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Immunodeficiency and Intermittent Dosing Promote Acquired Rifamycin Monoresistance in Murine Tuberculosis

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ABSTRACT More-permissive preclinical models may be useful in evaluating antituberculosis regimens for their propensity to select drug-resistant mutants. To evaluate whether acquired rifamycin monoresistance could be recapitulated in mice and, if so, to evaluate the effects of immunodeficiency, intermittent dosing, and drug exposures, athymic nude and BALB/c mice were infected. Controls received daily rifapentine alone or 2 months of rifampin, isoniazid, pyrazinamide, and ethambutol, followed by 4 months of rifampin/isoniazid, either daily or twice weekly with one of two isoniazid doses. Test groups received the same intensive regimen followed by once-weekly rifapentine or isoniazid/rifapentine with rifapentine doses of 10, 15, or 20 mg/kg of body weight plus one of two isoniazid doses. All combination regimens rendered BALB/c mouse cultures negative but selected mutants resistant to isoniazid (8.5%, 12/140) or rifampin (3.5%, 5/140) in nude mice ($P < 0.001$). Intermittently dosed intensive-phase therapy selected isoniazid and rifampin resistance in 10% (8/80, $P < 0.001$) and 20% (16/80, $P = 0.009$) of nude mice, respectively, compared to 0% treated with a daily regimen. Once-weekly rifapentine-containing continuation-phase regimens selected rifampin-resistant mutants at a rate of 18.0% (18/100, $P = 0.035$ compared to rifampin/isoniazid regimens). Higher isoniazid doses in the intermittent-treatment control regimen and higher rifapentine doses in once-weekly regimens were associated with less selection of isoniazid resistance. Acquired resistance, including rifamycin monoresistance, was more likely to occur in nude mice despite administration of combination therapy. These results recapitulate clinical outcomes and indicate that nude mice may be useful for evaluating the ability of novel regimens to prevent the selection of resistance.

KEYWORDS acquired rifamycin resistance, nude mouse, tuberculosis, intermittent therapy, immunodeficiency

Resistance to rifamycins greatly compromises outcomes of treatment of tuberculosis (TB) (1). Fortunately, acquired rifamycin monoresistance (ARR) is a rare occurrence. As illustrated by two clinical trials conducted by the TB Trials Consortium (TBTC) (2, 3), ARR is associated with advanced AIDS and intermittent treatment schedules, especially when a rifamycin with a long elimination half-life is used (4–9). As more novel drugs and regimens are considered for clinical trials, a new, more-permissive animal model may be useful to evaluate the propensity of new regimens to selectively amplify drug-resistant mutants.

We previously observed a surprising failure of daily (5 days per week [5/7]) combination treatment of immunodeficient athymic nude mice, but not immunocompetent BALB/c mice, with human-equivalent doses of rifampin (RIF [R]), isoniazid (INH [H]), and pyrazinamide (PZA [Z]) (10). Failure was due to acquired INH monoresistance (AHR) rather than to ARR, consistent with the much higher prevalence of AHR than of ARR among clinical isolates. To further validate nude mice as a stringent model for stress-

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TABLE 1 Lung CFU counts before and during treatment[a]

Treatment group	Drug regimen		Log_{10} CFU count in mice sacrificed at:				
	0–2 mo	2–6 mo	Mo 0.5	Mo 1	Mo 2[b]	Mo 4	Mo 6
BALB/c mice							
1	Untreated						
2	P 5/7	P 5/7		5.07 ± 0.50	Contaminated	0.50 ± 1.00[c]	0[c]
3	RHZE 5/7	RH 5/7	6.36 ± 0.06	4.97 ± 0.24	2.90 ± 0.14	0	0
5		PH_{50} 1/7				0	0
6	2wRHZE 5/7 + 6w$RH_{25}Z_{300}E_{200}$ 2/7	RH_{25} 2/7		5.12 ± 0.24	3.35 ± 0.07	0.89 ± 0.26	0
8		PH_{50} 1/7				0.14 ± 0.31	0
11	2wRH_5ZE 5/7 + 6w$RH_{12.5}Z_{300}E_{200}$ 2/7	$RH_{12.5}$ 2/7	6.36 ± 0.13	4.87 ± 0.13	2.70	0.34 ± 0.48	0
13		PH_{25} 1/7				0.14 ± 0.31	0
Nude mice							
1	Untreated						
2	P 5/7	P 5/7		5.55 ± 0.24	6.70	6.52[c]	ND
3		RH 5/7	6.43 ± 0.37	5.95 ± 0.19	3.77	0	0
4	RHZE 5/7	P 1/7				4.82 ± 0.86[c]	6.38
5		PH_{50} 1/7				2.44 ± 0.32	0.08
6		RH_{25} 2/7		6.15 ± 0.27	5.20	1.77 ± 0.58	0.16
7		P 1/7				6.63 ± 2.32[c]	6.50[c]
8	2wRHZE 5/7 + 6w$RH_{25}Z_{300}E_{200}$ 2/7	PH_{50} 1/7				2.09 ± 0.38[c]	0[c]
9		$P_{15}H_{50}$ 1/7				1.79 ± 0.30[c]	0
10		$P_{20}H_{50}$ 1/7				2.03 ± 0.42[c]	0
11		$RH_{12.5}$ 2/7	6.74 ± 0.25	6.16 ± 0.43	4.10	2.28 ± 1.04[c]	0.37[c]
12		P 1/7				6.82 ± 0.50[c]	6.30[c]
13	2wRH_5ZE 5/7 + 6w$RH_{12.5}Z_{300}E_{200}$ 2/7	PH_{25} 1/7				2.09 ± 0.08[c]	0.45[c]
14		$P_{15}H_{25}$ 1/7				1.39 ± 0.42	2.51[c]
15		$P_{20}H_{25}$ 1/7				0.74 ± 1.04[c]	0

[a]The log_{10} CFU counts at day 0 were 7.82 ± 0.22 for the BALB/c mice and 7.77 ± 0.18 for the nude mice. All members of group 1 of the BALB/c mice were dead before month 0.5; all members of group 1 of the nude mice were dead by day 9. Drug doses (in mg/kg) if not otherwise specified: rifampin (R), 10; rifapentine (P), 10; isoniazid (H), 10; pyrazinamide (Z), 150; ethambutol (E), 100. 2w, 2-week drug administration; 6w, 6-week drug administration; ND, not done due to death of all mice by month 4.

[b]Due to fungal contamination, only a few plates were available for analysis at month 2.

[c]CFU resistant to R or H coexisted at the indicated points between 2 and 6 months, but only CFU counts from mice without resistance are presented here.

testing regimens for their ability to prevent selection of drug-resistant mutants, we sought to recapitulate TBTC study 22 (2), which was closed prematurely to enrollment of HIV-infected subjects when 4 of 5 subjects who relapsed after receiving a once-weekly continuation-phase regimen of INH and rifapentine (RFP [P]) showed ARR. We hypothesized that selection of ARR rather than AHR in nude mice could be accomplished by increasing the rifamycin selection pressure and decreasing the INH counterselection pressure. The objective of the present study was to evaluate whether the emergence of ARR in TBTC study 22 could be recapitulated in mice and, if so, to evaluate the effects of immunodeficiency and of intermittently dosed initial-phase therapy as well as of INH and RPF doses on ARR occurrence.

(Part of this research was presented at the 2012 Conference on Retroviruses and Opportunistic Infections [CROI] [11]).

RESULTS

Lung CFU counts before treatment. The mean lung log_{10} CFU counts on the day of infection (day −17 [D−17]) were 3.54 (standard deviation, ±0.07) and 3.95 (±0.05) in BALB/c and nude mice, respectively. By D0, the mean CFU counts had increased to 7.82 (±0.22) log_{10} in BALB/c mice and 7.77 (±0.18) log_{10} in nude mice (Table 1).

Mortality during treatment. All untreated mice died or were euthanized due to moribund status within 4 weeks of infection. No mortality occurred in treated BALB/c mice. The mortality rates among nude mice during the initial 8-week intensive phase were due to gavage injuries and did not differ by regimen (Table 2). All mice dying in this phase had lung CFU counts that were similar to those observed at the closest planned sacrifice time points. Excess mortality in the continuation phase of treatment (between month 2 [M2] and M6) was observed with P monotherapy and with low-

TABLE 2 Mortality of nude mice during the treatment period[a]

Treatment group	Treatment regimen		No. of mice showing mortality during the treatment period/total no. of mice	
	0–2 mos	2–6 mos	0–2 mos	2–6 mos[b]
2	P 5/7	P 5/7	1/10	6/10 (6/6)
3		RH 5/7		0/10
4	RHZE 5/7	P 1/7	3/15	1/10 (1/1)
5		PH_{50} 1/7		0/10
6		RH_{25} 2/7		0/10
7		P 1/7		1/10 (1/1)
8	2wRHZE 5/7 + 6w$RH_{25}Z_{300}E_{200}$ 2/7	PH_{50} 1/7	2/10	0/10
9		$P_{15}H_{50}$ 1/7		0/10
10		$P_{20}H_{50}$ 1/7		0/10
11		$RH_{12.5}$ 2/7		4/10 (3/4)
12		P 1/7		3/10 (2/3)
13	2wRH_5ZE 5/7 + 6w$RH_{12.5}Z_{300}E_{200}$ 2/7	PH_{25} 1/7	3/15	1/10 (?)
14		$P_{15}H_{25}$ 1/7		0/10
15		$P_{20}H_{25}$ 1/7		1/10 (0/1)

[a]Drug doses (in mg/kg) if not otherwise specified: rifampin (R), 10; rifapentine (P), 10; isoniazid (H), 10; pyrazinamide (Z), 150; ethambutol (E), 100. The mortality data include only mice dead from any cause before scheduled sacrifice or sacrificed earlier due to severe illness.

[b]Numbers within parentheses indicate the proportions of dying mice harboring ≥1% resistant CFU. ?, not assessable.

dose-H-containing intermittent regimens (9 of 50 mice, groups 11 to 15) compared with high-dose-H-containing intermittent regimens (1 of 50 mice, groups 6 to 10; $P = 0.016$) (Table 2). Four deaths (of 2 mice with AHR [group 11], 1 mouse with ARR [group 12], and 1 mouse with both AHR and ARR but not multidrug resistance [MDR] [group 12]) in the low-H-dose groups were due to poorly controlled infection in the context of resistance, and 5 deaths were unrelated to resistance. One death in group 7 and all 6 deaths in group 2 (P monotherapy) were due to poorly controlled infection in the context of ARR (Table 2).

Lung CFU counts during treatment. (i) BALB/c mice. The daily and intermittently dosed intensive-phase regimens had similar levels of efficacy over the first 2 months in BALB/c mice (Fig. 1A). Due to difficulties with contaminated plates, only 1 to 2 mice per group contributed CFU counts at M2, so the differences were not statistically significant. Daily treatment for the entire intensive phase (group 3) was associated with

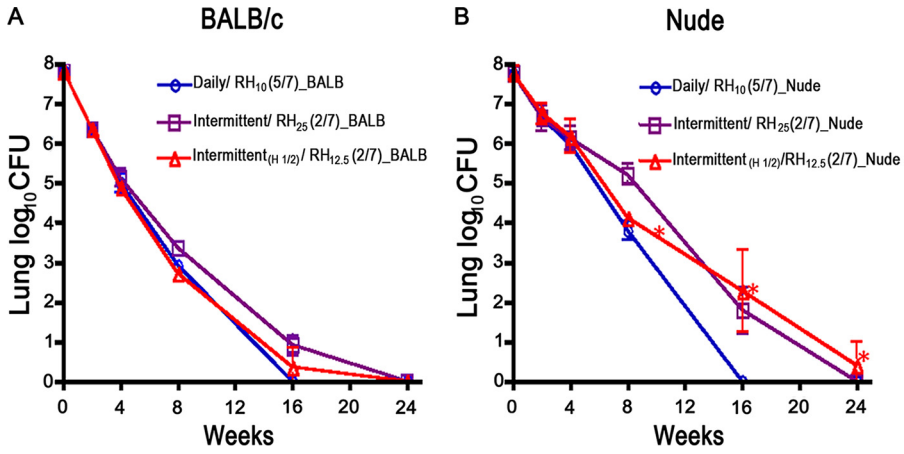

FIG 1 Lung $\log_{10}$ CFU counts before and after treatment with daily and intermittently dosed control (2RHZE/4RH) regimens in (A) BALB/c and (B) nude mice, omitting the results from mice in which resistance was selected. As explained in Table 5, group 3 received 5/7 treatments throughout. Groups 6 and 11 received 5/7 treatments for the first 2 weeks followed by twice-weekly therapy thereafter and differed only by the higher (group 6) or lower (group 11) dose of isoniazid (H). Drug doses were as follows unless otherwise noted: rifampin (R), 10 mg/kg; pyrazinamide (Z), 150 mg/kg; isoniazid (H), 10 mg/kg; ethambutol (E), 100 mg/kg. Asterisks (*) in Fig. 1B indicate the coexistence of CFU resistant to R or H at those points.

culture negativity in all mice after 4 months of treatment, whereas 5 of 5 and 2 of 5 mice receiving intermittently dosed control regimens (groups 6 and 11, respectively) remained culture positive after 4 months. P monotherapy showed substantial efficacy, rendering 3 of 5 and 4 of 5 mice culture negative after 4 and 6 months, respectively. However, in 1 of 5 mice at each of these time points, selection of ARR led to treatment failure (Table 1). All combination regimens rendered the lungs culture negative after 6 months of treatment (Fig. 1A).

(ii) Nude mice. Lung CFU counts obtained during treatment are shown in Table 1 (the data in the table omit the CFU counts from mice in which resistant mutants outgrew the susceptible population). The performance of the control regimens is likewise depicted graphically in Fig. 1B. The intensive-phase regimens were generally similar in their levels of efficacy over the first month, but their bactericidal activity was clearly lower in nude mice than in BALB/c mice, as previously observed for daily treatment (10). Only 2 months of daily treatment with ethambutol (EMB [E]) plus RHZ 5/7 followed by 2 months of daily treatment with RH (group 3) rendered all mice culture negative. Unlike the results seen in BALB/c mice, the results seen with the intermittently dosed control regimens (groups 6 and 11) were significantly worse than those seen with the daily control regimen after 4 months. P monotherapy failed in virtually all nude mice due to ARR. The P 1/7 continuation-phase regimen failed to contain bacterial growth, resulting in increasing CFU counts between months 2 and 6, irrespective of the initial-phase regimen and not all attributable to ARR. On the other hand, the addition of H to the once-weekly regimens in nude mice resulted in a continued bactericidal effect during the continuation phase as long as no selection of resistance occurred. However, these regimens were much less effective in nude mice. The intermittent RHZE intensive-phase treatment with low-dose H followed by treatment with P 20 mg/kg and high-dose H ($P_{20}H_{25}$) treatment 1 day per week (1/7) (group 15) rendered all mice culture negative after 6 months. No other regimen with the same intensive phase rendered lungs culture negative in nude mice despite 6 months of treatment.

Selection of drug-resistant mutants. BALB/c and nude mice began treatment with similarly sized H-resistant subpopulations (2.35 $\pm$ 1.05 and 2.43 $\pm$ 0.76 $\log_{10}$ CFU, respectively) representing the expected frequency of between 10^{-5} and 10^{-6}. Among the 4 BALB/c and 6 nude mice killed at D0, only 1 nude mouse had R-resistant CFU detected (lower limit of detection of 0.7 $\log_{10}$), again representing the expected frequency of $\leq 10^{-7}$. Whereas no selective amplification of resistance occurred in any of the 100 BALB/c mice receiving combination therapy, selective amplification was observed in 16 of 140 nude mice receiving the same regimens ($P < 0.001$) (Table 3). Examining AHR and ARR specifically, nude mice were more likely than BALB/c mice receiving the same regimens to experience selective amplification of H resistance (8.5% [12/140] versus 0%, $P = 0.001$) and R resistance (3.5% [5/140] versus 0%, $P = 0.06$). Among nude mice treated for at least 1 month, a predominantly intermittently dosed initial phase was associated with a greater proportion of mice with selective amplification of resistance than a daily initial phase (30.0% [24/80] versus 2.7% [1/37] for combined AHR and ARR, $P < 0.001$; 20% [16/80] versus 2.7% [1/37] for ARR alone, $P = 0.009$). Among nude mice, a once-weekly P-containing continuation-phase regimen was more likely to selectively amplify R-resistant mutants than an RH-containing regimen (18.0% [18/100] versus 3.3% [1/30], $P = 0.035$), but only if the once-weekly P-alone groups, where selective amplification of rifamycin resistance was observed in half the mice, were included. H dose size did not significantly impact the selective amplification of resistance in groups receiving once-weekly P-containing regimens. However, mice in the intermittently dosed control regimen group receiving low-dose H experienced more selective amplification of H resistance than those receiving high-dose H (40% [4/10] versus 0%, $P = 0.043$). Among nude mice receiving HP 1/7, a nonsignificant trend toward less selection of H resistance was seen with increasing P doses (20%, 15%, and 5% for 10, 15, and 20 mg/kg of body weight, respectively) (Table 3).

TABLE 3 Proportions of nude mice with selective amplification of resistance and emergence of resistance between month 2 and month 6

Treatment group	Drug regimen		No. of mice with indicated result/total no. of mice					
			Selective amplification only[a]			Resistance[b]		
	0–2 mos	2–6 mos	H	R	H or R	H	R	H or R
2	P 5/7	P 5/7	0/9	1/10	1/10	0/9	9/10	9/10
3		RH 5/7	0/10	0/10	0/10	0/10	0/10	0/10
4	RHZE 5/7	P 1/7	0/10	0/10	0/10	0/10	1/10	1/10
5		PH_{50} 1/7	0/10	0/10	0/10	0/10	0/10	0/10
6		RH_{25} 2/7	0/10	0/10	0/10	0/10	0/10	0/10
7		P 1/7	0/10	1/10	1/10	1/10	6/10	6/10[c]
8	2wRHZE 5/7 + 6w$RH_{25}Z_{300}E_{200}$ 2/7	PH_{50} 1/7	0/10	0/10	0/10	3/10	1/10	4/10
9		$P_{15}H_{50}$ 1/7	0/10	0/10	0/10	2/10	0/10	2/10
10		$P_{20}H_{50}$ 1/7	0/10	0/10	0/10	0/10	1/10	1/10
11		$RH_{12.5}$ 2/7	0/10	1/10[d]	1/10	4/10[d]	0/10	4/10
12		P 1/7	0/10	1/10	1/10	2/10[d]	5/10	5/10[d]
13	2wRH_5ZE 5/7 + 6w$RH_{12.5}Z_{300}E_{200}$ 2/7	PH_{25} 1/7	0/10	0/10	0/10	1/10	1/10	2/10
14		$P_{15}H_{25}$ 1/7	0/10	0/10	0/10	1/10	0/10	1/10
15		$P_{20}H_{25}$ 1/7	0/10	0/10	0/10	1/10	1/10	2/10

Drug doses (in mg/kg) if not otherwise specified: rifampin (R,10); rifapentine (P, 10); isoniazid (H, 10); pyrazinamide (Z, 150); ethambutol (E, 100).
[a]Selective amplification defined by $0.01 < H < 1\%$ or $0.001 < R < 1\%$ of resistance on H (0.2 μg/ml) or R (1.0 μg/ml).
[b]Resistance defined by resistant CFU comprising ≥1% of total CFU.
[c]One isolate was multidrug resistant (MDR) which was resistant to both H (0.2 μg/ml) and R (1.0 μg/ml).
[d]One isolate had heterogenous strains which were resistant to either H (0.2 μg/ml) or R (1.0 μg/ml) but not both.

Drug susceptibility testing (DST) and mutational analysis of isolates harboring >1% resistance. The majority of resistant isolates were available for DST testing and mutational analysis. All H- or R-resistant isolates tested were resistant to 16 μg/ml of the respective drugs except one R-resistant isolate having an MIC of 2 to 4 μg/ml (Table 4). Seven of 9 H-resistant isolates had a point mutation, insertion, or deletion in *katG*,

TABLE 4 MIC and mutational analysis of isolates harboring >1% resistance[a]

Treatment group and regimen	Duration of treatment (wks)	Resistant CFU[b] (%)		MIC (μg/ml)		*katG* mutation		*rpoB* mutation	
		H	R	H	R	Nucleotide	Amino acid	Nucleotide	Amino acid
BALB/c mice									
G2, P 5/7	16	0	97		>16			1592 C→T	S531L
	24	0	77		>16			1576 C→T	H526Y
Nude									
G2, P 5/7	4	<0.01	2		>16			1577 A→G	H526R
	13	<0.01	100		>16			1576 C→T	H526Y
	13	<0.01	2		2–4			1598 T→C	L533P
	13	0	100		>16			1576 C→T	H526Y
	13	<0.01	20		>16			1577 A→G	H526R
	16	0	6		>16			1565 C→T	S522L
G7, $_{(2+6)w}$RHZE + 4P 1/7	16	<0.01	3		>16			1592 C→G	S531W
	16	0.06	0.25	>16[c]	>16[c]	1513 T→C	W505R	1592 C→T	S531L
	24	<0.01	6		>16			1576 C→T	H526Y
G8, $_{(2+6)w}$RHZE + 4PH_{50} 1/7	16	33	0	>16		765 G→C	M255I		
	16	100	0	>16		691 A ins	Frame shift		
	24	0	100		>16			1592 C→T	S531L
G9, $_{(2+6)w}$RHZE + 4$P_{15}H_{50}$ 1/7	16	100	0	>16		902 AG del	Frame shift		
	16	75	0	>16		WT			
G11, $_{(2+6)w}$$RH_5ZE$ + 4$RH_{12.5}$ 2/7	19	100	0.0014	>16		853 G ins	Frame shift		
G13, $_{(2+6)w}$$RH_5ZE$ + 4PH_{25} 1/7	16	0	100		>16			1592 C→T	S531L
G14, $_{(2+6)w}$$RH_5ZE$ + 4$P_{15}H_{25}$ 1/7	16	16	0	>16		1856 T→G	L619R		
	24	100	0	>16		1643 G→A	G548D		
G15, $_{(2+6)w}$$RH_5ZE$ + 4$P_{20}H_{25}$ 1/7	16	30	0	>16		WT			
	16	0	100		>16			1592 C→T	S531L

[a]Drug doses (in mg/kg) if not otherwise specified: rifampin (R), 10; rifapentine (P), 10; isoniazid (H), 10; pyrazinamide (Z), 150; ethambutol (E), 100. (2 + 6)w, combination of 2-week plus 6-week drug administration; G, group; ins, insertion; del, deletion; WT, wild type.
[b]Resistance defined by growth on H (0.2 μg/ml) or R (1 μg/ml).
[c]MIC measured only with resistant isolates on H (0.2 μg/ml) or R (1 μg/ml).

each different from the others. None harbored *inhA* promoter mutations. The remaining 2 isolates had wild-type *katG* and *inhA* promoter sequences. All R-resistant isolates tested had *rpoB* mutations of the type commonly observed in clinical isolates. The S531L (1592 C→T) mutation was most common, followed by H526Y (1576 C→T). The vast majority of mutations occurred in these 2 codons (42.8% each). The mutant with low-level resistance harbored the L533P mutation that is known to be associated with low-level resistance (31, 32). In one nude mouse from group 7, selective amplification of a MDR mutant (0.002% of total CFU) occurred.

DISCUSSION

Previous clinical studies have suggested that the following factors are associated with ARR: advanced immunodeficiency, intermittent intensive-phase therapy, highly intermittent continuation-phase therapy with long-lived rifamycins, and very low H concentrations (2–9). The present study confirmed and extended these findings in mice by demonstrating that all of these risk factors combined in an additive way to increase the probability of ARR. Thus, the optimal method of prevention of ARR is likely to require a multipronged approach that addresses each factor. Although athymic nude mice do not precisely recapitulate the immune defects of human immunodeficiency virus (HIV) infection, their extreme T-cell immunodeficiency leads to poor containment of bacillary growth, reduced response to first-line TB drugs, and outgrowth of drug-resistant mutants (10). In this sense, they may well represent patients with advanced HIV-TB coinfection. Indeed, the selection of ARR in 18.0% of nude mice and 0% of BALB/c mice receiving once-weekly P-containing regimens in the present experiment was remarkably similar to the 13.3% and 0% observed with the same regimen in HIV-positive and HIV-negative subjects, respectively, in TBTC study 22 (2, 3).

Administration of intermittent regimens to patients with advanced immunodeficiency has selected ARR in multiple settings (2, 8, 12, 14, 15) and did so in this preclinical study. A daily (5/7) intensive-phase regimen prevented ARR despite a continuation phase of once-weekly P monotherapy in nude mice, while an intermittent initial-phase regimen followed by the same once-weekly P continuation phase resulted in ARR in 50% of mice. This finding indicates that the intensive phase, by eradicating actively replicating bacilli and spontaneous drug-resistant subpopulations, is a critical period for preventing the selection of drug-resistant mutants. It reinforces recommendations to avoid intermittent initial-phase therapy in HIV-infected patients (13).

Development of ARR requires both sufficient rifamycin pressure to drive selective amplification of spontaneous rifamycin-resistant mutants and the absence of H and other companion drug exposures sufficient to counter this selection by killing the rifamycin-resistant mutants. In TBTC study 22, this was illustrated by the occurrence of ARR in subjects with very low plasma H concentrations but not low rifamycin exposures (16). Therefore, we hypothesized that, compared to prior results in nude mice in which daily RHZ/RH therapy selected only AHR (10), greater selection of ARR would result from increasing the rifamycin selection pressure by replacing R with P and by decreasing H exposure through intermittent dosing and dosage reduction to represent rapid H acetylation phenotypes. The observed association of ARR with once-weekly P-containing regimens and very low, especially zero, H concentrations supports this hypothesis. While our lower dose of H was not associated with ARR, the absence of H in the continuation phase was. Together with the evidence of very low H concentrations in 2 subjects with ARR in TBTC study 22 (16), this result provides further evidence that very low H exposures resulting from pharmacokinetic (PK) variability put such HIV-TB patients at greater risk of ARR with intermittent therapy. The magnitude of PK variability for INH and the rifamycins in the human population is profound and is increasingly appreciated as a key driver of the selection of drug-resistant mutants (17).

Rifamycin concentrations may also be an important driver of ARR, as exemplified in TBTC study 23 (18). However, higher rifamycin exposures may be expected to limit the selective amplification only of mutations conferring low levels of rifamycin resistance (19), as the most common rifamycin resistance mutations confer high-level resistance

that is unlikely to be overcome even by the higher rifamycin doses now being studied in clinical trials, including the most commonly observed mutations, such as S531L, H526Y, and S522L, which were also observed in this study. As such, there was no observed effect of P dose on ARR in the present study. However, as expected, a trend of decreasing AHR with increasing doses of P was observed. Thus, optimizing the dose of both the H and rifamycin components will be key to preventing both ARR and AHR and thereby to blocking the pathway of sequential mutations leading to MDR TB. In the present study, MDR *Mycobacterium tuberculosis* was selected in one nude mouse from group 7. Although the proportion of MDR colonies was below the clinical breakpoint of 1%, it was substantially higher than the spontaneous frequency of an MDR mutant in the lungs of any single nude mouse at the initiation of treatment, which was estimated to be approximately $<10^{-12}$ from our day 0 data. The MDR phenotype is caused by the sequential accumulation of mutations in different genes involved in individual drug resistance (20). Given that the total number of bacteria present in the entire nude mouse population at the start of treatment was $\leq 10^{10}$, it is unlikely that the MDR mutant was present at the initiation of treatment. As H-resistant mutants comprised 0.7% and 3.4% of the total CFU in 2 of the 9 mice from groups 6 and 11 sacrificed between weeks 8 and 10 of treatment, it is more likely that an H-resistant mutant was initially selectively amplified during the intermittent initial phase and that a second *rpoB* mutation was acquired and amplified during the once-weekly P continuation phase.

All ARR in our experiment was associated with *rpoB* mutations commonly observed among clinical isolates, including 85.7% caused by mutations in codons 531 and 526 (21). Three of the 4 cases of ARR among HIV-positive subjects in study 22 shared the same mutations with all 6 tested ARR isolates from nude mice receiving the same regimens (2). Seven of nine H-resistant isolates had detectable mutations in the *katG* gene and not in the *inhA* promoter. Unlike H resistance in clinical isolates, which is dominated by *katG* 315T mutations, AHR in the current experiment was caused by a variety of unique *katG* mutations. This is consistent with our prior published observations (10, 22) and our unpublished observations in BALB/c and C3HeB/FeJ mice treated with a range of H doses and in the hollow-fiber infection model and suggests that either the fitness cost of most *katG* mutations is higher in humans than in these other models or additional fitness costs imposed by transmission also influence the distribution of H resistance mutations in clinical isolates (23). The high peak concentrations produced by the H doses used for intermittent regimens in our experiment were likely sufficient to prevent selective amplification of the low-level resistance associated with *inhA* promoter mutations and perhaps the lower level of resistance associated with the 315T mutation compared to the higher level of resistance expected with more complete disruption of KatG (24, 25). Two H-resistant isolates showed no *katG* or *inhA* promoter mutation. The findings are similar to previous results (10) and may be explained by other loci associated with H resistance.

The delayed attainment of culture negativity with the intermittent-treatment control regimens in nude mice supports recommendations to avoid twice-weekly HR in patients with advanced HIV (1) and the conclusion of a recent systematic review suggesting that administration of at least 8 months of therapy with daily dosing in the initial phase might improve outcomes in HIV-TB coinfected patients (26).

In conclusion, the results of this preclinical study indicate that the high rate of ARR among HIV-positive subjects in TBTC study 22 (2, 3) resulted from a "perfect storm" of risk factors: advanced HIV infection, intermittent initial-phase therapy, once-weekly continuation-phase therapy, and barely detectable H concentrations. They serve to emphasize the complex interplay between host immunity, the frequency of spontaneous resistance mutations and their fitness costs, the composition and scheduling of the treatment regimen, and key drug exposures in determining whether or not resistance to a given drug will emerge. A single-animal model cannot be expected to encompass the heterogeneity in each of these factors encountered in human TB. Our results support the use of nude mice as a more stringent model for stress-testing the ability of

TABLE 5 Experimental scheme[a]

Treatment group	Drug regimen		Both strains or nude mice only?[b]	No. of mice sacrificed/total no. of mice					
	0–2 mos	2–6 mos		Mo 0.5	Mo 1	Mo 2	Mo 4	Mo 6	Total
1	Untreated		+/+		5/10				5/10
2	P 5/7	P 5/7	+/+		5/5	5/5	5/5	5/5	20/20
3		RH 5/7	+/+	5/5	5/5	5/5	5/5	5/5	25/25
4	RHZE 5/7	P 1/7	0/+				0/5	0/5	0/10
5		PH_{50} 1/7	+/+				5/5	5/5	10/10
6		RH_{25} 2/7	+/+		5/5	5/5	5/5	5/5	20/20
7		P 1/7	0/+				0/5	0/5	0/10
8	2wRHZE 5/7 + 6w$RH_{25}Z_{300}E_{200}$ 2/7	PH_{50} 1/7	+/+				5/5	5/5	10/10
9		$P_{15}H_{50}$ 1/7	0/+				0/5	0/5	0/10
10		$P_{20}H_{50}$ 1/7	0/+				0/5	0/5	0/10
11		$RH_{12.5}$ 2/7	+/+	5/5	5/5	5/5	5/5	5/5	25/25
12		P 1/7	0/+				0/5	0/5	0/10
13	2wRH_5ZE 5/7 + 6w$RH_{12.5}Z_{300}E_{200}$ 2/7	PH_{25} 1/7	+/+				5/5	5/5	10/10
14		$P_{15}H_{25}$ 1/7	0/+				0/5	0/5	0/10
15		$P_{20}H_{25}$ 1/7	0/+				0/5	0/5	0/10
Total			125/200[c]	15/20	20/20	20/20	35/70	35/70	125/200

[a]Drug doses (in mg/kg) if not otherwise specified: rifampin (R), 10; rifapentine (P), 10; isoniazid (H), 10; pyrazinamide (Z), 150; ethambutol (E), 100.
[b]+/+, arm included both BALB/c and nude mice; 0/+, arm included nude mice only.
[c]Four BALB/c mice sacrificed on day −17 and 6 nude mice sacrificed on day 0 were not included.

novel regimens to prevent the selection of drug resistance and studying the pharmacological factors associated with resistance selection, at least as it may pertain to patients with TB-HIV coinfection. As a number of novel treatment regimens are now being evaluated or considered in phase 2/3 clinical trials, new opportunities for evaluating the role of nude mice in regimen development will arise and warrant further study.

MATERIALS AND METHODS

Antimicrobials and mycobacterial strain. INH, RIF, PZA, EMB, and RFP were formulated and administered *per os*, as previously described (10). *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was subjected to passage in mice, frozen in aliquots, and subcultured prior to infection (10).

Mice and aerosol infection. Six-week-old female BALB/c mice ($n = 133$) and athymic nu/nu (nude) Swiss mice ($n = 212$) (Charles River, Wilmington, MA) were aerosol infected with *M. tuberculosis* H37Rv using a log-phase broth culture (4×10^7 CFU/ml). Four BALB/c mice and three nude mice were sacrificed on the same day of infection. Mice were assigned to each treatment group by block randomization by run. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Chemotherapy. Treatment began 17 days after infection. All treatment regimens consisted of an 8-week intensive phase followed by a 16-week continuation phase (Table 5). Intensive-phase regimens consisted of RHZE administered 5/7 for the entire 8 weeks or administered 5/7 for the first 2 weeks and twice weekly (2/7) thereafter. A variety of continuation-phase regimens followed each intensive-phase regimen, as detailed below and in Table 5. BALB/c mice served as immunocompetent controls and were allocated to a subset of treatment regimens. Drug doses selected to match the average area under the plasma concentration-time curve were as follows unless otherwise noted: for R, 10 mg/kg of body weight; for Z, 150 mg/kg; for H, 10 mg/kg; for E, 100 mg/kg; for P, 10 mg/kg (10, 22, 27). For 2/7 dosing, the Z, H, and E doses were increased to 300, 25, and 200 mg/kg, respectively. For 1/7 dosing, the H dose was 50 mg/kg and P was dosed at 10, 15, or 20 mg/kg. Group 1 was an untreated negative-control group, consisting of mice expected to succumb to infection before month 1. Group 2 was a control group consisting of mice receiving daily (5/7) P monotherapy to confirm that selection of ARR was possible. Groups 3, 4, and 5 received the standard first-line regimen administered 5/7 during the intensive phase followed by RH 5/7, P 1/7, and HP 1/7, respectively, in continuation phase. Groups 6 to 10 received a predominantly intermittent initial-phase regimen of RHZE administered 5/7 for 2 weeks and then 2/7 for 6 weeks, as allowed in the control arm in TBTC study 22. Group 6 received the continuation-phase regimen of RH 2/7, like the control arm in TBTC study 22. Group 7 received only P 1/7 during the continuation phase. Groups 8, 9, and 10 received PH 1/7, the continuation-phase regimen associated with ARR in TBTC study 22, with P doses of 10, 15, and 20 mg/kg, respectively, to investigate the effect of increasing P doses on resistance selection. Groups 11 to 15 had the same intermittent intensive-phase regimen as groups 6 to 10, respectively, except that the H dose was reduced by 50% to simulate the median plasma exposures in humans with a fast acetylation phenotype and to investigate its effect on selective amplification of resistant mutants (22).

Assessment of treatment efficacy. Treatment efficacy was assessed on the basis of lung CFU counts at predetermined time points (Table 5), the proportions of mice with selective amplification of resistant subpopulations and mortality during treatment. Lungs were homogenized, and serial 10-fold dilutions were plated in duplicate on selective 7H11 agar (10). Additional plates were supplemented with H (0.2 μg/ml) or R (1.0 μg/ml) to quantify the H- and R-resistant subpopulations. All plates were incubated for 28 days. The proportion of resistant colonies was calculated either from these screening plates or from indirect DST performed as described below. Isolates were considered to be resistant when the proportion of CFU growing on drug-containing media was $\geq$1% of the CFU count on drug-free media (28), whereas selective amplification of resistance was defined by the presence of resistant colonies whose proportion was at least 10 times higher than the baseline proportion of spontaneous resistant mutants (i.e., between 0.01% and 1% on INH-containing media and between 0.001% and 1% on RIF-containing media).

Drug susceptibility testing. DST for H and R was performed on resistant isolates obtained by direct plating of lung homogenates on H- and R-containing media with INH concentrations of 0.05, 0.2, 1, 4, and 16 μg/ml and R concentrations of 0.25, 1, 4, and 16 μg/ml. Colonies on drug-free and drug-containing agar plates were scraped together and pooled for each plate and were separately subjected to DST. Lung isolates showing growth on both H- and R-containing screening plates at the same time underwent additional susceptibility testing on agar mixed with both INH (0.2 μg/ml) and RIF (1.0 μg/ml) to identify multidrug resistance. The stock *M. tuberculosis* H37Rv strain used for the original infection was used as a control. The MIC was defined as the lowest drug concentration preventing at least 99% of the growth observed on drug-free plates.

Mutation analysis of resistant genes. Isolates resistant to H ($\geq$0.2 μg/ml) and/or R ($\geq$1 μg/ml) were subjected to mutation analysis. Two or three colonies from plates containing H (1 μg/ml), R (4 μg/ml), or H and R (0.2 and 1 μg/ml, respectively) were evaluated independently. The entire *katG* gene, the *inhA* promoter region, and the rifamycin resistance-determining region of the *rpoB* gene were amplified by PCR and sequenced (Genewiz, South Plainfield, NJ) (22, 29, 30).

Statistical analysis. CFU counts were $\log_{10}$ transformed before analysis. Group means were compared by one-way analysis of variance (ANOVA) with Dunnett's or Bonferroni's posttest, as appropriate. Proportions were compared using Fisher's exact test. GraphPad Prism (5.0; GraphPad, San Diego, CA) was used for all analyses.

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S.-W.P. and E.L.N. conceived and designed the study. S.-W.P., R.T., P.J.C., and E.L.N. performed the experiments and analyzed or interpreted the data. S.-W.P. and E.L.N. wrote the manuscript.

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