Expression of katG in Mycobacterium tuberculosis Is Associated with Its Growth and Persistence in Mice and Guinea Pigs

Zhongming Li, Cynthia Kelley, Frank Collins, David Rouse, and Sheldon Morris

The molecular mechanisms associated with the pathogenesis of tuberculosis are not well understood. The present study evaluated the role of catalase-peroxidase as a potential virulence factor for Mycobacterium tuberculosis. Growth and persistence of M. tuberculosis H37Rv in intravenously infected BALB/c mice were compared with katG-deleted, isoniazid-resistant M. tuberculosis H37RvINH\(^6\). Transformation of M. tuberculosis H37Rv (TBkatG) or Mycobacterium intracellulare (MACkatG) genes into M. tuberculosis H37RvINH\(^6\) restored its catalase-peroxidase activities and the ability of the recombinants to persist in spleens of mice and guinea pigs. Transformation with the TBkatG gene with the codon 463 R\(\rightarrow\)L mutation also restored catalase-peroxidase activity and enhanced persistence. However, transformants with the codon 275 T\(\rightarrow\)P mutant expressed low levels of enzymatic activity and failed to persist in guinea pig spleen, although they did survive in mouse tissues. These results indicate that KatG contributes to the ability of M. tuberculosis to grow and survive within the infected host tissues.

Pulmonary tuberculosis remains the leading life-threatening bacterial infection we face in the world today. The World Health Organization estimates that nearly 3 million people, including 200,000 children and infants, die from tuberculosis annually [1]. Many who are infected with the human immunodeficiency virus (HIV) develop rapidly disseminating mycobacterioses in a lethal disease combination that is greatly complicating tuberculosis control measures worldwide [2]. The convergence of the tuberculosis and HIV epidemics in homeless and drug-abuse populations has also been associated with an increasing incidence of multidrug-resistant tuberculosis, which is having its own devastating impact on the implementation of preventive public health measures against this disease [3].

The magnitude of this widening tuberculosis epidemic underscores the need for an improved understanding of the pathogenesis of this highly infectious disease if we are to develop new and improved strategies to prevent its further spread, especially in severely immunocompromised individuals. However, despite a great deal of study, we still know surprisingly little about the virulence factors associated with the establishment of active lung disease in individuals exposed to a tuberculous infection.

One correlate of virulence seems to be the loss of catalase activity seen in many isoniazid-resistant (INH\(^6\)) strains of Mycobacterium tuberculosis, along with a marked decrease in their virulence for guinea pigs compared with their drug-susceptible counterparts [4]. There is a surprising dearth of good epidemiologic data on the virulence of drug-resistant strains of M. tuberculosis in human populations, so it is unclear how much this reduction in guinea pig virulence applies to other experimental animals [5] or to humans [6]. The increasing number of human isolates of isoniazid-resistant tubercle bacilli from both HIV-positive and -negative patients strongly suggests that some of these resistant organisms retain much of their infectivity and virulence, yet the effect of specific gene mutations associated with reduced susceptibility to isoniazid on human virulence has not been thoroughly documented [7, 8].

Genetic analysis indicates that many isoniazid-resistant strains of tubercle bacilli have deletions or missense mutations in the katG gene, which encodes the catalase-peroxidase bi-functional enzyme [9, 10]. Transformation of katG-defective mycobacteria with a functional katG gene has been shown to restore drug susceptibility to the recombinant [11]. Given a defined role for the katG gene in determining levels of susceptibility to isoniazid and the inverse correlation between drug resistance and guinea pig virulence, Wilson et al. [12] reported that integration of a functional katG gene into an isoniazid-resistant strain of Mycobacterium bovis fully restored its guinea pig virulence, thereby establishing KatG as a virulence factor in this organism.

In the present study, the KatG protein was examined as a putative virulence factor in mice and guinea pigs exposed to M. tuberculosis infection. Transformation of katG genes from M. tuberculosis and Mycobacterium intracellulare into the avirulent M. tuberculosis H37RvINH\(^6\) increased the ability of this organism to multiply and persist in both mice and guinea pigs.

Material and Methods

Bacterial cultures. M. tuberculosis H37Rv (ATCC 27294), H37RvINH\(^6\) (ATCC 35822), and H37Ra (ATCC 25177) were ob-
M. tuberculosis H37RvINHβ is catalase-negative and isoniazid-resistant to at least 50 μg/mL. It was originally selected from the drug-sensitive M. tuberculosis H37Rv by a single-step procedure using an isoniazid gradient plate [13]. Strain H37RvINHβ (ATCC 35822) expresses ‘‘low’’ virulence in both mice and guinea pigs [14].

Stock cultures for virulence testing were grown in broth (Middlebrook 7H9; Difco, Detroit) enriched with 10% albumin-dextran-catalase additive (Becton Dickinson, Cockeysville, MD) and 0.05% Tween 80 for 8–10 days at 37°C and stored at −70°C in 1-mL ampules until required. The viability of the frozen suspension was determined by thawing randomly selected ampules, with brief sonication to break up any clumped bacilli. Then we did serial 10-fold dilution in 0.05% Tween-saline, which was plated on agar (Middlebrook 7H11; Difco), with or without 50 μg/mL kanamycin. The plates were incubated at 37°C in sealed plastic bags for 3–4 weeks, when the colonies could be readily counted [5].

M. tuberculosis H37RvINHβ recombinants. The katG gene of M. tuberculosis H37Rv (TBkatG) was cloned into the shuttle vector pMD31, and site-directed mutagenesis of the gene was carried out as described elsewhere [15, 16]. The M. intracellulare katG gene (MACkatG) was cloned into the HindIII site of pMD31 following the addition of HindIII linkers, using a standard protocol [17]. The pMD31 plasmid constructs were electroporated into H37RvINHβ, and the kanamycin-resistant transformants were selected by plating the suspension on Middlebrook 7H11 agar enriched with 50 μg/mL kanamycin.

Genetic characterization of katG deletion by polymerase chain reaction (PCR) analysis. The extent of the chromosomal deletion in M. tuberculosis H37RvINHβ upstream of katG was mapped by PCR amplification using previously described synthetic oligonucleotides corresponding to a portion of a 2-kb KpnI fragment known to be present 10 kb upstream of the TBkatG gene [18, 19]. DNA from H37Rv and H37RvINHβ was extracted as described [20], and appropriate fragments from the katG gene locus were amplified by PCR [10]. Amplification was carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide.

Virulence testing. Specific pathogen-free BALB/c female mice, 6–8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained under barrier conditions. They were fed sterilized commercial mouse chow and water ad libitum. Groups of 5 mice were infected intravenously via a lateral tail vein with serial 10-fold dilutions of each mycobacterial suspension, ranging from 107 to 109 cfu in 0.2 mL of Tween-saline. The mice were examined daily for up to 12 weeks and any deaths noted. Moribund animals were euthanized, autopsied, and checked macroscopically for signs of tuberculosis infection. The lungs and spleen of the surviving mice were checked for viable mycobacteria by plating the homogenized organs on Middlebrook 7H11 agar [5].

Outbred female Hartley guinea pigs were obtained from Charles River Farms (Wilmington, MA), housed in groups of 4, and fed commercial guinea pig pellets and water ad libitum. They weighed 300–350 g at the time of infection. Groups of 4 guinea pigs were injected intramuscularly in the right hind leg with 5 × 109 cfu of mycobacteria suspended in 0.5 mL of Tween-saline. At 12 weeks, the animals were euthanized by an overdose of pentobarbital.

The site of inoculation, draining inguinal lymph node, lungs, liver, and spleen were examined macroscopically, and the number and size of visible tubercles were used to estimate a total organ score (total possible, 100). The maximum score for the spleen was set at 40, the liver at 30, the lungs at 20, and the site of inoculation and lymph node combined as 10. These proportions are based on long experience showing that the major tuberculocellular involvement occurred in the spleen, followed by the liver and then the lungs [21]. For obvious reasons, the caseous lesions at the injection site and its draining lymph node were given the lowest score. Any moribund animals were sacrificed and their survival time in days noted.

The total score for each animal was divided by its survival time in days, and the square root of this number provided a root index of virulence (RIV) for each organism that could vary from a low of 0 for M. tuberculosis H37Ra to a high of 1.2 for M. tuberculosis Erdman (Collins FM, unpublished data). In addition, the spleen was removed aseptically from each animal and homogenized separately in cold saline; the mean number of viable mycobacteria was determined by plate count [22].

Bacterial enumeration in vivo. BALB/c mice were infected intravenously with 5 × 107 to 5 × 109 cfu of each recombinant. The spleens and lungs were removed aseptically from 5 randomly selected mice at increasing time intervals and homogenized separately in 5 mL of cold Tween-saline in a blender (Seward Stomacher 80; Tekmar, Cincinnati, OH). The homogenates were diluted in 10-fold steps, plated on Middlebrook 7H11 agar (with or without kanamycin), and incubated at 37°C in sealed plastic bags for 3 weeks, when the colonies could be counted. The counting error for 5 replicate determinations was usually <10% of the mean.

Characterization of mycobacterial transformants. The isoniazid MIC was determined using the absolute concentration method [23]. The catalase assay procedure used to measure oxygen bubble evolution from a whole cell suspension has been described [16]. Cell suspensions for the peroxidase assay were prepared by culturing the recombinants in 10 mL of medium (Long’s synthetic; Quality Biologicals, Rockville, MD) with or without 50 μg/mL kanamycin. The cultures were incubated at 37°C and harvested in midlog phase by centrifugation at 3000 g for 8 min at 4°C. After being washed, the cells were resuspended in 0.7 mL of PBS supplemented with 2 mM 4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride (ICN Biochemicals, Aurora, OH) and sonicated on ice for four 30-s cycles interspersed with 30-s rest periods. The sonicate was centrifuged at 8000 g for 2 min to remove the insoluble fraction, and the total protein concentration in the supernatant fluid was determined using an assay reagent (Bio-Rad, Richmond, CA).

The peroxidase activity of the extract was assayed using a previously described method [16]. Briefly, the assay was carried out in a buffer prepared by adding 25.7 mL of 0.2 M sodium phosphate to 24.3 mL of 0.1 M citric acid and adjusting the pH to 5.0, before bringing to a final volume of 100 mL with deionized water. A 10-μg tablet of 2,2’-azino-bis-(3-ethylbenzthiazole 6-sulfonic acid) diammonium salt (Sigma, St. Louis) was added as substrate. Hydrogen peroxide (25 μL; 30% vol/vol) was added to 25 μL of substrate solution, followed by the addition of 30 μL of cell extract/mL of mixture. Absorbance at 405 nm was monitored at 2-min intervals for up to 12 min in a spectrophotometer. Three determinations were made per assay carried out on three separate occasions.

Statistical analysis. The data were analyzed using Student’s t-test and an analysis of variance between two of several independent
Table 1. Catalase and peroxidase activity of recombinant M. tuberculosis H37RvINH\textsuperscript{b} bearing various katG genes compared with levels of isoniazid sensitivity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase activity\textsuperscript{a}</th>
<th>Peroxidase activity\textsuperscript{a}</th>
<th>MIC\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>+</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b}</td>
<td>−</td>
<td>0.0</td>
<td>800</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b} [TBkatG]</td>
<td>+</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b} [MACkatG]</td>
<td>+</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b} [TBkatG(275)]</td>
<td>−</td>
<td>0.1</td>
<td>800</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b} [TBkatG(463)]</td>
<td>+</td>
<td>0.9</td>
<td>2</td>
</tr>
<tr>
<td>H37RvINH\textsuperscript{b} [pMD31]</td>
<td>−</td>
<td>0.0</td>
<td>800</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Visible bubbles at 25°C.

\textsuperscript{b} Relative peroxidase activity = (A\textsubscript{ast} Sample − A\textsubscript{ast} Vector control)/(A\textsubscript{ast} H37Rv − A\textsubscript{ast} Vector control).

\textsuperscript{c} MIC, µg of isoniazid/mL.

means by a statistical program (InStat Statistics; GraphPad Software, San Diego).

Results

Characterization of the katG transformants. In an earlier study [24], we demonstrated by Southern hybridization that M. tuberculosis H37RvINH\textsuperscript{b} had undergone a deletion encompassing the entire katG gene. Subsequent PCR analysis using primers designed from the published katG sequence confirmed the complete absence of the katG gene from H37RvINH\textsuperscript{b}. To further map the deletion, primers were designed from a species-specific KpnI fragment that is located 10 kb upstream. Failure to amplify PCR products using the KpnI fragment primers suggests that H37RvINH\textsuperscript{b} has a deletion of at least 10 kb upstream of the katG locus. Previous studies have demonstrated that the ahpC gene in the H37RvINH\textsuperscript{b} strain is up-regulated [25]. However, overexpression of ahpC failed to restore virulence to a number of isoniazid-resistant strains of M. tuberculosis and should therefore not confound the interpretation of these virulence assays [26].

To assess whether expression of the katG gene could complement the avirulent phenotype and restore isoniazid sensitivity to H37RvINH\textsuperscript{b}, pMD31 plasmid constructs containing either TBkatG or MACkatG genes were electroporated into H37RvINH\textsuperscript{b}. In addition, transformants using pMD31 constructs having a T→P mutation at codon 275, TBkatG(275), or an R→L mutation at codon 463, TBkatG(463), were prepared and evaluated for catalase activity using whole cell suspensions and for isoniazid resistance by the absolute concentration method [23]. Cell extracts of these transformants were also assayed for peroxidase activity (table 1). Transformation with both the TBkatG and MACkatG plasmid constructs restored catalase and peroxidase activities to H37RvINH\textsuperscript{b}, while reducing its isoniazid MIC 160- to 400-fold. Levels of catalase and peroxidase activities of the TBkatG transformed H37RvINH\textsuperscript{b} and the wild-type H37Rv were virtually identical. Moreover, the 463 R→L mutation did not significantly affect the enzymatic activity of the TBkatG(463) recombinant or the capacity of katG to increase isoniazid sensitivity [15, 16]. In contrast, the recombinants transformed with the codon 275 T→P mutant expressed little catalase or peroxidase activity and remained highly resistant to isoniazid (table 1).

Growth of the katG recombinants in mouse lung. Mice infected intravenously with 10\textsuperscript{9} cfu of H37RvINH\textsuperscript{b} survived for at least 12 weeks with no sign of morbidity or mortality, whereas controls challenged with 10\textsuperscript{7} cfu of H37Rv all died within 8 weeks (figure 1). None of the mice challenged with 2 × 10\textsuperscript{8} viable H37RvINH\textsuperscript{b} (TBkatG or MACkatG, data not shown) died during the 3-month observation period. Mice infected with a standard dose (1–2 × 10\textsuperscript{8} cfu) of these transformants showed a substantial amount of early growth in the lung, followed by a prolonged phase of persistence lasting for the remainder of the experiment (figure 2).

Although both recombinants multiplied extensively in vivo, neither grew as aggressively as the H37Rv control (2 log increase vs. 3.5 log). Of interest, the transformants expressing the katG(275) and katG(463) mutant genes showed growth patterns in mouse lung that were remarkably similar to those of the TBkatG and MACkatG recombinant strains. Both mutants showed an initial 100- to 200-fold increase in viability, followed by persistence for the remainder of the experiment. Somewhat surprisingly, the vector control also showed this early growth phase but was then rapidly and progressively eliminated from both the lungs and spleen, so that by 12 weeks, the residual viable counts were significantly lower (P < .01) than those for the katG transformants (figure 2). The vector
control curve was indistinguishable from that for mice infected with a similar number of H37Rv INH (data not shown).

Replicate plating of these organ homogenates on Middlebrook 7H11 agar with and without kanamycin indicated that at least 80% of the transformants had retained their plasmids in the absence of continued antibiotic selection. The level of in vivo stability shown by these katG recombinants is consistent with previously published results [27].

Growth of the katG recombinants in mouse spleen. The temporal growth profile for the H37Rv INH katG transformants in the spleen generally resembled that seen for the wild-type H37Rv (figure 3). However, the splenic counts for all of the katG recombinants remained at least 100-fold higher than for the vector control at 12 weeks. The spleen counts for the H37Rv control increased up to 50-fold during the first 14 days of infection before stabilizing into a prolonged stationary phase (figure 3). In contrast, the vector control counts declined sharply during this period, so that the residual counts observed at 12 weeks represented <10% of the original inoculum. These vector control counts were significantly lower than all of those with the katG transformants (P < .01) and resembled those seen in mice infected with M. tuberculosis H37Ra, in which little or no in vivo growth could be observed at any time; the counts declined steadily to reach undetectable levels by 12 weeks [28].

Virulence of the katG recombinants in guinea pigs. Guinea pigs infected intramuscularly with ~5 x 10⁶ cfu of the katG recombinants were examined after 12 weeks for macroscopic signs of systemic tuberculous disease. Recombinants bearing the TBkatG and MACkatG genes expressed RIV [21] values of 0.62 and 0.46, respectively, which compared favorably with the 0.88 obtained for the H37Rv control (table 2). These RIV values were all substantially higher than that for the vector control and were consistent with the splenic counts for these recombinants, indicating a partial restoration of virulence. The splenic counts for the virulent H37Rv control (4 x 10⁶ cfu) were significantly higher (P < .01) than those for the TBkatG (5.8 x 10⁴ cfu) and the MACkatG transformants (1.8 x 10² cfu). These counts are substantially higher than those for the vector control or for H37Rv INH, both of which were undetectable at 12 weeks after infection (table 2). Of interest, the transformant expressing the mutant katG(463) gene showed some restoration in guinea pig virulence (RIV = 0.46), whereas that achieved by the katG(275) mutant was much lower (RIV = 0.19). Moreover, while viable katG(463) were present in the splenic homogenates prepared 12

<table>
<thead>
<tr>
<th>Strain</th>
<th>Root index of virulence*</th>
<th>Spleen (cfu ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>0.88</td>
<td>4.0 ± 0.37 x 10⁶</td>
</tr>
<tr>
<td>H37Rv INH</td>
<td>0.0</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>H37Rv INH[TBkatG]</td>
<td>0.62</td>
<td>5.8 ± 0.36 x 10⁴</td>
</tr>
<tr>
<td>H37Rv INH[MACkatG]</td>
<td>0.40</td>
<td>1.8 ± 0.24 x 10³</td>
</tr>
<tr>
<td>H37Rv INH[katG(275)]</td>
<td>0.19</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>H37Rv INH[katG(463)]</td>
<td>0.46</td>
<td>1.4 ± 0.21 x 10³</td>
</tr>
<tr>
<td>H37Rv INH[pMD31]</td>
<td>0.0</td>
<td>&lt;10²</td>
</tr>
</tbody>
</table>

NOTE. 4 animals per organism sacrificed at 12 weeks.
* See Materials and Methods for further details.
weeks after challenge, no viable organisms could be detected in animals infected with the katG(275) mutant.

**Discussion**

Mycobacteria are rapidly phagocytosed by the resident macrophages once they enter the alveolar tissues. Virulent tubercle bacilli are able to survive and grow in this hostile intracellular environment for an extended period of time, whereas less virulent or attenuated strains multiply for a shorter period of time before being eliminated from the tissues by the cell-mediated immune response they induce in the host [28]. In response to this intracellular stimulus, the activated macrophage generates a variety of reactive oxygen intermediaries, including superoxide, hydroxyl radical, and hydrogen peroxide, the latter of which interacts with lipids and proteins within the phagosomal membrane to produce a variety of potentially lethal organic peroxides [29]. Pathogenic mycobacteria seem to protect themselves from these activated oxygen compounds by producing virulence factors that allow them to continue to proliferate within the phagolysosome.

One factor associated with the survival of *M. tuberculosis* in vivo may be the catalase-peroxidase enzyme complex [16]. Middlebrook was the first to draw attention to the simultaneous loss of catalase activity and guinea pig virulence shown by many isoniazid-resistant strains of *M. tuberculosis* [4]. Recently, Sherman et al. [25] showed that the expression of *katG* by the tubercle bacillus can protect it from the toxic effects of these organic peroxides. This finding is consistent with data reported by Wilson et al. [12], showing that integration of a functional *katG* gene into a catalase-negative avirulent strain of *M. bovis* completely restored its guinea pig virulence.

The present study confirms the role of *katG* in the growth and survival of *M. tuberculosis* in both the murine and guinea pig models of human tuberculosis. Transformation of plasmid constructs containing *katG* genes taken from *M. tuberculosis* or *M. intracellulare* into the avirulent *katG*-deleted H37RvINH<sup>8</sup> at least partially restored its ability to survive in guinea pigs (table 2) and mice (figures 2, 3). Transformants expressing the TBkatG gene bearing the 463 R→L mutation (the most common *katG* alteration found in isoniazid-resistant clinical isolates) also persisted in mouse and guinea pig spleens substantially better than did the vector control or H37RvINH<sup>8</sup>. This was not unexpected, since the 463 perturbation has only a minimal effect on enzymatic activity and isoniazid sensitivity [15, 16].

In contrast, the persistence of the transformant expressing the mutant KatG protein with a T→P alteration at the 275 codon (a mutation associated with a dramatic reduction in catalase-peroxidase activities as well as high isoniazid resistance) varied depending on the host species. Recombinants expressing the codon 275 mutant persisted within the mouse spleen and lung significantly better than the vector control. However, when this recombinant strain was tested in guinea pigs, a difference in virulence was noted (table 2). Compared with the TBkatG recombinant, the *katG*(275) transformant showed minimal dissemination from the injection site, and no viable bacilli were detected in splenic homogenates prepared after 12 weeks of infection.

The discordance between these two animal models is not without precedence. Several mycobacterial strains have substantial attenuation in the guinea pig while still retaining their virulence for mice [30, 31]. The physiologic mechanisms allowing the *katG*(275) transformant to persist in mouse tissues, but not in guinea pig organs, have yet to be clarified. Recently, we reported that the *katG*(275) mutant showed a markedly reduced, though not totally ablated, enzymatic activity [16]. Expression of this mutant gene in *katG*-defective *Mycobacterium smegmatis* and *M. bovis* BCG hosts yielded only 10% and 30% of the wild-type peroxidase activity, respectively.

In the present study, cell extracts of H37RvINH<sup>8</sup> transformed with *katG*(275) exhibited about 10% of the wild-type peroxidase activity. Although sharply reduced, this low level of catalase-peroxidase activity may still exceed the threshold required for survival by the TBkatG(275) recombinant in the mouse. Alternatively, the in vitro assay used to measure peroxidase activity may not reflect the "real world" enzymic kinetics between the *katG*(275) mutant and its as yet unknown in vivo substrate.

The correlation between low levels of peroxidase activity and the in vivo survival of this organism in the mouse model may provide an explanation for the observed variability in the virulence of a number of clinical isolates of *M. tuberculosis* that have been classified as catalase-negative. Ordway et al. [32] reported that 2 catalase-negative strains (as determined by the whole-cell bubble assay) were avirulent when tested in mice, whereas 2 other catalase-negative strains were highly mouse-virulent. The latter strains may have a substantially reduced level of enzymatic activity (insufficient to produce visible oxygen bubbles), yet retain sufficient residual activity to allow their increased survival when introduced into normal mice.

The role of *katG* as a virulence gene for human isolates of tubercle bacillus is even less clear. The identification of *katG* defects in 50%–70% of isoniazid-resistant patient isolates of *M. tuberculosis* indicates that mutants in *katG* can persist in human tissues [9, 10, 33]. However, most of these strains have missense mutations in *katG*, which reduces but does not eliminate their catalase-peroxidase activity [9, 16]. In fact, only a small fraction of isoniazid-resistant *M. tuberculosis* isolates are found to be completely catalase-negative. The relative absence of catalase-negative isolates (and especially *katG*-deleted strains) suggests that selection of this *katG*-negative phenotype is not favored in the patient. In addition, recent epidemiologic evidence suggests that some isoniazid-resistant strains with a *katG* mutation may be less transmissible than their drug-sensitive counterparts (van Soolingen D, personal communication). Further molecular epidemiologic studies will be needed before the impact of these *katG* mutations on the infectivity and persistence of these isoniazid-resistant tubercle bacilli within the community can be fully assessed.

Although the expression of TBkatG by the H37RvINH<sup>8</sup> recombinant did significantly increase its persistence in mice and
guinea pigs, its virulence was never completely restored to the level exhibited by the wild-type *M. tuberculosis* H37Rv. The inability of the katG gene to completely complement the virulence defect seen in H37Rv INH*R* is best illustrated by the mouse lung growth curves (figure 2), in which the wild-type H37Rv counts increased 3.5 logs over the 3-month experimental period, whereas the corresponding increase for the TBFkatG recombinant was only ~100-fold. The fact that the normal mouse defenses were able to limit the growth of recombinants transformed with either the TBFkatG or MACKatG gene within the lung is an important observation, since expression of virulence by *M. tuberculosis* H37Rv has been associated with its progressive growth within the lung long after growth has been brought under control in the liver and spleen [28].

Since the levels of peroxidase activity are nearly identical with the TBFkatG transformant and the wild-type H37Rv, it is improbable that failure to restore complete virulence resulted from an inadequate expression of katG from the recombinant plasmid. The incomplete complementation of virulence in the TBFkatG recombinants suggests the deletion of other virulence genes located near the katG gene locus in the drug-resistant mutant. Studies are in progress to identify these additional virulence determinants by examining whether expression of chromosomal genes linked to katG (such as furA, an iron-uptake regulator gene recently shown to be immediately up-stream of katG) can restore full virulence to the TBFkatG transformants of H37Rv INH*R*.

**References**