1.9	−90.0			0.96	0.59	38.5		
	+	+	1.17	2.3	−139.3	−114.5 ± 24.7	3.2	1.03	0.62	39.8	42.9 ± 6.6	103.2
			1.12	2.4	−114.3			1.01	0.50	50.5		
J774.16			0.86	1.5	−74.4			0.81	0.31	61.7		
	–	–	0.91	1.4	−52.1	−68.8 ± 14.7	6.33	0.96	0.54	43.8	55.2 ± 9.9	182.6
			0.89	1.6	−79.8			1.00	0.40	60.0		
			0.89	1.3	−46.1			–	–	–		
	+	–	0.95	1.4	−47.4	−67.0 ± 24.7	3.2	0.70	0.70	0	5.0 ± 7.0	39.7
			0.95	1.8	−89.5			0.90	0.81	10.0		
			0.94	1.8	−91.5			0.78	0.48	38.5		
	+	+	0.97	1.4	−44.3	−52.7 ± 35.4	3.2	0.99	0.60	39.4	41.7 ± 4.7	102.9
			0.90	1.1	−22.2			0.85	0.45	47.1		

Macrophages were infected at an MOI of 1–2:1. The number of bacilli used to infect macrophages was 1.6 × 10^5. Direct assessment of the effect of the antimycobacterial effect of RNI-generating macrophages by quantitating CFU indicates that these cells are mycobacteriocidal because culturing *M. tuberculosis* with activated macrophages for 2 d resulted in a decrease in the input CFU by 41.4% to 55.2%. Data are shown as means of triplicate samples and SDs.

of nitrate occurs in mammalian cells (31), and that macrophages are a major source of mammalian nitrate synthesis (32), have spurred intensive research efforts leading to the discovery of the L-arginine-dependent metabolic pathway used to generate reactive nitrogen intermediates (for review, see references 13, 14, 33, and 34). The biological significance of this novel pathway is underscored by the participation of its products, NO or its related species, in a wide range of physiological processes. NO accounts for the activity of endothelial-derived relaxing factor, which mediates vasodilation through interaction with the heme prosthetic group of cytosolic guanylate cyclase; it functions as a neurotransmitter in the central nervous system; and it inhibits platelet aggregation (for review, see references 13, 14, 33, and 34). In the immune system, the L-arginine-dependent pathway offers a new microbicidal mechanism for resistance to infectious pathogens (for review, see references 13, and 14). Since the report that antitumor activity of activated macrophages is related to the L-arginine-dependent metabolic pathway (10–12), the significance of this novel cytotoxic mechanism has been extended to macrophage antimicrobial defense against a wide variety of organisms (for review, see references 13 and 14). The present study provides evidence that: (*a*) the mycobacteriocidal activity of cytokine-activated murine macrophages strongly correlates with the induction of the L-arginine-dependent generation of RNI; (*b*) the effector molecule(s) that could participate in mediating this antimycobacterial function are toxic RNI, including NO, NO_2, and HNO_2; (*c*) RNI generated chemically are mycobacteriocidal; (*d*) ROI are unlikely to be significantly involved in killing *M. tuberculosis*; (*e*) the oxygen radical scavenger SOD perturbs NO_2^- production and must be used with caution in discriminating between cytocidal mechanisms involving ROI and RNI.

The significance of the L-arginine-dependent cytotoxic mechanism for macrophage inhibition against another pathogenic mycobacterium, *M. leprae*, has been reported recently (35). Further, our results confirm and considerably extend recent reports that implicated RNI in resistance to *M. tuberculosis* (20) and BCG (36), but that did not provide direct evidence on the cytotoxic and cytocidal effects of RNI against mycobacteria. It must be stated, however, that the assessment of the growth and killing of *M. tuberculosis* in macrophage cultures is technically difficult (37, 38). *M. tuberculosis* is a serious pathogen requiring high-level containment, it clumps

Table 4. *Reactive Nitrogen Intermediate-generating BALB/c Peritoneal Macrophages Are Mycobacteriocidal*

Time of harvest (postinfection)	NMMA	L-Arginine	−IFN-γ/LPS CFU 0 h	−IFN-γ/LPS CFU Time of harvest	−IFN-γ/LPS Decrease in CFU	−IFN-γ/LPS Decrease in CFU (mean ± SD)	−IFN-γ/LPS NO_2^-	+IFN-γ/LPS CFU 0 h	+IFN-γ/LPS CFU Time of harvest	+IFN-γ/LPS Decrease in CFU	+IFN-γ/LPS Decrease in CFU (mean ± SD)	+IFN-γ/LPS NO_2^-
h			× 10^5		%	%	*nmol*	× 10^5		%	%	*nmol*
48			2.11	4.7	−123.8			2.02	0.77	62.0		
	−	−	2.15	3.8	−76.7	−100.3 ± 33.3	5.95	2.03	0.91	55.2	58.3 ± 3.4	202.4
			−	−	−			1.96	0.83	57.7		
			2.74	3.7	−65.2			2.04	2.2	−7.8		
	+	−	1.97	3.3	−67.5	−77.2 ± 18.8	0	1.92	2.1	−9.4	−8.6 ± 1.1	17.9
			1.96	3.9	−98.9			−	−	−		
			1.99	3.6	−80.9			1.81	0.62	65.7		
	+	+	1.99	3.1	−55.9	−68.4 ± 17.7	0	1.88	0.86	54.3	60.7 ± 5.3	160.7
			−	−	−			2.00	0.76	62.0		
96			1.97	7.1	−260.4			2.06	1.05	49.0		
	−	−	1.97	5.6	−184.3	−222.4 ± 53.8	23.8	1.92	0.90	53.1	56.2 ± 9.2	273.8
			−	−	−			1.94	0.65	66.5		
			2.03	9.1	−348.3			2.11	6.0	−184.4		
	+	−	2.05	8.9	−334.1	−332.1 ± 17.3	0	2.07	5.8	−180.2	−204.5 ± 38.6	29.8
			2.03	8.4	−313.8			2.02	7.4	−249.0		
			1.96	8.1	−413.3			1.94	0.59	69.6		
	+	+	1.98	10.1	−410.1	−374.8 ± 63.9	0	1.88	0.71	62.2	65.9 ± 5.2	267.9
			1.92	7.7	−301.0			−	−	−		

Macrophages were infected at an MOI of 1–2:1. The number of bacilli used to infect macrophages was 2.7 × 10^5. Direct assessment of the effect of the antimycobacterial effect of RNI-generating macrophages by quantitating CFU indicates that these cells are mycobacteriocidal, decreasing the number of input CFU by 58.3% at 48 h postinfection. Similar degree of killing was observed when organisms were cultured with activated macrophages for 96 h (56.2%). Data are shown as means of triplicate samples and SDs.

in culture and in macrophages, confounding assessment of growth and killing of single cells, and it requires 3–4 wk for colonies to grow. Commonly encountered technical problems were taken into careful consideration in these experiments. (*a*) No antibiotics were used in cultures. (*b*) Extracellular organisms were minimized by carefully washing out cultures after 4–6 h of infection. The medium used for washing as well as the original culture supernatants were pooled and the number of CFU determined. Since the number of CFU used to infect cultures was known, the input of CFU could be determined precisely for each culture well. (*c*) After infections were set up, cultures were not manipulated until harvest. (*d*) At the end of the experiment, after careful removal of the culture supernatant, 0.1% saponin was used to lyse cells to minimize errors from clumping. The removed supernatant was centrifuged to assure retrieval of any cells that were removed inadvertently. The cell pellet was lysed with saponin, and pooled together with lysed cells from the corresponding culture for determination of CFU.

It is generally accepted that failure of bacteria to form colonies on nutrient media is the most reliable measure of killing or nonviability. Dormant states are conceivable but not easily demonstrated. Once removed from macrophages, growth-inhibited but viable mycobacteria would be expected to form colonies after 4 wk of culture on nutrient-enriched medium, and correlatively, failure to do so means the organisms are not, by the standard criterion, viable. In preliminary experiments, agar plates were kept and counted for CFU periodically for as long as 2 mo, thereby excluding the possibility of undercounting as a result of a slower growth rate of reversibly inhibited organisms. Therefore, our data (Tables 3 and 4) that RNI-generating macrophages decreased the number of viable input organisms by quantifying CFU on agar plates indicated that these phagocytes are cytocidal for *M. tuberculosis* in this in vitro system. The more efficient macrophage antimycobacterial activity seen in experiments using radiolabeling (~90% suppression; Table 1) versus direct counting of CFU (~50% decrease in CFU compared with input intracellular organisms; Tables 3 and 4) as in index for assessment suggests that killing is not complete and that there

is likely to be a significant component of reversible intracellular bacteriostasis in our in vitro system.

The effector molecules mediating mycobacteriocidal activity that result from acidification of NO_2^- could be either NO, NO_2, or HNO_2, or any combination of the three. The antibacterial effect of sodium nitrite, a compound that can form various oxides of nitrogen at acidic pH (12, 24), has been known for years (39). The mechanisms for this effect had been studied extensively in the past as a result of the widespread use of NO_2^- in meat curing. Data from these studies had shown that the ability of sodium nitrite to kill and to inhibit certain microorganisms was pH dependent, reaching a maximum at the pH range of 4.5–5.5 (24, 39). The reactive nitrogen species directly responsible for the toxicity had been proposed to be NO/NO_2 or HNO_2 (24, 40). Data from the present study indicate that sodium nitrite effectively killed virulent *M. tuberculosis* at acidic pH (Table 2). At pH 4.5, 1 mM NO_2^- resulted in >1-log decrease in CFU, reaching 3 log and total killing at 2 and 5 mM, respectively. The pH-dependent mycobacterial effect of NO_2^- may be of relevance in vivo in the acidic environment of phagolysosomes of activated macrophages (as low as pH 4.5) (41). Indeed, the significance of the microbicidal effect of RNI in vivo has been demonstrated recently in a murine cutaneous leishmaniasis model (14, 42). In our system, the difference in efficiency between the mycobacteriocidal activity of RNI generated by acidification of NO_2^- in vitro (Table 2) and by cytokine-activated macrophages (Tables 3 and 4) could be used to challenge the significance of this mechanism in killing *M. tuberculosis* in vivo. Although it is impossible to determine the dosage of bacilli necessary to initiate infection, data obtained from animal studies and clinical observations suggest that human beings under natural conditions probably inhale no more than a few organisms (43, 44). In contrast, the dose of bacilli used to infect macrophage cultures in our experiments was routinely $1–2 \times 10^5$. This large dose of bacilli is nonphysiological, and it is likely that in vivo resistance can be achieved when RNI-generating macrophages are challenged with but a few *M. tuberculosis*. Indeed, it is well known that the character and the course of tuberculous infection in animals depends to a large extent on the dose of *M. tuberculosis* used for infection (43). It is, however, important to bear in mind that the purpose of testing the antimycobacterial effect of acidified NO_2^- was to establish, in a cell-free system independent of the complex events within macrophages, whether chemically produced RNI are mycobacteriocidal. The amount of NO_2^- added in the cell-free system (Fig. 6 and Table 2) is clearly greater than that produced by macrophages stimulated in vitro as measured by quantifying the amount of NO_2^- in culture supernatants. It is, however, possible that the effective concentrations in the cell-free system can be achieved within particular intracellular compartments. Quantitative comparison of the macrophage and cell-free killing systems are, at present, not possible because the actual cytocidal species of RNI within macrophages is not known, nor is its intracellular half-life, concentration, and compartmentalization. Similarly, for the cell-free system, information on the relative concentrations of the various RNI (including NO, NO_2, HNO_2, etc.), and their stability are not known.

In contrast to the potent antimycobacterial activity of RNI, our data suggest that ROI probably do not play a significant role in macrophage antimycobacterial activity. (*a*) The ROI-deficient D9 mutant kills *M. tuberculosis* as effectively as its parental line, J774.16, which generates oxygen radicals efficiently (Tables 3 and 4). (*b*) The antimycobacterial effect of activated macrophages strongly correlates with the amount of RNI generated (Figs. 3 and 7, and Tables 1, 3, and 4). (*c*) The antimycobacterial effect of nonactivated D9 in the presence of SOD correlates with NO_2^- production (Fig. 3). (*d*) Scavengers for O_2^-, H_2O_2, OH·, and $^1O_2$ do not affect the antimycobacterial activity of macrophages (Fig. 3). (*e*) *M. tuberculosis* is highly resistent to killing by H_2O_2 generated enzymatically by the glucose/glucose oxidase system (Fig. 5). Consistent with the view that ROI may not contribute significantly to macrophage antimycobacterial activity is evidence that *M. tuberculosis* can parasitize mononuclear phagocytes without eliciting the oxidative burst by gaining entry via the C3 receptor (45). Further, we and others have shown that major cell wall–associated and secreted glycolipids of mycobacteria are efficient scavengers of oxygen radicals (17, 46, 47).

Obviously, our data in vitro do not entirely exclude a role for ROI in defense against *M. tuberculosis*, since D9 cells are capable of generating a low level of oxygen radicals. In addition, the ROI scavengers used may not have removed all the reactive oxygen species generated by activated macrophages, and may produce some nonspecific effect. Indeed, one of the scavengers used in the present study was adventitiously found to affect macrophage NO_2^- production (Figs. 3 and 4). Addition of SOD to D9 cells not exposed to cytokines resulted in significant production of NO_2^-. Superoxide anion is known to react with NO to form peroxynitrite anion, which decomposes rapidly once protonated to form OH· and NO_2, and subsequently nitrate (48). In a system used to evaluate the vasodilatory effect of NO derived from endothelial cells, SOD has been shown to increase the stability of this RNI by scavenging O_2^- (49). Therefore, it is possible that SOD, by virtue of its ability to scavenge O_2^-, stabilizes a basal level of NO released by nonactivated D9 macrophages, thus rendering these cells inhibitory to mycobacteria. The effect of SOD on NO_2^- production was also observed in J774.16 cells (data not shown). The precise mechanisms by which SOD affects NO_2^- production by macrophages are currently unknown; though the fact that this effect was not seen with boiled enzymes suggests that native conformation of the enzyme, which is most likely required for the scavenging of O_2^- by SOD, is important. Thus, SOD, one of the simplest tools for discriminating between microbicidal mechanisms dependent on ROI or RNI perturbs the production of RNI and can lead to significant misinterpretation of data. The biological significance of the effects of SOD on macrophage NO_2^- production remains to be determined, and the relationship between the respiratory burst and the L-arginine-dependent cytotoxic pathway merits reexamination.

Finally, the relevance of the present findings on the antimycobacterial activity of RNI produced by murine macrophages to human tuberculous infections requires comment. It must be stated that convincing evidence that human monocytes produce sufficient amounts of RNI for antimicrobial activity has, despite efforts in several laboratories, not been forthcoming. Thus, the importance of the L-arginine-dependent cytotoxic mechanism in resistance to human pathogens remains to be established.

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