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Susceptibility of a Panel of Virulent Strains of *Mycobacterium tuberculosis* to Reactive Nitrogen Intermediates

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Murine bone marrow-derived macrophages were infected with a panel of virulent isolates of *Mycobacterium tuberculosis* including laboratory strains Erdman and H37Rv and various clinical isolates in order to determine the sensitivity of each of these strains to the antimycobacterial activities of macrophage-generated reactive nitrogen intermediates (RNI). All of the *M. tuberculosis* strains grew in murine bone marrow-derived macrophages; however, gamma interferon-primed macrophages limited the initial growth of intracellular bacilli. Some of the mycobacterial strains, including Erdman, were killed over the first 4 days of infection, as evidenced by significant decreases in the number of viable intracellular bacilli determined by a CFU assay. Other mycobacterial strains were not killed during this same period, and some isolates, including CSU 24 and CSU 31, grew steadily in activated macrophages. The accumulation of nitrite on infected monolayers was measured, and it was found that inhibitory levels of RNI did not vary among infections with the different strains. Nitrite tolerance was determined in a cell-free system for each of the strains in order to compare susceptibilities of the strains to RNI. All of the strains tested were killed by levels of RNI generated by the acidification of 10 mM $NaNO_2$ to pH 6.5 or 5.5, and the strains exhibited a range of tolerance to lower concentrations of RNI. No correlations were observed between such cell-free RNI tolerances and the capacity of bacilli to resist macrophage RNI-mediated killing. These results indicate that under stringent conditions, RNI can kill *M. tuberculosis*, but that under less harsh, more physiological conditions, the effects of RNI range from partial to negligible inhibition.

A protective response to infection with *Mycobacterium tuberculosis* involves the production of gamma interferon (IFN-γ) by mycobacterial antigen-specific T cells which activates infected macrophages to control the growth of intracellular bacilli (10, 40). Intracellular bacilli resist several IFN-activated defenses of macrophages (41). Phagosome acidification is hindered by *M. avium* and *M. tuberculosis* (5, 11, 50), and phagosome-lysosome fusion (22, 26, 36) only partially occurs following phagocytosis of live bacilli. Also, mycobacteria are not susceptible to the solitary effects of macrophage-generated reactive oxygen intermediates (4, 13, 20). Rather, most evidence suggests that IFN-activated murine macrophages control *M. tuberculosis* via a nonoxidative, arginine-dependent mechanism which involves the generation of reactive nitrogen intermediates (RNI) (9, 19, 21).

For a variety of microbial pathogens, murine macrophage antimicrobial activity is correlated with the production of nitrite from L-arginine, and the inhibition of macrophage nitrite production by arginine analogs is correlated with the loss of antimicrobial activity (1, 3, 6, 20, 25, 29). Microbial and endogenous signals stimulate expression of inducible nitric oxide synthase (iNOS) in macrophages (12, 43, 48, 55), and this enzyme cleaves a terminal guanidino group from L-arginine, thereby liberating the nitric oxide radical, NO, and a by-product, L-citrulline (reviewed by Marletta [30] and Nathan et al. [33, 34]). NO is quickly oxidized to other RNI, including nitrogen dioxide and peroxynitrite, and ultimately to end products, nitrite (NO_2^-) and nitrate (NO_3^-), which are relatively stable under normal physiological conditions. RNI chemically generated in bacterial growth media are directly inhibitory for the growth of a wide range of bacteria (45), including mycobacteria (9, 15, 21, 35, 52). Acidification of nitrite is antibacterial, and the effects are due to the conversion of nitrite to the more reactive species, nitrous acid (HNO_2) or NO (15, 45, 49). Reactivity of these radicals toward transition metals, thiol groups, Fe-S groups, lipids, and DNA bases (reviewed by Farrell and Blake [17] and Nathan [32]) results in a general inhibition of the energy conservation of bacteria through interaction with enzymes involved in proton-dependent active transport, oxygen uptake, and oxidative phosphorylation that are inactivated (56) in aerobic bacteria.

Reports conflict as to the exact nature of RNI-mediated control exerted by IFN-activated, murine macrophages on mycobacteria. In vitro infections with *M. avium* (4, 6) and *M. lepraemurium* (31) are not always controlled, but similarly stimulated murine macrophages control *M. tuberculosis* and *M. bovis* (1, 9, 20) via RNI. Also the various macrophage models differ in the ability to control mycobacterial infections via RNI. For instance, human macrophages require stimuli other than IFN-γ to control *M. avium* (7, 8, 14) and *M. tuberculosis* (16, 44). Furthermore, data also conflict as to whether macrophage RNI represent a mycobacteristatic (4, 13, 14, 21) or mycobactericidal (4, 9, 14) defense in murine and human macrophages. Mycobacterial strain-related differences may be responsible for some of these contradictory results (4, 14, 15, 35, 46).

To clarify the nature of RNI-mediated control of *M. tuberculosis*, we infected a well-characterized murine bone marrow-derived macrophage model with a panel of virulent isolates, with or without IFN priming. In this report, we provide further evidence of strain-related differences in the ability of *M. tuberculosis* to resist macrophage RNI-mediated control. Macrophage killing of some virulent strains was linked to RNI pro-

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TABLE 1. Characterization of *M. tuberculosis* strains used

Strain	MDR[a]	Virulence[b]	Source[c]
CSU 15	No	Low	K
CSU 21	Yes	Low	C
CSU 22	Yes	High	C
CSU 24	No	Moderate	C
CSU 25	No	High	C
CSU 31	No	High	N
CSU 32	Yes	High	N
CSU 35	Yes	High	N
CSU 39	Yes	High	N
CSU 44	No	High	N
CSU 46	No	Moderate	N
H37Rv	No	Moderate	TMCC 102
Erdman	No	Moderate	TMCC 107

[a] Strains were classified as multiple drug resistant (MDR) if they were resistant to six or more of the following drugs: isoniazid, ethambutol, rifampin, streptomycin, kanamycin, cycloserine, *para*-amino salicylic acid, rifabutin, ethionamide, amikacin, capreomycin, and pyrazinamide. Drug resistance was determined by the proportional method.

[b] Determined by the ability to grow in the lungs of C57BL/6 mice exposed to a low-dose aerosol of bacilli. Growth was classified relative to that of the type strain, Erdman.

[c] K, James Kilburn, Centers for Disease Control and Prevention, Atlanta, Ga. (isolates from the United States); C, Ray Cho, Seoul, Republic of Korea (isolates from Asia); N, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo. (isolates from the United States); TMCC, Trudeau Mycobacterial Culture Collection.

duction, while other virulent strains resisted such killing and grew, albeit at a lower rate than in control macrophages. We show that these strains varied in their induction of RNI in unprimed macrophages, but all strains induced similarly inhibitory levels of RNI in IFN-primed macrophages. The results of this study therefore suggest that while most virulent strains of *M. tuberculosis* are susceptible to RNI, the consequences of such exposure to physiologic concentrations of RNI within host macrophages are for the most part bacteriostatic.

MATERIALS AND METHODS

Bacteria. Virulent *M. tuberculosis* type strains Erdman (TMCC 107) and H37Rv (TMCC 102) and a variety of human clinical isolates were grown to mid-log phase in Proskauer-Beck or glycerine alanine salts medium containing 0.04% Tween 80 and stored in 2-ml aliquots at −70°C. Frozen stocks were counted by serial dilution in saline and plating onto Middlebrook 7H11 agar. Each strain had been passaged in media fewer than three times since obtaining seed stocks or original collection. The strains varied by drug resistance and virulence in mice, as shown in Table 1. The drug resistance for each mycobacterial strain had been determined at time of collection as described elsewhere (38). The relative level of virulence in a low-dose aerosol mouse model had been determined previously for several of the mycobacterial strains as described previously (38, 42).

Mice. Bone marrow-derived macrophages were harvested from 8- to 12-week-old female specific-pathogen-free C57BL/6 mice purchased from Charles River Laboratories, Wilmington, Mass.

Media and reagents. The complete cell culture medium (cCM) was Dulbecco's modified Eagle medium that contained 10 mM HEPES, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 250 ng of amphotericin B per ml, and minimal essential medium nonessential amino acids supplemented with 10% L-929 fibroblast-conditioned medium and 10% heat-inactivated, endotoxin-low fetal calf serum (Summit Biotechnologies, Inc., Fort Collins, Colo.). Incomplete cell culture medium (iCM) lacked the 2-mercaptoethanol, antibiotics, antimycotics, and L929-conditioned medium. ^{N}G-monomethyl-L-arginine(L-NmmA) and ^{N}G-monomethyl-D-arginine (D-NmmA) were from Chem-Biochem Research, Inc., and Cyclopss Biochem (Salt Lake City, Utah). Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) was autoclaved, and 100 ml of filter-sterilized oleic acid-dextrose complex (OADC; 0.81% NaCl, 5.0% bovine albumin fraction V [Intergen Company, Purchase, N.Y.], 2.0% dextrose [Fisher Scientific, Fairlawn, N.J.], 0.06% oleic acid [J. T. Baker Chemical Co., Phillipsburg, N.J.] [pH 7.0]) was added to 900 ml of 7H9 broth. Sodium nitrite was purchased from J. T. Baker. Middlebrook 7H11 agar also contained the OADC. Tween 80 was from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Recombinant murine IFN-γ was purchased from Genzyme (Boston, Mass.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Bone marrow-derived macrophage culture. Mice were killed by cervical dislocation, and the femurs were aseptically removed. Marrow was flushed out of the femurs by using ice-cold cCM. Bone marrow cells were plated at 10^6/well in 24-well tissue culture-treated dishes (Falcon, Becton Dickinson Labware, Lincoln Park, N.J.) in 1 ml of cCM and incubated at 37°C in 5% CO_2. After 24 h, 1 ml of cCM was added, and nonadherent cells were removed after another 24 h by replacing the medium with fresh cCM. Cells were incubated for another 6 days with one change of medium, and cCM was replaced with iCM on day 8 of culture. Monolayers were primed and infected within another 48 h.

Experimental infection and bacterial enumeration. Bone marrow-derived macrophages were primed by the addition of murine recombinant IFN-γ (100 U/ml), and L-NmmA or D-NmmA was added to a final concentration of 1 mM. Control macrophages received an equal volume of iCM alone. After 18 to 24 h, each strain of *M. tuberculosis* was resuspended in iCM, and approximately 10^6 viable bacilli were added to each well in a final volume of 2 ml. After 4 h, extracellular bacilli were removed with three washes of sterile saline, and a 2-ml volume of iCM containing the appropriate combinations of IFN-γ (50 U/ml), L-NmmA, and D-NmmA (1 mM) was added to the infected monolayers. Culture supernatants were collected and frozen at −70°C at various times after infection, and the monolayers were lysed with double-distilled H_2O containing 0.1% saponin. Viable bacilli were serially diluted in saline containing 0.04% Tween 80 and plated onto Middlebrook 7H11 agar. The colonies which appeared on the plate after 3 to 4 weeks of incubation at 37°C were counted to determine the $\log_{10}$ viable bacilli per milliliter.

Measurement of nitrite. Nitrite production by the macrophage monolayers was determined by the Griess assay as described elsewhere (47). Briefly, macrophage supernatants were thawed and added to a 96-well enzyme-linked immunosorbent assay plate (Falcon) in duplicate, and an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine, 2.5% H_3PO_4) was added. The absorbance at 550 nm was measured on an enzyme-linked immunosorbent assay reader, and the concentration of nitrite was calculated by comparing optical density values to a standard curve of $NaNO_2$ in iCM.

Mycobacterial nitrite tolerance. The 7H9 broth was acidified to pH 4.5, 5.5, or 6.5 with the addition of 2 N HCl and filter sterilized. Sodium nitrite was added from a sterile 1 M stock to final concentrations ranging between 0.1 and 10 mM. Sodium nitrite was not added to control cultures. The modified 7H9 broth was added to sterile, capped glass tubes in 1-ml aliquots. Mycobacterial stocks were thawed, and any clumps were dispersed by gentle and repeated aspiration through a 26-gauge needle. Approximately 10^5 viable bacilli in a 50-μl volume were added to each tube. The cultures were incubated at 37°C, and serial dilutions were plated onto Middlebrook 7H11 agar at the indicated days after infection and counted as described above.

The MIC of nitrite was determined for each mycobacterial strain by calculating the concentration of added $NaNO_2$ required to generate a growth curve with an average slope of zero over the course of 10 days of incubation at pH 6.5. The MBC of nitrite was determined by calculating the concentration of added $NaNO_2$ required to reduce the original mycobacterial inoculum by 99.9% over the same course of 10 days of incubation at pH 6.5 or 5.5.

Data presentation and statistical analysis. All infected macrophage cultures were performed in triplicate, and data are shown as means ± 1 standard deviation (SD) from a representative of two to four independent experiments, and statistical analysis was performed by using homoscedastic Student *t* tests. A one-tailed test was used to determine the significance of changes between time points in the same group, and a two-tailed test was used for comparison between groups. Cell-free mycobacterial cultures were performed in triplicate, and data are shown as means ± 1 SD; statistical analysis was performed by using both the analysis of variance *F* test and a two-tailed, paired Student *t* test.

RESULTS

Growth of *M. tuberculosis* in bone marrow-derived macrophages. To determine if the strains are controlled similarly, murine bone marrow-derived macrophages were infected with a panel of strains of *M. tuberculosis*, and all of the strains were virulent for our macrophage model since they grew within macrophages over 10 days (Fig. 1 and 2). Infections with the type strain, Erdman, and clinical isolates CSU 15, CSU 25, and CSU 39 were initially killed ($P < 0.05$ between days 1 and 4) in IFN-primed macrophages (Fig. 1A). These strains subsequently began to grow in the activated macrophages after 4 to 7 days of infection. Other clinical isolates were not killed in the IFN-activated macrophages; numbers of viable CSU 21 and CSU 35 remained at the level of the inoculum, while CSU 31 and CSU 24 grew in IFN-primed macrophages as shown in Fig. 2A). However, the intracellular growth of these four strains was significantly slower in IFN-primed macrophages than in

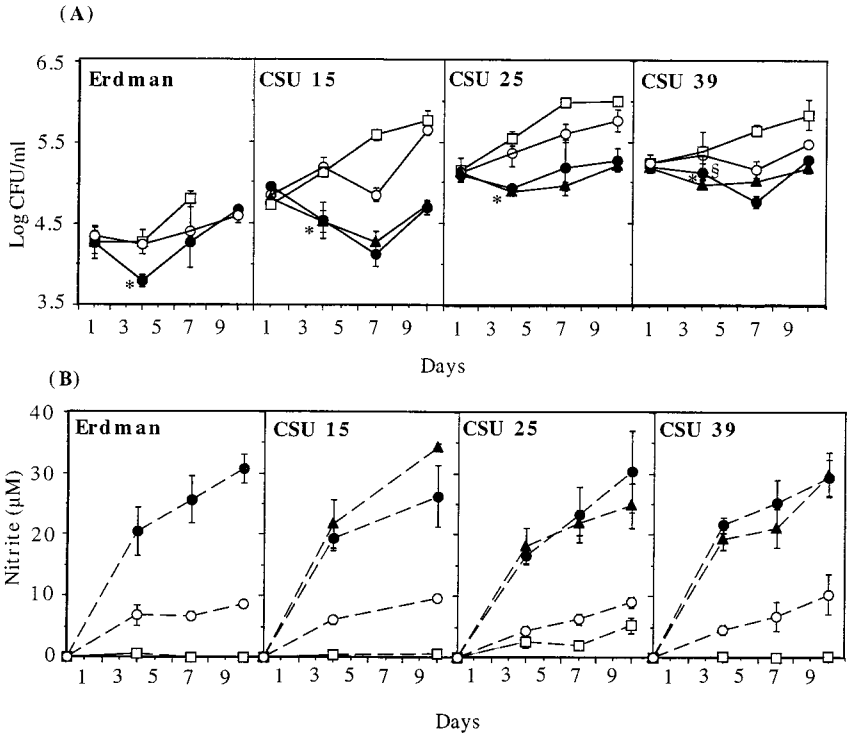

FIG. 1. Several virulent strains of *M. tuberculosis* are initially killed in murine IFN-primed macrophages, and mycobacterial growth can be restored by blocking the RNI metabolism of infected macrophages. (A) Bone marrow-derived macrophages were primed with murine IFN-γ (●) in the presence of L-NmmA (○) or D-NmmA (▲) 18 to 24 h prior to infection with 10^6 viable bacilli; control monolayers were infected without IFN priming (□). The number of viable bacilli remaining in the monolayers at the indicated days following infection was determined by plating serial dilutions of macrophage lysate. Bacterial colonies were counted, and the $\log_{10}$ CFU per milliliter is presented. (B) Nitrite accumulation was determined in culture supernatants collected from macrophages prior to lysing. □, control; ●, IFN primed; ○, IFN primed plus L-NmmA; ▲, IFN primed plus D-NmmA. Each value represents the mean of three determinations ± SD. Differences between means of viable bacilli in IFN-primed and control cultures were significant for all infections after 4 or more days where comparisons could be drawn ($P < 0.005$) (§, $P < 0.05$). Asterisks indicate significant differences ($P < 0.05$) in the mean number of viable bacilli between days 1 and 4 of infection in IFN-primed monolayers.

control monolayers after 7 or 10 days of culture ($P \leq 0.005$). Extracellular growth of bacilli was negligible, as determined by plating the monolayer supernatants over the course of infection (data not shown).

The observed antimycobacterial activity of activated macrophages was partially blocked by addition of L-NmmA, which blocks RNI metabolism in macrophages (37). Coculture of IFN-primed monolayers with L-NmmA restored the number of viable bacilli retrieved to levels similar to those of control macrophages, and the growth of all of the strains *M. tuberculosis* was restored regardless of the nature of control by IFN-primed macrophages. Coculture with the inactive D-enantiomer of NmmA did not restore the number of viable bacilli to control levels. The viability of treated macrophage monolayers was monitored by trypan blue exclusion to determine whether any of the treatments could decrease monolayer viability, which could affect the intracellular growth of *M. tuberculosis* (data not shown). A 1 mM concentration of L-NmmA blocked 70 to 100% of nitrite accumulation without adversely affecting macrophage viability, while higher levels of L-NmmA were toxic to the monolayers. The same concentration of D-NmmA failed to significantly reduce nitrite levels from infected monolayers and was also nontoxic for the monolayers. IFN priming reduced infected monolayer confluency approximately 15 to 30% after 7 days of infection, and as many as 40% of IFN-primed macrophages showed evidence of cell death after 10 days of infection. However, the number of viable bacilli was not significantly altered by these effects, since the addition of L-NmmA restored the number of viable bacilli to that seen in viable, unprimed macrophages. Furthermore, neither enantiomer of NmmA enhanced the growth of *M. tuberculosis* in unprimed macrophages (data not shown).

The viable bacilli retrieved from the treated macrophages showed evidence of morphological variation when plated onto 7H11 agar. Colonies from IFN-primed macrophages were noticeably smaller and slightly less opaque than control colonies retrieved from control macrophages after 3 to 5 weeks of incubation at 37°C (data not shown). Bacilli retrieved from IFN-primed macrophages cultured in the presence of L-NmmA formed colonies which were similar in size and opacity to control colonies. Addition of D-NmmA to infected macrophages did not restore colony morphology to that of the controls.

Production of RNI by *M. tuberculosis*-infected murine macrophages. It was possible that different levels of RNI were induced by the individual strains of *M. tuberculosis* to account for observed differences in their intracellular growth. To test this, the accumulation of a stable end product of the reactive nitrogen metabolism, nitrite, was measured in the macrophage culture medium. As shown in Fig. 1B and 2B, strains CSU 15, CSU 35, CSU 39, and Erdman failed to consistently induce detectable levels without IFN priming. On the other hand, strains CSU 21, CSU 24, CSU 25, and CSU 31 routinely induced between 5 and 15 μM nitrite accumulation within 7 days of infection in unprimed monolayers. Upon priming with IFN-γ, all of the strains induced greater nitrite accumulation, ranging between 20 and 40 μM; however, there were no significant differences between the individual strains. IFN priming

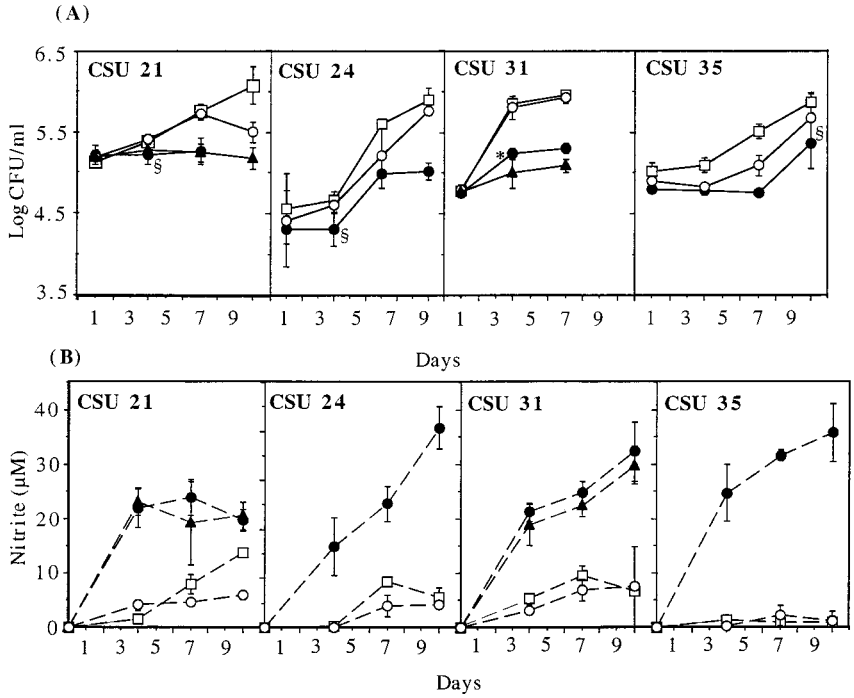

FIG. 2. Other virulent strains of *M. tuberculosis* are not initially killed in IFN-primed macrophages, yet they exhibit reduced growth which can also be reversed by blocking the RNI metabolism of infected macrophages. (A) Bone marrow-derived macrophages were primed with murine IFNγ (●) in the presence of L-NmmA (○) or D-NmmA (▲) 18 to 24 h prior to infection with 10^6 viable bacilli; control monolayers were infected without IFN priming (□). The number of viable bacilli remaining in the monolayers at the indicated days following infection was determined by plating serial dilutions of macrophage lysate. Bacterial colonies were counted, and the $\log_{10}$ CFU per milliliter is presented. (B) Nitrite accumulation was determined in culture supernatants collected from macrophages prior to lysing. □, control; ●, IFN primed; ○, IFN primed plus L-NmmA; ▲, IFN primed plus D-NmmA. Each value represents the mean of three determinations ± SD. Differences between means of viable bacilli in IFN-primed and control cultures were significant for all infections after 4 or more days where comparisons could be drawn ($P < 0.005$) (§, $P < 0.05$). Asterisks indicate significant differences ($P < 0.05$) in the mean number of viable bacilli between days 1 and 4 of infection in IFN-primed monolayers.

of uninfected control macrophages did not stimulate the release of nitrite (data not shown).

Nitrite tolerance of *M. tuberculosis* in 7H9 broth. To test the possibility that strains of *M. tuberculosis* vary in susceptibility to the toxic effects of RNI, their relative susceptibilities to nitric oxide in a cell-free system were calculated. Under acidic conditions, nitrite, which is itself not toxic, becomes nitrous acid, which subsequently dismutes to the toxic radical, nitric oxide (49). *M. tuberculosis* was cultured in mycobacterial culture broth acidified to pH 4.5, 5.5, or 6.5 in increasing concentrations of nitrite over 10 days. All of the strains exhibited similar responses to the conditions generated, as represented by CSU 25 in Fig. 3. Acidification of the 7H9 broth to pH 4.5 was toxic to *M. tuberculosis*, and the numbers of viable bacilli fell to below detectable levels within 4 days of inoculation. The strains also exhibited significantly reduced growth at pH 5.5

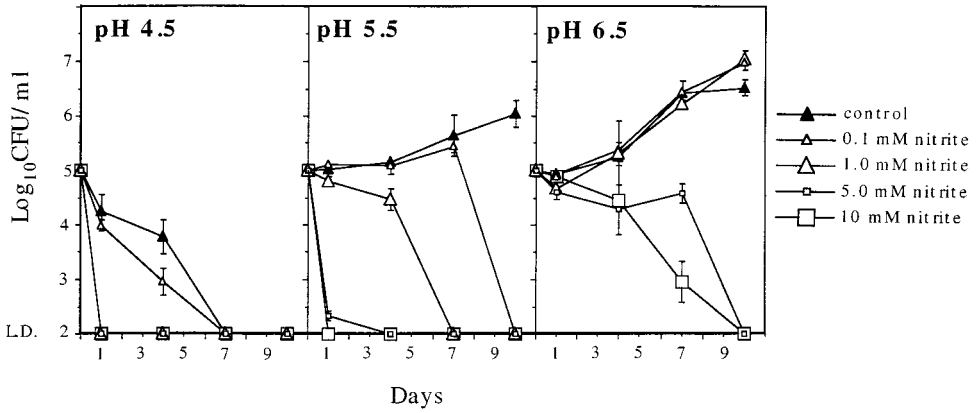

FIG. 3. *M. tuberculosis* is killed in 7H9 broth in conditions of low pH as well as in the presence of acidified nitrite. Middlebrook 7H9-OADC broth was acidified to pH 4.5, 5.5, or 6.5, and $NaNO_2$ was added to the final concentrations indicated. Approximately 10^5 viable *M. tuberculosis* CSU 25 cells were inoculated into 1-ml aliquots of the modified broth and incubated at 37°C over 10 days. Cultures were serially diluted and plated onto 7H11 agar at the indicated times after inoculation for determination of the $\log_{10}$ CFU of viable bacilli per milliliter. Responses of the strains of *M. tuberculosis* listed in Table 2 were similar to that demonstrated by CSU 25. The limit of detection (L.D.) for the dilution series used was 2 logs CFU/ml. Each value represents the mean of three counts ± SD.

TABLE 2. Nitrite tolerance of *M. tuberculosis* strains in 7H9 broth

Strain	Growth[a] (log CFU/ml)	MIC (mM)[b]	MBC[c] (mM)	
			pH 6.5	pH 5.5
CSU 31[d]	1.22 ± 0.00	1.64 ± 0.08	4.58 ± 0.03	0.12 ± 0.02
H37Rv	2.29 ± 0.10	2.16 ± 0.06	5.35 ± 0.08	0.27 ± 0.15
CSU 22	2.67 ± 0.16	2.44 ± 0.03	5.30 ± 0.06	0.31 ± 0.05
CSU 39[e]	2.47 ± 0.05	2.44 ± 0.13	5.36 ± 0.11	0.93 ± 0.39
Erdman[e]	3.05 ± 0.00	2.69 ± 0.08	5.31 ± 0.21	0.01 ± 0.00
CSU 21[d]	2.65 ± 0.10	2.62 ± 0.14	5.60 ± 0.09	0.26 ± 0.01
CSU 46	2.95 ± 0.19	3.27 ± 0.12*	6.15 ± 0.28*	0.11 ± 0.02
CSU 24[d]	1.64 ± 0.21	2.79 ± 0.16*	6.64 ± 0.42*	0.02 ± 0.00
CSU 25[e]	1.52 ± 0.12	3.11 ± 0.08*	7.20 ± 0.33*	0.24 ± 0.01
CSU 15[e]	2.51 ± 0.04	4.02 ± 0.09**	8.24 ± 0.16**	0.54 ± 0.23**
CSU 44	2.63 ± 0.07	4.46 ± 0.08**	9.39 ± 0.14**	ND
CSU 32	2.45 ± 0.31	4.57 ± 0.26**	9.88 ± 0.76**	ND
CSU 35[d]	1.80 ± 0.02	4.40 ± 0.33**	10.53 ± 0.84**	0.17 ± 0.01

[a] Numerical values represent the $\log_{10}$ increase in the number of viable bacilli above that of the original inoculum for a period of 10 days. SDs are also indicated.

[b] MICs at pH 6.5 indicate the concentrations of sodium nitrite required to prevent growth of the original inoculum for a period of 10 days.

[c] MBCs represent the concentrations of sodium nitrite requires to kill 99.9% of the original inoculum within 10 days. SDs for all concentrations are indicated. Asterisks indicate groups that differ significantly with respect to MICs and MBCs at pH 6.5 (P of < 0.001). ND, not determined.

[d] Strains of *M. tuberculosis* that resisted killing in IFN-primed macrophages, as shown by the results presented in Fig. 2.

[e] Strains that were killed in IFN-primed macrophages, as shown by the results presented in Fig. 1.

from growth at pH 6.5 (analysis of variance F test; $\Pr > F = 0.0001$). All of the strains grew at pH 6.5, as shown in Table 2.

The MBC at pH 5.5 of added $NaNO_2$ was determined, and at pH 6.5, the MBC as well as the MIC was calculated for each mycobacterial strain. At pH 6.5, MICs ranged from 1.64 ± 0.08 to 4.57 ± 0.26 mM, and the concentration of added nitrite required to kill 99.9% of the mycobacterial cultures ranged from 4.58 ± 0.03 to 10.53 ± 0.84 mM (Table 2). The MBCs were significantly reduced by decreasing the pH from 6.5 to 5.5. Bacilli which were retrieved from media in which the cultures were dying in the presence of RNI generally formed colonies which were smaller and less opaque than colonies formed by growing cultures (data not shown). The differences in MICs and MBCs could not be attributed to variability in the growth rates of the strains in the absence of nitrite. The pH of the cultures was monitored over 10 days and found to change by less than 0.2 pH unit (data not shown). The strains were grouped according to their MICs ($P < 0.05$) and MBCs ($P < 0.001$) for acidified nitrite at pH 6.5. The same grouping did not hold for the MICs at pH 5.5, likely due to a significant interaction between the toxic effects of RNI and pH.

DISCUSSION

The results of this study show that a panel of virulent clinical isolates of *M. tuberculosis* exhibited a wide range of susceptibility to the effects of exposure to RNI generated by IFN-primed murine macrophages. Some strains appeared to be only marginally affected by the presence of RNI, while others showed evidence of bacteriostasis when RNI were present. Still other strains exhibited an initial reduction in bacterial numbers in activated macrophages. In no instance were the numbers of intracellular bacilli reduced by more than 1.5 logs, suggesting that the observed antimycobacterial activity of IFN-primed macrophages may be better characterized as static rather than cidal. The possibility arises that our in vitro system limited the mycobactericidal potential of bone marrow-derived macrophages, as it has been shown that L-arginine may become limiting in cultures after 3 to 4 days, effectively stopping RNI generation (48, 54). However, all IFN-primed, infected macrophage cultures, with the exception CSU 21-infected cultures, continued to accumulate nitrite for up to 10 days, albeit at different lower rates. In the case of the CSU 39 infections of IFN-primed macrophages, the rates of nitrite accumulation were the same between days 4 and 7 and between days 7 and 10; however, intracellular bacilli were killed between days 4 and 7, and they grew between days 7 and 10, making it unlikely that L-arginine depletion was responsible for resumed growth. For CSU 21-infected, IFN-primed macrophages, cessation of nitrite accumulation was not associated with resumed mycobacterial growth, which suggests that other factors in the activated macrophages also control mycobacterial growth. Under harsher conditions in a nitrite tolerance test, all strains were inhibited. Here again, variation in tolerance was observed; however, there was no consistent correlation between the tolerance of a strain in the cell-free nitrite tolerance test and its ability to withstand the effects of RNI in murine macrophages, illustrating that the ability of the bacilli to survive within IFN-primed macrophages is multifactorial. Collectively, these data indicate that *M. tuberculosis* is susceptible to RNI, but that under in vitro conditions, the amount of RNI to which intracellular bacilli are exposed results only in stasis or transient killing of a proportion of the infecting inoculum, whereas other isolates may be only marginally affected.

Our data support the conclusions of others that *M. tuberculosis* is killed by direct exposure to chemically generated RNI and that strains can vary widely in their susceptibility. A modified nitrite tolerance test was used to compare the susceptibilities of the strains to RNI. Similar work was originally approached by inoculating bacilli into acidic mycobacterial culture broth or onto agar in the presence of increasing concentrations of sodium nitrite (52). In this way, mycobacteria were exposed to RNI arising from nitrite under acidic conditions (15, 45, 49, 51), and the ability of each mycobacterial species to tolerate the RNI was determined by observing growth after 4 to 6 weeks of incubation. With the accumulating evidence that RNI play an important role in the control of virulent mycobacteria (2, 9, 19, 21), others have used a nitrite tolerance approach to show that *M. tuberculosis* is killed by chemically generated RNI (9, 35). O'Brien et al. (35) inoculated a panel of strains into cell culture media containing 0.072 to 3.62 mM nitrite and compared the number of viable bacilli at 0 and 24 h. Chan et al. (9) inoculated only the Erdman strain into mycobacterial 7H9 broth containing 0.25 to 10 mM nitrite and compared both the decrease in [^{3}H]uracil incorporation and the CFU counts after 24 h. We chose an approach designed to observe the effects of RNI on a panel of growing cultures of *M. tuberculosis*. Mycobacterial 7H9 broth, a medium designed specifically for the determination of MICs of antimycobacterial agents, was inoculated with *M. tuberculosis* over a wide range of nitrite, 0.1 to 10 mM. Where others had shortened the duration of exposure from a matter of weeks to 24 h, we measured mycobacterial growth in the presence of RNI over 10 days. This enabled us to calculate MICs and MBCs based on the growth rate of the cultures. Unlike the findings of earlier studies, *M. tuberculosis* was shown to be sensitive to acidification of the control media. It has been demonstrated that oleate is toxic to *M. tuberculosis* under acidic conditions (53), and it is likely that the strains were sensitive to the OADC supplement in the broth preparation.

Virulent strains were susceptible to a narrow range of acidified nitrite, and this finding is supported by the reports of

others that pathogenic slow growers including *M. tuberculosis*, *M. bovis*, and *M. avium* were eradicated by less than 14.5 mM nitrite (52). In the present study, MICs and MBCs at pH 6.5 varied significantly among the strains such that they could be classified into low-, moderate-, and high-tolerance groups for acidified nitrite. MBCs were lower at pH 5.5, as would be expected when a higher concentration of protons shifts the equilibrium farther toward HNO_2/NO. However, we were less confident of the MBCs at pH 5.5, since mycobacterial growth was significantly hindered at this lower pH. A comparison of the multidrug resistance status of the clinical isolates did not reveal any correlation to the susceptibility to acidified nitrite. Likewise, strains CSU 31 and CSU 21 exhibit the highest resistance to isoniazid (INH), 33 and 100% resistance, respectively, at 5 μg of INH per ml, yet CSU 39, with no resistance to the same concentration of INH, exhibits MICs and MBCs for acidified nitrite which fall between the values for CSU 31 and CSU 21.

While all of the *M. tuberculosis* strains are virulent in mice, they displayed a range of virulence shown previously by their ability to achieve high numbers in the lungs within the first 20 days after aerosol challenge (38). We have shown here that all of the strains tested could resist some level of RNI generated in a cell-free assay, yet there was no correlation with their virulence for mice and the level of tolerance of acidified nitrite. This finding is reminiscent of earlier work (23, 27) comparing H_2O_2 susceptibility to virulence in guinea pigs of human isolates of *M. tuberculosis*. While there was a strong association of attenuation with H_2O_2 susceptibility in macrophages, there were also attenuated strains which were resistant to H_2O_2, supporting the belief that resistance to multiple antimycobacterial properties of activated macrophages is necessary for in vivo virulence. Differences in the individual nitrite tolerances among the strains could not explain the abilities of some to withstand RNI-mediated killing in IFN-primed macrophages. Strains CSU 31 and CSU 35 were not resistant to RNI-mediated killing, yet these two isolates exhibited the lowest (4.58 ± 0.03 mM) and highest (10.53 ± 0.84 mM), respectively, MBCs for nitrite at pH 6.5. A likely explanation for this observation is that intracellular bacilli were exposed to different levels of RNI even though similar levels of nitrite were measured from all of the IFN-primed, infected monolayers. If some of the strains were to hinder the acidification of the phagosomes in which they resided (11, 50), this would favor conversion of NO into nitrite and nitrate species in such a compartment, thereby reducing mycobacterial exposure to the protonated, more lethal RNI species. In such an instance, macrophage iNOS activity is not affected, thereby allowing similar amounts of nitrite to accumulate in the supernatant regardless of a mycobacterial modification of phagosomes. In fact, we have previously shown that acidification of *M. avium*-infected macrophages augments IFN-activated control of the intracellular bacilli (4).

In support of our contention that macrophage RNI are mycobacteristatic, we noted that the surviving bacilli which were retrieved from IFN-primed macrophages seemed to replicate at a lower rate. These bacilli formed smaller colonies, slightly less opaque and of the same shape, on the 7H11 plates compared to bacilli retrieved from unprimed macrophages or from IFN-primed macrophages cultured in L-NmmA. The exact mechanism for a decrease in colony size is unknown, although the lower replication rate could have been due to RNI interference with bacterial energy metabolism (56) or damage to the bacterial genome (28). Alternatively, it is also possible that in order to survive within the activated monolayers, the bacilli which were exposed to macrophage RNI underwent adaptive physiological changes that changed their rate of replication on agar.

The data also showed that the variation in degree of IFN-activated control of *M. tuberculosis* was not due to the induction of different levels of RNI in the infected macrophages. Some of the strains of *M. tuberculosis* induced low-level RNI production in macrophages without IFN priming, but these low levels of RNI were not clearly inhibitory toward the intracellular bacilli since all of the strains grew within unprimed macrophages. We have not determined the mechanism by which *M. tuberculosis* induces RNI in unprimed macrophages. It has been previously shown that some clinical isolates of *M. tuberculosis* induce secretion of tumor necrosis factor alpha (TNF-α) from infected bone marrow-derived macrophages without IFN priming (42). Since TNF-α functions in an autocrine manner to induce nitric oxide (18, 24), perhaps the infected macrophages produced sufficient TNF-α to activate iNOS gene expression. In any event, those strains which induced RNI in the absence of IFN priming did not induce different inhibitory levels of RNI in IFN-primed macrophages compared to the other strains. Therefore, induction of different levels of RNI could not explain the ability of some strains to withstand RNI-mediated killing within macrophages.

The observation here suggesting that murine macrophage-generated RNI represent a primarily bacteriostatic defense is in fact highly consistent with events in the mouse lung following exposure to low-dose aerosol infections (38, 39). Following the containment of the pulmonary infection after 20 or so days, there may follow a small degree of bacterial elimination, but overall the infection assumes a state of chronic disease. There is certainly no evidence for the occurrence of wholesale bacterial destruction that could be attributed to killing of bacilli by RNI.

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