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Strains of *Mycobacterium tuberculosis* Differ in Susceptibility to Reactive Nitrogen Intermediates In Vitro

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The effects on the viability of *Mycobacterium tuberculosis* strains and one *Mycobacterium bovis* strain from exposure to sodium nitrite for 24 h, in both neutral and acidic media, were tested. The in vitro resistance of mycobacteria to reactive nitrogen intermediates, generated at an acidic pH, was found to have a significant ($P < 0.05$) positive correlation to the virulence of strains in guinea pigs.

Strains of *Mycobacterium tuberculosis* can have large differences in virulence in animals. The factors explaining this variation in virulence remain to be determined, but virulence in guinea pigs does correlate with resistance to hydrogen peroxide. However, other antimicrobial mechanisms exist and we have previously shown that the killing of *M. tuberculosis* by guinea pig alveolar macrophages does not require hydrogen peroxide (19).

Reactive nitrogen intermediates (RNI), which include nitric oxide (NO·), are an alternative to peroxide as an antimicrobial agent. Although the role of NO· in microbial killing by alveolar macrophages is unknown, the production of NO· by activated murine peritoneal and bone marrow-derived macrophages has been shown to be an effector mechanism against a number of pathogens (2, 11–13, 15, 25), including *Mycobacterium leprae* (1), *Mycobacterium bovis* BCG (9), and *M. tuberculosis* (3, 4).

We have compared strains of *M. tuberculosis* and one *M. bovis* strain with different levels of virulence in guinea pigs for their susceptibilities to killing by RNI.

Eight strains of *M. tuberculosis* (H37RaHR, H37Ra, B1453, 79112, 79500, H37Rv, I2646, and 79499) (14) and one strain of *M. bovis* (81470) were cultured from frozen Lowenstein-Jensen slopes kept in the Leicester University Culture Collection. Mycobacteria were maintained in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with ADC (Difco) at 37°C and were subcultured every 7 days. In each experiment, a 7-day-old culture was used. Prior to use, mycobacteria were washed twice by centrifugation in 0.01% (vol/vol) Tween 80 (Sigma, St. Louis, Mo.), resuspended in medium (see below), sonicated for three 5-s bursts at 40 W (Ultrasonic Engineering, London, United Kingdom) to disrupt bacterial clumps, and counted with a Thoma chamber. Mycobacteria were then diluted to the appropriate working concentration in medium.

To test the effects of RNI on mycobacterial survival, experiments were performed in reconstituted powdered Eagle's minimum essential medium without phenol red, glutamine, and sodium bicarbonate (Flow Laboratories, Costa Mesa, Calif.). The medium was supplemented with sodium pyruvate (110 mg/liter) (Sigma), glucose (3.5 g/liter) (Fisons, Loughborough, United Kingdom), glutamine (584 mg/liter) (Sigma), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (15 mM) (Sigma), and 10% (vol/vol) heat-inactivated newborn calf serum (Gibco, Paisley, United Kingdom). pH was adjusted to 5.0 or 7.0, and medium was filter sterilized and stored at 4°C for use within 7 days. This final growth medium is referred to as MEM.

Sodium nitrite (Sigma) was diluted from a stock solution of 5 mg/ml to give solutions with final concentrations of 2.5, 1.25, 0.5, and 0.05 mg/ml of sterile nanopure water. Fresh NO_2^- solutions were prepared for each experiment. Mycobacteria were prepared as described above in MEM, pH 5 or pH 7, at a concentration of 10^8 bacilli per ml. Bacteria (10^7) were added to 100 μl of each NO_2^- solution and 800 μl of MEM, pH 5 or pH 7, as appropriate. Controls consisted of 10^7 bacteria plus 900 μl of MEM, pH 5 or pH 7. Suspensions were incubated at 37°C and 5% CO_2 for 24 h. pHs of both media did not change during the course of incubation. Viable count assays were performed after 0 and 24 h by plating serial dilutions of suspension onto 7H11 agar (Difco) with OADC enrichment (Difco). The number of colony-forming units was then counted after a 3-week incubation at 37°C. Before dilution, mycobacteria were sonicated to ensure that single cells were plated.

Data were analyzed by analysis of variance. The significance of the regression coefficient was determined by the *t* test.

At an acidic pH, NO_2^- is converted to nitrous acid; subsequent decomposition of nitrous acid generates other RNI, including NO· (24). As NO· is considered to be the most potent RNI (17), it was decided to test both neutral and acidic NO_2^- solutions for antimycobacterial activity. The fate of mycobacteria incubated in MEM, pH 5 or pH 7, in the presence of a range of concentrations of NO_2^- is shown in Tables 1 and 2. There was no change in the number of mycobacteria incubated in either neutral MEM or acidic MEM alone for 24 h. At pH 7, none of the concentrations of nitrite tested significantly affected any strain of tubercle bacilli, except strain 79500 (Table 1). There was a significant ($P < 0.05$) decrease in the survival of this strain at each nitrite concentration tested. In contrast, all the strains of *M. tuberculosis* tested showed susceptibility to RNI at pH 5 in a dose-dependent manner. However, the extent of killing by RNI varied according to the strain of *M. tuberculosis*. For example, at a concentration of 125 μg of NO_2^- per ml, strain 79499 showed a 34% decrease in survival, whereas strain B1453 exhibited a 58% decrease. *M. bovis* was resistant to NO_2^- at all the concentrations tested. In other words, the concentration of NO_2^- at which the killing became statistically significant varied according to the strain. For example, the killing of H37Ra was significant ($P < 0.05$) at

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TABLE 1. Effects of NO_2^- on the viability of mycobacteria incubated in MEM at pH 7.0

Strain	RIV[a]	% Survival at indicated $NaNO_2$ concn (μg/ml)[b]:				
		250	125	50	5	0
M. tuberculosis						
H37RaHR	0.11	96 ± 7	105 ± 5	97 ± 16	106 ± 2	110 ± 9
H37Ra	0.52	143 ± 7	170 ± 16	149 ± 5	202 ± 4	154 ± 25
B1453	0.77	152 ± 42	130 ± 9	120 ± 31	142 ± 49	136 ± 34
79112	0.92	115 ± 6	121 ± 14	124 ± 14	95 ± 15	103 ± 2
79500	0.98	67 ± 5	84 ± 3	93 ± 2	109 ± 8	131 ± 15
H37Rv	1.01	108 ± 5	107 ± 6	122 ± 15	141 ± 10	142 ± 13
I2646	1.17	81 ± 3	88 ± 5	109 ± 6	95 ± 5	93 ± 4
79499	1.29	114 ± 1	115 ± 6	102 ± 15	123 ± 11	101 ± 9
M. bovis	>1.29	102 ± 2	103 ± 1	106 ± 11	103 ± 22	107 ± 3

[a] Strains showing RIVs in guinea pigs of ≥ and ≤1.0 were termed high- and low-virulence strains, respectively. RIV is a measurement of the rate at which pathological lesions develop in organs in vivo (1a, 14, 16).

[b] Each value represents the percentage (± SD) of mycobacteria remaining after a 24-h incubation in MEM at pH 7 in the presence of various concentrations of NO_2^- at 37°C and 5% CO_2 and is the average of three experiments.

50 μg/ml, but significant ($P < 0.05$) killing of H37Rv was not seen until 125 μg/ml was used.

When the percentage survival after 24-h exposure to 125 μg of sodium nitrite per ml at pH 5 was plotted against the root index of virulence (RIV) for each strain (Fig. 1), there was a significant ($P < 0.05$; regression coefficient, 0.633) positive correlation between resistance to RNI in vitro and the degree of in vivo virulence in guinea pigs. When survival data at nitrite concentrations of above and below 125 μg/ml were analyzed, no correlation was seen. At 250 μg/ml, the concentration of nitrite was high enough to overcome the resistance of the majority of strains. At 50 μg/ml, differences in susceptibility were not manifested because the concentration of nitrite was sufficiently low for susceptible strains to survive.

This demonstration of a toxic effect of RNI is in agreement with Chan et al. (3), who also found that RNI generated from acidic NO_2^- were lethal for *M. tuberculosis* (Erdman strain). The range of sodium nitrite concentrations we tested included the maximum concentrations of NO_2^- that others have found in culture supernatants of activated murine macrophages (23) and were those demonstrated to inhibit the growth of *M. bovis* BCG and *M. tuberculosis* (Erdman strain) in a cell-free system at an acidic pH (3, 9).

It has been reported that gamma interferon-activated murine macrophages exert antimycobacterial activity via RNI (1, 3, 4, 9). However, differences in the susceptibility of *M. tuberculosis* and *Mycobacterium avium* strains to killing by these macrophages have been reported (6, 8). Doi and colleagues (6) proposed that the differences in susceptibility to the antimicrobial mechanisms of activated macrophages might be a reflection of differences in susceptibility to RNI. They went on to show that this was true for *M. avium* (6). Three strains within the *M. avium* complex differed in their susceptibilities to RNI derived from gamma interferon-activated macrophages or from acidification of nitrite. We have now confirmed the hypothesis that strains of *M. tuberculosis* also differ in their susceptibilities to RNI and, furthermore, have shown that this phenomenon is related to virulence. Our observations, as well as those of Doi and colleagues (6), indicate that in experiments designed to identify antimycobacterial mechanisms of phagocytes, care should be taken in interpreting data collected with a single strain.

The reason for the heightened sensitivities of avirulent strains to RNI is unknown. It could be that the target molecules are more sensitive or at a more critical threshold of availability or that avirulent strains have less efficient detoxifying systems.

The identity of the mycobacterial target of NO· is unclear. In tumor cells, NO· inactivates the iron- and sulfur-dependent enzymes involved in respiration, energy production, and cell multiplication (7, 26). Iron-sulfur complexes in *Clostridium botulinum* react with NO_2^- to form iron-nitrosyl complexes (20), and NO· produced by murine macrophages has been detected by its reaction with the iron-sulfur protein, ferredoxin,

TABLE 2. Effects of NO_2^- on the viability of mycobacteria incubated in MEM at pH 5.0

Strain	RIV[a]	% Survival at indicated $NaNO_2$ concn (μg/ml)[b]:				
		250	125	50	5	0
M. tuberculosis						
H37RaHR	0.11	0 ± 0	20 ± 9	37 ± 5	70 ± 12	103 ± 4
H37Ra	0.52	5 ± 0.5	49 ± 4	74 ± 1	100 ± 1	108 ± 0.5
B1453	0.77	13 ± 11	42 ± 18	81 ± 32	92 ± 5	105 ± 32
79112	0.92	0 ± 0	41 ± 4	65 ± 15	102 ± 23	95 ± 9
79500	0.98	0 ± 0	45 ± 5	59 ± 7	66 ± 4	128 ± 20
H37Rv	1.01	64 ± 11	68 ± 3	103 ± 2	124 ± 6	124 ± 2
I2646	1.17	4 ± 5	29 ± 6	54 ± 9	97 ± 3	92 ± 2
79499	1.29	53 ± 2	66 ± 5	68 ± 1	127 ± 23	102 ± 20
M. bovis	>1.29	99 ± 22	100 ± 15	104 ± 10	108 ± 7	121 ± 18

[a] See Table 1, footnote *a*.

[b] Each value represents the percentage (± SD) of mycobacteria remaining after a 24-h incubation in MEM at pH 5 in the presence of various concentrations of NO_2^- at 37°C and 5% CO_2 and is the average of three experiments.

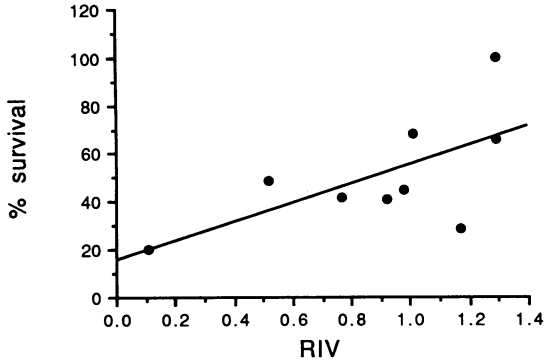

FIG. 1. The relationship between the survival of mycobacteria over a 24-h period in the presence of 125 μg of $NaNO_2$ per ml in MEM, pH 5, and their virulence in guinea pigs.

of *Clostridium pasteurianum* (22). The addition of iron sulfate plus L-cysteine reversed the RNI-mediated trypanostatic activity of both activated murine peritoneal macrophages and NO· gas (25) and inhibited the killing of *Schistosoma mansoni* by murine peritoneal macrophages (15). The effect of NO· on intracellular microorganisms is therefore likely to be similar to its effect on tumors.

The sensitivity of mycobacterial iron-sulfur centers to NO· is not known. However, Rook and coworkers (21) found that activated murine macrophages could strongly inhibit the replication of both *M. tuberculosis* H37Rv and *M. bovis* BCG in the presence or absence of ferric ammonium citrate. If RNI were solely responsible for this antimycobacterial effect, this would indicate that they were not inactivating only iron-sulfur-containing enzymes.

Denis (5) has suggested that iron-dependent superoxide dismutase (SOD) was the target of NO· in an avirulent *M. avium* strain. Fe-SOD is unlikely to be the explanation here. All the strains of *M. tuberculosis* tested contain cyanide-resistant SOD, which is of the Mn-Fe type (14). Furthermore, similar amounts of SOD are found in each of these *M. tuberculosis* strains (14).

Besides the correlation between virulence and RNI resistance demonstrated here, the same group of strains shows a correlation between virulence and hydrogen peroxide resistance (10). This suggests that susceptibility to RNI is governed not by the amount or quality of the target molecule but by the detoxification system. In this study, the catalase-negative strains B1453 and H37RaHR were among the most susceptible to RNI. This suggests that catalase plays a role in resistance to RNI. Possibly, RNI interact with peroxide, generated by mycobacterial cell metabolism, to produce more bactericidal products such as singlet oxygen (18). Alternatively, the iron within catalase may interact with, and thus detoxify, NO·. However, this cannot be the total explanation. Strains 79112 and H37Ra are catalase positive but highly sensitive to RNI. Therefore, other detoxification systems must exist. Exactly the same situation was seen when peroxide susceptibility was investigated (14). Hydrogen peroxide susceptibility correlated well with small amounts of catalase in isoniazid-resistant strains but not in low-virulence, isoniazid-sensitive, catalase-positive strains. These data suggest common mechanisms for peroxide and RNI resistance. Since both agents are likely to operate via the generation of free radicals, strain variations in a repair system, such as that for DNA, might provide the common link.

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REFERENCES

1. **Adams, L. B., S. G. Franzblau, Z. Vavrin, J. B. Hibbs, Jr., and J. L. Krahenbuhl.** 1991. L-Arginine dependent macrophage effector functions inhibit metabolic activity of *Mycobacterium leprae*. J. Immunol. **147:**1642–1646.

1a. **Allen, B. W. (Hammersmith Hospital, London, United Kingdom).** Personal communication.

2. **Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue.** 1993. Release of nitric oxide during the T cell independent pathway of macrophage activation. Its role in resistance to *Listeria monocytogenes*. J. Immunol. **150:**888–895.

3. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.

4. **Denis, M.** 1991. Interferon gamma treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. **132:**150–157.

5. **Denis, M.** 1991. Tumour necrosis factor and granulocyte macrophage colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium*: killing effector mechanisms depend on the generation of reactive nitrogen intermediates. J. Leukocyte Biol. **49:** 380–387.

6. **Doi, T., M. Ando, T. Akaike, M. Suga, K. Sato, and H. Maeda.** 1993. Resistance to nitric oxide in *Mycobacterium avium* complex and its implication in pathogenesis. Infect. Immun. **61:**1980–1989.

7. **Drapier, J. C., C. Pellat, and Y. Henry.** 1991. Generation of EPR-detectable nitrosyl-iron complexes in tumour target cells cocultured with activated macrophages. J. Biol. Chem. **266:**10162–10167.

8. **Flesch, I. E. A., and S. H. E. Kaufmann.** 1987. Mycobacterial growth inhibition by interferon-γ-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J. Immunol. **138:**4408–4413.

9. **Flesch, I. E. A., and S. H. E. Kaufmann.** 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect. Immun. **59:**3213–3218.

10. **Goren, M. B., J. M. Grange, V. R. Aber, B. W. Allen, and D. A. Mitchison.** 1982. Role of lipid content and hydrogen peroxide susceptibility in determining the guinea pig virulence of *Mycobacterium tuberculosis*. Br. J. Exp. Pathol. **63:**693–700.

11. **Granger, D. L., J. B. Hibbs, Jr., J. R. Perfect, and D. T. Durak.** 1988. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J. Clin. Invest. **81:**1129–1136.

12. **Green, S. J., M. S. Meltzer, J. B. Hibbs, Jr., and C. A. Nacy.** 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine dependent killing mechanism. J. Immunol. **144:**278–283.

13. **Green, S. J., C. A. Nacy, R. D. Schreiber, D. L. Granger, R. M. Crawford, M. S. Meltzer, and A. H. Fortier.** 1993. Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in *Mycobacterium bovis* BCG-treated mice. Infect. Immun. **61:**689–698.

14. **Jackett, P. S., V. R. Aber, and D. B. Lowrie.** 1978. Virulence and resistance to superoxide, low pH and hydrogen peroxide among the strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104:**37–45.

15. **James, S. L., and J. Glaven.** 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine dependent production of reactive nitrogen intermediates. J. Immunol. **143:**4208–4212.

16. **Mitchison, D. A., J. G. Wallace, A. L. Bhatia, J. B. Selkon, T. V. Subbaiah, and M. C. Lancaster.** 1960. A comparison of the virulence in guinea pigs of South Indian and British tubercle bacilli. Tubercle **41:**1–22.

17. **Moncada, S.** 1992. The L-arginine:nitric oxide pathway. Acta

Physiol. Scand. **145:**201–227.
18. **Noronha-Dutra, A. A., M. M. Epperlein, and N. Woolf.** 1993. Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing. FEBS Lett. **321:**59–62.
19. **O'Brien, S., and P. W. Andrew.** 1991. Guinea pig alveolar macrophage killing of *Mycobacterium tuberculosis* does not require hydrogen peroxide or hydroxyl radical. Microb. Pathog. **11:**229–236.
20. **Reddy, D., J. R. Lancaster, Jr., and D. P. Cornforth.** 1983. Nitrite inhibition of *Clostridium botulinum*: electron spin resonance detection of iron-nitric oxide complexes. Science **221:**769.
21. **Rook, G. A. W., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma interferon on human monocytes and murine peritoneal macrophages. Immunology **59:**333–338.
22. **Stuehr, D. J., S. S. Gross, I. Sakuma, R. Levi, and C. F. Nathan.** 1989. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium derived relaxing factor and the chemical reactivity of nitric oxide. J. Exp. Med. **169:**1011–1020.
23. **Stuehr, D. J., and M. A. Marletta.** 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon gamma. J. Immunol. **139:**518–525.
24. **Taylor, T. W. J., E. W. Wignall, and J. F. Cowley.** 1927. The decomposition of nitrous acid in aqueous solution. J. Chem. Soc. **11:**1923–1930.
25. **Vincendeau, P., S. Daulouede, B. Veyret, M. L. Darde, B. Bouteille, and J. L. Lemesre.** 1992. Nitric oxide-mediated cytostatic activity on *Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei*. Exp. Parasitol. **75:**353–360.
26. **Wharton, M., D. L. Granger, and D. T. Durack.** 1988. Mitochondrial iron loss from leukaemia cells injured by macrophages. A possible mechanism for electron transport chain defects. J. Immunol. **141:**131–137.