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In Vitro and In Vivo Activities of the Benzoxazinorifamycin KRM-1648 against *Mycobacterium tuberculosis*

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The in vitro and in vivo activities of a new benzoxazinorifamycin, KRM-1648 (KRM), against *Mycobacterium tuberculosis* were studied. The MIC at which 50% of the isolates are inhibited (MIC_{50}) and the MIC_{90} of KRM for 30 fresh isolates of *M. tuberculosis* measured by the BACTEC 460 TB System were 0.016 and 2 μg/ml, respectively. These values were much lower than those for rifampin (RMP), which were 4 and >128 μg/ml, respectively, and considerably lower than those for rifabutin (RBT), which were 0.125 and 8 μg/ml, respectively. A correlational analysis of the MICs of these drugs for the clinical isolates revealed the presence of cross-resistance of the organisms to KRM and either RMP or RBT although the MICs of KRM were distributed over a much lower range than were those of the other two drugs. KRM and RMP at concentrations of 1 to 10 μg/ml almost completely inhibited the bacterial growth of RMP-sensitive strains ($H_{37}Rv$, Kurono, and Fujii) of *M. tuberculosis* phagocytosed in macrophage-derived J774.1 cells. KRM was more active than RMP in inhibiting the growth of the RMP-resistant (MIC = 8 μg/ml) Kurata strain but failed to show such an effect against the RMP-resistant (MIC >128 μg/ml) Watanabe strain. When KRM was given to *M. tuberculosis*-infected mice at dosages of 5 to 20 mg/kg of body weight by gavage, once daily six times per week from day 1 after infection, it was much more efficacious than RMP against infections induced in mice by the RMP-sensitive Kurono strain, as measured by a reduction of rates of mortality, a reduction of the frequency and extent of gross lung lesions, histopathological changes in lung tissues, and a decrease in the bacterial loads in the lungs and spleens of infected mice. KRM also displayed significant therapeutic efficacy against infection induced by the RMP-resistant Kurata strain, while neither KRM nor RMP was efficacious against infection by the RMP-resistant Watanabe strain. In the case of infection with the Kurono strain, the efficacy of the drugs in prolonging the time of survival was in the order KRM, RBT, RMP. KRM was much more efficacious than RMP, when given at 1- to 4-week intervals. These findings suggest that KRM may be useful for the clinical treatment of tuberculosis contracted through RMP-sensitive strains, even when it is administered at long intervals.

Increases in the incidence of tuberculosis coincident with the AIDS epidemic have been reported in some countries in Africa (22) and in certain regions in the United States (1, 4). The recent emergence of multidrug-resistant tuberculosis (MDR-TB) (2, 5, 10, 14, 17, 18), defined as infection with *Mycobacterium tuberculosis* having an in vitro resistance to both isoniazid and rifampin (RMP) (6, 10, 17), in patients infected with the human immunodeficiency virus (HIV) has resulted in extremely high mortality rates ranging from 72 to 89%, with median intervals from diagnosis to death of 4 to 16 weeks in such patients (5, 7, 8, 11, 21). Thus, MDR-TB in immunocompromised hosts such as HIV-infected patients is almost entirely intractable to treatment. It is recommended that, in treating MDR-TB, clinicians should select for use at least three new drugs (usually four) which the patient has not received and to which the current isolate is susceptible (6, 17). For this type of use, the following drugs are candidates: pyrazinamide, ethambutol, ethionamide, cycloserine, streptomycin, capreomycin, kanamycin, amikacin, clofazimine, ofloxacin, ciprofloxacin, sparfloxacin, thiacetazone, and para-aminosalicylate. New quinolones in particular are recommended for use in such cases (6). However, RMP and isoniazid are considered the most effective drugs for the treatment of tuberculosis. Therefore, for clinical treatment of HIV patients with MDR-TB, certain new rifamycin derivatives can be also recommended, provided that they have much greater in vivo activities against MDR-tubercle bacilli than RMP. We previously found that a newly synthesized drug, KRM-1648 (KRM), 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl) benzoxazinorifamycin (29), displayed much more potent in vitro antimicrobial activity against slowly growing mycobacteria, including *M. tuberculosis*, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, and *Mycobacterium avium-Mycobacterium intracellulare* complex, than did RMP and rifabutin (RBT) (19, 25, 27). In addition, KRM was strongly efficacious against mycobacterial infections due to *M. avium*, *M. intracellulare*, *M. marinum*, and *Mycobacterium leprae* induced in mice, rats, and rabbits (9, 20, 24, 26, 28).

Although a few studies of the therapeutic efficacy of KRM against experimental *M. tuberculosis* infections induced in mice have been performed (13, 15), the profile of the anti-*M. tuberculosis* activity of this drug remains to be determined in detail. In particular, it is of interest to determine the therapeutic efficacy of KRM against infections with RMP-resistant *M. tuberculosis* strains, which frequently cause intractable tuberculosis in HIV-infected persons (6, 17). In this study, the in vitro activity of KRM against clinical isolates of *M. tuberculosis* was determined by a radiometric method (16, 25). We also evaluated the therapeutic efficacy of KRM against murine infections

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due to *M. tuberculosis* with varying levels of RMP resistance and its therapeutic effect in mice treated at various intervals.

MATERIALS AND METHODS

Organisms. For in vitro studies, 30 fresh isolates of *M. tuberculosis* from patients and two stock cultures ($H_{37}Rv$ and Kurono) in our laboratory were used. For in vivo studies, *M. tuberculosis* $H_{37}Rv$, Kurono, and Fujii (RMP-susceptible strains) and Kurata, Okamoto, Ii, Yokoyama, Nagai, and Watanabe (RMP-resistant strains) were used. Test organisms were subcultured in Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.).

Mice. Six-week-old female BALB/c mice, purchased from Japan Clea Co., Osaka, Japan, were used.

Drugs. KRM (KANEKA Corporation, Japan), RBT (Farmitalia Carlo Erba, Italy), and RMP (Daiichi Pharmaceutical Co., Japan) were used. For in vitro studies, these drugs were dissolved in dimethyl sulfoxide. Working solutions were prepared from the stock solutions by dilution with distilled water.

MIC determination. MICs of test drugs were determined with the BACTEC 460 TB System (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), as reported by Lee and Heifets (16).

Determination of MBC. Test organisms were inoculated into tubes containing 5 ml of Kirchner medium supplemented with 0.05% Tween 80, with or without the addition of serial twofold dilutions of test drugs. After a 2-week cultivation at 37°C, bacterial growth was observed to determine MICs. For MBC determination, the number of CFU in the 2-week-old cultures with test drugs at concentrations higher than or the same as those used to determine the MICs were counted on 7H11 agar (Difco) plates and compared with the CFU at time 0. MBC was defined as the minimum concentration of test drug which killed 99.9% of the organisms during the 2-week incubation. Experiments for determining individual MICs and MBCs were repeated at least three times.

Antimicrobial activities of test drugs against organisms ingested by macrophages. The intracellular killing of *M. tuberculosis* organisms phagocytosed by macrophages (Mϕs) because of test rifamycins was measured by the method of Blanchard et al. (3) with some modifications. The Mϕ-derived J774.1 cell line was maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, Md.). The Mϕs (10^5 cells) harvested in the middle-log phase were incubated in 0.2 ml of 1% FBS–RPMI 1640 medium containing *M. tuberculosis* (multiplicity of infection = 5) in 96-well culture dishes (Corning Glass Works, Corning, N.Y.) at 37°C for 4 h in a CO_2 incubator (95% air–5% CO_2). The Mϕs were then washed with 2% FBS–Hanks balanced salt solution and cultured in 1% FBS–RPMI 1640 medium with or without test drugs at 37°C in a CO_2 incubator for 2 days. After rinsing with 2% FBS–Hanks balanced salt solution, the Mϕs were lysed with 0.27% saponin, and subsequently the uptake of [^{3}H]glycerol (New England Nuclear Corp., Boston, Mass.) by surviving *M. tuberculosis* cells was measured as follows. To the resulting Mϕ lysate (100 μl), 100 μl of 7H9 medium containing 10 μCi of [^{3}H]glycerol per ml was added. After a 24-h cultivation at 37°C, the organisms were collected onto glass-fiber filter paper and radioactivity was determined in a liquid scintillation counter (Tri-Carb liquid scintillation spectrometer; Packard Instrument Co., Downers Grove, Ill.).

In vivo activities of rifamycin derivatives. *M. tuberculosis* $H_{37}Rv$, Kurono, Fujii, Kurata, Okamoto, Ii, Yokoyama, Nagai, or Watanabe was grown in 7H9 broth containing 0.05% Tween 80 at 37°C until its optical density (at 540 nm, 1.5 cm light path) reached about 0.3. The bacterial suspension was centrifuged at 1,000 rpm for 3 min to remove large bacterial clumps, and the upper layer was gently sonicated with a Handy Sonic sonicator (model 20P; Tomy Seiko Co., Tokyo, Japan). The resultant bacterial suspension was diluted with 7H9 medium to obtain an optical density of 0.2 at 540 nm and then used as an inoculum for infection. Mice were infected intravenously with the organisms at doses from 1.5×10^7 to 3.6×10^8 per mouse. The mice were given KRM, RMP, or RBT, finely emulsified in 2.5% gum arabic–0.2% Tween 80, by gavage, once daily six times per week from day 1 for up to 8 weeks after infection or until the end of the experiment (see the footnotes to Tables 1 to 5 for details). The therapeutic efficacy of a test drug was determined on the basis of the reduction of mortality rates of infected animals, the incidence of gross lesions in the visceral organs, and bacterial load at sites of infection, especially the lungs and spleens. Tissue homogenates in distilled water prepared with a glass homogenizer were decontaminated with NaOH at a final concentration of 0.2%, for 20 s, after which the homogenates were immediately neutralized with 0.5 N HCl, prior to serial dilution. The numbers of CFU in the visceral organs were counted by inoculating a serial 10-fold dilution with distilled water containing the tissue homogenates onto 7H11 agar plates and culturing them at 37°C for 2 weeks.

Histopathology. *M. tuberculosis*-infected mice with or without treatment with KRM were killed 8 weeks after infection. The lungs were removed and fixed in 10% formalin. Tissue sections were prepared and stained with hematoxylin and eosin and by the Ziehl-Neelsen technique.

Statistical evaluation. Student's *t* test and the χ^2 test were performed to determine the significance of differences in log CFU and survival rates between individual regimens, respectively.

TABLE 1. MICs of KRM, RBT, and RMP for clinical isolates and some reference strains of *M. tuberculosis*

Drug	MIC (μg/ml)[a] for:			
	Clinical isolates[b] at:		Reference strain	
	50% inhibition	90% inhibition	$H_{37}Rv$	Kurono
KRM	0.016	2	0.004	0.002
RBT	0.063	8	0.016	0.016
RMP	4	>128	0.125	0.063

[a] MICs were determined by the BACTEC 460 TB System.
[b] Thirty strains were tested for each MIC.

RESULTS

In vitro activity of KRM against *M. tuberculosis*. The in vitro anti-*M. tuberculosis* activity of KRM was compared with those of RBT and RMP. Table 1 shows the MICs of these drugs for clinical isolates and some reference strains of *M. tuberculosis*. KRM displayed more than 64-fold greater activity than RMP and four- to eightfold greater activity than RBT on the basis of the MICs at which 50 and 90% of the isolates were inhibited. As shown in Fig. 1A, a correlational analysis of the MICs for KRM (MIC_{KRM}) for individual test strains with those for RMP (MIC_{RMP}) revealed that the organisms showed cross-resistance to KRM and RMP, although the MICs of KRM were distributed over a significantly lower range than were those of RMP. Similar results were obtained for MICs of KRM and RBT, although the difference in the MIC ranges of the two drugs was not so large as in the above-described case (Fig. 1B). It is noteworthy that most RMP-resistant strains ($MIC_{RMP} \geq$ 128 μg/ml) were intermediately resistant to KRM (MIC_{KRM} = 0.5 to 4 μg/ml) (Fig. 1A). Figure 2 shows the susceptibility distribution of the test strains of *M. tuberculosis* to KRM, RBT, and RMP. MICs of these drugs were distributed as follows: KRM, ≤0.002 to 4 μg/ml; RBT, 0.004 to 16 μg/ml; RMP, 0.008 to >128 μg/ml. It is noteworthy that about 70 and 55% of test strains were susceptible to 0.125 μg of KRM and RBT per ml, respectively, while only 30% of the strains were sensitive to the same concentration of RMP. Moreover, there was no strain which was resistant to 8 μg of KRM per ml, although about 30% of the strains were highly resistant to RMP ($MIC_{RMP} \geq$ 128 μg/ml).

The MBCs and MICs of KRM and RMP were determined for five strains of *M. tuberculosis*, which were used as inocula to induce infection. The MBCs and MICs of the two drugs were measured by the broth dilution method with Kirchner medium. KRM and RMP each had a low MBC/MIC ratio of 2, demonstrating potent microbicidal activities against the strains of *M. tuberculosis* tested (data not shown).

Inhibitory effect of KRM on intracellular *M. tuberculosis*. The antimicrobial activities of KRM and RMP against the organisms phagocytosed in J774.1 Mϕ cells were examined. In our assay system, the change in vitality of phagocytosed bacteria during a 2-day cultivation was measured in terms of the residual ability of recovered organisms from Mϕs lysed with saponin to take up [^{3}H]glycerol. Thus, this method mainly assesses the extent of intracellular growth of test organisms after Mϕ phagocytosis. As shown in Table 2, both KRM and RMP almost completely inhibited the intracellular growth of RMP-sensitive *M. tuberculosis* (strains $H_{37}Rv$, Fujii, and Kurono). In the case of the RMP-resistant (MIC = 8 μg/ml) Kurata strain, the level of inhibitory activity of RMP but not of KRM was greatly decreased. In the case of the RMP-resistant (MIC >

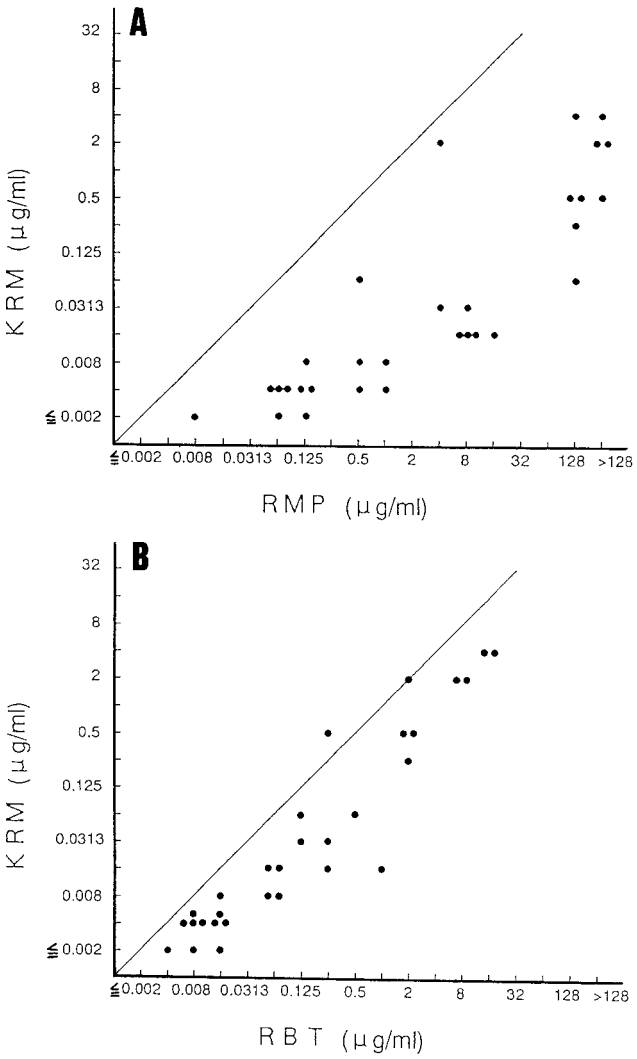

FIG. 1. Correlation between MICs of KRM and those of RMP (A) or RBT (B) for 30 clinical isolates of *M. tuberculosis*. MICs were determined by the BACTEC 460 TB System.

128 μg/ml) Watanabe strain, bacterial growth was only minimally affected by RMP, while KRM displayed decreased but still significant growth inhibitory activity.

Therapeutic efficacy of KRM against infections induced by RMP-sensitive or RMP-resistant strains. The therapeutic efficacy of KRM against murine infection due to the RMP-sensitive (MIC = 0.063 μg/ml) Kurono or the RMP-resistant Kurata strain was determined by measuring the prolongation of survival of infected animals. Infected mice (10 mice per regimen) were given KRM or RMP for 8 weeks from day 1 after infection. At week 8, 5 of 10 animals per regimen were killed, the incidence and extent of gross lung lesions were scored, and the bacterial loads in the lungs and spleens were counted. All of 10 untreated control mice died during the first 8 weeks. The mice that died were autopsied and subjected to the same examination described above at the time of death. The remaining five mice per regimen were observed for up to 30 weeks to determine their survival rates.

Table 3 shows the therapeutic efficacies of KRM and RMP against infections induced by either the RMP-sensitive Kurono strain or the RMP-resistant Kurata strain during an 8-week chemotherapy after infection. KRM reduced the incidence of gross lung lesions in mice infected with the RMP-sensitive Kurono strain or the RMP-resistant Kurata strain. The efficacy of KRM in this respect was much greater than that of RMP. Notably, KRM still significantly reduced the incidence of lung lesions in animals infected with the RMP-resistant Kurata strain, while RMP had no such effect.

With respect to the efficacies of KRM and RMP in reducing bacterial loads in the visceral organs during the 8 week-chemotherapy, the following results were obtained. In the case of infection due to the RMP-sensitive Kurono strain, both KRM and RMP displayed significant and dose-dependent efficacies in reducing the bacterial load in the lungs and spleens ($P < 0.01$, Student's t test), as compared with that of untreated control mice. Efficacy was observed even at the lowest dose of only 5 mg/kg of body weight. At doses of 5 and 10 mg/kg, the efficacy of KRM was significantly greater than that of RMP ($P < 0.01$). In addition, it was noted that KRM at doses of 5 to 20 mg/kg caused a steady elimination of bacteria in the lungs and spleens as follows. Bacterial load in the lungs at day 1 (5.5 log units) was decreased to 2.6 to 3.4 log units after an 8-week treatment with KRM. Similarly, the number of bacterial CFU in the spleens at day 1 (6.5 log units) was reduced to <1.8 to 2.8

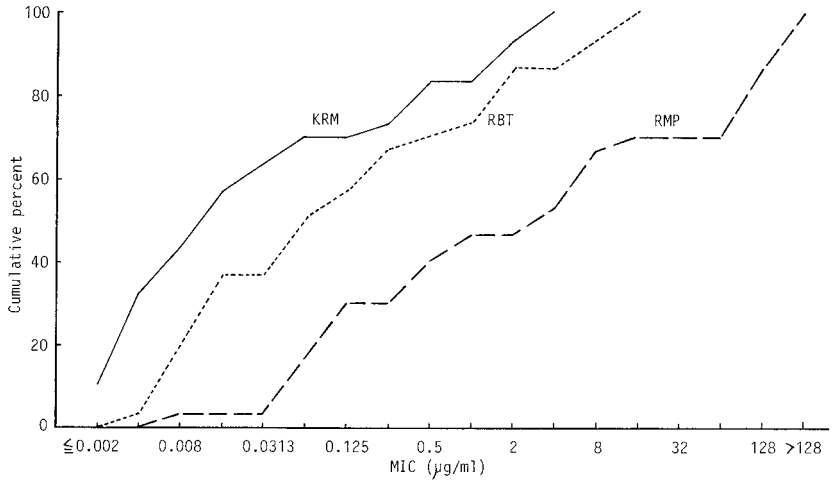

FIG. 2. Susceptibility distribution of 30 strains of clinically isolated *M. tuberculosis* to KRM (solid line), RBT (dotted line), and RMP (dashed line).

TABLE 2. Growth inhibition of *M. tuberculosis* phagocytosed in J774.1 Mϕs by KRM and RMP[a]

Strain	MIC (μg/ml)		[^{3}H]glycerol uptake by control culture (cpm)	Growth inhibition (%)[b] with:					
	KRM	RMP		KRM at (μg/ml):			RMP at (μg/ml):		
				1	5	10	1	5	10
$H_{37}Rv$	0.004	0.125	12,069	97.4	97.3	98.8	95.7	95.2	98.5
Kurono	0.002	0.063	15,163	95.6	95.4	96.9	91.5	93.0	96.1
Fujii	0.002	0.063	5,354	94.6	96.1	97.5	92.5	94.1	96.8
Kurata	0.016	8	2,163	83.4	87.6	90.8	53.3	72.8	83.3
Watanabe	4	>128	7,427	8.3	31.1	32.9	5.2	−4.5	−0.8

[a] J774.1 Mϕ cells infected with the indicated strains of *M. tuberculosis* were cultured for 2 days in the medium containing the indicated concentrations of either KRM or RMP. After cell lysis, [^{3}H]glycerol uptake by surviving organisms was measured as described in Materials and Methods.

[b] Growth inhibition (%) was calculated as growth inhibition (%) = (1 − ^{3}H uptake by the test culture with drugs/^{3}H uptake by the control culture without drugs) × 100. The means of four incubations are given. Variations of the estimated value of ^{3}H uptake (cpm) for each regimen with or without the test drugs ranged from 1.9 to 19.2% (the mean: 8.2 ± 0.7%) of each original volume. The rates at which growth of strains $H_{37}Rv$, Kurono, Fujii, and Kurata was inhibited by KRM and RMP at the indicated concentrations were all significant ($P < 0.05$ or 0.01). In the case of strain Watanabe, only the values for KRM at 5 and 10 μg/ml were significant ($P < 0.05$).

log units at week 8. A similar degree of bacterial elimination in the organs was observed only in mice given RMP at the dose of 20 mg/kg.

On the other hand, in mice infected with the RMP-resistant Kurata strain, the efficacy of each of the two drugs was markedly reduced. A significant reduction of bacterial loads, compared with that of untreated control mice, was observed only for higher doses of KRM (10 and 20 mg/kg) ($P < 0.01$). RMP did not significantly reduce bacterial loads. In this experiment, KRM (but not RMP) given at the dose of 20 mg/kg caused a slow elimination of bacteria in the spleen tissues during chemotherapy. The bacterial load at day 1 (6.4 log units) was decreased to 5.3 log units after an 8-week treatment with KRM. RMP was not efficacious in causing bacterial elimination from the sites of infection.

These findings indicate that both KRM and RMP were less effective against the RMP-resistant strain than against the RMP-sensitive strain. These results also indicate that KRM was potentially efficacious in eradicating tubercle bacilli of an RMP-sensitive nature and that its efficacy was much greater than that of RMP.

For each regimen five mice, which were given an 8-week treatment with either KRM or RMP as described above, were observed to have a prolonged survival time of up to 30 weeks after infection (Fig. 3 and 4). Both KRM and RMP markedly prolonged the survival time of mice infected with the RMP-sensitive Kurono strain (Fig. 3). The therapeutic efficacy of KRM was much greater than that of RMP in terms of prolonging the survival time of infected mice. A significant difference was observed between the number of surviving mice given

TABLE 3. Therapeutic efficacies of KRM and RMP against *M. tuberculosis* infection induced in mice in terms of reducing the frequency and extent of gross pulmonary lesions and the bacterial loads in their lungs and spleens[a]

Strain and MIC	Drug	Dose (mg/kg)	No. of mice	No. of mice with gross lung lesions in the amt of:[b]			Log CFU/organ (mean ± SE)[c] in:	
				None	1+ to 2+	3+ to 4+	Lungs	Spleens
Kurono	None	None	10	0	0	10	8.46 ± 0.07	7.31 ± 0.09
MIC_{KRM}, 0.002 μg/ml	KRM	5	5	5	0	0	3.41 ± 0.04[d,e]	2.84 ± 0.40[d,e]
	KRM	10	5	5	0	0	3.17 ± 0.12[d,e]	2.58 ± 0.44[d,e]
	KRM	20	5	5	0	0	2.56 ± 0.40[d]	<1.77[d,e]
MIC_{RMP}, 0.063 μg/ml	RMP	5	5	0	0	5	6.78 ± 0.07[d]	5.88 ± 0.11[d]
	RMP	10	5	0	5	0	5.23 ± 0.10[d]	4.59 ± 0.22[d]
	RMP	20	5	5	0	0	3.39 ± 0.24[d]	2.56 ± 0.28[d]
Kurata	None	None	5	0	0	5	6.61 ± 0.13	6.30 ± 0.15
MIC_{KRM}, 0.016 μg/ml	KRM	5	4	3	1	0	6.29 ± 0.14	6.29 ± 0.07
	KRM	10	4	4	0	0	5.98 ± 0.11[d,e]	5.91 ± 0.09
	KRM	20	5	5	0	0	4.97 ± 0.25[d,e]	5.28 ± 0.06[d,e]
MIC_{RMP}, 8 μg/ml	RMP	5	5	0	1	4	6.83 ± 0.16	6.48 ± 0.24
	RMP	10	5	0	1	4	6.89 ± 0.16	6.43 ± 0.21
	RMP	20	5	0	1	4	6.74 ± 0.15	6.40 ± 0.12

[a] Mice infected with either the RMP-sensitive Kurono strain (2.0×10^7 CFU) or the RMP-resistant Kurata strain (1.5×10^7 CFU) were given or not given the indicated doses of KRM or RMP for 8 weeks from day 1 after infection. The mice were killed at week 8; their lungs were subjected to scoring of gross lesions and their visceral organs were subjected to counting of bacterial loads. Untreated control mice infected with the Kurono strain died during days 43 to 52 (on average, they survived 48.2 days). Mice that died were subjected to the same examinations at the time of death.

[b] None, no macroscopic lesions; 1+, less than 20 small nodules; 2+, more than 20 small nodules; 3+, many small nodules with a few large nodules; 4+, many large nodules.

[c] Bacterial loads (log CFU) of the lungs and spleens on day 1 after infection were 5.50 ± 0.07 and 6.51 ± 0.03, respectively, for strain Kurono and 5.52 ± 0.05 and 6.43 ± 0.04, respectively, for strain Kurata.

[d] Significantly different from untreated control mice ($P < 0.01$).

[e] Significantly different from RMP-treated mice ($P < 0.01$).

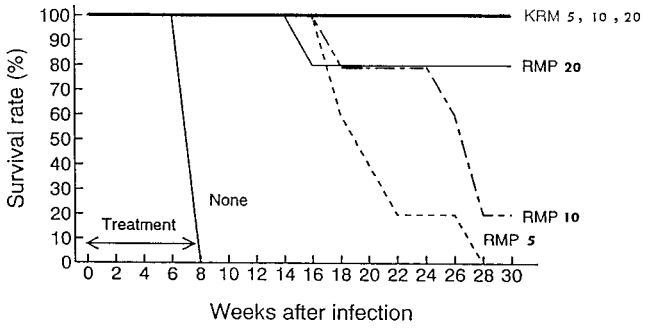

FIG. 3. Therapeutic efficacy of KRM and RMP against RMP-sensitive *M. tuberculosis* Kurono infection induced in mice in terms of reducing the rate of mortality of infected animals. Mice infected with 2.0×10^7 CFU of organisms were given the indicated doses of either KRM or RMP for 8 weeks from day 1 after infection (Table 3) and thereafter were observed until week 30 for their ability to survive.

KRM and those given RMP at doses of 5 mg/kg ($P < 0.05$, χ^2 test with Yate's modification). Figure 4 shows the results of the study of infection by the RMP-resistant Kurata strain. In this case, KRM prolonged the survival time of infected mice in a dose-dependent manner. KRM at doses of 10 and 20 mg/kg increased the number of survivors, while RMP failed to display such an effect. Moreover, a significant difference was observed between the number of surviving mice given KRM and those given RMP at the 20-mg/kg dose ($P < 0.05$).

Next, we assessed therapeutic activity of these drugs on the basis of the eradication of tubercle bacilli in mice which were given the drug during the first 8 weeks; thereafter the mice were observed for survival rates for up to 30 weeks after infection. As shown in Table 4, in the case of RMP-sensitive Kurono strain infection, all the mice that were given KRM treatment and that survived for 30 weeks after infection were free of gross lung lesions and displayed markedly reduced bacterial loads in their lungs and spleens even at the lowest dose (5 mg/kg). In this case, we observed no obvious bacterial growth at the sites of infection (lungs and spleens) in mice given 5 to 20 mg of KRM per kg during the 22 weeks subsequent to the discontinuation of chemotherapy: there were 2.6 to 3.4 log units of bacterial CFU in the lungs at week 8 versus 2.4 to 2.8 log units at week 30 and there were <1.8 to 2.8 log units of bacterial CFU in spleens at week 8 versus 2.1 to 2.7 log units at week 30.

RMP also decreased the incidence and extent of gross lung lesions and reduced the bacterial loads in survivors; however, its therapeutic efficacy was much less than that of KRM in these respects. In four mice which survived because of RMP treatment at the dose of 20 mg/kg, bacterial growth during the observation period was suppressed compared with that of mice that died in spite of treatment with a low dose of RMP (5 or 10 mg/kg). The results were as follows: there were 3.4 log units of bacterial CFU in the lungs of RMP-treated mice at week 8 versus 4.0 log units at week 30 (for dead mice, the results were 7.5 log units) and there were 2.6 log units of bacterial CFU in the spleens at week 8 versus 3.9 log units at week 30 (for dead mice, the results were 6.4 to 6.7 log units).

In the case of RMP-resistant Kurata strain infection, KRM did not reduce the incidence of gross lung lesions in survivors at week 30 but did reduce the extent of their lung lesions. All of the infected mice given RMP died during weeks 16 to 28. In the two types of infection, mice that died during the observation period despite KRM or RMP treatment showed similar extents of lung lesions and bacterial loads in their lungs and spleens.

In separate experiments, KRM and RMP each failed to display any significant efficacy in reducing gross lung lesions or bacterial loads in visceral organs, in the case of infections due to RMP-resistant *M. tuberculosis* ($MIC_{RMP} \geq 128$ μg/ml) strains, including Okamoto ($MIC_{KRM} = 0.063$ μg/ml), Ii ($MIC_{KRM} = 0.25$ μg/ml), Yokoyama ($MIC_{KRM} = 0.5$ μg/ml), Nagai ($MIC_{KRM} = 0.5$ μg/ml), and Watanabe ($MIC_{KRM} = 4.0$ μg/ml) (data not shown).

Comparison of therapeutic efficacies of KRM, RBT, and RMP. Figure 5 shows the therapeutic efficacies of KRM, RBT, and RMP against *M. tuberculosis* Kurono infection as measured by the prolongation of survival of infected mice. KRM and RBT at 2.5 and 5.0 mg/kg and RMP at 5.0 mg/kg each completely eliminated mortality among infected mice. KRM at 0.5 mg/kg exhibited significantly higher efficacy in retarding the death of mice than did RBT ($P < 0.05$, χ^2 test) and RMP ($P < 0.01$) at the same dose. The efficacy of RMP at 2.5 mg/kg was similar to that of RBT at 0.5 mg/kg. KRM, RBT, and RMP at 0.25 mg/kg significantly prolonged survival time ($P < 0.01$) beyond that of untreated mice in the order KRM, RBT, RMP. These findings indicate the superiority of KRM to RBT and RMP in reducing mortality among mice infected with RMP-sensitive *M. tuberculosis* organisms.

Therapeutic effect of KRM given at various frequencies of dosing. Table 5 shows the therapeutic efficacy of KRM and RMP given at various frequencies of dosing for up to 8 weeks after infection with *M. tuberculosis* Kurono. The following results were obtained. First, KRM reduced the severity of lung lesions even when given only once per 4 weeks. In addition, KRM decreased the incidence of lung lesions when given once per 2 weeks. Complete inhibition of the incidence of lung lesions was observed when KRM was given once weekly. In contrast, RMP reduced only the severity of lung lesions even when administered biweekly or daily, six times per week.

Second, KRM completely eliminated the bacterial loads in the lungs and spleen when given daily, six times per week, whereas RMP given at the same frequency reduced but did not eliminate bacterial loads (3.7 and 4.2 log decreases for lungs and spleens, respectively). In addition, KRM given once per 4 weeks still caused 2.3 and 1.9 log decreases in the bacterial loads in the lungs and spleens, respectively, compared with those in untreated control mice ($P < 0.01$, Student's t test),

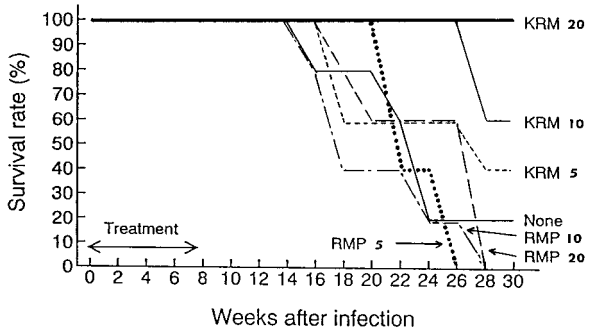

FIG. 4. Therapeutic efficacy of KRM and RMP against RMP-resistant *M. tuberculosis* Kurata infection induced in mice in terms of reducing the rate of mortality of infected animals. Mice infected with 1.5×10^7 CFU of organisms were given the indicated doses of either KRM or RMP for 8 weeks from day 1 after infection (Table 3) and thereafter were observed until week 30 for their ability to survive.

TABLE 4. Therapeutic efficacies of KRM and RMP against *M. tuberculosis* infection induced in mice in terms of reducing the frequency and extent of gross lung lesions and bacterial loads in the lungs and spleens[a]

Strain and MIC	Drug	Dose (mg/kg)	Survival time (weeks)	Fate	No. of mice	No. of mice with gross lung lesions in the amt of:			Log CFU/organ (mean ± SE) in:	
						None	1+ to 2+	3+ to 4+	Lungs	Spleens
Kurono	None	None	8	Died	10	0	0	10	8.46 ± 0.07	7.31 ± 0.09
MIC_{KRM}, 0.002 μg/ml	KRM	5	30	Killed	5	5	0	0	2.73 ± 0.26[b]	2.66 ± 0.22[b]
	KRM	10	30	Killed	5	5	0	0	2.81 ± 0.26[b]	2.17 ± 0.25[b]
	KRM	20	30	Killed	5	5	0	0	2.43 ± 0.28[b,c]	2.07 ± 0.30[b,c]
MIC_{RMP}, 0.063 μg/ml	RMP	5	18–28	Died	5	0	1	4	7.48 ± 0.10[b]	6.66 ± 0.33
	RMP	10	30	Killed	1	0	0	1	5.61	3.32
			18–28	Died	4	0	3	1	7.53 ± 0.10[b]	6.41 ± 0.37
	RMP	20	30	Killed	4	3	1	0	4.04 ± 0.45[b]	3.90 ± 0.45[b]
			18	Died	1	0	0	1	7.75	5.77
Kurata	None	None	30	Killed	1	0	0	1	6.78	5.18
MIC_{KRM}, 0.016 μg/ml			16–24	Died	4	0	0	4	7.01 ± 0.20	6.88 ± 0.34
	KRM	5	30	Killed	2	0	2	0	5.95 ± 0.23	4.35 ± 0.02
			18–28	Died	3	0	1	2	7.59 ± 0.05	6.61 ± 0.44
	KRM	10	30	Killed	3	0	3	0	6.27 ± 0.75	4.70 ± 0.42
			28	Died	2	0	0	2	7.52 ± 0.09	6.58 ± 0.09
	KRM	20	30	Killed	5	0	5	0	5.72 ± 0.20	4.98 ± 0.37
MIC_{RMP}, 8 μg/ml	RMP	5	22–26	Died	5	0	0	5	7.03 ± 0.11	6.44 ± 0.27
	RMP	10	16–28	Died	5	0	1	4	7.32 ± 0.17	7.28 ± 0.17
	RMP	20	20–28	Died	5	0	3	2	7.23 ± 0.28	6.74 ± 0.25

[a] Mice infected with the *M. tuberculosis* Kurono or Kurata strain were given or not given KRM or RMP for 8 weeks as described in Table 3 and thereafter observed for an additional 22 weeks for their rate of survival (Fig. 3 and 4). At the time of the death, the lung tissue of each mouse was observed for gross lesions and bacterial loads in the lungs and spleens were counted. Surviving mice were killed at week 30 and subjected to the same examination. Untreated control mice were the same as those represented in Table 3. The other details of the experiment are as stated in Table 3.

[b] Significantly different from untreated control mice ($P < 0.01$).

[c] Significantly different from RMP-treated mice ($P < 0.01$).

while RMP given by the same protocol failed to exert such a significant efficacy. Notably, KRM given once per 2 weeks decreased bacterial loads in the lungs and spleens (2.8 and 2.2 log decreases, respectively), as in the case with RMP given biweekly (2.3 and 2.1 log decreases, respectively). These findings indicate that KRM is much more efficacious than RMP, when given at a very low frequency of dosing ($P < 0.01$, Student's t test).

Lung histopathology. Figure 6 shows the histopathological findings for hematoxylin- and eosin-stained lung tissue of mice treated or not treated with KRM, at week 8 after infection with the *M. tuberculosis* Kurono strain. The lungs of untreated mice (Fig. 6A) displayed prominent and diffuse infiltration of mononuclear phagocytes and alveolar exudation of fibrin, although lesions lacked obviously demarcated tubercles, Langhans cells, and necrosis. Nearly confluent granulomatous foci were observed. A number of acid-fast bacilli were found in the lesions on Ziehl-Neelsen-stained sections (data not shown). On the other hand, in the lungs of mice treated with KRM (Fig. 6B), alveolar tissue and bronchi were completely free of infiltration by inflammatory cells and no tubercle bacilli were found in the lesions (data not shown). These findings indicate that KRM was efficacious in preventing an inflammatory reaction in the lungs of mice infected with the RMP-sensitive *M. tuberculosis* Kurono strain. In infected mice treated with RMP, sporadic tubercle foci with mononuclear phagocyte accumulation were observed (Fig. 6C). A small number of acid-fast bacilli were observed in such foci (data not shown).

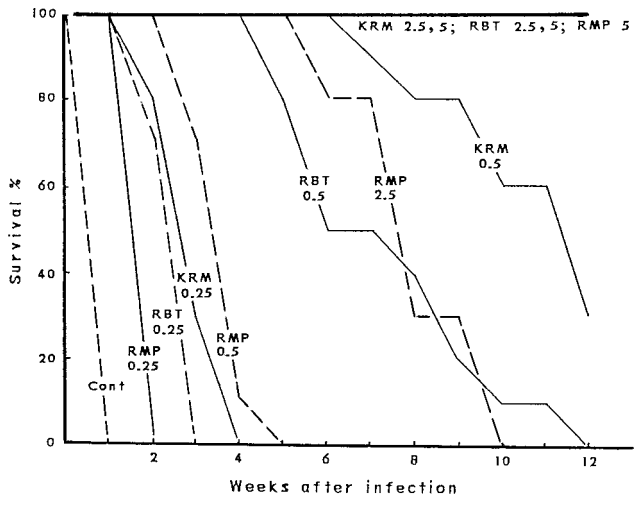

FIG. 5. Therapeutic efficacy of KRM, RBT, and RMP against *M. tuberculosis* Kurono infection induced in mice as measured by the prolongation of the survival time of infected animals. Mice infected with 3.6×10^8 CFU of organisms were given the indicated doses of either KRM, RBT, or RMT for 12 weeks from day 1 after infection.

DISCUSSION

In this study, we found that KRM had much more activity in vitro against clinical isolates of *M. tuberculosis* than did RMP and RBT. In addition, KRM showed excellent efficacy in the treatment of experimental tuberculosis in mice induced by RMP-sensitive *M. tuberculosis*, as measured by the prolongation of survival of host mice, a reduction of the frequency and/or extent of lung lesions, and bacterial eradication in visceral organs. The efficacy of KRM was much greater in this respect than that of RMP. Moreover, KRM also had significantly greater efficacy than RBT in reducing the mortality of infected mice. Kuze et al. (15) reported promising activity of KRM against *M. tuberculosis* $H_{37}Rv$ infection in ddY outbred

TABLE 5. Therapeutic efficacies of KRM and RMP given at various intervals against *M. tuberculosis* Kurono infection induced in mice[a]

Drug	Dose (mg/kg)	Administration protocol (times/week)	No. of mice with gross lung lesions in the amt of:[b]			Log CFU/organ (mean ± SE)[c] in:	
			None	1+ to 2+	3+ to 4+	Lungs	Spleens
None	None	0/0	0	0	5	8.12 ± 0.10	7.88 ± 0.04
KRM	10	6/1	5	0	0	<1.77	<1.77
	10	2/1	5	0	0	2.28 ± 0.26[d,e]	4.02 ± 0.32[d,e]
	10	1/1	5	0	0	3.46 ± 0.08[d,e]	4.53 ± 0.22[d,e]
	10	1/2	3	2	0	5.36 ± 0.35[d,e]	5.64 ± 0.11[d,e]
	10	1/4	0	5	0	5.85 ± 0.03[d,e]	6.02 ± 0.06[d,e]
RMP	10	6/1	3	2	0	4.46 ± 0.06[d]	3.64 ± 0.19[d]
	10	2/1	0	4	1	5.84 ± 0.10[d]	5.78 ± 0.06[d]
	10	1/1	0	0	5	7.29 ± 0.18[d]	6.66 ± 0.09[d]
	10	1/2	0	0	5	7.13 ± 0.14[d]	7.00 ± 0.19[f]
	10	1/4	0	0	5	7.93 ± 0.10	7.22 ± 0.07[d]

[a] Mice infected with 2.7×10^7 CFU of the RMP-sensitive Kurono strain were given a 10 mg/kg dose of either KRM or RMP at various intervals as indicated here for up to 8 weeks from day 1 after infection. Mice were killed at week 8.
[b] The amounts are defined in Table 3. The number of mice tested for each protocol was five.
[c] Bacterial loads (log CFU) of visceral organs on day 1 after infection were as follows: lungs, 5.56 ± 0.06; spleens, 6.81 ± 0.02.
[d] Significantly different from untreated control mice ($P < 0.01$).
[e] Significantly different from RMP-treated mice ($P < 0.01$).
[f] Significantly different from untreated control mice ($P < 0.05$).

mice, in the form of an increased rate of survival of infected mice compared with that of untreated control mice. More recently, Klemens et al. (13) reported that KRM was more active than either RMP or RBT in reducing bacterial loads in the lungs and spleens of outbred CD-1 mice, when the drug was given at a dose of 20 mg/kg by gavage, 5 days per week for 4 weeks from week 1 after infection. Thus, our study confirmed and expanded these previous findings. Furthermore, KRM exerted significant therapeutic efficacy even when given to mice at a very low frequency such as once per 4 weeks. The high durability of the in vivo activity of KRM might be due not only to its extremely potent antimicrobial activity but also to the peculiar nature of its pharmacokinetics. As reported by Yamane et al. (29) for a murine model, KRM showed pharmacokinetics comparable to those of RMP, especially in distribution to the lung tissue, gradually reaching a peak concentration there of 6.71 μg/g at 12 h after the administration of a single 20-mg/kg dose by gavage. In this case, the rate of accumulation of KRM in the lungs was much lower than that of RMP, which peaked (9.35 μg/g) within 1 h after administration. In the spleen, KRM gradually accumulated and peaked (16.3 μg/g) at 12 h, while RMP displayed a low and transient peak (2.25 μg/g) around 3 h after administration. These situations for in vivo activity of KRM will permit the establishment of a new protocol of tuberculosis therapy with long intervals of drug administration. Moreover, very short-term treatment with KRM of patients with tuberculosis may be possible, as proposed by Klemens et al. (13).

The analysis of correlation of MICs of KRM for individual isolates of *M. tuberculosis* with those of RMP and RBT indicated the presence of cross-resistance in most organisms to KRM and RMP and to KRM and RBT. In our recent study of the mechanism of the in vitro antimycobacterial activity of KRM, this drug displayed a level of inhibitory activity against RNA polymerase of *M. avium* similar to that of RMP but exhibited a markedly increased permeability into mycobacterial cytoplasms through the cell wall and/or cell membrane (13). This indicates that the activity of KRM against mycobacterial organisms, which is superior to that of RMP, is largely dependent upon its increased ability to accumulate at the target sites inside bacteria. This is consistent with our finding concerning the cross-resistance of test organisms of *M. tuberculosis* to KRM and RMP. In fact, Telenti et al. (23) recently showed that most populations (ca. 97%) of RMP-resistant *M. tuberculosis* had mutations in the gene *rpoB*, which encodes the RNA polymerase subunit β, within a region corresponding to 23 amino acids. This means that such *M. tuberculosis* organisms with mutations in gene *rpoB* might acquire a mutated RNA polymerase which is rendered resistant to KRM as well as to RMP, since the mode of inactivation of RNA polymerase by KRM seems to be the same as that of RMP described above. Further discussion on this problem must wait for a determination of the specific nature of the RMP resistance in the test strains used in this study by *rpoB* gene sequencing.

As expected from the finding of cross-resistance to KRM and RMP, the in vivo activity of KRM against RMP-resistant organisms in our murine model of tuberculosis was inferior to that against RMP-sensitive organisms, despite the facts that KRM showed excellent efficacy against tuberculosis induced by RMP-sensitive organisms and that its activity was much greater than those of RMP and RBT. However, it should be emphasized that KRM displayed significant efficacy against infections induced by RMP-resistant (MIC = 8 μg/ml) *M. tuberculosis*, while RMP lacked entirely this in vivo activity against this organism.

It should in any case be emphasized that tuberculosis due to RMP-resistant organisms is nearly intractable to treatment even when KRM, which shows excellent in vivo activity against a variety of mycobacteria, including *M. tuberculosis* and *M. avium* complex, is used (9, 13, 26, 28). This fact may indicate the unavoidable seriousness of the recent problem with the clinical treatment of MDR-TB. In the treatment of patients with RMP-resistant tubercle bacilli, appropriate protocols must be devised for KRM therapy, such as, for example, the use of drug-delivery systems or the combined use of KRM with other drugs. In fact, an additive therapeutic effect was found by Klemens et al. (13) with the use of KRM plus pyrazinamide or KRM plus isoniazid in the treatment of *M. tuberculosis* infection induced in mice. To solve these problems, in particular how to increase the efficacy of KRM against infections due to RMP-resistant tubercle bacilli, further detailed studies will be required.

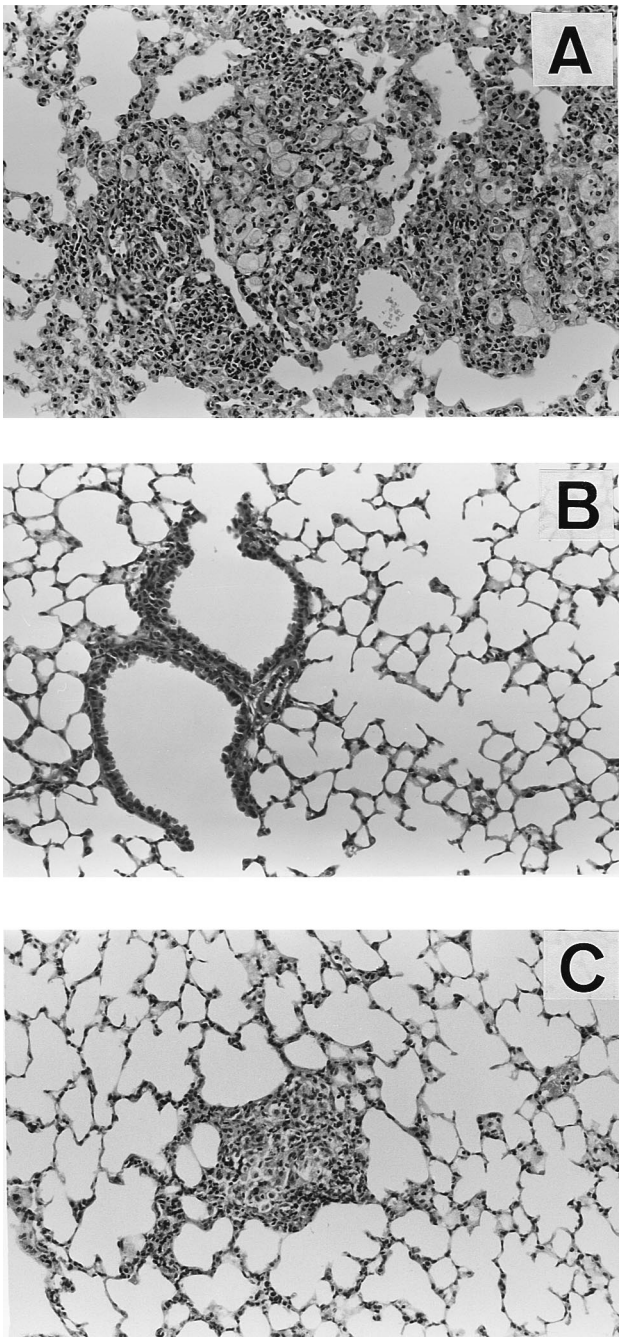

FIG. 6. Histopathology of the lungs of *M. tuberculosis*-infected mice with or without KRM treatment. Mice infected with 2.7×10^7 CFU of RMP-sensitive Kurono organisms were given 10 mg of KRM per kg for 8 weeks, and their lung tissue sections were subjected to microscopic observation after hematoxylin and eosin staining. (A) Untreated control mice. Magnification, ×79. (B) KRM-treated mice. Magnification, ×79. (C) RMP-treated mice. Magnification, ×79.

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