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Antagonism between Isoniazid and the Combination Pyrazinamide-Rifampin against Tuberculosis Infection in Mice

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Mice that had been inoculated intravenously with 6.30 $\log_{10}$ *Mycobacterium tuberculosis* H37Rv 14 days earlier were administered one of three combinations of drugs, i.e., isoniazid (INH)-rifampin (RMP)-pyrazinamide (PZA), INH-RMP, and RMP-PZA, during an initial 2-month period to mimic the initial phase of chemotherapy for human tuberculosis and during a later 4-month period to mimic the continuation phase of chemotherapy. At the end of the initial phase, all three combined regimens were found to have been highly effective in terms of the number of CFUs in the spleens of infected mice. The bactericidal activities of INH-RMP-PZA and INH-RMP were similar, whereas that of RMP-PZA was significantly greater. The spleens of all of the mice that had been treated initially with INH-RMP-PZA were culture negative by the end of 6 months of treatment, regardless of the regimen employed during the continuation phase. However, after an additional period of 6 months without treatment, the proportion of spleen culture positivity, or relapse rate, was significantly smaller in the subgroup treated with RMP-PZA during the continuation phase than in the subgroups treated with INH-RMP-PZA or INH-RMP; the relapse rate did not differ significantly between the latter two subgroups. These results suggest that antagonism occurs between INH and the combination RMP-PZA during both the initial and continuation phases of chemotherapy, compromising the benefit conferred by the addition of PZA to the combined regimen. The preliminary pharmacokinetic analysis suggested that the pharmacological interaction between INH and RMP was very likely to be involved in the mechanism of antagonism, as concomitant treatment with INH had significantly reduced the peak serum level and the area under the serum concentration-time curve of RMP in mice.

At present, the duration of short-course chemotherapy for tuberculosis may be as brief as 6 months. During an initial intensive phase of 2 months, patients are treated with daily isoniazid (INH), rifampin (RMP), and pyrazinamide (PZA), with or without ethambutol or streptomycin; then they are treated with daily or twice-weekly INH-RMP during a continuation phase of 4 months (6). Without the use of PZA in the initial phase, the relapse rate is unacceptable, therefore RMP and PZA appear to be obligate components of the initial phase of the 6-month regimen (6). However, it was reported that PZA did not enhance the therapeutic activity of the combination INH-RMP in humans during the continuation phase (2).

Our previous experiments on short-course preventive chemotherapy for tuberculosis in mice demonstrated that the sterilizing activity of RMP was significantly enhanced by the addition of PZA, and 2 months of treatment with the combination RMP-PZA was significantly more effective in terms of sterilizing organs and preventing subsequent disease than treatment with INH for 6 months (11). A surprising finding was that the combination RMP-PZA was more effective than the combination INH-RMP-PZA, suggesting antagonism between INH and the combination RMP-PZA (11). Confirmation of this finding by additional animal experiments would suggest the need to reconsider the composition of the regimens employed in the treatment for human tuberculosis. In an attempt to confirm the occurrence of this antagonism and to evaluate its possible cause, we have compared the activities of INH-RMP-PZA, INH-RMP, and RMP-PZA as both initial and continuation chemotherapies in established tuberculosis infection of mice and the major pharmacokinetic parameters of RMP and PZA in mice receiving or not receiving INH concomitantly.

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MATERIALS AND METHODS

Mice. Two-hundred and twenty outbred female Swiss mice 28 days of age were purchased from the Janvier Breeding Center, le Genest Saint-Isle, France.

Drugs. RMP (batch X0337) and PZA (batch 8005) were gifts of Merrel Dow, France, and INH (batch BA 290097) was a gift of Roche, France.

Infection with *Mycobacterium tuberculosis*. Mice were infected by intravenous inoculation of 0.5 ml of a suspension in Dubos broth diluted to contain 6.30 $\log_{10}$ *M. tuberculosis* H37Rv.

Chemotherapy. To assess the extent of *M. tuberculosis* infection before instituting chemotherapy, CFUs were enumerated in the individual spleens of 10 mice that had been inoculated with *M. tuberculosis* 14 days earlier. The remaining mice were randomly allocated to three groups, and treatment was initiated immediately; a group of 170 mice was treated with the combination INH-RMP-PZA, and two groups of 20 mice each were treated with the combination INH-RMP or RMP-PZA. Drugs were administered per os by an esophageal cannula (gavage) six times weekly in the following dosages chosen to provide serum concentrations in the mice comparable to those obtained in humans: INH, 25 mg/kg (5); RMP, 10 mg/kg (5); and PZA, 150 mg/kg (3, 5). After an initial period of treatment for 2 months to mimic the initial phase of chemotherapy, 20 mice of the group treated with INH-RMP-PZA and all of the mice of the remaining two groups were sacrificed for the enumeration of CFUs in the

spleens. The remaining mice of the group that had been initially treated with INH-RMP-PZA were randomly allocated to three subgroups with equal numbers of mice, each of which was treated with one of the combinations INH-RMP-PZA, INH-RMP, or RMP-PZA, for an additional period of 4 months to mimic the continuation phase of chemotherapy. At the end of the continuation phase, 10 mice of each subgroup were sacrificed for spleen cultures and the remaining mice were held without chemotherapy for an additional 6 months, after which all were sacrificed for spleen cultures.

Enumeration of CFUs and spleen cultures. Spleens were aseptically removed and homogenized by a standard procedure (7). To enumerate CFUs in the spleen, appropriate dilutions of the homogenates were plated onto Löwenstein-Jensen medium. At the end of the continuation phase chemotherapy and the 6-month follow-up period without chemotherapy, the entire homogenate of each spleen was plated without dilution onto 15 to 20 tubes of Löwenstein-Jensen medium. The results of the cultures were recorded after incubation at 37°C for 6 weeks (11).

Pharmacokinetic analysis. Female Swiss mice weighing 27 ± 2 g were divided into three groups: the untreated control and treated groups I and II. Mice of group I were treated daily by gavage with RMP-PZA, and the group II mice were treated with INH-RMP-PZA. The dosages of these compounds were exactly the same as for chemotherapy, i.e., INH, 25 mg/kg; RMP, 10 mg/kg; and PZA, 150 mg/kg. Twenty-four hours after the eighth dose of treatment, blood samples were collected from three mice of each group, pooled separately, and designated as 0-h samples; blood samples were again collected from three mice of each group at 0.25, 0.5, 1, 2, 4, 8, and 16 h after the ninth dose and pooled separately. In addition, blood samples were also collected from three mice of the untreated control group and pooled. The experiments were repeated in triplicate. Serum INH, RMP, and PZA levels were determined by high-pressure liquid chromatographic methods (9, 10, 13). The area under the serum concentration-time curve (AUC) was calculated by using the linear trapezoidal rule up to the final measured concentration and then extrapolating to infinity (4). The terminal half-life ($t_{1/2}$) was determined by regression analysis. The peak serum level (C_{max}) was defined as the highest drug concentration among all the samples; and T_{max} was the corresponding time of sampling when C_{max} occurred.

Statistical analysis. The results obtained from each group or subgroup were compared by means of the Student *t* test and the chi-square test; differences were considered significant at the 95% level of confidence.

RESULTS

Mortality. As shown in Table 1, 10 and 13 mice died during the initial and continuation phases, respectively, and 20 mice died during the 6-month follow-up period without chemotherapy. Because of postmortem necrosis, it was possible to culture the spleens of only three animals that died during the follow-up period: one negative, one positive, and one contaminated. The relatively small number of colonies of *M. tuberculosis* isolated from the spleens of mice sacrificed at the end of the follow-up period suggested that it is unlikely indeed that the deaths were caused by the infection of *M. tuberculosis*. Because the mortality rates did not differ significantly among the three groups and three subgroups ($P > 0.05$), whatever the cause of the deaths, the mortality did not affect the comparison of antimicrobial activities among different groups or subgroups.

TABLE 1. Numbers of mice in study groups and number that died during each phase of the study

Phase and treatment group[a]	No. of mice		
	Enrolled[b]	Died[c]	Killed[d]
Initial			
INH-RMP-PZA	170	10	20
INH-RMP	20	0	20
RMP-PZA	20	0	20
Continuation			
INH-RMP-PZA	46	4	10
INH-RMP	47	4	10
RMP-PZA	47	5	10
Follow-up			
INH-RMP-PZA	32	7[e]	25
INH-RMP	33	4	29
RMP-PZA	32	9[f]	23

[a] Phases are described in the text. All three subgroups during the continuation and follow-up phases were treated with INH-RMP-PZA during the initial phase.
[b] Number of mice enrolled at the beginning of the phase.
[c] Number of mice that died during the phase.
[d] Number of mice sacrificed at the end of the phase.
[e] Two spleens from the dead mice were cultured; one was found to be *M. tuberculosis* negative, and the other was contaminated.
[f] One spleen from a dead mouse was cultured and found to be positive for *M. tuberculosis*.

Antibacterial activities of the chemotherapy combinations during the initial phase. At the start of chemotherapy, 14 days after inoculation of the mice, cultures from the spleens of all 10 mice were *M. tuberculosis* positive; the mean number of CFUs was 6.65 ± 0.19 $\log_{10}$ per spleen, indicating that the infection of *M. tuberculosis* was well established. At the end of the initial phase of chemotherapy, *M. tuberculosis* was isolated from the spleens of all but one each of the mice treated with the combinations INH-RMP-PZA and RMP-PZA and from all of the mice treated with INH-RMP. The numbers of CFUs in the spleens were (mean ± standard deviation) 2.79 ± 0.73 $\log_{10}$, 3.00 ± 0.54 $\log_{10}$, and 0.86 ± 0.44 $\log_{10}$ for the mice treated with INH-RMP-PZA, INH-RMP, and RMP-PZA, respectively; all of these values are significantly smaller than the pretreatment value ($P < 0.001$). The difference between the CFUs in the spleens of mice treated with INH-RMP-PZA and those treated with INH-RMP was not significant ($P > 0.05$), whereas the CFUs in the spleens of mice that had been treated with RMP-PZA were significantly smaller than those of the other two groups ($P < 0.001$).

Antibacterial activities of the chemotherapy combinations during the continuation phase. *M. tuberculosis* was not isolated from the spleen of any of the mice sacrificed after the initial treatment with INH-RMP-PZA and continuation treatment with the same combination, with INH-RMP, or with RMP-PZA.

Relapse after cessation of chemotherapy. Relapse, defined as the isolation of *M. tuberculosis* from a spleen obtained during or at the end of 6 months of follow-up without chemotherapy, was found to have occurred in 9 of 26 (34.6%), 11 of 29 (37.9%), and 2 of 24 (8.3%) mice that had been treated during the continuation phase with INH-RMP-PZA, INH-RMP, or RMP-PZA, respectively. Relapses were significantly less frequent among the mice that had been

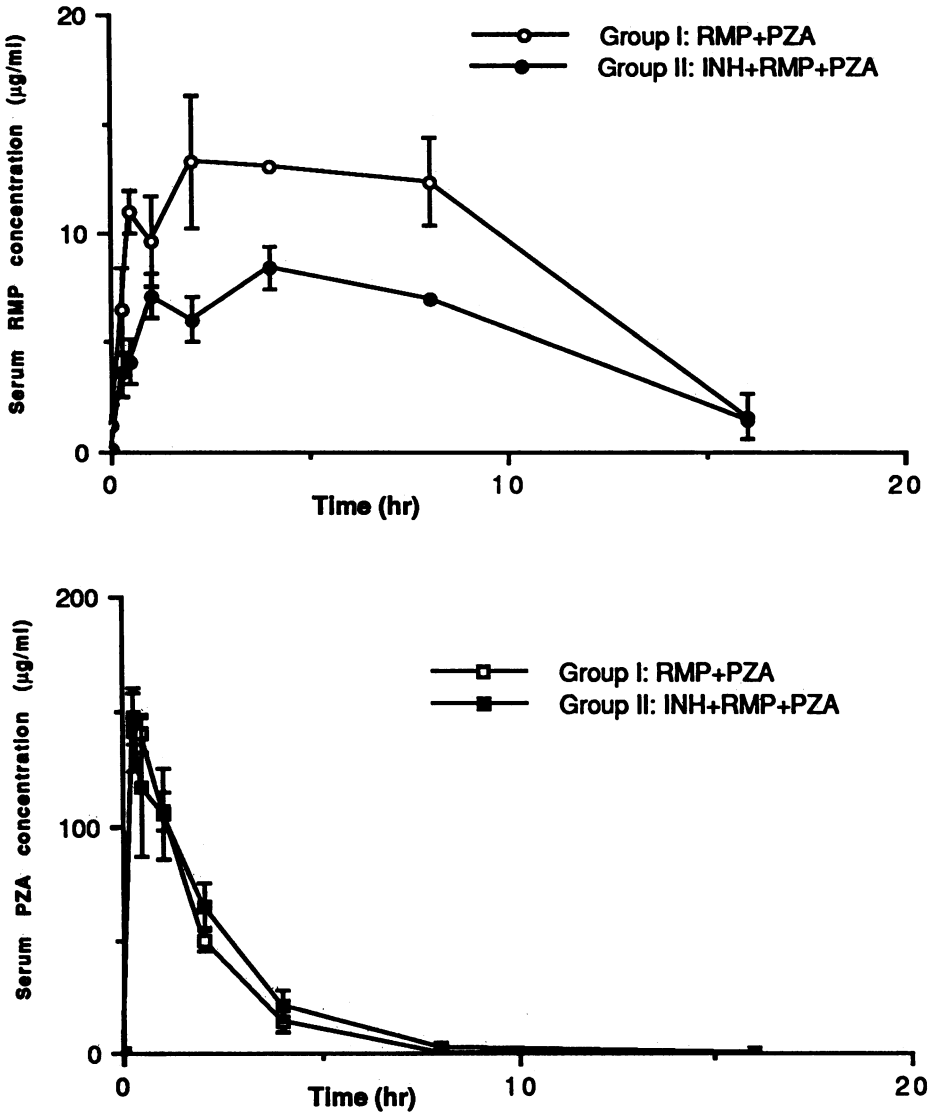

FIG. 1. Serum concentration-time curves of RMP and PZA in mice treated with RMP (10 mg/kg)-PZA (150 mg/kg) (group I) and with INH (25 mg/kg)-RMP (10 mg/kg)-PZA (150 mg/kg) (group II). Mice had been pretreated with the regimen for 8 daily gavages. Blood samples were collected from three mice of each group at 24 h after the eighth dose, pooled separately, and designated as 0-h samples; they were collected again from three mice of each group at 0.25, 0.5, 1, 2, 4, 8, and 16 h after the ninth dose and pooled separately. Serum RMP and PZA concentrations were determined by high-pressure liquid chromatographic methods. Each point represents the mean of three experiments; bars represent standard deviations.

treated with RMP-PZA than among the mice treated with either of the other two combinations ($P < 0.05$).

Major pharmacokinetic parameters of RMP and PZA in mice receiving or not receiving INH concomitantly. No RMP or PZA was detected in untreated control mice. The serum concentration-time curves of RMP and PZA in groups I and II are shown in Fig. 1. The major pharmacokinetic parameters of RMP and PZA in mice receiving or not receiving INH concomitantly are summarized in Table 2. Concomitant treatment with INH did not affect the pharmacokinetic parameters of PZA except that it slightly prolonged the elimination half-life of PZA. However, concomitant treatment with INH significantly reduced the C_{max} and AUC of RMP ($P < 0.01$).

DISCUSSION

An important feature of our current experiment is that the mice were infected with a larger number of virulent *M. tuberculosis* organisms than in our previous experiment in preventive therapy (11), and when treatment was begun, the infection was well established, with a larger bacillary population. Under these conditions, the great majority of mice are expected to die as the result of serious infection within 4 weeks after inoculation if they are not treated effectively (12).

The results of this experiment indicate that an initial 2-month treatment with any of the three combined regimens was highly effective in reducing the bacillary population. The

TABLE 2. Major pharmacokinetic parameters of INH, RMP, and PZA in mice treated with or without INH concomitantly

Treatment	Result (mean ± SD) for the following parameter:			
	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC (mg · h/liter)
INH	28.20 ± 3.8	0.25 ± 0	1.7 ± 0.17	52.2 ± 2.2
RMP				
−INH	14.7 ± 1.7	4.7 ± 3.1	2.8 ± 1.2	160.6 ± 15.2
+INH	8.4 ± 1.2	4.0 ± 0	3.5 ± 0.3	97.4 ± 9.0
P value	<0.01	>0.05	>0.05	<0.01
PZA				
−INH	146.1 ± 13.0	0.42 ± 0.2	1.05 ± 0.14	303.8 ± 17.9
+INH	147.4 ± 11.3	0.25 ± 0	1.31 ± 0.06	355.2 ± 49.2
P value	>0.05	>0.05	<0.05	>0.05

combination INH-RMP was as bactericidal as INH-RMP-PZA, but both of these combinations were less active than RMP-PZA. These results are consistent with those of our previous experiments in preventive therapy (11), except that no significant difference was observed in our previous study between the 2-month treatments with RMP-PZA and INH-RMP-PZA, perhaps because of the smaller bacillary population present at the beginning of treatment.

At the end of the continuation phase of chemotherapy, no difference in antimicrobial activity was detected among the three subgroups treated with the combinations INH-RMP-PZA, INH-RMP, or RMP-PZA; in fact, all the spleens of treated mice were culture negative. After a follow-up of 6 months without chemotherapy, however, numbers of mice were found to have relapsed, since *M. tuberculosis* was cultured from the spleens of these animals. The relapse rate was significantly smaller in the subgroup that had been administered RMP-PZA during the continuation phase than in the subgroups administered INH-RMP-PZA or INH-RMP; no difference in relapse rate was found between the mice of the latter two subgroups. Thus, the sterilizing activity of RMP-PZA was superior to that of the combinations INH-RMP-PZA and INH-RMP, a result entirely consistent with the results of the initial phase of chemotherapy.

It is also interesting that, despite the larger bacillary populations in the mice of this experiment, the relapse rates of the mice that had been treated with RMP-PZA (8.3%) or INH-RMP-PZA (34.6%) were significantly lower than those of the corresponding groups in the previous experiment (56.0 and 95.0%, respectively) (11) ($P < 0.01$). That the mice of the present experiment were treated for a total of 6 months, whereas those of the previous experiment had been treated for only 2 months, suggests that the relapse rate can be significantly reduced by more-prolonged treatment.

PZA is capable of preventing selection in mice of RMP-resistant mutants of *M. tuberculosis* (8). It is not yet clear whether RMP-PZA is more active than the other two combinations with respect to preventing the selection of resistant mutants. However, the results of our experiments demonstrate that RMP-PZA produces a stronger bactericidal activity than either of the other two combinations: 2 $\log_{10}$ more killing during the initial phase and a 30% lower relapse rate. Thus, we have confirmed the occurrence of antagonism between INH and the combination RMP-PZA in the chemotherapy of *M. tuberculosis* infection in mice. As a result of the antagonism, the activity of the combination INH-RMP-PZA was reduced to the same level as that of INH-RMP, and the benefit of adding PZA to the latter combination was compromised.

The nature of antagonism between INH and the combination RMP-PZA in mice could be pharmacological or microbiological. A preliminary pharmacokinetic analysis suggested that the pharmacological interaction between INH and RMP was very likely to be involved in the mechanism of the antagonism, as concomitant treatment with INH significantly reduced the C_{max} and AUC of RMP. However, it is unclear whether the antagonism can be entirely attributed to a pharmacological interaction between INH and RMP, as the C_{max} of RMP (8 μg/ml) in mice receiving INH concomitantly was still very significantly greater than the MIC of RMP against *M. tuberculosis* (0.06 to 0.25 μg/ml) (1). Further pharmacokinetic and microbiological studies should be conducted.

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