MAJOR ARTICLE

Isoniazid's Bactericidal Activity Ceases because of the Emergence of Resistance, Not Depletion of *Mycobacterium tuberculosis* in the Log Phase of Growth

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Background. It is believed that the cessation of isoniazid's early bactericidal activity during the initial phase of antituberculosis therapy is due to the depletion of *Mycobacterium tuberculosis* in the exponential phase of growth. We examined the veracity of this cornerstone belief.

Methods. We used an in vitro infection model in which *M. tuberculosis* was exposed to isoniazid concentration–time profiles encountered in human patients. Experiments were performed to examine the time-related changes in the total bacterial population, the isoniazid-susceptible subpopulation, and the isoniazid-resistant subpopulation.

Results. The cessation of microbial kill occurred between days 3 and 4 of isoniazid therapy, as occurs in patients. There were multiple logs of organisms in the exponential phase of growth remaining at the time when bactericidal activity ceased. The isoniazid-susceptible subpopulation was replaced by an isoniazid-resistant subpopulation after 80 h of therapy. The size of the isoniazid-susceptible subpopulation continued to decrease after the total population had ceased to decrease, whereas the resistant subpopulation remained in the exponential phase of growth. Resistance was due to single point mutations in the catalase-peroxidase gene and to reserpine-inhibitable efflux pumps.

Conclusions. The age-old hypothesis that isoniazid's microbial killing of *M. tuberculosis* during log-phase growth ceases because of the depletion of this bacillary population needs to be modified.

Tuberculosis is the most common chronic infectious cause of death. Reactivated tuberculosis manifests as chronic cavitary pneumonia. The population burden of *Mycobacterium tuberculosis* inside these pulmonary cavities is estimated to be between 7 and 9 $\log_{10}$ cfu/mL [1]. Thus, the total bacterial burden in a pulmonary cavity of 3 cm in diameter is expected to be >9 $\log_{10}$ cfu. Large portions of this bacillary population are coughed out when the pulmonary cavities rupture.

Some of the best insights into the effects of antituberculosis compounds on this bacillary population were made >2 decades ago by Jindani et al. [2]. They demonstrated that, during therapy, the decrease in the microbial population in sputum was biphasic. The first phase, which is mediated by isoniazid, is characterized by a rapid bacillary decrease during the first 2 days of therapy. The second phase, which is mediated by rifampin and pyrazinamide, is a much slower decrease in bacterial density that occurs between days 3 and 14. The conclusion was that "rapidly growing bacilli in cavitary lesions are killed, or at least prevented from growing, during the first 2 days of treatment. The speed of this process is determined by drug action, with priority given to the drug with greatest bactericidal activity against log-phase growth organisms. After 2 days, the remaining, persisting bacilli are killed in a process of slow sterilization, in which speed is determined by bac-

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terial factors rather than drug action" (p. 1352) [3]. Restated, the belief is that isoniazid kills the largest subpopulation of bacilli that are in the exponential phase of growth during the first 2 days of therapy. When this population is depleted, early bactericidal activity ceases [2–7]. This microbial killing during the first 2 days of therapy has been developed into an index that is often the first clinical test of the efficacy of new antituberculosis drugs [8–11]. In the present study, we tested the underlying hypothesis that the cessation of the isoniazid microbial killing is due to the depletion of *M. tuberculosis* in the exponential-phase growth. If this were true, microbial killing would cease only after the population of bacilli in the exponential-phase growth was extinct.

The effect of the emergence of resistance on early bactericidal activity studies has been inadequately explored [2, 12–14]. However, chromosomal mutations arise in a stochastic process that results in a population of resistant mutants whose size is proportional to the product of the mutation frequency ($\sim 10^{-6}$ for isoniazid) [15] and the total bacterial population present in pulmonary cavities. Mathematically, a considerable population of isoniazid-resistant isolates should exist before therapy, given the large bacillary loads in the chest cavity. Indeed, isoniazid-resistant subpopulations have been demonstrated in patients not treated with isoniazid [16]. Moreover, in vitro [17] and in patients [18], *M. tuberculosis* resistance to other antituberculosis agents has been shown to arise rapidly. Therefore, we examined the effect of the emergence of resistance on early bactericidal activity.

MATERIALS AND METHODS

Bacterial isolate. *M. tuberculosis* H37Ra (ATCC 25177; American Type Culture Collection) was used in our studies. Bacterial cultures were stored at −80°C in Middlebrook 7H9 broth with 10% oleic acid albumin dextrose catalase and 0.025% Tween 80 (Becton Dickinson), hereafter called "medium," and aliquots were thawed for each study. The cultures were incubated in 5% CO_2 at 37°C in medium for 4 days to achieve exponential-phase growth.

Drugs. Isoniazid (Sigma-Aldrich) was dissolved in sterile water to the desired drug concentrations. Reserpine (Sigma-Aldrich) was dissolved in dimethylsulfoxide and then diluted to the desired concentration in media.

MIC. Determination of isoniazid MICs was performed using methods recommended by the Clinical and Laboratory Standards Institute [19].

Hollow-fiber pharmacodynamic model of tuberculosis (HFM). The HFM has been described elsewhere [17, 20]. Hollow fibers are capillary semipermeable tubes (molecular-weight cutoff, 20 kDa) that are encased in a plastic cartridge. The cartridge space outside the capillary lumina is termed the "extracapillary space" (figure 1). *M. tuberculosis* cultures grow in

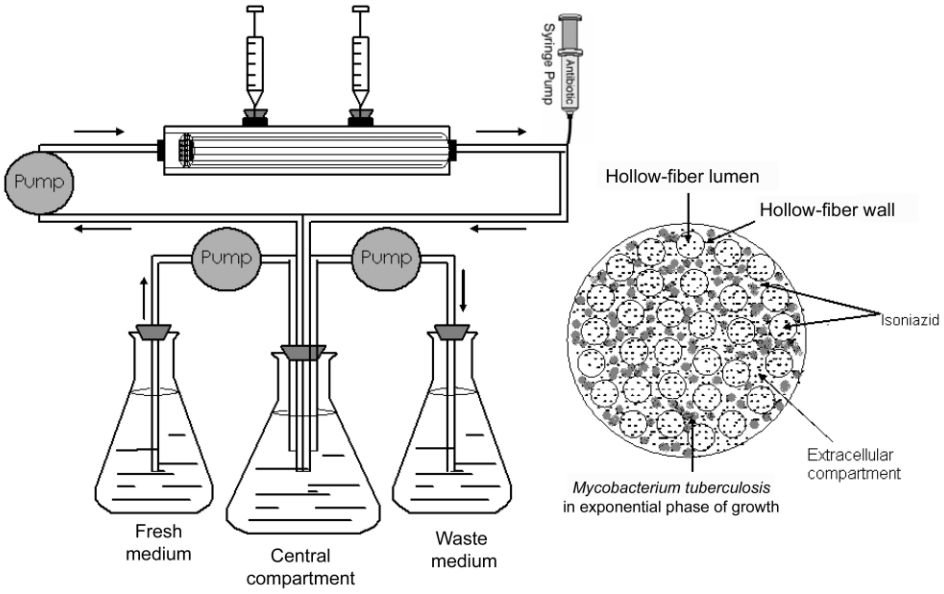

Figure 1. Hollow-fiber pharmacodynamic model of tuberculosis. A schematic of the model, together with a cross-section of the cartridge, is shown. *Mycobacterium tuberculosis* grow in the extracapillary space but are too big to cross into the central compartment. Isoniazid is administered to the central compartment. Medium circulates in the central compartment in the direction shown by the arrows. Fresh medium is added to and waste medium isovolumetrically removed from the central compartment. The rate of medium flow through the system determines the rate of decrease, and therefore the half-life, of isoniazid. The isoniazid in the central compartment diffuses into and out of the extracapillary compartment by first-order kinetics.

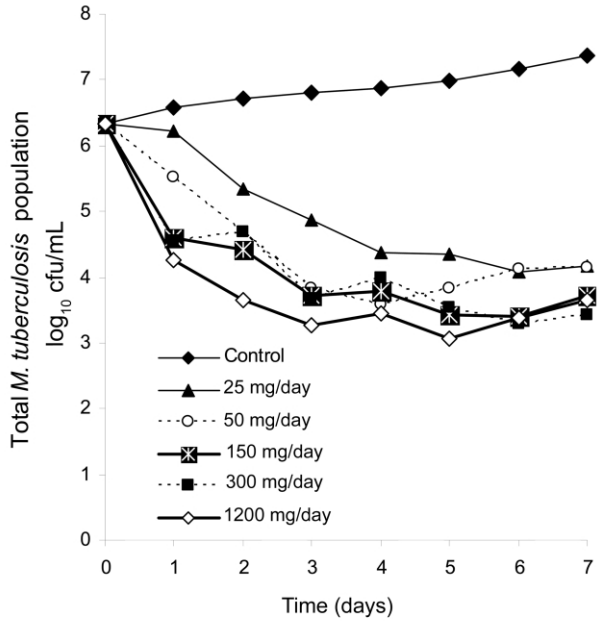

Figure 2. Day-to-day changes in total *Mycobacterium tuberculosis* populations in response to isoniazid therapy. The day-to-day changes in total *M. tuberculosis* populations in hollow-fiber systems treated with isoniazid exposures simulating 5 isoniazid doses are shown. Between days 3 and 4, the decrease in bacterial density stopped.

the extracapillary space but are too big to cross into the central compartment. Fresh medium was circulated within the central compartment, and nutrients, which are small molecules, diffused from the central into the extracapillary compartment. Used medium was removed from the system at a rate equal to the inflow of fresh medium. Isoniazid was administered to the central compartment via a syringe pump and diffused across the hollow fibers into and out of the extracapillary compartment by first-order kinetics. The isoniazid concentration in the central compartment decreased with a half-life ($t_{1/2}$) that mimicked that encountered in humans. Because of single-nucleotide polymorphisms in the N-acetyltransferase-2 gene, the serum isoniazid concentration in humans decreases at 1 of 2 half-lives: 0.9–1.8 h in fast acetylators and 2.2–4.4 h in slow acetylators [21]. We mimicked these 2 elimination phenotypes by varying the flow rates of medium into and out of the HFM. The flow rates were a solution to the equation: flow rate = central compartment volume $\times \ln(2)/t_{1/2}$. We set the central compartment volume at 250 mL. To mimic half-lives of 1.8 and 4.1 h, the flow rates of fresh medium through the system were 96.3 and 42.3 mL/h, respectively.

Isoniazid dose-range studies. For each experiment, *M. tuberculosis* cultures in medium were incubated under shaking conditions and 5% CO_2 at 37°C. On day 4 of growth, the cultures were used to determine the prevalence of resistant mutants that would grow on Middlebrook 7H10 agar supplemented with 0.2 or 1.0 mg/L isoniazid (concentrations defining low- and high-level resistance). Then, 15 mL of 5×10^6 cfu/mL of *M. tuberculosis* in the log phase of growth were inoculated into the extracapillary compartment of preconditioned HFM that were incubated at 37°C and 5% CO_2. Computer-controlled isoniazid infusions were started 24 h after bacterial inoculation and administered once daily for 7 days.

In the first dose-ranging study, *M. tuberculosis* cultures were inoculated into the extracapillary compartment of 6 hollow-fiber systems. We simulated serum concentration–time profiles of isoniazid doses in slow acetylators treated with 0, 25, 50, 150, 300, and 1200 mg/day. These doses were chosen so that a wide dose range could initially be examined. For the results to have clinical meaning, we examined doses ⩽1200 mg, because the acute ingestion of 1500 mg of isoniazid is toxic to patients. Starting from the first isoniazid infusion, the central compartment of each HFM was sampled 12 times over the course of 48 h, to validate that intended serum concentration–time profiles were achieved. *M. tuberculosis* cultures in the extracapillary compartment were sampled daily. Samples (0.6 mL) were washed twice, to prevent drug carryover, by suspending them in 6 mL of warm medium and then vigorously mixing. The contents were then centrifuged for 10 min at 3000 *g*, supernatant discarded, and contents resuspended in 6 mL of medium, and the process was repeated. Pilot studies with known bacillary densities demonstrated that this process does not change quantitative counts. The cultures were then resuspended in 0.6 mL of saline, serially diluted, and plated onto Middlebrook 7H10 agar for enumeration of the total microbial population and onto agar that contained 0.2 mg/L isoniazid for enumeration of the resistant subpopulation. Plates were read after 3 weeks of incubation.

On the basis of the results of the first dose-ranging study described above, *M. tuberculosis* cultures in 8 hollow-fiber systems were treated with isoniazid exposures simulating oral doses of 0, 25, 100, and 300 mg/day. For each dose, slow acetylator and fast acetylator concentration-time profiles were simulated. In addition, 600 mg administered to fast acetylators was also simulated. Sampling was as described above. To capture any possible isoniazid resistance due to the induction of efflux pumps [22, 23], day 7 cultures were also plated onto agar that contained 0.2 mg/L isoniazid and 20 mg/L reserpine. This concentration of reserpine was chosen because it has no effect on the growth of *M. tuberculosis* but inhibits isoniazid-induced efflux pumps [22]. Three isoniazid-resistant isolates per treatment regimen per day were selected for DNA sequencing of a 209-bp region of the catalase-peroxidase *(katG)* gene, using methods described by Parsons et al. [24].

Measurement of isoniazid concentration. Isoniazid concentrations were measured using the assay described by Jayaram et al. [25]. The assay was linear over a 100-fold range of 0.05–

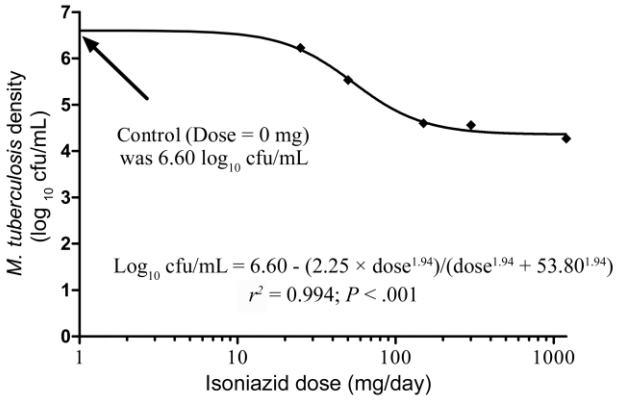

Figure 3. Inhibitory sigmoid E_{max} curve describing the relationship between isoniazid dose and microbial killing. Doses of ⩾150 mg/day were associated with maximal microbial killing.

10 mg/L ($r^2 > 0.99$), with a lower limit of detection of 0.05 mg/L. Samples expected to have concentrations <0.05 mg/L were concentrated to 16:1 by evaporation before assay. The between-day coefficient of variation for the assay was 0.08%–2.09%.

Mathematical modeling. All mathematical modeling and simulations were performed using the ADAPT II software of D'Argenio and Schumitzky [26]. Inhibitory sigmoid E_{max} analysis was performed for microbial density versus dose. The inhibitory sigmoid E_{max} model is defined by the Hill equation: $E = E_{con} - \{E_{max} \times (C)^H / [(C)^H + (C_{50})^H]\}$, where E is the observed *M. tuberculosis* $\log_{10}$ cfu/mL at a specific time point, E_{con} is the bacillary $\log_{10}$ cfu/mL in the control hollow-fiber system, E_{max} is the maximum reduction in bacillary density ($\log_{10}$ cfu/mL) during isoniazid therapy, C is the isoniazid drug exposure, C_{50} is the drug exposure for which there is 50% of maximum killing, and H is the Hill constant.

The observed isoniazid-susceptible subpopulation is the difference between the total population and the isoniazid-resistant subpopulation. Changes in isoniazid concentration and the resultant changes in isoniazid-susceptible and -resistant subpopulations with time were described using a mathematical model previously described in detail in the *Journal of Infectious Diseases* [17]. We used Bayesian parameters derived from the model in a mathematical simulation of the changes in the total population, the isoniazid-susceptible population, and the emergence of resistance expected with the recommended isoniazid clinical dose of 300 mg/day administered to slow acetylators.

RESULTS

The isoniazid MIC in the *M. tuberculosis* isolate used in our studies was 0.0156 mg/L. In the first study, maximum isoniazid concentrations were achieved in 1 h, and isoniazid decreased with a half-life of 4.1 h, as occurs in patients [21]. As shown in figure 2, microbial killing of the total *M. tuberculosis* population in each of the HFM treated with isoniazid effectively ceased between days 3 and 4. When the microbial killing ceased, the total bacterial population had not reached extinction. On the other hand, bacilli in the control arm remained in the exponential phase of growth during the entire experiment. Inhibitory sigmoid E_{max} modeling for isoniazid dose and microbial killing for the first 2 days of therapy is shown in figure 3. The steep section of the exposure-effect curve occurred between the dose range of 25 and 150 mg/day, whereas doses of 150, 300, and 1200 mg/day achieved similar extents of microbial killing (figure 3). The prevalence of mutants resistant to 0.2 mg/L isoniazid in the inoculum was 1 in 1.8×10^5 cfu. By day 7 of therapy, this subpopulation with low-level resistance had virtually replaced the total population, especially at doses of ⩽300 mg (figure 4), whereas the controls exhibited a resistant subpopulation at a fraction equivalent to that of the inoculum.

Next, we simulated isoniazid doses of 0, 25, 100, and 300 mg in fast and slow acetylators, so that several doses would lie on the steep portion of the dose-effect curve depicted in figure 3. We achieved a $t_{1/2}$ of 4.2 h for slow acetylators and 1.8 h for fast acetylators. Similar to the results of the first study, microbial killing ceased after day 3. When microbial killing ceased, the *M. tuberculosis* density had only decreased by slightly >2 $\log_{10}$ cfu/mL from the day-0 counts. The cessation of killing coincided with the increase in size of both the low-level and high-level isoniazid-resistant subpopulations. We comodeled isoniazid concentrations from all dose groups with the changes in the drug-resistant and drug-susceptible subpopulations over

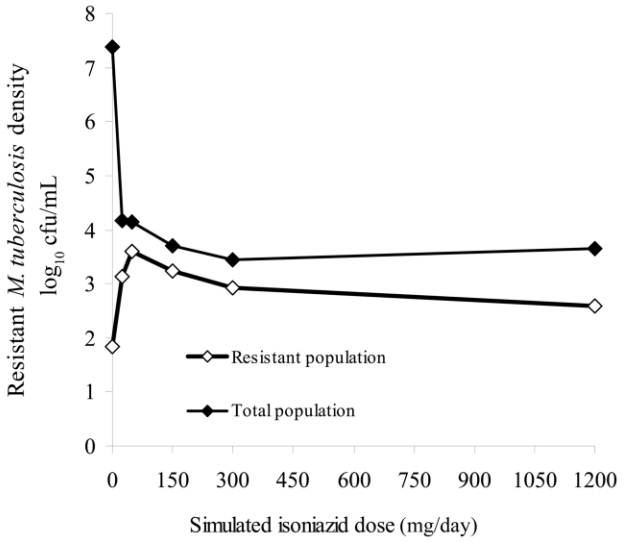

Figure 4. Day-7 changes in total and low-level isoniazid-resistant populations. The resistant subpopulation was higher in all isoniazid-treated hollow-fiber systems, compared with controls. At isoniazid doses of 50–300 mg/day, the resistant population accounted for a substantial portion of the total population. The size of the resistant subpopulation was bigger than in controls, even at an isoniazid dose of 1200 mg/day.

Table 1. Mean parameter estimates for the isoniazid population.

Parameter	Slow acetylator	Fast acetylator
Clearance rate, L/h	15.84 ± 3.27	55.82 ± 15.30
Volume of central compartment, L	95.08 ± 15.85	148.29 ± 43.06
$K_{gmax\text{-}S}$, $\log_{10}$ cfu/mL/h	0.01 ± <0.001	0.01 ± <0.001
$C_{50g\text{-}S}$, mg/L	3.24 ± 1.34	3.42 ± 3.12
$H_{g\text{-}S}$	6.07 ± 3.43	3.77 ± 2.33
$K_{gmax\text{-}R}$, $\log_{10}$ cfu/mL/h	0.02 ± 0.01	0.02 ± 0.01
$C_{50g\text{-}R}$, mg/L	4.19 ± 2.78	6.65 ± 4.09
$H_{g\text{-}R}$	4.77 ± 2.27	5.13 ± 2.30
$K_{kmax\text{-}S}$, $\log_{10}$ cfu/mL/h	11.95 ± 2.96	16.81 ± 2.53
$C_{50k\text{-}S}$, mg/L	11.92 ± 2.33	10.23 ± 3.90
$H_{k\text{-}S}$	1.27 ± 0.08	1.29 ± 0.02
$K_{kmax\text{-}R}$, $\log_{10}$ cfu/mL/h	7.49 ± 2.46	1.56 ± 1.24
$C_{50k\text{-}R}$, mg/L	17.69 ± 2.14	17.19 ± 2.42
$H_{k\text{-}R}$	5.88 ± 3.12	9.22 ± 1.39
POPMAX, $\times 10^9$ cfu/mL	4.12 ± 0.15	4.42 ± 0.82

NOTE. Data are mean ± SD values. K_{gmax} is the rate constant for maximum bacterial growth, K_{kmax} is the rate constant for maximum bacterial killing, C_{50k} is the drug concentration needed to achieve 50% of the maximum killing rate, C_{50g} is the drug concentration needed to achieve 50% effect on the maximum growth rate, H_k is the sigmoidicity constant for microbial killing, and H_g is the sigmoidicity constant for drug effect on microbial growth. These are shown for sensitive (S) and resistant (R) subpopulations. POPMAX is the estimated maximum size of the bacterial density in the control hollow-fiber system after bacterial growth enters the stationary phase.

time. Our mathematical model explained the drug concentrations adequately ($r^2 = 0.97$ and 0.93 for fast and slow acetylators, respectively) for both the total bacterial population (r^2 of 0.97 and 0.95, respectively) and the resistant subpopulation ($r^2 = 0.47$ and 0.67, respectively). $P < .001$ for drug concentrations and both bacterial populations. Population parameter estimates derived from the mathematical model are shown in table 1. These parameters were used in a mathematical simulation, which predicted that the isoniazid clinical dose of 300 mg/day would result in amplification of the preexistent resistant subpopulation to a degree that it equaled the size of the drug-susceptible population by 80 h of therapy (figure 5). Subsequently, the susceptible population continued to be killed but was replaced by resistant mutants, so that observation of the total population would lead one to conclude that killing ceased at ~80 h (3.3 days) of isoniazid therapy.

Seventy-three percent of resistant isolates from the HFM experiment that we tested had single point mutations in the *katG* gene. There were mutations in codon 300 of resistant isolates cultured from the bacterial suspension used to inoculate the HFM, the nontreated control arm, and all of the isoniazid treatment arms. However, *katG* mutations were identified in codons 315, 321, and 322 only in the isoniazid-treated arms, starting on day 3 of therapy. At day 7, the density of the low-level isoniazid-resistant subpopulation was 4.40–71.61 times higher in all treatment HFM, compared with the control HFM. However, when agar containing isoniazid was further supplemented with 20 mg/L reserpine, the density of the isoniazid-resistant subpopulation in the isoniazid treatment HFM was only 1.15–3.72 times that in the controls (figure 6). Reserpine alone had no effect on the total *M. tuberculosis* population in any of the regimens. These data imply that reserpine-inhibitable efflux pumps play a role as a mechanism contributing to resistance.

DISCUSSION

The goals of modern antituberculosis therapy are (1) an initial rapid elimination of *M. tuberculosis* in the exponential phase of growth, (2) an elimination of slowly replicating and nonreplicating organisms, and (3) prevention of resistance [5]. Isoniazid is the principal drug used for the first goal. The effect of the drug on bacilli in the exponential phase of growth has traditionally been measured using the index of early bactericidal activity. Early bactericidal activity is the average rate of decrease in the number of bacilli in sputum during the first 2–5 days of therapy. The maximum early bactericidal activity encountered in patients with tuberculosis who are treated with isoniazid is 0.62 ± 0.24 $\log_{10}$ cfu/mL/day [13]. This compares well with a 3-day decrease of 2.13 $\log_{10}$ cfu/mL (figure 2), which is an average of 0.71 $\log_{10}$ cfu/mL/day in our in vitro HFM. In addition, clinical studies of early bactericidal activity have demonstrated that the steep portion of the dose-effect curve lies between 0 and 150 mg/day [13], whereas doses of ⩾150 mg

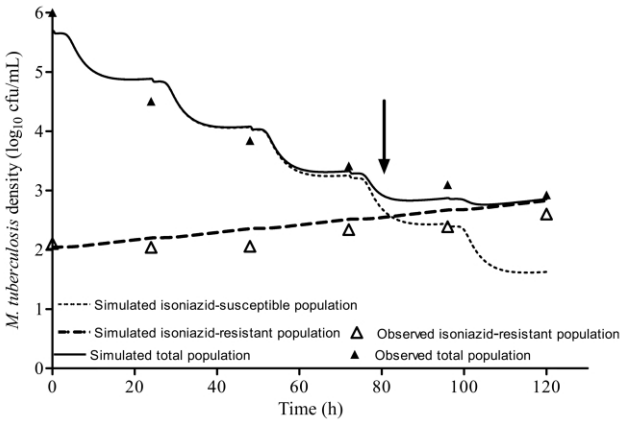

Figure 5. Mathematical model–derived changes in total, susceptible, and resistant *Mycobacterium tuberculosis* populations in response to 300 mg/day isoniazid. The parameters used for the simulation were calculated from data on the 8 doses, as shown in table 1. At the 80-h time point or day 3.3 *(vertical arrow),* the resistant subpopulation, which was growing rapidly, surpassed the susceptible population. At that time, the total population (which was in decline) started to increase, heralding the end of effective microbial killing. The actual total and isoniazid-resistant populations that we observed demonstrated that our simulations closely described actual events in the hollow-fiber system.

have the same bactericidal activity [2]. This is identical to observations in our in vitro HFM (figure 3). Thus, our in vitro HFM model closely reflects pharmacodynamic events in patients. By contrast, some studies have demonstrated a relapse of tuberculosis in some patients treated with isoniazid doses of 900 mg administered once to twice per week [27, 28]. However, this reflects sterilization failure and not early bactericidal activity. Furthermore, the average daily isoniazid exposures achieved in patients who relapsed were less than the average exposures expected in patients treated with isoniazid 150 mg every day.

We used our in vitro experimental model to examine the veracity of the notion that early bactericidal activity ceases because of the depletion of bacilli in the exponential phase of growth. In our HFM, the decrease in the total microbial population in response to isoniazid stopped at a time point similar to when early bactericidal activity ceases in humans. The changes in the total microbial population were due to an interaction of microbial killing of the drug-susceptible population and resistance amplification. During the first 3 days, the changes in the total bacterial population were driven by the rapid decrease in the susceptible subpopulation, which at that time made up most of the total population. The end of the decrease in size of the total microbial population occurred long before the susceptible population reached extinction. Mathematical simulations, as well as direct observation, revealed that the apparent cessation of microbial killing occurred at the point when the subpopulation of drug-resistant isolates, which were undergoing exponential-phase growth, exceeded drug-susceptible organisms (figure 5). The isoniazid-susceptible population continued to die long after the total population ceased to decrease. Therefore, we conclude that the termination of isoniazid's microbial killing was, for the most part, caused by the emergence of resistance. The resistance was due to *katG* mutations that are typical of those encountered in isoniazid-resistant clinical isolates [29–31] and to the recently described isoniazid-related induction of multidrug resistance pumps [22, 23]. Other mechanisms of resistance, such as error-prone replication due to isoniazid-associated free radicals [32, 33], as well as to a host of other isoniazid-induced metabolic products [34], should be studied in the future to determine whether they contribute to the rapid emergence of resistance. However, the main point is that isoniazid's time-delimited activity was due to an intertwining of the microbial killing of the drug-susceptible subpopulation (bactericidal activity) and the emergence of resistance caused by multiple mechanisms. This means that it is disadvantageous to separate bactericidal activity (goal 1) from the prevention of resistance (goal 3), as is the case in classic tuberculosis literature. This is similar to the lesson learned regarding other bacteria, in which resistance to antibiotics emerges rapidly and abolishes microbial kill [35].

The implications of our findings are that the early bactericidal activity of each compound will vary depending on how quickly resistance to the compound emerges. For example, the equivalent of the mean exposure achieved by 400 mg/day of moxifloxacin was associated with a monophasic decrease in *M. tuberculosis* density, with no emergence of resistance, during 7 days of therapy in the HFM [17]. This is different from our results with isoniazid. Johnson et al. [36] compared the rates of bacillary decrease in sputum from patients treated for 7 days with isoniazid 300 mg/day versus moxifloxacin 400 mg/day. Isoniazid mediated the usual biphasic response: a decrease of 0.67 ± 0.17 $\log_{10}$ cfu/mL/day between days 0 and 2 and of 0.14 ± 0.16 $\log_{10}$ cfu/mL/day between days 2 and 7 (with virtually no decrease between days 4 and 7). However, moxifloxacin mediated a bacillary decrease of 0.33 ± 0.39 $\log_{10}$ cfu/mL/day between days 0 and 2 and of 0.24 ± 0.17 $\log_{10}$ cfu/mL/day between days 2 and 7, which is essentially a monophasic decrease. Thus, the duration of early bactericidal activity in patients differed between those who received moxifloxacin versus isoniazid treatment. The 2–3-day delimitation of events is probably specific to the pharmacologic properties of isoniazid. We propose that preclinical models be used to estimate doses of antituberculosis drugs that have maximum microbial killing but can also limit resistance amplification for longer durations of therapy. Such doses can then be examined in clinical studies in which a long duration of early bactericidal activity can be favorably exploited. This could lead to an accelerated rate of sputum conversion in patients. Finally, our experiments illus-

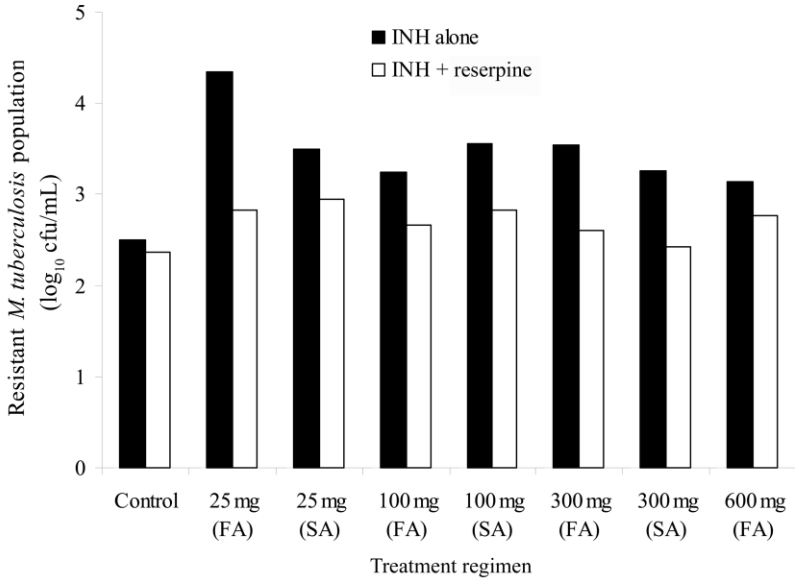

Figure 6. Effect of reserpine on the size of the resistant population. Results were from cultures on day 7 of therapy. Reductions in the size of the resistant subpopulation for bacterial samples taken from the hollow-fiber model of tuberculosis and cultured on agar that contained 0.2 mg/L isoniazid (INH) with and without 20 mg/L reserpine are shown. In the INH-treated arms, there was a substantial reduction in the population with low-level INH-resistance, compared with controls. Reserpine alone had no effect on the total microbial population in any of the treatment arms (data on total populations are not shown for reasons of clarity). In pilot studies, reserpine had no effect on the population of isolates with low-level INH resistance. Although it is possible that reserpine may have interfered with the activation of INH, a prodrug, the most likely explanation is that INH treatment induced reserpine-inhibitable efflux pumps. FA, fast acetylators; SA, slow acetylators.

trate the need to reexamine many of the assumptions that underlie modern antituberculosis chemotherapy. We suggest that all accepted dogmas be cast in the form of a falsifiable hypothesis and then examined in experimental systems that use modern pharmacokinetic-pharmacodynamic methods. The role that resistance plays in different *M. tuberculosis* metabolic populations, other than those in exponential-phase growth, is a subject of future studies.

In early bactericidal activity studies, new drugs administered as monotherapy are compared with isoniazid monotherapy. However, even when combined with ethambutol, rifampin, and pyrazinamide, early bactericidal activity is still mostly derived from isoniazid [2, 3]. Pyrazinamide has virtually no early bactericidal activity [2, 3], and efflux pumps associated with isoniazid resistance also confer drug tolerance to ethambutol [23], whereas ethambutol antagonizes other drugs in the combination regimen [2, 3]. Therefore, it is doubtful that pyrazinamide and ethambutol could delay the emergence of isoniazid resistance. Nevertheless, studies need to be performed in the future to determine whether combination therapy, especially with rifampin, delays the time to the emergence of isoniazid resistance.

Given the rapid emergence of isoniazid resistance, why has resistance not been commonly documented during studies of early bactericidal activity? One possible reason may be the fact that, in the case of isoniazid induction of efflux pumps, the resistance is transient [22]. Thus, resistance would probably not show if the cultures were first cultured in medium that does not contain the drug and then subcultured onto agar that has been supplemented with isoniazid, as is the common practice. Another possible explanation may be that the currently accepted definition of resistance is when $\geqslant$1% of the total population of *M. tuberculosis* grows in the presence of either 0.2 or 1 mg/L isoniazid [19]. However, it is clear from an examination of figure 5 that, by the end of early bactericidal activity studies on day 2, the resistant subpopulation is still <1% of the total. Because current methods are designed to capture resistance at the 1% threshold, they would underestimate the presence of resistant mutants. Moreover, molecular methods have recently demonstrated, in clinical sputum samples, that isoniazid-susceptible bacilli often coexist with an isoniazid-resistant population whose size is far in excess of the mutation frequency [37]. However, standard diagnostic methods may obscure the existence of this resistant subpopulation [37]. Finally, it may be that host immunologic conditions, such as macrophages, may decrease the burden of resistant *M. tuberculosis* in patients, making it more difficult to detect resistant isolates in sputum. In summary, we demonstrate that the cessation of isoniazid's bactericidal activity is due to the rapid

emergence of resistance and not to the depletion of *M. tuberculosis* in the exponential phase growth, as has been previously assumed.

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