Treatment of Tuberculosis with Rifamycin-containing Regimens in Immune-deficient Mice

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Rationale: Daily rifapentine plus isoniazid-pyrazinamide in mice infected with *Mycobacterium tuberculosis* produces cure in 3 months. Whether cure corresponds to latent infection contained by host immunity or true tissue sterilization is unknown.

Objectives: To determine the length of treatment with rifapentine-isoniazid-pyrazinamide or rifampin-isoniazid-pyrazinamide needed to prevent relapse in immune-deficient mice.

Methods: Aerosol-infected BALB/c and nude mice were treated 5 days per week with either 2 months of the rifapentine-based regimen followed by rifapentine-isoniazid up to 12 months or the same regimen with rifampin instead of rifapentine. Cultures of lung homogenates were performed during the first 3 months and then every 3 months. Relapse rates were assessed after 3, 6, 9, and 12 months of treatment in BALB/c ($\pm$ 1 mo of cortisone) and nude mice.

Measurements and Main Results: All rifapentine-treated mice were lung culture-negative at 3 months but 13% of BALB/c that received cortisone and 73% of nude mice relapsed. After 6, 9, and 12 months of treatment no mouse relapsed. Rifampin-treated BALB/c mice remained culture positive at 3 months. All were culture negative at 6, 9, and 12 months. None, including those receiving cortisone, relapsed. Rifampin-treated nude mice harbored more than 4 log₁₀ lung cfu at Month 2 and approximately 6 log₁₀ cfu with isoniazid resistance at Month 3. A supplementary experiment demonstrated that 7 days a week treatment did not prevent isoniazid resistance, whereas addition of ethambutol did.

Conclusions: In nude mice, sterilization of tuberculosis is obtained with rifapentine-containing treatment, whereas failure with development of isoniazid resistance is obtained with rifampin-containing treatment.

Keywords: tuberculosis; immune-deficient mice; rifapentine; rifampin; drug resistance

The current short-course regimen for tuberculosis (TB) requires a four-drug combination of rifampin (R) + isoniazid (H) + pyrazinamide (Z) + ethambutol (E) for 2 months followed by RH for 4 months (1), resulting in cure for most patients. Whether cure corresponds to real sterilization of lesions or only to a state where remaining persisters are contained by specific immunity, at least in immune-competent hosts, is unknown. In the Cornell persistence model, treatment of *Mycobacterium tuberculosis*-infected mice with HZ in the diet reduced the bacterial burden to an

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Recent evidence from immune-deficient murine models of tuberculosis suggests that daily treatment with rifapentine-containing regimens sterilized tuberculosis, whereas daily treatment with rifampin-containing regimens failed with development of isoniazid resistance.

What This Study Adds to the Field

In an immune-deficient host, true sterilization of tuberculosis can be obtained with daily rifapentine but failure of treatment, associated with acquired resistance to isoniazid, happens with daily rifampin.

undetectable level. After therapy cessation, a proportion of mice spontaneously relapsed and the proportion was increased by corticosteroid administration (2–5). In other mouse studies, 9–12 months of treatment with RH, without Z, prevented spontaneous relapse in all mice but most mice relapsed after receiving 2 months of high-dose cortisone on treatment completion (6, 7). Thus, in mice, cure (i.e., lack of spontaneous relapse) does not necessarily mean true sterilization but only immune containment.

Patients with TB with advanced HIV infection seem to be at increased risk for relapse, especially those not receiving antiretroviral therapy (8–10). Thus, in humans as in mice, immune containment of latent and persistent bacilli is likely preventing TB reactivation.

In previous mouse studies, replacing R with the long-lasting rifamycin derivative rifapentine (P) accelerated the elimination of persisters and achieved cure in 12 weeks instead of the 24 weeks required with R-containing regimens (11, 12). To determine if potent P-containing regimens could achieve true sterilization, we treated, in Study 1, congenitally athymic nude mice (nu/nu) 5 days per week with daily P 10 mg/kg + H 10 mg/kg + Z 150 mg/kg for 2 months followed by PH up to 12 months. Because nude mice lack mature T cells and cannot mount specific immunity against TB, we assumed they would relapse if any bacilli remain alive on treatment cessation. In parallel, we treated immune-competent BALB/c mice with the same P-containing regimen and on treatment completion administered high-dose cortisone to half of them to decrease their immune status, as done by McCune and coworkers (2–5) and Grumbach (7).

To assess the sterilizing potential of P over that of R, the same mouse models were used, infected the same day with the same *M. tuberculosis* strain and treated with the same drug regimens in which R was substituted for P. Because treatment with RHZ failed in nude mice with development of H resistance, a 3-month supplementary experiment (Study 2) was performed in immune-competent and nude mice to determine probable causes of failure

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(i.e., treatment given only 5 days a week or absence of ethambutol in the basic scheme of treatment with 2RHZ/RH, or both).

Some of the results of these studies have been previously reported in the form of abstracts (13, 14).

METHODS

Antimicrobials

H, R, Z, and E were purchased from Sigma (St. Louis, MO). All drugs were either dissolved or suspended in water before oral gavage. P tablets (PRIFTIN containing 150 mg of the active ingredient rifapentine per tablet; Bridgewater, NJ), donated by Sanofi-Aventis, were ground into a fine powder, suspended in water, and briefly sonicated before use. Drug solutions were prepared weekly and stored at 4°C.

Mice and Aerosol Infection

Six-week-old female BALB/c mice ($n = 190$) and 6-week-old congenitally athymic nu/nu (nude) Swiss mice ($n = 185$) were purchased from Charles River (Wilmington, MA). They were aerosol infected with *M. tuberculosis* H37Rv (ATCC 27294) (15) using the Middlebrook Inhalation Exposure System (Glas-Col, Terre Haute, IN) with a log-phase broth culture containing $8.93 \log_{10}$ cfu per milliliter. BALB/c and nude mice were infected in four consecutive runs of close to 100 animals each. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. The *in vitro* prevalence of drug-resistant mutants in the H37Rv strain of *M. tuberculosis* was 2.61×10^{-6} for H 0.2 µg/ml and 2.2×10^{-8} for R 1 µg/ml.

Chemotherapy

Treatment began 14 days after infection for Studies 1 and 2. Drugs were administered by gavage at the following doses: H, 10 mg/kg; R, 10 mg/kg; P, 10 mg/kg; Z, 150 mg/kg; and E, 100 mg/kg. Dosages for R, P, H, Z, and E were chosen to produce serum area under the concentration curve (AUC) in mice equivalent to the AUC obtained with currently recommended human dosages (12, 16, 17).

Study 1. All drugs were administered by gavage 5 days a week (5/7). Mice were treated with RHZ or PHZ for the first 2 months, then with either RH or PH (2RHZ/RH or 2PHZ/PH), respectively, for a total of 12 months according to the experimental scheme presented in Table 1. Negative controls included 20 BALB/c mice and 20 nude mice, and five of each was killed the day after infection (D-13) and the day of treatment initiation (D0). The remaining 10 mice from each group were kept to assess mortality. Mice treated with 2RHZ/RH included 80 BALB/c and 75 nude mice. At Month 6 of treatment, 30 BALB/c and 15 nude mice were withdrawn from treatment and followed-up for 3 months to assess the relapse rate. Half of the 30 BALB/c mice received cortisone during the whole month after treatment cessation. At months 9 and 12 of treatment, 15 BALB/c and 15 nude mice were

withdrawn from treatment and followed-up for 3 months to assess the relapse rate. The BALB/c mice received cortisone during the first month after treatment cessation.

Study 2. The scheme of Study 2, which included 80 BALB/c mice and 80 nude mice, is presented in Table 2. R, H, and Z were administered to all mice for 2 months at the same doses as in Study 1, but either 5 days a week (5/7) or 7 days a week (7/7). In addition, half of the mice, either BALB/c or nude, received an oral 2-month supplement of 100 mg/kg of ethambutol (E). During the third month of therapy, mice received only RH.

Cortisone administration. To compare the influence of corticosteroids versus that of genetic immune-deficiency on the reactivation rate of TB after treatment cessation, a cohort of immune-competent mice (BALB/c) received 5 days a week 1 mg of cortisone in 0.2-ml volume by the subcutaneous route during the first 4 weeks after completion of anti-TB treatment.

Assessment of Treatment Efficacy

Study 1. Treatment efficacy was assessed on the basis of lung cfu counts during treatment and the proportion of mice with culture-positive relapse after treatment completion. To monitor the bacterial multiplication and establish baseline cfu counts, untreated mice were killed the day after aerosol infection and again 14 days later (D0), at the initiation of treatment. Subsequently, mice from each treatment group were killed at monthly intervals during the first 3 months of treatment and then at 3-month intervals to assess bactericidal activity or confirm the culture-negative state. After killing, lungs were placed in phosphate-buffered saline, homogenized, and plated on 7H11 agar enriched with 10% oleic acid-albumin-dextrose-catalase and supplemented with cycloheximide (10 µg/ml), carbenicillin (50 µg/ml), polymyxin B (25 µg/ml), and trimethoprim (20 µg/ml), to prevent contamination. Plates were incubated for 28 days at 37°C before cfu were enumerated.

The proportion of culture-positive relapses was determined by killing cohorts of 15 mice 3 months after the completion of treatment. Mice were considered to be culture-positive if greater than or equal to 1 cfu was identified after plating the entire lung homogenate obtained at killing. In the first study (Table 1) the proportion of mice with culture-positive relapse was planned to be determined after 6, 9, and 12 months of treatment for mice receiving the 2RHZ/RH regimen, and after 3, 6, 9, and 12 months of treatment for mice receiving the 2PHZ/PH regimen. Because nude mice treated with 2RHZ/RH exhibited a sharp increase in lung cfu counts associated with acquired resistance to H, the investigation of relapse rate in these mice became meaningless. As a consequence, after the Month 3 time-point, nude mice on treatment with 2RHZ/RH were continued on RH and used for monthly lung cfu counts with drug susceptibility testing (DST) of the isolates, and for performing pharmacokinetic analyses of R, H, and Z.

Drug susceptibility testing and mutation analysis. DST to H and R was performed by the indirect proportion method, scraping colonies grown on first-culture plates and testing their drug susceptibility on

TABLE 1. SCHEME OF STUDY 1

Drug Regimens and Mice		Number of Mice Killed on							Total Mice
		D-13	D0	M1	M2	M3 (+3)	M6 (+3)	M9 (+3)	
Untreated									
(1) BALB/c mice		5	5	+ 10 for mortality					20
(2) Nude mice		5	5	+ 10 for mortality					20
2RHZ/RH									
(3a) BALB/c mice				5	5	5	5 (15)		35
(3b) + cortisone*							(15)	(15)	45
(4) Nude mice				5	5	5	5 (15)	5 (15)	75
2PHZ/PH									
(5a) BALB/c mice				5	5	5 (15)			30
(5b) + cortisone*						(15)	(15)	(15)	60
(6) Nude mice				5	5	5 (15)	5 (15)	5 (15)	90
Total BALB/c		5	5	20	10	10 (30)	5 (45)	(30)	190
Total Nude		5	5	20	10	10 (15)	10 (30)	10 (30)	185

Definition of abbreviations: D = day; H = isoniazid, 10 mg/kg; M = month of 4 weeks; P = rifapentine, 10 mg/kg; R = rifampin, 10 mg/kg; Z = pyrazinamide, 150 mg/kg.

All drugs were given orally 5 days a week. R and P were administered 1 hour before other drugs.

Months 3 (+3), 6 (+3), 9 (+3), and 12 (+3) indicate that subgroups of 15 mice were killed 3 months after completion of 3, 6, 9, or 12 months of treatment.

* 1 mg/day for 4 weeks after completion of antibiotic treatment, and mice were killed 2 months later.

TABLE 2. SCHEME OF STUDY 2

Mice	Regimen		Number of Mice Killed on					Total
	Drug	Rhythm	D-13	D0	M1	M2	M3	
BALB/c	Untreated	—	5	5	+10 for mortality			20
	2RHZ/RH	5/7			5	5	5	15
	2RHZ/RH	7/7			5	5	5	15
	2RHZE/RH	5/7			5	5	5	15
	2RHZE/RH	7/7			5	5	5	15
Nude	Untreated	—	5	5	+10 for mortality			20
	2RHZ/RH	5/7			5	5	5	15
	2RHZ/RH	7/7			5	5	5	15
	2RHZE/RH	5/7			5	5	5	15
	2RHZE/RH	7/7			5	5	5	15
Total mice	BALB/c		5	5	20	20	20	80
	Nude		5	5	20	20	20	80

Definition of abbreviations: D = day; E = ethambutol, 100 mg/kg; H = isoniazid, 10 mg/kg; M = month of 4 weeks; R = rifampin, 10 mg/kg; Z = pyrazinamide, 150 mg/kg.

All drugs were given orally 5 days a week (5/7) or 7 days a week (7/7). R was administered 1 hour before other drugs.

10% oleic acid-albumin-dextrose-catalase-containing 7H11 agar with H concentrations of 0.1, 0.2, 1, 5, and 10 $\mu\text{g/ml}$ and R concentrations of 0.5, 1, and 2 $\mu\text{g/ml}$, according to standard procedures (18–20). Representative samples of *M. tuberculosis* colonies (~ 3) isolated from nude mouse lungs and resistant to 1 $\mu\text{g/ml}$ of H were subjected to mutation analysis. The entire *katG* gene was amplified by polymerase chain reaction using four overlapping primer sets. The *inhA* promoter region was also amplified using specific primers. The resulting polymerase chain reaction products were then sequenced to determine the presence of mutations.

Study 2. After the same procedures as in the first study, 80 BALB/c and 80 nude mice were aerosol infected and initiated on treatment 14 days later for 3 months with either 2RHZ/RH or 2RHZE/RH. According to the experimental scheme depicted in Table 2, the treatment was administered for half of them 5 days a week (5/7), whereas for the other half the treatment was administered 7 days a week (7/7). The comparative bactericidal activity of each drug regimen and its ability to prevent the selection of H-resistant mutants was assessed in lungs in five mice per time-point monthly by cfu counts performed on 7H11 selective agar with and without 0.2 $\mu\text{g/ml}$ of H or 1 $\mu\text{g/ml}$ of rifampin. H-resistant strains isolated in this study were subjected to mutation analysis using the same procedures as described previously.

Pharmacokinetic Analyses

Pharmacokinetic analyses were performed in nude mice that had been infected with *M. tuberculosis* and treated for 4 months with 2RHZ/2RH. A total of 27 mice were gavaged with a single dose of R at 10 mg/kg, H at 10 mg/kg, and Z at 150 mg/kg. R was administered 1 hour before H and Z. Three mice per time-point were anesthetized with isoflurane and exsanguinated by cardiac puncture at 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 24 hours after R dosing. Blood was collected in microcentrifuge tubes, held for 30 minutes at room temperature, and centrifuged at 6,500 rpm for 10 minutes to obtain serum. Serum was frozen at -80°C before being shipped overnight on dry ice to Dr. Peloquin's Pharmacokinetics Laboratory. Drug concentrations were determined using validated HPLC assays. Single-dose serum-concentration time curves were determined using noncompartmental techniques (WinNonLin Software; PharSight, Sunnyvale, CA).

Statistical Analysis

Cfu counts were $\log_{10}$ transformed before analysis, expressed as mean $\log_{10}$ cfu $\pm$ SD, and compared using one-way analysis of variance followed by Dunnett multiple comparison test (STATA 8.2; StataCorp LP, College Station, TX). The proportions of mice relapsing after completing treatment were compared using Fisher exact test. Bonferroni procedure was used to adjust the type I error rate for multiple comparisons (STATA 8.2).

RESULTS

Study 1

Lung cfu counts before treatment. The day after infection (D-13) the mean lung $\log_{10}$ cfu count ($\pm$ SD) was 4.40 ± 0.09 and 4.24 ± 0.15 in BALB/c and nude mice, respectively. By the initiation of treatment on D0, the mean $\log_{10}$ cfu counts had increased to 7.84 ± 0.13 in BALB/c mice and 7.83 ± 0.11 in nude mice. One week later, on Day 7, all untreated mice were very sick and the lung $\log_{10}$ cfu counts had reached 9.20 ± 0.20 and 9.14 ± 0.21 in the three BALB/c and three nude mice that were killed, respectively. All remaining untreated BALB/c mice died of overwhelming TB infection by Day 8 (i.e., 22 d after infection), whereas all untreated nude controls died by Day 12 (i.e., 26 d after infection).

Lung cfu counts during treatment with rifapentine-containing regimen. During the first 2 months of treatment (Figure 1), the decrease in lung cfu counts was significantly more rapid in immune-competent BALB/c than in immune-deficient nude mice: at Month 1, the $\log_{10}$ cfu counts were 3.92 ± 0.27 and 4.84 ± 0.25 , respectively ($P = 0.0006$); at Month 2, the corresponding counts were 0.92 ± 0.67 and 2.03 ± 0.49 ($P =$

Figure 1. $\log_{10}$ lung cfu counts the day after infection (–0.5), at treatment initiation (0), and after 1, 2, and 3 months of 5 days a week treatment with an initial 2 months of rifapentine, 10 mg/kg, isoniazid, 10 mg/kg, and pyrazinamide, 150 mg/kg followed by 1 month of rifapentine and isoniazid in *Mycobacterium tuberculosis*-infected athymic nude and BALB/c mice.

0.0173). However, all mice were culture negative at 3 months and remained culture negative at Months 6, 9, and 12 of treatment.

Culture status 3 months after completion of treatment with rifapentine-containing regimen. After 3 months of treatment, 30 BALB/c and 15 nude mice were withdrawn from treatment and followed for 3 more months. Half of the 30 BALB/c mice received cortisone for 4 weeks. On completion of 3-months follow-up, none of the BALB/c mice was culture positive (i.e., relapsed), whereas two (13%) of the BALB/c mice that received cortisone and 11 (73%) of the nude mice relapsed. Actually, by error BALB/c mice received only 0.4 mg (20 mg/kg) daily cortisone instead of 1 mg (50 mg/kg). The percentage of cortisone-induced reactivations should therefore be considered as the minimum probable.

Subgroups of mice that were continued on treatment after the first 3 months were withdrawn from treatment at Months 6, 9, and 12; kept under observation for 3 more months as was done at the 3-months treatment time-point; and then killed for lung culture. None of them relapsed, even the nude mice, indicating that true sterilization of TB was obtained between 3 and 6 months of treatment with 2PHZ/PH.

Lung cfu counts in mice treated with rifampin-containing regimen. BALB/C MICE. During the first 3 months of treatment with 2RHZ/RH the log₁₀ lung cfu counts decreased rapidly according to the usual pattern and were 4.94 ± 0.07 , 3.04 ± 0.08 , and 0.79 ± 0.43 at Months 1, 2, and 3, respectively. Related to baseline cfu counts of 7.84 ± 0.13 log₁₀ the corresponding reductions in counts were 2.9, 4.8, and 7.05 log₁₀.

At Month 6 of treatment, all five mice killed for lung cfu counts were culture negative. At this time-point, 30 mice were withdrawn from treatment and followed-up for 3 months. Half of them received daily cortisone, 1 mg for 4 weeks; among them one died during administration of cortisone. On completion of the 3-month follow-up, all mice were killed and their entire lung plated for culture. All were culture-negative, suggesting that sterilization might have been obtained. This conclusion is supported by the 9- and 12-month results because at these time-points of treatment, the same procedure was repeated and again all lung cultures were negative.

NUDE MICE. Similar to the observation in nude mice treated with PHZ, the reduction in lung cfu counts induced by RHZ was significantly slower in nude mice than in BALB/C mice. Actually, the log₁₀ cfu counts were 5.43 ± 0.14 at Month 1 and 4.36 ± 0.90 at Month 2 (i.e., 1.6 log₁₀ higher in nude than in BALB/c mice) ($P < 0.0001$). It is noticeable that the 2-month log₁₀ cfu counts in nude mice ranged from 4.02–5.18 and were premonitory, as recognized later, of the rise in the cfu counts after the initial fall, which is characteristic of the selection of drug-resistant mutants (21).

At Month 3, the data were striking (Figure 2). The log₁₀ lung cfu counts sharply increased to 6.10 ± 1.07 , ranging from 5.30–7.70. DST performed on the *M. tuberculosis* isolates demonstrated for each of them susceptibility to R but resistance to H with 1–10% mutants resistant to 0.2 µg/ml of H.

Because of the Month 3 unexpected increase in cfu and also because some nude mice were sick, it was decided to change the initial protocol, continue RH treatment for all mice, and monitor monthly the outcome of the lung cfu counts in the nude mice, sacrificing in priority mice that were sick.

At Month 4, the lung log₁₀ cfu counts in nude mice reached a peak value of 7.52 ± 0.75 , ranging from 6.47–8.85. Among the five killed mice, two with 8.08 and 8.85 lung log₁₀ cfu, respectively, were moribund. All *M. tuberculosis* isolates harbored 100% of mutants resistant to 0.2 µg/ml of H but remained fully susceptible to R.

Figure 2. Log₁₀ lung cfu counts the day after infection (–0.5), at treatment initiation (0), and at monthly intervals during 5 days a week treatment with an initial 2 months of rifampin, 10 mg/kg, isoniazid, 10 mg/kg, and pyrazinamide, 150 mg/kg, followed by 4 months of rifampin and isoniazid in *Mycobacterium tuberculosis*-infected athymic nude and BALB/c mice.

At Month 5, the lung cfu counts plateaued at 7.35 ± 1.43 log₁₀, ranging from 4.88–8.56. Among the five killed mice, two with 8.00 and 8.56 lung log₁₀ cfu, respectively, were moribund. Two mice died during weekends and their lungs could not be plated. DST was performed to 0.1, 0.2, 1, and 10 µg/ml of H, and 0.5 and 2 µg/ml of R. All isolates grew uniformly on plates containing 0.1, 0.2, and 1 µg/ml of H but neither on 10 µg/ml of H nor on 0.5 and 2 of R.

At Month 6, the lung cfu counts were 6.61 ± 3.72 log₁₀, ranging from 0 to 8.85. Two mice died during weekends and their lungs could not be plated. Among the five killed mice, three with 8.85, 8.45, and 8.08 cfu, log₁₀ were moribund and the one with negative culture was in apparent good health. As before all isolates were H-resistant and R-susceptible.

At Month 7, the lung cfu counts were 4.63 ± 3.24 log₁₀ with respective counts of 0.70 (two colonies), 2.49, 4.25, 7.18, and 8.53 in the five killed mice, the latter mouse being moribund. All isolates, even from the one with only two colonies, were H-resistant and R-susceptible. At Month 7 plus 1 week a mouse from the remaining nude mice seemed moribund and was killed for lung cfu counts and DST. It harbored 8.94 log₁₀ cfu and the isolate was H-resistant and R-susceptible.

From the remaining eight nude mice, all in apparent good health, four were killed on Month 8 and four on Month 9. All of them were lung culture negative. Therefore, out of the 48 nude mice on treatment with 2RHZ/RH, which were used for cfu counts during treatment, 9 (19%; one at Month 6, four at Month 8, and four at Month 9) had converted their lung culture to negative, suggesting that the 2RHZ/RH combination has also a sterilizing potential as long as the selection of H-resistant mutants does not occur.

Pharmacokinetics of rifampin, isoniazid, and pyrazinamide in nude mice. The total drug serum concentrations of R, H, and Z were performed in nude mice, which were on treatment for 4 months with 2RHZ/RH. The peak serum concentration (C_{max}) in milligrams per liter and the area under the concentration curve (AUC_{0-24}) in milligrams hour per liter were 16.08 ± 3.53 and 116.1, respectively, for R; 6.67 ± 1.00 and 7.1, respectively, for H; and 180.39 ± 12.36 and 400.4, respectively, for Z. These values are comparable with values in BALB/c mice and in patients receiving 10 mg/kg of R, 5 mg/kg of H (in rapid inactivators), and 20–30 mg/kg of Z (12), and strongly suggest that the pharmacokinetics of the tested drugs is not the cause of the unexpected response of nude mice to the 2RHZ/RH treatment.

Study 2

The aerosol infection implanted more than 4.5 log₁₀ cfu in the lungs of both BALB/c and nude mice. Fourteen days later, at treatment initiation, both groups of mice harbored close to 8 log₁₀ cfu in their lungs (Table 3). The reduction of the cfu counts in lungs of BALB/c and nude mice during treatment provides crucial information on the impact of the initial supplement of ethambutol, the 7/7 rhythm of treatment, and immune deficiency.

In BALB/c mice, the lung cfu counts were similar at Months 1, 2, and 3 in those that received the initial supplement of E and in those that did not receive the supplement, confirming that E does not add bactericidal activity to RHZ. In nude mice, the results followed the same trend but were clearly more favorable at Month 3 in mice that had received the initial supplement of E, whether the treatment was administered 5/7 or 7/7. The explanation for these findings is provided by Table 4, which shows that selection of H-resistant mutants occurred in 5 of the 15 mice treated with 2RHZ/RH 5/7 and in 5 of the 15 mice treated with 2RHZ/RH 7/7 and was the cause of the reduced efficacy in nude mice that did not receive E during the first 2 months.

In BALB/c as in nude mice the reduction in cfu counts was significantly better at each monthly time-point in mice treated 7/7 than in mice treated 5/7. Treatment with 2RHZ/RH given 7/7 was not more able than 2RHZ/RH given 5/7 to prevent the selection of H-resistant mutants in the nude mice. Consequently, a reliable comparison of the better bactericidal activity of 7/7 treatment over 5/7 can only be done in BALB/c and in nude mice treated with 2RHZE/RH (Figure 3). At each monthly time-point and for each mouse species, 7/7 treatment was more effective than 5/7 treatment. It is worth noting that the response to treatment was, as seen in Study 1, inferior in nude mice compared with BALB/c mice. For example, the cfu counts at Month 3 in nude mice treated 5/7 were similar to the cfu counts at Month 2 in BALB/c mice treated 5/7 and one log more at the same Month 2 than in BALB/c mice treated 7/7. The impact of the rhythm of drug administration and of the immune status of mice on treatment efficacy was thus spectacular.

Mutation Analysis

Study 1. At month 5, all five killed mice yielded positive cultures with 100% mutants resistant to 1 µg/ml H. For analysis, three colonies grown on 1 µg/ml H were randomly selected from lung cultures of each mouse. Colonies from four of the five mice had mutations in the *katG* gene (Table 5). In the remaining mouse, the

sequence encoding the proximal 222 amino acids of the *katG* gene could not be amplified despite repeated polymerase chain reaction attempts. This may indicate a deletion or a mutation in the primer binding site.

Study 2. At Month 2, mutants resistant to H 0.2 µg/ml were selected from two out of the five mice killed from the 2RHZ/RH 5/7 treatment group (Table 4). Three colonies were selected from mouse 1 and a single colony from mouse 2. All colonies had mutations in *katG* (Table 5). At Month 3, H-resistant mutants were also selected from one mouse of the 2RHZ/RH 5/7 treatment group and four mice of the 2RHZ/RH 7/7 treatment group. All tested colonies except those from two mice of the 2RHZ/RH 7/7 treatment group had mutations in the *katG* gene. In none of the tested resistant colonies were there mutations in the *inhA* promoter.

DISCUSSION

The first and crucial result of the reported experiments is the demonstration that TB infection can apparently be sterilized in nude mice by combination therapy including 10 mg/kg of P, 10 mg/kg of H, and 150 mg/kg of Z given 5 days a week for 3–6 months. Such a spectacular result raises several issues. The first issue is the reality of the sterilization. In the past, McCune and coworkers (3) and Grumbach (7) used immune-competent mice injected with cortisone to reactivate persisters after treatment completion and succeeded in reactivating TB in a high percentage of mice. We also used immune-competent mice injected with cortisone and, after 3 months of treatment with 2PHZ/PH, succeeded in reactivating TB in a proportion of mice but a proportion much lower than the spontaneous reactivations observed in nude mice. Our results confirm that nude mice, which lack mature T cells and are thus deprived of cell-mediated immunity, are more prone to reactivate TB than normal mice given cortisone. Therefore, the absence of TB reactivation in nude mice is likely attributable to the killing of all persisters (i.e., to true sterilization of TB infection by antibiotic treatment).

The second issue is to determine the antibiotics responsible for the sterilization. Among the rifamycins, P seems more potent than R not only because it was able to prevent the selection of H-resistant mutants in nude mice when given in combination with HZ, but also because culture conversion was consistently (12) obtained in BALB/c mice earlier with P-containing regimens than with R-containing regimens. However, it should not be overlooked that 20% of nude mice treated with 2RHZ/RH survived and became culture negative, suggesting that provided selection of H-resistant mutants was prevented this regimen

TABLE 3. LUNG CFU COUNTS BEFORE AND DURING TREATMENT IN STUDY 2

Mice	Drug Regimens	Log ₁₀ cfu in Lungs of Mice* at the Following Time-points				
		D–13	D0	Month 1	Month 2	Month 3
BALB/c	Untreated	4.51 ± 0.19	7.72 ± 0.07	All were dead by Day 22 after infection		
	RHZ 5/7			5.05 ± 0.11	3.15 ± 0.42	0.85 ± 0.58
	RHZ 7/7			4.38 ± 0.27	2.25 ± 0.22	0.08 ± 0.18
	RHZE 5/7			4.96 ± 0.19	3.1 ± 0.45	0.78 ± 0.48
	RHZE 7/7			4.33 ± 0.23	1.91 ± 0.08	0.08 ± 0.18
Nude	Untreated	4.6 ± 0.09	7.83 ± 0.13	All were dead by Day 26 after infection		
	RHZ 5/7			5.92 ± 0.20	4.46 ± 0.85	3.42 ± 2.06
	RHZ 7/7			5.34 ± 0.05	3.37 ± 0.27	3.79 ± 2.65
	RHZE 5/7			5.85 ± 0.27	4.12 ± 0.29	2.76 ± 0.28
	RHZE 7/7			5.17 ± 0.16	3.71 ± 0.11	0.97 ± 0.69

Definition of abbreviations: D = day; E = ethambutol, 100 mg/kg; H = isoniazid, 10 mg/kg; M = month of 4 weeks; R = rifampin, 10 mg/kg; Z = pyrazinamide, 150 mg/kg.

All drugs were given orally 5 days a week (5/7) or 7 days a week (7/7). R was administered 1 hour before other drugs.

* Five mice at each time point for each treatment group.

TABLE 4. SELECTION OF ISONIAZID-RESISTANT MUTANTS IN TUBERCULOSIS-INFECTED NUDE MICE IN RELATION TO THE RHYTHM OF TREATMENT AND THE ADDITION OF ETHAMBUTOL

Drug Regimens	Total Mice	No. of Nude Mice with Selection of Mutants Resistant to H, 0.2 µg/ml			
		Total	At Month 1	At Month 2	At Month 3
2RHZ/RH 5/7	15	5/15	2/5*	2/5†	1/5‡
2RHZ/RH 7/7	15	5/15	1/5§	0/5	4/5¶
2RHZE/RH 5/7	15	0/15	0/5	0/5	0/5
2RHZE/RH 7/7	15	0/15	0/5	0/5	0/5

Definition of abbreviations: E = ethambutol, 100 mg/kg; H = isoniazid, 10 mg/kg; R = rifampin, 10 mg/kg; Z = pyrazinamide, 150 mg/kg; 5/7 = 5 days per week; 7/7 = 7 days per week.

* One mouse harbored 0.4% and the other harbored 1% resistant mutants.

† One mouse harbored 3% and the other 10% resistant mutants.

‡ With 50% resistant mutants.

§ With 0.2% resistant mutants.

¶ One mouse harbored 25% and the other three mice harbored 50% resistant mutants.

seemed also to have the potential to cure nude mice, although after treatment of longer duration. No relapse study could be performed with these mice but it is possible that they also were sterilized. An argument in favor of such a hypothesis is provided by the BALB/c mice treated with 2RHZ/RH. All mice were culture-negative at 6 months and all of them did not reactivate despite receiving high-dose cortisone after treatment completion. Although BALB/c mice injected with cortisone might not be as permissive in reactivating persisters as nude mice, they are much more able to do so than normal mice without cortisone and, consequently, support the hypothesis that a proportion if not all BALB/c mice treated for 6 months with 2RHZ/RH were sterilized. If it is the case, our data differ from those of McCune and coworkers (3) and Grumbach (7). McCune and coworkers (3) did not succeed in sterilizing mice with the combination of H and high doses of Z, and Grumbach (7) did not succeed in sterilizing mice with the combination of R and H. In both cases, one of two key sterilizing drugs, R or Z, was missing. One may thus speculate that

Figure 3. Log₁₀ lung cfu counts the day after infection (–0.5), at treatment initiation (0), and after 1, 2, and 3 months of treatment with an initial 2 months of rifampin (R), 10 mg/kg, isoniazid (H), 10 mg/kg, pyrazinamide (Z), 150 mg/kg, and ethambutol (E), 100 mg/kg, followed by 1 month of rifampin and isoniazid in *Mycobacterium tuberculosis*-infected athymic nude and BALB/c mice. Treatment was administered either 5 days a week (5/7) or 7 days a week (7/7).

TABLE 5. MUTATIONS IN THE *KATG* GENE OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES RESISTANT TO ISONIAZID

Study	Month	Treatment Group	Mouse No.	Colony No.	katG	
					Nucleotide	Amino Acid
1	5	2 RHZ/RH	1	1	1023 G→C	W341C
				2	1023 G→C	W341C
				3	1023 G→C	W341C
			2	1	1685 A→DEL	Frameshift
				2	1685 A→DEL	Frameshift
				3	1685 A→DEL	Frameshift
			3	1	539 C→G	T180R
				2	1314 G→A	W438Stop
				3	539 C→G	T180R
			4	1	678 G→DEL	Frameshift
				2	678 G→DEL	Frameshift
				3	678 G→DEL	Frameshift
			5	1	Not amplified	?
				2	Not amplified	?
				3	Not amplified	?
2	2	2RHZ/RH 5/7	1	1	830 G→A	G277D
				2	830 G→A	G277D
				3	830 G→A	G277D
	3	2RHZ/RH 5/7	1	1	1314 G→A	W438Stop
				2	1235 G→C	W412S
				3	1235 G→C	W412S
	3	2RHZ/RH 7/7	1	1	WT	
				2	WT	
				3	WT	
			2	1	WT	
				2	WT	
				3	WT	
			3	1	WT	
				2	WT	
				3	WT	
4	1	440 T→C	L147P			
	2	440 T→C	L147P			
	3	440 T→C	L147P			

Definition of abbreviations: A = adenine; C = cytosine; DEL = deletion; E = ethambutol, 100 mg/kg; G = guanine; H = isoniazid, 10 mg/kg; R = rifampin, 10 mg/kg; T = thymine; WT = wild type; Z = pyrazinamide, 150 mg/kg; 5/7 = 5 days per week; 7/7 = 7 days per week.

→ indicates the substitution of C for G, G for C, G for A, A for G, or C for T, respectively; or indicates DEL.

Amino acid codes: C = cysteine; D = aspartate; G = glycine; L = leucine; P = proline; R = arginine; S = serine; T = threonine; W = tryptophan.

the potent sterilizing effect of 2PHZ/PH should be attributed not totally to P but also to Z.

An unexpected result of the reported experiments is the failure of the 2RHZ/RH regimen in a high proportion of nude mice and its association with H resistance. Because of the numerous reports (22–26) dealing with the association between mono-resistance to rifamycins and HIV-related TB, R resistance was expected to be found in nude mice that failed on treatment with the 2RHZ/RH regimen. When H and not R resistance was identified it was thought that the pharmacokinetics of R was deficient in the nude mice compared with normal mice. Actually, the pharmacokinetics of R, H, and Z were similar in nude and BALB/c mice and were comparable with human pharmacokinetics. Therefore, failure with selection of H resistance in nude mice was not related to lower C_{max} and AUC_{0-24} of R and Z. Likely the inability of R to prevent resistance to H is related to the limited R exposure provided by the administration of 10 mg/kg of R 5 days a week. According to Rosenthal and coworkers (12), one may assume that the percentage of the time during which the serum concentration of R is above minimum inhibitory concentration is no more than 60% of the week. Mice being rapid inactivators of H, one may also assume that the weekly exposure to H is far less

than permanent. This means that the bacilli were not exposed to active drug concentrations for long and multiple periods of time every week. In an immune-competent host, the bacillary population is likely inhibited from growing by host immunity and the postantibiotic effect (27) during the period of time when bacilli are not exposed to antibiotics. However, in a host deprived of T-cell immunity like the nude mouse, bacillary regrowth may have occurred as soon as the exposure to antibiotic and the postantibiotic effect ceased. Therefore, one may speculate that in nude mice treated 5/7 with RHZ there were successive cycles of killing and regrowth and these cycles favored the selection of mutants resistant to H, which is the most bactericidal drug and the drug with longest postantibiotic effect, especially after repeated exposures (28), as proposed by Mitchison (29). This explanation is supported by the fact that treating nude mice 7 days a week with RHZ, a dosing rhythm that is also not sufficient to ensure permanent R exposure (12) and permits cycles of killing and regrowth, did not prevent more than the 5 days a week treatment the selection of H-resistant mutants. Paradoxically, the rifamycin-monoresistance observed in patients treated with intermittent dosing of P and H may be explained symmetrically by the same phenomenon: P in these patients was the drug that ensured the longest drug exposure (30) and consequently the strongest selective pressure. One may predict that increasing the dose of R or its rhythm of administration to twice daily would ensure permanent rifamycin exposure as the P-containing regimen did (12), prevent the selection of H-resistant mutants in nude mice, and also lead to sterilization.

All H-resistant isolates with detectable mutations had mutations in the *katG* gene and not in the *inhA* promoter, probably because nude mice were treated with 10 mg/kg doses of H, which resulted in a high peak concentration and in the selection of mutants with a high level of H resistance. Such a hypothesis is supported by the findings of a previous study (14) in which immune-competent BALB/c mice were treated with a range of H doses, 1.56–50 mg/kg, and in which the selection of the *inhA* promoter mutation was only observed in mice treated with the lowest effective dose of 3.125 mg/kg. It should also be noted that in three mice of Study 2 (Table 5) the colonies resistant to H did not have any identifiable mutations in the *katG* gene or the *inhA* promoter region suggesting that mutations have taken place in other genes that were not investigated, as observed in other studies of the genetic background of H resistance (31). At last, it is remarkable that we did not find in the present study and in the previous one (14) the most common *katG* mutation found in human specimens, S315T (31, 32). No explanation can be given for this absence but one can speculate that the laboratory strain H37Rv used to infect mice (33) does not produce as easily S315 mutations in the *katG* gene as a clinical strain (e.g., of the Beijing family) (34, 35). One may speculate also that peak serum concentrations of H being different in mice and humans, the selective pressure of H is different in mice and humans and results in the selection of different mutants. In the laboratory mouse, a compliant model host with a highly controlled diet and environment, and an entirely intracellular habitat for the bacilli, the drug selective pressure may be different from that in humans where drug resistance occurs in very diverse operational conditions, in a highly genetically diverse host whose bacilli are typically extracellular at the time of treatment (36).

It is reassuring that the addition of ethambutol (E) to the 2RHZ/RH regimen given 5 or 7 days a week prevented the selection of H-resistant mutants in nude mice, as previously demonstrated in immune-competent mice (37). The effect of E is not related to an increase in the overall antimicrobial potency of the drug regimen because the addition of E to RHZ did not accelerate the reduction of cfu counts in nude mice and in BALB/c

mice. Likely, in combination with RZ, its bacteriostatic activity prevented the outgrowth of H-resistant mutants more effectively than RZ alone. Thus, E seems to be an essential component of the standard 2RHZE/RH treatment of TB in immune-deficient hosts.

One may wonder how far the present results obtained in BALB/c and nude mice can be extended to the treatment of patients. Likely, the better bactericidal activity of 7/7 treatment over 5/7 treatment in both strains of mice is consistent with clinical findings. The greater the drug exposure, the better the antimicrobial activity (38, 39). Intermittent regimens or 5/7 treatments are not an ideal but rather a realistic compromise to promote supervised treatment (40). The lack of beneficial impact of an E supplement to RHZ during the 2-month initial phase of treatment in BALB/c mice reflects the bacteriostatic activity of E and is also valid in immune-competent patients infected with fully drug susceptible *M. tuberculosis*. E is combined with RHZ during the initial phase as a protective drug in case of unexpected H or R mono-resistance (1). More surprising was its protective effect against H resistance in nude mice. There are no comparative data in immune-deficient patients with TB but it is reasonable to think that these patients also benefit from the initial E supplement even though they harbor drug-susceptible *M. tuberculosis*. The final and crucial issue to address is the possibility to extrapolate totally or partly the treatment response in nude mice to the treatment response in immune-deficient patients. Clearly, nude mice are at the worst extremity of the scale of the immune-deficient hosts. They lack any CD4 or CD8, T cells and recapitulate the loss of CD4⁺ T cells in persons living with advanced AIDS, a condition that is less and less frequent thanks to highly active antiretroviral treatment. Our results in nude mice should therefore be considered as “proof of concept” of the negative impact of immune deficiency on the response to anti-TB treatment. They are, however, in favor of more intensive anti-TB treatment in HIV-positive patients with TB.

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