In Vitro Model of Mycobacterial Growth Arrest Using Nitric Oxide with Limited Air

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An in vitro model of mycobacterial growth arrest was developed using Mycobacterium bovis BCG. When an exponentially growing culture was transferred to an evacuated tube, growth continued; treatment with a source of nitric oxide (diethylenetriamine-nitric oxide adduct [DETA-NO] at 50 μM) halted growth immediately, and aeration restored growth. When the period of growth arrest exceeded 4 h, a time lag occurred before aeration could restore growth. The lag time was maximal (24 h) after 16 h of growth arrest. These time lags indicated that one transition period was required for cells to achieve full arrest of growth and another for them to recover fully from growth arrest. DETA-NO-induced growth arrest failed to protect from the lethal effects of anaerobic shock, which caused rapid lysis of both growing and growth-arrested cells. While growth arrest had little effect on the lethal action of rifampin, it eliminated isoniazid lethality. Growth arrest reduced but did not eliminate fluoroquinolone lethality. Two fluoroquinolones, moxifloxacin and gatifloxacin, were equally lethal to exponentially growing cells, but moxifloxacin was more active during growth arrest. This difference is attributed to the fluoroquinolone C-7 ring structure, the only difference between the compounds. Collectively these data characterize a new system for halting mycobacterial growth that may be useful for evaluating new antituberculous agents.

Chemotherapy of tuberculosis requires long treatment periods in which logistical problems and adverse reactions make it difficult for patients to adhere to therapy. Poor patient adherence probably contributes to the selective amplification of resistant bacterial subpopulations and to the emergence of multidrug-resistant strains of Mycobacterium tuberculosis. The need for long treatment periods is attributed in part to a fraction of the infecting bacteria entering a dormant (persistent) state (6) in which antimicrobial susceptibility is thought to diminish (21, 22). A way to shorten treatment time would be to find new compounds or strategies that kill dormant cells more effectively. Such an effort would be facilitated by the availability of experimental models of dormancy. The most commonly used in vitro model involves the gradual depletion of oxygen from liquid cultures of M. tuberculosis (22). An animal model involves low-dose aerosol infection of mice. In the mouse system, an initial acute infection (exponential bacterial growth) shifts to a chronic state (arrested bacterial growth) due to stress imposed by the host immune system (13). Both models suffer from (i) the fact that the stress triggering growth arrest begins well before arrest, at a poorly defined time, and (ii) the requirement for specialized containment facilities. Eliminating these deficiencies could be important for finding new compounds.

Nitric oxide appears to be a key to the arrest of M. tuberculosis growth in mice: the shift from acute to chronic infection fails to occur in mice deficient in inducible nitric oxide synthase, an enzyme involved in the synthesis of nitric oxide (7, 12). An encouraging attempt to model the phenomenon in vitro involves treatment of M. tuberculosis with diethylenetriamine-nitric oxide adduct (DETA-NO), a generator of nitric oxide (18). Concentrations of DETA-NO that are transiently bacteriostatic are found, with growth resuming as DETA-NO and nitric oxide are degraded. If the nitric oxide generated from DETA-NO could be stabilized, the period of growth arrest might be significantly lengthened.

In the present work we describe growth arrest of Mycobacterium bovis BCG, a slowly growing mycobacterium that does not require biosafety level 3 containment facilities. By placing cultures in evacuated tubes, we were able to sustain growth arrest for 72 h with a single treatment of DETA-NO. Exposure to air reinstated growth. DETA-NO-mediated growth arrest eliminated lethality for isoniazid, had an intermediate effect for fluoroquinolones, and had little effect for rifampin (rifampicin). Since fluoroquinolone lethality was sensitive to drug structure, the system should be useful for identifying derivatives with improved ability to kill nongrowing mycobacteria.

MATERIALS AND METHODS

Bacterial cells and growth conditions. M. bovis BCG, substrain Pasteur, isolate KD1295 (1), was grown in Middlebrook 7H9 liquid medium (catalog no. 271310; Difco) or on 7H10 agar medium (catalog no. 262710; Difco) supplemented with 10% albumin dextrose complex, 0.2% glycerol, and 0.05% Tween 80 (4). The incubation temperature was 37°C.

Chemicals and reagents. Rifampin, isoniazid, and DETA-NO were obtained from Sigma-Aldrich Chemical Corp. (St. Louis, MO). Fresh 10 mM solutions of DETA-NO were prepared in 10 mM sodium hydroxide immediately before being added to bacterial cultures. Moxifloxacin was obtained from Bayer Corp. (West Haven, CT), and gatifloxacin was from Bristol-Myers Squibb (Wallington, CT). Fluoroquinolones were dissolved in 0.1 ml of 1 N NaOH (1/10 of the final volume), and then sterile water was added to obtain a final concentration of 10 mg/ml. Solutions were kept at −20°C for several weeks during the experiments.

Assay of bacterial growth. To obtain exponentially growing cultures, frozen aliquots of M. bovis BCG (30 μl) were diluted to 3 ml and incubated for 3 days

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Assay of bacterial growth. To obtain exponentially growing cultures, frozen aliquots of M. bovis BCG (30 μl) were diluted to 3 ml and incubated for 3 days
with shaking in a screw-cap tube. Then 1 ml of 7H9 medium was added for an additional 2 days, followed by additional medium to obtain a turbidity reading of not more than 30 with a Klett-Summerson colorimeter (about 2 × 10^6 CFU/ml). Cells were grown to a turbidity reading of 100 (about 9 × 10^6 CFU/ml), and 14.5 ml of culture was placed in a 15-ml Vacutainer tube (catalog no. BD 36641; Becton Dickinson, Franklin Lakes, NJ) using a syringe. DETA-NO was added by syringe with minimal addition of air. Cultures were agitated by rolling. Recovery from growth arrest was attained by removal of 7 ml of culture by using a syringe to avoid introducing air into the tube. The cells removed from the Vacutainer tube were then placed in a 200-ml screw-cap Falcon bottle (Becton Dickinson, Franklin Lakes, NJ), mixed vigorously for 1 min, and placed on a low-profile roller (Stoval Co., Greensboro, NC) for aerated growth.

Anaerobic shock. Cells were grown to exponential phase in 7H9 medium supplemented as described above but with additional glucose to 1% and equimolar monobasic/dibasic sodium phosphate buffer (pH 6.8) to 100 mM as previously described for anaerobic growth of Escherichia coli (3). Cells were then shifted to anaerobic conditions by passing (bubbling) a mixture of 85% N_2, 10% H_2, and 5% CO_2 through the culture for 5 min (9). Traces of oxygen were removed as described in reference 9. Relative to CFU counts at the time of drug addition. Colonies were counted after incubation for 4 weeks; survival was calculated the drug concentrations and for the times indicated in the figures, cells were split into aliquots; half of the aliquots were treated with DETA-NO at 50 μM; and then rifampin, isoniazid, or fluoroquinolone was added. After incubation at the drug concentrations and for the times indicated in the figures, cells were diluted in 7H9 medium and applied to 7H10 agar plates lacking any drug. Colonies were counted after incubation for 4 weeks; survival was calculated relative to CFU counts at the time of drug addition.

RESULTS

Effect of DETA-NO on M. bovis BCG growth and survival. M. bovis BCG grew exponentially in evacuated (Vacutainer) tubes (Fig. 1A). Addition of DETA-NO at a moderate concentration (50 μM) to early-log-phase cultures blocked growth for at least 72 h (Fig. 1A); higher concentrations (100 and 500 μM) caused loss of CFU, and lower concentrations (5 and 25 μM) allowed growth (Fig. 1A). Growth arrest was also observed when relative turbidity was measured (Fig. 1B); in this case, lethal DETA-NO concentrations were not distinguished from bacteriostatic concentrations, indicating that lethal doses do not cause cell lysis. When tubes containing air were used, DETA-NO at 50 μM failed to sustain growth arrest (Fig. 1B inset). Thus, it was important to place cultures in evacuated tubes prior to the addition of DETA-NO in order to establish the growth arrest model.

To determine whether growth-arrested cells can resume growth, cultures were treated with 50 μM DETA-NO for 16 h and transferred to air-containing tubes. Cultures were then aerated by vigorous shaking for 1 min, followed by aerobic incubation on a roller. Growth resumed after a lag of 24 h (Fig. 2A). We next measured the length of the lag period associated with the exit from growth arrest after various times in 50 μM DETA-NO. More than 4 h of DETA-NO treatment was required before resumption of growth exhibited a lag (Fig. 2A inset and Fig. 2B), suggesting that a metabolic shift occurs beginning at 4 h of DETA-NO treatment. The lag became maximal (24 h) after about 16 h of DETA-NO treatment (Fig. 2B). These data indicated that the metabolic shift was complete after 16 h of DETA-NO treatment.

Effect of DETA-NO on susceptibility to anaerobic shock. Previous work indicated that removal of oxygen from cultures of M. tuberculosis reduces culture turbidity and viability unless the removal rate is slow (19, 20, 22). To determine whether growth arrest induced by DETA-NO protects M. bovis BCG from lethality generated by anaerobic conditions, we challenged M. bovis BCG by passing an anaerobic gas mixture through cultures for 5 min. This anaerobic shock caused cell death, as indicated by a 4-log-unit drop in CFU for both growing and growth-arrested cells (Fig. 3A). When culture turbidity was measured after anaerobic shock, it was seen to drop dramatically (Fig. 3B). Thus, anaerobic shock causes extensive cell lysis that is not blocked by pretreatment with DETA-NO.

Effects of growth arrest on the lethality of rifampin and isoniazid. When rifampin was added at various concentrations to cultures of M. bovis BCG that had been transferred to evacuated tubes and treated with 50 μM DETA-NO for 16 h, bacterial survival was similar to that observed for cultures from
which DETA-NO had been omitted (Fig. 4A). These data are consistent with RNA polymerase, the intracellular target of rifampin, being active during growth arrest (22). When isoniazid was added to cultures of M. bovis BCG, a 10-fold drop in viability occurred after 24 h at the highest drug concentration tested (Fig. 4B). Pretreatment with 50 μM DETA-NO for 16 h blocked the lethal action of isoniazid (Fig. 4B). Thus, growth arrest had different effects on rifampin and isoniazid.

Effect of DETA-NO on fluoroquinolone lethality. When M. bovis BCG was growing exponentially following transfer to an evacuated tube, two fluoroquinolones, moxifloxacin and gatifloxacin, exhibited equal killing kinetics at 3 times the MIC (Fig. 5A). Following a 16-h DETA-NO treatment, measurement of kinetics showed that moxifloxacin killed M. bovis BCG more extensively than gatifloxacin (Fig. 5B). When fluoroquinolone concentrations were varied, the two compounds were indistinguishable with exponentially growing cells (Fig. 6A), as observed with the kinetics experiment (Fig. 5A). After a 16-h DETA-NO treatment, moxifloxacin retained more activity than gatifloxacin (Fig. 6B). Thus, survival following fluoroquinolone treatment is more sensitive to quinolone structure with nonreplicating cells than with exponentially growing cells. At the highest fluoroquinolone concentration tested, growth arrest partially protected M. bovis BCG from being killed by moxifloxacin (3-fold protection) and gatifloxacin (10-fold protection) (Fig. 6C and D). These data indicate that the DETA-NO system is sensitive to drug structure, a feature required for an assay intended to identify compounds with superior activity.
The work presented here describes a novel model of in vitro mycobacterial growth arrest that arises when exponentially growing *M. bovis* BCG is placed in an evacuated tube and treated with DETA-NO, a generator of nitric oxide. Growth stopped immediately, as measured by relative turbidity or by the number of CFU (Fig. 1). Vigorous aeration restored growth (Fig. 2). However, restoration of growth required a period of adaptation if growth arrest extended for more than 4 h. These data are consistent with growth arrest being associated with a metabolic shift that must be reversed before the presence of air can restore growth. That shift appears to be complete after about 16 h of growth arrest (Fig. 2B), since longer periods of arrest fail to lengthen the lag before recovery begins. A lag in recovery is also seen when the growth of *M. tuberculosis* is arrested by gradual depletion of oxygen (22).

Since no arrest of growth was observed when *M. bovis* BCG was transferred to tubes containing air and then treated with DETA-NO (Fig. 1B inset), the use of evacuated tubes is important for the model. Presumably the reduced head volume and tight seal limit the loss of nitric oxide, since the arrest period was much shorter (17 h) when *M. tuberculosis* was treated with 500 μM DETA-NO in a vented flask (18). Data essentially identical to those shown in Fig. 1 were obtained with *M. tuberculosis* H37Rv (data not shown). We plan to use the evacuated tube model of DETA-NO-induced growth arrest to study the metabolic shift that occurs when *M. tuberculosis* growth is halted (15, 16).

In previous work, transfer of exponentially growing *M. tuberculosis* to an anaerobic chamber caused cell death (half-life, 10 h); in that case, partial adaptation to anaerobic growth protected cells from the lethal effects of anaerobiosis (20). In the present case, anaerobic shock caused cell lysis within minutes, and viable counts dropped by at least 3.5 log units in 5 min (data not shown). Growth arrest failed to protect *M. bovis* BCG from this cell lysis (Fig. 3). We are now investigating anaerobic shock as a novel treatment for tuberculosis.

Mycobacterial growth arrest had little (twofold) effect on rifampin lethality (Fig. 4A) but blocked killing by isoniazid (Fig. 4B). Other models of growth arrest also reveal that rifampin lethality (Fig. 4A) but blocked killing by isoniazid (Fig. 4B). Other models of growth arrest also reveal that rifam 
fampin retains much more of its lethal activity than isoniazid (5, 17, 22). The present model offers the advantages of (i) simple, inexpensive logistics, (ii) growth arrest beginning at the time that stress is applied, and (iii) temporally well defined stages of growth arrest. It will now be interesting to examine new compounds, such as diarylquinolines, for lethal activity with the DETA-NO-mediated growth arrest system described above, since one of the diarylquinolines shows excellent activity with several dormancy models (5).

Data obtained with fluoroquinolones emphasize that with some compounds, changes in drug structure can improve lethal activity following growth arrest (Fig. 5 and 6). That may be important, since widespread resistance is likely to develop if fluoroquinolone use for tuberculosis becomes more extensive (2). Since the two agents we compared, moxifloxacin and gatifloxacin, differ only in their C-7 ring structures, the C-7 substituent must be important for killing nongrowing cells. A similar but more dramatic difference between moxifloxacin and gatifloxacin was observed following growth arrest by treatment of Mycobacterium smegmatis and M. tuberculosis with chlorophenicol (8, 10). We have suggested that cell death can arise from drug-mediated destabilization of gyrase-DNA complexes and chromosome fragmentation (11). We are now modifying the fluoroquinolone C-7 ring structure to improve lethal activity when mycobacterial growth is arrested.

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