Biphasic Kill Curve of Isoniazid Reveals the Presence of Drug-Tolerant, Not Drug-Resistant, Mycobacterium tuberculosis in the Guinea Pig

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The marked reduction in the potent early bactericidal activity of isoniazid during the initial phase of anti-tuberculosis (anti-TB) therapy has been attributed not only to the depletion of logarithmically growing bacilli but also to the emergence of isoniazid resistance. We studied the anti-TB activity of isoniazid and its ability to select for drug-resistant mutant strains in guinea pigs, in which the histopathology of TB closely resembles that of human TB. Prior mouse passage did not appear to enhance the virulence of Mycobacterium tuberculosis in guinea pigs. The human-equivalent dose of isoniazid was determined to be 60 mg/kg. Although isoniazid therapy caused rapid killing of bacilli in guinea pig lungs during the first 14 days of administration and rescued guinea pigs from acute death, its activity was dramatically reduced thereafter. This reduction in activity was not associated with the emergence of isoniazid-resistant mutant strains but, rather, with the selection of phenotypically tolerant “persisters.”

Isoniazid has biphasic activity against Mycobacterium tuberculosis in patients [1], with significant bactericidal activity during the first 2 days of therapy but reduced activity thereafter. Consistent with its ability to inhibit mycolic acid synthesis [2], isoniazid has potent bactericidal activity against exponentially growing M. tuberculosis in vitro and in animal models of tuberculosis (TB), as well as in humans with active TB [1]. However, the drug has minimal activity against dormant tubercle bacilli deprived of nutrients [3] or oxygen [4] in vitro. In addition, isoniazid has reduced sterilizing activity relative to rifampin in humans [5], in the standard mouse model [6], and in a mouse hypoxic granuloma model of latent TB infection [7, 8]. These observations have led to the prevailing concept that the reduced bactericidal activity of isoniazid is attributable to rapid initial killing of actively multiplying bacilli and selection of genetically drug-susceptible, non-replicating “persisters,” which exhibit phenotypic tolerance to isoniazid.

This dogma has been challenged recently by observations in an in vitro model simulating isoniazid concentration-time profiles encountered in humans [9]. In this model, M. tuberculosis killing by isoniazid ceased when the size of a drug-resistant subpopulation undergoing exponential-phase growth unimpeded by isoniazid exceeded the size of a drug-susceptible population that was being eliminated by isoniazid. The authors concluded that the marked reduction in isoniazid’s potent early bactericidal activity in tuberculous patients is primarily attributable to the emergence of drug resistance.
The rapid emergence of isoniazid resistance has not been observed commonly in studies of early bactericidal activity in humans [10, 11]. We have addressed this controversy in the guinea pig model, in which the histopathology of lung lesions closely resembles that of human TB lesions [12]. First, we determined the most suitable M. tuberculosis wild-type strain and inoculum for establishing acute infection with a large bacillary burden (≥10⁹ bacilli), analogous to that in the lung cavities of humans with TB [13]. Next, we identified the human-equivalent dose of isoniazid in guinea pigs. We then assessed the activity of isoniazid against M. tuberculosis in guinea pig lungs. Having observed biphasic killing typical of isoniazid, we investigated whether the second phase of greatly reduced activity was attributable to the emergence of drug resistance or to the selection of phenotypically tolerant “persisters.”

MATERIALS AND METHODS

M. tuberculosis strains. Three wild-type M. tuberculosis strains were used: a CDC1551 strain, which was passaged twice in mice (CDC1551); an H37Rv strain, which was passaged twice in mice (H37Rv-JHU); and an H37Rv strain, which was not animal passaged (H37Rv-TAMU; kindly provided by Dr. David McMurray). Before aerosol infection, cultures were grown to log phase (optical density at 600 nm, ∼0.5) in Erlenmeyer flasks containing supplemented Middlebrook 7H9 broth (Difco Laboratories) [14].

Animals. Female outbred Hartley guinea pigs with or without jugular vein vascular catheters (250–300 g) were purchased from Charles River Laboratories. Animals were maintained under pathogen-free conditions and fed water and chow ad libitum. All procedures followed protocols approved by the Institutional Animal Care and Use Committee at Johns Hopkins.

Pharmacokinetics experiments. Separate groups of 3 guinea pigs each were given a single dose of isoniazid (Sigma) at 30 or 60 mg/kg. Doses were prepared in 40% sucrose (weight/volume) in a final volume of 0.5 mL and delivered in the posterior oropharynx by an automatic pipette with a disposable tip. Blood (∼0.3 mL) was removed serially from guinea pigs through the intravenous catheter at the following time points after antibiotic dosing: 30 min and 1, 2, 4, 6, 8, 12, and 24 h. Serum was separated and stored at 70°C. Samples were mailed overnight on dry ice to C.A.P. Serum isoniazid concentrations were determined using a validated high-performance liquid chromatography (HPLC) assay comprising a ThermoFinnigan P4000 HPLC pump with model AS1000 fixed-volume autosampler, a model UV2000 ultraviolet detector, a Gateway Series e computer, and the ChromQuest HPLC data management system. The plasma standard curve for isoniazid ranged from 0.5 to 20 µg/mL. The absolute recovery of isoniazid from plasma was 61%. The within-day precision (percent coefficient of variation) of validation quality control samples was 1%–6%, and the overall validation precision was 6%–10%. Quality control sample concentrations were 13, 6, and 0.8 µg/mL. Serum concentration data were entered into a WinNonlin worksheet (WinNonlin software, version 4.0; Pharsight) and were analyzed by means of standard noncompartmental techniques to determine the relevant pharmacokinetic parameters.

Aerosol infections. Guinea pigs were aerosol-infected with M. tuberculosis by means of the Inhalation Exposure System (Glas-Col). Three animals from each group were killed on days 1, 7, 14, 21, 28, or 56 after infection. Lungs were examined grossly for lesions, and small, randomly selected sections were formalin-fixed for histopathology. The remainder of each lung was homogenized, as described elsewhere [8]. Undiluted lung homogenates were plated on 7H11 plates containing cycloheximide (50 µg/mL), carbenicillin (100 µg/mL), polymyxin B (200 U/mL), and trimethoprim (20 µg/mL), and diluted samples were plated on 7H10 plates for enumeration of colony-forming units (CFU).

Antibiotic therapy. Oral therapy with isoniazid (60 mg/kg; 5 doses/week) was initiated 14 days after infection in guinea pigs infected with ∼10⁹ bacilli of H37Rv-TAMU. Isoniazid-treated guinea pigs were killed on days 7, 14, 42, or 84 after therapy, and lungs were homogenized and plated for CFU enumeration. In addition, diluted and undiluted lung homogenates were plated on 7H11 antibiotic-containing plates along with isoniazid 0.2 µg/mL, to quantify the number of isoniazid-resistant colonies. After dilution, the entire lung homogenate was plated (lower limit of detection of isoniazid-resistant mutants, <5 CFU/lung).

Lung decontamination and assessment of drug-resistant mutants. To remove any adverse effect of antimicrobials on the growth of drug-resistant mutant strains, for subsequent isolation of drug-resistant mutant strains at the peak lung CFU burden (day 14 after infection), lung homogenates were decontaminated by equal-volume addition of 2% sulfuric acid and incubated for 10 min at room temperature before the addition of 2 mL of sodium hydroxide. Processed lung samples were plated on 7H10 plates containing isoniazid at 0.05, 0.1, 0.2, or 1.0 µg/mL or streptomycin at 0.5, 1.0, 2.0, or 10.0 µg/mL. Colonies were counted 28 days after incubation at 37°C (lower limit of detection of drug-resistant mutant strains, <10 CFU/lung).

RESULTS

Assessment of virulence of wild-type M. tuberculosis strains in guinea pig lungs. Before initiation of antibiotic studies, we sought to characterize the virulence of several different wild-type strains of M. tuberculosis in guinea pigs, as well as to identify the inoculating dose required to kill untreated animals during acute infection. First, guinea pigs were aerosol-infect ed with 10²–10³ bacilli of CDC1551, H37Rv-JHU, or H37Rv-
TAMU strain. All guinea pigs in each group survived with similar weight gain over the next 56 days (data not shown). As demonstrated in figure 1, animals infected with either CDC1551 or H37Rv-TAMU had \(10^9\) bacilli implanted per lung on day 1 after aerosol infection, whereas the inoculated dose of H37Rv-JHU was a mean (\(\pm\) standard deviation [SD]) of 2.2 \(\pm\) 0.3 log\(_{10}\) CFU/lung.

Each strain grew exponentially during the first 14 days of infection. Bacillary growth ceased thereafter in animals infected with H37Rv-JHU or H37Rv-TAMU, whereas mean (\(\pm\)SD) lung CFU counts increased from 6.7 \(\pm\) 0.3 log\(_{10}\) to 7.7 \(\pm\) 0.2 log\(_{10}\) CFU/lung between days 14 and 21 in CDC1551-infected animals. However, by day 28, lung CFU counts in the CDC1551 group decreased to 6.9 \(\pm\) 0.2 log\(_{10}\) CFU/lung, and there was no statistically significant difference between lung CFU counts in this group and those in the implantation-matched H37Rv-TAMU group at days 28 or 56 after aerosol infection. Gross examination of the lungs at day 28 after infection revealed discrete tubercles, which were equivalent in number and size to those in the H37Rv-TAMU and CDC1551 groups and more extensive than those in the H37Rv-JHU group (figure 2A–2C), consistent with the greater bacillary burden in the former 2 groups. Histological examination of the lungs at the same time point revealed granulomas with central caseation in the H37Rv-JHU group and diffuse peribronchiolar inflammation comprising lymphocytes and histiocytes without distinct granulomas in the H37Rv-TAMU and CDC1551 groups (figure 2D–figure 2F).

Next, we compared the virulence of each strain and its ability to kill guinea pigs acutely after aerosol infection at a higher inoculum. As demonstrated in figure 3, each strain was implanted equally (\(\sim\)10\(^5\) bacilli/lung) in guinea pigs. All CDC1551-infected animals succumbed to infection or were euthanized owing to their moribund condition by day 24, and all H37Rv-JHU– and H37Rv-TAMU–infected animals succumbed to infection or were euthanized by day 28 after infection. Each strain grew exponentially during the first 14 days of infection. Mean lung CFU counts increased between days 14 and 21 from 8.6 log\(_{10}\) to 9.4 log\(_{10}\) CFU/lung in H37Rv-JHU–infected animals and from 8.0 \(\pm\) 0.3 log\(_{10}\) to 9.8 \(\pm\) 0.3 log\(_{10}\) CFU/lung in CDC1551–infected animals. In H37Rv-TAMU–infected animals, lung CFU counts increased from 9.0 \(\pm\) 0.1 log\(_{10}\) to 9.8 \(\pm\) 0.7 log\(_{10}\) CFU/lung between days 14 and 28 after infection. Differences in lung CFU counts between the 3 infected groups were not statistically significant at the final time point assessed (figure 3). Gross examination of the lungs at the final time point revealed diffuse inflammation without discrete lesions in all groups, and histopathological evaluation revealed an acute lung injury pattern with thickening of the interalveolar septa by a mononuclear cell infiltrate and fibroblastic proliferation. There was intraalveolar deposition of fibrin and formation of hyaline membrane, reminiscent of diffuse alveolar damage in humans. No discrete granulomas were identified in any of the groups (data not shown).

Identification of the human-equivalent dose of isoniazid in guinea pigs. Although early studies suggested the human-equivalent dose of isoniazid in guinea pigs to be 10 mg/kg [15, 16], more-recent studies [17] showed that this dose underestimates drug exposures in humans receiving conventional isoniazid doses [18]. Table 1 shows the main pharmacokinetic parameters of isoniazid in guinea pigs, compared with known corresponding values in humans and mice. After oral administration of isoniazid in guinea pigs, the mean (\(\pm\)SD) peak serum concentrations were 9.0 \(\pm\) 3.2 and 16.8 \(\pm\) 3.5 \(\mu\)g/mL after 30 and 60 mg/kg doses, respectively. Mean values (\(\pm\)SD) for the area under the serum concentration–time curve (AUC\(_{\text{area}}\)) were 22.9 \(\pm\) 5.5 and 34.1 \(\pm\) 4.9 mg\(\cdot\)h/L for isoniazid doses of 30 and 60 mg/kg, respectively. Although extensive pharmacodynamic data are lacking, the available data suggest that AUC/minimum inhibitory concentration is the parameter most closely correlated with the bactericidal activity of isoniazid [20]. Therefore, based on AUC data in humans, the human-equivalent dose of isoniazid in the guinea pig model was determined to be 60 mg/kg.

Assessment of bactericidal activity of isoniazid in heavily infected guinea pigs. One group of H37Rv-TAMU–infected guinea pigs was treated with isoniazid (60 mg/kg daily, 5 days/week) beginning 14 days after aerosol infection. Isoniazid therapy reduced the lung CFU burden by \(\sim\)4 log\(_{10}\) CFU/lung (figure 4) during the first 14 days and significantly ameliorated lung pathology both grossly (figure 5) and histologically (data not shown). However, the bactericidal activity of isoniazid was
greatly diminished thereafter, causing a further reduction of \(< 2 \log_{10} \text{CFU/lung}\) during the next 28 days of therapy (figure 4). Although mean lung CFU values could not be calculated at day 84 because of sample contamination, the total lung CFU count from a single animal at that time point was 3.2 \(\log_{10} \text{CFU/lung}\).

**Lack of selection of isoniazid-resistant mutants in guinea pig lungs.** To investigate the basis of the biphasic killing curve of isoniazid against *M. tuberculosis* in guinea pig lungs, we tested for the presence of isoniazid-resistant mutant strains in lungs after isoniazid monotherapy. Isoniazid-resistant colonies were not isolated at days 7, 14, or 42 of isoniazid therapy. Of interest, despite the high bacillary burden at the start of therapy (\(\sim 10^9 \text{CFU/lung}\)), isoniazid-resistant mutants were not detected at day 0.

The frequency of spontaneous isoniazid resistance is reported to be \(\sim 10^{-8}\) per bacterium per generation in *M. tuberculosis* cultures grown in vitro [21]. To determine whether the intrinsic frequency of isoniazid resistance for the strains tested was different from that reported elsewhere, H37Rv-TAMU and H37Rv-JHU were grown to midexponential phase in complete Middlebrook 7H9 broth and then plated on 7H10 plates containing isoniazid. Isoniazid-resistant mutants of H37Rv-JHU were isolated at frequencies of 6.5 colonies per \(10^5 \text{CFU}\), 4.9 colonies per \(10^5 \text{CFU}\), and 2.8 colonies per \(10^5 \text{CFU}\), whereas isoniazid-resistant mutants of H37Rv-TAMU were isolated at frequencies of 8.4 colonies, 4.7 colonies, and 2.4 colonies per \(10^5 \text{CFU}\) when grown on plates containing isoniazid at a concentration of 0.2 \(\mu \text{g/mL}\), 1 \(\mu \text{g/mL}\), and 10 \(\mu \text{g/mL}\), respectively.

Next, to determine whether spontaneous isoniazid-resistant mutants in the H37Rv background are unable to survive and multiply in guinea pig lungs, we aerosol-infected guinea pigs with H37Rv-TAMU or H37Rv-JHU, using broth cultures containing a mean (\(\pm \text{SD}\)) of 7.9 \(\pm 0.04 \log_{10} \text{CFU/mL}\) and 7.5 \(\pm 0.02 \log_{10} \text{CFU/mL}\), respectively, to deliver \(\sim 10^6 \text{CFU}\) into the lungs of infected animals. On day 14 after infection, total lung CFU counts were 8.7 \(\log_{10} \text{CFU/lung}\) and 9.0 \(\log_{10} \text{CFU/lung}\) in the H37Rv-TAMU and H37Rv-JHU groups, respectively. Despite the high lung bacillary burden in both groups, isoniazid-resistant mutants could not be recovered from either group on plates containing 0.2 \(\mu \text{g/mL}\) isoniazid.

**Figure 2.** Guinea pig lung pathology 28 days after low-dose aerosol infection with *Mycobacterium tuberculosis*, including the gross (A–C) and histological (D–F) appearance of lungs from animals infected with strain H37Rv-JHU (A, D), CDC1551 (B, E), or H37Rv-TAMU (C, F).

**Figure 3.** Growth of *Mycobacterium tuberculosis* in guinea pig lungs after high-dose aerosol infection. Animals were infected via aerosol with \(\sim 10^6 \text{CFU}\) of H37Rv-JHU, CDC1551, or H37Rv-TAMU strain.
Finally, to test the hypothesis that isoniazid-resistant mutants are selectively deficient for survival in guinea pig lungs, we compared the frequency of recovery of isoniazid-resistant and streptomycin-resistant mutant strains from heavily infected guinea pig lungs, because the rate of spontaneous resistance to streptomycin in M. tuberculosis cultures is reported to be very similar to that of spontaneous resistance to isoniazid [22]. Eight guinea pigs were aerosol-infected with H37Rv-TAMU, with use of a broth culture containing a mean (± SD) of 7.8 ± 0.04 log_{10} CFU/mL to deliver ∼10^6 CFU into the lungs of infected animals. On day 14 after infection, the total mean lung CFU burden (± SD) was 8.0 ± 0.2 log_{10} CFU. Although streptomycin-resistant mutant strains were isolated at the expected frequency, isoniazid-resistant mutant strains again were not detected (table 2).

**DISCUSSION**

In this study, we developed a guinea pig model of TB chemotherapy in which the peak M. tuberculosis burden approaches that observed in human pulmonary cavities [13] to test the bactericidal activity of isoniazid and its ability to select for drug-resistant mutants. Our results show that the infectious dose required to achieve a peak burden of ∼10^9 organisms and to cause rapid killing of infected animals is ∼10^8 CFU and that prior mouse passaging probably does not alter the virulence of wild-type M. tuberculosis. As observed in human early bactericidal activity studies [1, 23, 24], isoniazid given at human-equivalent doses produced a biphasic killing curve in guinea pig lungs, with strong bactericidal activity during the first 14 days of therapy and significantly reduced activity thereafter. Of interest, we show, to our knowledge for the first time, that, unlike streptomycin monotherapy [25], isoniazid monotherapy does not select for drug-resistant mutants in guinea pig lungs. Our results are consistent with reduced bactericidal activity of isoniazid caused by depletion of exponentially growing M. tuberculosis and selection of phenotypically tolerant “persisters.”

Our in vitro data indicated a high probability of isoniazid-resistant mutants being present in the initial implanted inoculum in guinea pig lungs after high-dose aerosol infection. Moreover, after 14 days of exponential bacillary growth, when the total lung CFU burden was 10^5–10^6 CFU/lung, we expected to recover ∼10^5–10^6 isoniazid-resistant mutants. We were surprised to find that isoniazid-resistant mutants could not be detected. In contrast, streptomycin-resistant mutants were isolated at the expected frequency from the same heavily infected lungs. We believe that the absence of detectable isoniazid-resistant mutants before the initiation of therapy largely explains the lack of selection of isoniazid-resistant mutants after isoniazid monotherapy. Human studies of isoniazid monotherapy have shown that isoniazid resistance is related to peak plasma concentrations and not to the AUC of the drug, with progressively lower degrees of resistance with increasing doses [26].

Spontaneous mutations associated with isoniazid resistance are most commonly found in the katG gene, accounting for up to 80% of isoniazid-resistant clinical isolates [27]. The high peak serum concentrations associated with the 60-mg/kg isoniazid dose in the guinea pig model may preferentially select against katG point mutations that preserve some catalase function or mutations in inhA or its promoter region, all of which confer lower levels of drug resistance [28], compared with point

**Figure 4.** Antituberculosis activity of isoniazid in heavily infected guinea pigs. Animals were infected via aerosol with ∼10^8 colony-forming units (CFU) of H37Rv-TAMU strain and were either left untreated (solid line) or treated with isoniazid (60 mg/kg) (dashed line) beginning on day 14 after infection. Data are representative of 2 separate experiments.

<table>
<thead>
<tr>
<th>Test species</th>
<th>Drug dosage, mg/kg</th>
<th>C_{max} µg/mL</th>
<th>T_{max} h</th>
<th>t_{1/2} h</th>
<th>AUC_{0→r} mg h/L</th>
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<tbody>
<tr>
<td>Guinea pig</td>
<td>30</td>
<td>9.0 ± 3.2</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>22.9 ± 5.5</td>
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<td>60</td>
<td>16.76 ± 3.5</td>
<td>0.75 ± 0.03</td>
<td>0.90 ± 0.2</td>
<td>34.09 ± 4.9</td>
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<tr>
<td>Mouse</td>
<td>25</td>
<td>28.2 ± 3.8</td>
<td>0.25 ± 0</td>
<td>1.7 ± 0.17</td>
<td>52.2 ± 2.2</td>
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<tr>
<td>Human</td>
<td>6.2 ± 6</td>
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| Rapid acetylators | 5.4 ± 20 | 1.1 ± 0.5 | 1.54 ± 0.31 | 19.9 ± 6.1 |
| Slow acetylators  | 7.1 ± 1.9| 1.1 ± 0.6 | 3.68 ± 0.59 | 48.2 ± 1.5 |

**Table 1.** Pharmacokinetic Values for Isoniazid in Guinea Pigs, Mice, and Humans

<table>
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**NOTE.** Data are mean (± standard deviation), unless otherwise indicated. AUC_{0→r}, area under the serum concentration–time curve; C_{max}, mean peak serum concentration; T_{max}, time to peak concentration; t_{1/2}, half-life.

* Data adapted from Grosset and Ji [19].
mutations or deletions in katG, which abolish catalase function and confer higher levels of drug resistance. Long before the katG gene was shown to encode a catalase-peroxidase enzyme responsible for isoniazid resistance [29], it was known that catalase-deficient isoniazid-resistant strains are significantly less virulent than are drug-susceptible strains in the guinea pig TB model, leading to regression and healing of lesions and prolonged animal survival [30, 31]. In contrast, catalase-positive isoniazid-resistant strains and those resistant to other drugs, including streptomycin, appear to retain full virulence in guinea pigs [25, 31–33].

More recently, KatG was shown to protect M. tuberculosis from the toxic effects of organic peroxides generated by the phagocyte nicotinamide adenine dinucleotide phosphate-oxidase [34, 35], and integration of a functional katG gene into avirulent catalase-negative strains of Mycobacterium bovis [36] and M. tuberculosis [37] restored virulence in guinea pigs. Therefore, we hypothesize that a small population of katG-deficient, isoniazid-resistant mutants cannot be amplified in guinea pig lungs even after isoniazid monotherapy, because such mutants are killed by host immune mechanisms involving oxidative stress, perhaps within alveolar macrophages [38] or necrotic lung tissue. However, the heavily infected guinea pig lungs did not show a significant degree of tissue necrosis, possibly reflecting an abundance of secondary lesions. Unlike primary lesions, secondary lesions arise from hematogenous seeding [39] and elaborate the anti-inflammatory cytokine transforming-growth-factor-β [40].

Of interest, at least some catalase-deficient isoniazid-resistant mutants appear to retain virulence in the mouse model [41]. It is not surprising, therefore, that isoniazid-resistant mutants can be selected readily at the expected frequency after isoniazid monotherapy in mice [42]. Unlike mouse TB lesions, guinea pig TB granulomas more closely approximate their human counterparts with respect to cellular composition, granuloma architecture, and the presence of caseation necrosis [43]. However, katG-deficient isoniazid-resistant mutants are readily detectable from the sputum of infected patients, suggesting that phagocytes in guinea pigs may mount a more vigorous oxidative burst than do their human counterparts, leading to enhanced killing of catalase-deficient M. tuberculosis strains. Alternatively, the observed differences between humans and

### Table 2. Detection of Drug-Resistant Mutants in Heavily Infected Guinea Pig Lungs on Day 14 after Aerosol Infection

<table>
<thead>
<tr>
<th>Antibiotic, concentration</th>
<th>Inoculum, mean log_{10} CFU/lung (± standard deviation)</th>
<th>Frequency of drug-resistant mutants, colonies per 10⁵ CFU plated</th>
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<tr>
<td>Streptomycin</td>
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<tr>
<td>0.5 μg/mL</td>
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<td>11.8</td>
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<tr>
<td>1.0 μg/mL</td>
<td>8.2 ± 0.2</td>
<td>7.5</td>
</tr>
<tr>
<td>2.0 μg/mL</td>
<td>8.2 ± 0.2</td>
<td>4.3</td>
</tr>
<tr>
<td>10.0 μg/mL</td>
<td>8.2 ± 0.2</td>
<td>0⁶</td>
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<tr>
<td>Isoniazid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 μg/mL</td>
<td>7.9 ± 0.2</td>
<td>0⁶</td>
</tr>
<tr>
<td>0.1 μg/mL</td>
<td>7.9 ± 0.2</td>
<td>0⁶</td>
</tr>
<tr>
<td>0.2 μg/mL</td>
<td>7.9 ± 0.2</td>
<td>0⁶</td>
</tr>
<tr>
<td>1.0 μg/mL</td>
<td>7.9 ± 0.2</td>
<td>0⁶</td>
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**NOTE.** Each treatment group included 4 guinea pigs.

* Lower limit of detection of drug-resistant mutants, <10 colony-forming units (CFU)/lung.
guinea pigs may be related to the formation of cavities in humans, which may provide more permissive growth conditions for catalase-negative mutants and perhaps allow for the development of compensatory mutations, including overexpression of the alkyl hydroperoxide reductase AhpC [44], to further enhance survival and multiplication. Although it has been suggested that catalase-peroxidase enzyme deficiency may confer reduced virulence of *M. tuberculosis* in humans [45], this remains to be proved.

Our observation that the biphasic killing curve of isoniazid occurs in guinea pigs without selection of resistant mutants is contrary to that of Gumbo et al, who used an in vitro hollow-fiber model simulating human isoniazid serum concentration–time profiles to conclude that strong early bactericidal activity by isoniazid in humans is greatly reduced owing to the emergence of drug resistance [9]. Specifically, in these studies, the microbial kill of isoniazid ceased between days 3 and 4 of therapy, coinciding with the replacement of the decreasing isoniazid-susceptible population by a growing isoniazid-resistant subpopulation. The absence of selective amplification of isoniazid-resistant mutants despite isoniazid monotherapy in the guinea pig model provided a unique opportunity to show that emergence of drug resistance is not necessary for the marked reduction in strong early bactericidal activity by isoniazid. On the contrary, selection of phenotypically tolerant bacilli is sufficient to reproduce the biphasic killing curve of isoniazid.

Although the emergence of resistance to isoniazid undoubtedly occurs during monotherapy in humans, animal models and clinical experience indicate that it does not emerge and replace the drug-susceptible population as rapidly as it does in the hollow-fiber model [10]. This is because the in vitro hollow-fiber model consists of a relatively homogeneous population of logarithmically growing bacilli in the absence of host immune effector mechanisms. Under such permissive conditions, the resistant subpopulation is rapidly amplified even when the responsible mutations would confer a selective disadvantage for in vivo growth. On the other end of the spectrum, isoniazid-resistant mutants are unable to survive in the guinea pig model, so that amplification of isoniazid-resistant mutants does not occur. It is likely that the development of cavitary disease in human TB creates more permissive conditions, allowing for the outgrowth of isoniazid-resistant mutants on isoniazid monotherapy, yet not with the rapidity seen in the in vitro hollow-fiber model. These observations do not diminish the usefulness of that model. Although it cannot simulate the complex host-pathogen interactions underlying the phenomenon of persistence or potential immune-based preferential killing of drug-resistant mutants, it remains an important model for investigating the pharmacodynamics of anti-TB drugs and resistance suppression. No experimental model can reproduce faithfully all aspects of human TB, and the strengths and limitations of each model must be recognized.

In conclusion, using an in vivo model in which isoniazid-resistant *M. tuberculosis* mutants cannot be amplified, we demonstrated that isoniazid shows biphasic killing not unlike that seen in clinical trials evaluating the early bactericidal activity of isoniazid. The marked reduction in bactericidal activity by isoniazid in this model appears to reflect phenotypic antibiotic tolerance. These data are compatible with those of human studies, which have not documented drug resistance as the primary mechanism for the cessation of that early bactericidal activity [10, 11].

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