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Preventive Chemotherapy of Tuberculosis in Cornell Model Mice with Combinations of Rifampin, Isoniazid, and Pyrazinamide

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The efficacies of rifampin-containing preventive regimens were measured in Cornell model mice in which an initially severe infection with *Mycobacterium tuberculosis* H37Rv was first treated for 7 weeks with 25 mg of isoniazid and 1,000 mg of pyrazinamide per kg of body weight in the diet and then with one of four test regimens given by daily oral gavage for 6 weeks. These regimens were 15 mg of rifampin per kg alone (R), rifampin plus 25 mg of isoniazid per kg (RH), rifampin plus 150 mg of pyrazinamide per kg (RZ), or rifampin plus isoniazid and pyrazinamide (RHZ). The interval between the rifampin gavage and the gavage with the other drugs ranged from 10 to 45 min, so that interference with rifampin absorption did not occur. Mice were sacrificed at 11 and 20 weeks after the termination of chemotherapy, with each killing being preceded by 3 weeks of high-dose dihydrocortisone treatment. Entire spleens and lungs were cultured. The proportions of mice with positive spleens at either killing time were 74% of 43 mice treated with R, 63% of 41 mice treated with RH, 65% of 43 mice treated with RZ, and 53% of 45 mice treated with RHZ, a just significant ($P = 0.04$) trend for fewer positive spleens with increasing numbers of drugs in the regimen. However, no trend was found in the corresponding proportions of mice with positive spleens or lungs, which were 81, 63, 65, and 71% for mice treated with R, RH, RZ, and RHZ, respectively. Thus, in the Cornell model, R alone, RH, RZ, and RHZ all had similar efficacies.

An influential French study of preventive treatment of chronic murine tuberculosis found that isoniazid (INH) alone was least effective, while the efficacies of regimens containing rifampin (RMP) were highest when RMP was used with pyrazinamide (PZA), the next highest efficacy was found with RMP alone, and the least efficacy was found with RMP plus PZA and INH (12). These findings were later attributed, at least in part, to interference with the absorption of RMP caused by the presence in the gavage dose of PZA and INH (5). A reduction in the absorption of RMP was also reported when it was given to mice together with INH, PZA, ethambutol (EMB), and particularly, INH-PZA-EMB; the reduced absorption could be prevented in part by giving RMP first and then giving a second gavage with the other drugs 10 to 15 min later (3, 6). Although this pharmacokinetic interference with the absorption of RMP in mice could explain, at least in part, the superiority of the regimen of RMP-PZA over the regimen of RMP-INH-PZA in the protection experiments, it could not account for the apparent considerable superiority of RMP-PZA over RMP alone, a finding that has stimulated trials of RMP-PZA in the preventive treatment of tuberculosis in human immunodeficiency virus-positive subjects (7, 16). The French study used a model in which the mice were first vaccinated with *Mycobacterium bovis* BCG and then infected with a moderate dose of a virulent culture of the H37Rv strain of *Mycobacterium tuberculosis*, after which the CFU counts in the spleen remained constant. We were doubtful about the validity of this model for application to the human situation (14), and we therefore decided to run an additional experiment with RMP-containing regimens for two reasons. First, we thought that mice with a dormant infection induced by treatment with INH and a high dosage of PZA, as originally described by investigators from Cornell University Medical School (13), might be an alternative and possibly superior model of dormancy in humans. Second, there was a need to retest regimens in such a manner as to reduce or eliminate the pharmacokinetic influence of other drugs on the absorption of RMP to see whether this pharmacokinetic effect accounted for the entire antagonistic action of INH-PZA on the activity of RMP. We describe here our experience with regimens of RMP alone (R group), RMP-INH (RH group), RMP-PZA (RZ group), and RMP-INH-PZA (RHZ group) in Cornell model mice, with the dose of RMP preceding the doses of the other drugs tested.

MATERIALS AND METHODS

Selective culture media. The 7H11 plates and liquid Kirchner medium were made selective by the addition of one antibiotic tablet (Selectatab; Mast Laboratories, Bootle, United Kingdom) to 500 ml of medium to produce final concentrations of 100 μg of carbenicillin per ml, 200 U of polymyxin B per ml, 20 μg of trimethoprim per ml, and 10 μg of amphotericin B per ml (15).

Chemoprophylaxis experiment in mice. A total of 233 BALB/c mice, each weighing 18 to 22 g, were infected intravenously with 8.8×10^5 CFU of mouse-passaged *M. tuberculosis* H37Rv in 0.2 ml of 0.1% gelatin in saline. The disease was allowed to progress without treatment for 2 weeks, when the mean viable counts of bacilli were 1.02×10^7 and 6.76×10^7 CFU per organ in spleens and lungs of a sample of five mice, respectively. The mice were then put on a pelletted diet containing INH and PZA, estimated on the basis of the consumption of 4 g of diet per mouse per day, to dose them with 25 mg of INH per kg of body weight and 1,000 mg of PZA per kg daily (the HZ regimen). This diet was continued for 7 weeks, when the mean viable counts were 6.17×10^2 and 1.51×10^3 CFU per organ in the spleens and lungs of five mice, respectively. Four groups, each of 47 mice, were then treated with the test RMP-containing regimens, which were given by daily gavage (6 days per week) for an additional 6 weeks. These regimens were R, RH, RZ, and RHZ. The doses were 15 mg of RMP per kg of body weight, 25 mg of INH per kg, and 150 mg of PZA per kg. The mice were first treated by gavage with RMP in 0.1-ml volumes and then, after an interval, with the remaining drugs in 0.25-ml volumes. A control group of 10 mice received INH and PZA in the diet over the entire 13 weeks, after which positive cultures

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TABLE 1. Serum RMP concentrations in groups of five mice

Expt	Treatment group	AUC (μg · h/ml)[a]	Geometric mean concn (μg/ml)	95% Confidence limits
A	R	217	8.73	7.85–9.71
	RH	220	8.67	7.80–9.64
	RZ	210	9.21	8.28–10.2
	RHZ	182	6.85	6.15–7.62
B	R	225	9.27	8.55–10.1
	RH	183	8.71	8.04–9.44
C	R	213	9.92	8.72–9.99
	RHZ	215	9.34	9.27–10.61

[a] AUC, area under the concentration-time curve.

were obtained from cultures of 3 of 10 lungs and 6 of 10 spleens in selective Kirchner medium. About one-third of the mice which were given the test regimens were sacrificed at 26 weeks, and the remaining two-thirds were sacrificed at 35 weeks. For 3 weeks prior to sacrifice, the mice were given intramuscular dihydrocortisone three times weekly at 0.75 mg/kg for the mice sacrificed at 26 weeks or 0.5 mg/kg for the mice sacrificed at 35 weeks.

Experiments on separating the RMP dose from the doses of the other drugs. The effect of separating the gavage with RMP from the gavage with the remaining drugs was explored in separate experiments, which were also performed in BALB/c mice. Blood was taken by tail snip from five mice at each of several intervals after the gavages, and the RMP content in the serum was assayed microbiologically by a diffusion plate assay with *Staphylococcus aureus* as the test organism (6). The area under the concentration-time curve was calculated by the trapezoidal method.

CFU counts in organs. The methods of counting the numbers of CFU in organs have been described elsewhere (6). In brief, the spleen and lungs were removed aseptically and were homogenized in 5 ml of water in a motor-driven Fluon glass grinder. Serial 10-fold dilutions of the homogenized suspensions were added to segments of selective 7H11 medium plates. If only a proportion of the organs were expected to yield viable bacilli, the entire suspension was divided between two 28-ml screw-cap bottles, each containing 10 ml of selective liquid Kirchner medium. Subcultures were made after incubation for about 4 weeks onto slopes of Lowenstein-Jensen medium. The plates, packed in polythene bags, were incubated at 37°C for 3 to 4 weeks, and the Lowenstein-Jensen medium slopes were incubated for 6 weeks.

Statistical methods. The concentrations of the drugs in serum were log transformed and examined by analysis of variance with Tukey's test for individual means. Chi-square and Fisher's exact two-tail tests were run on proportions of organs with positive cultures.

RESULTS

Before the start of the main experiment, RMP was given 10 to 15 min before the other drugs were given to mice in the various treatment groups, and the concentrations of the drugs in serum were measured at 1.5, 3, 6, and 24 h after administration of the RMP dose. The results, expressed as areas under the concentration-time curves and geometric mean concentrations with their confidence limits, are provided in Table 1 (experiment A). The means for the R, RH, and RZ groups were similar, but in each case, they were greater than the mean for the RHZ group (by Tukey's test, $P < 0.05$). For this reason, the interval between gavages with RMP and the remaining drugs was set at 10 to 15 min for the RH group, 25 to 30 min for the RZ group, and 40 to 45 min for the RHZ group. After the main experiment, two further comparisons showed similar concentrations of drug in serum in the R group and the RH and RHZ groups, respectively, by using the same gavage intervals used in the main experiment (Table 1, experiments B and C).

Of the 288 mice in the four treatment groups, 12 died, mainly from labyrynthitis (presumptively because of infection with *Streptobacillus moniliformis*), evenly distributed between the treatment groups and during the course of the experiment. An additional four mice died from the effects of the higher (0.75 mg/kg) dose of dihydrocortisone, but the lower dihydrocortisone dose of 0.5 mg/kg was not lethal. The results of the culture of whole organs in selective liquid Kirchner medium (Table 2) showed no difference, as indicated by the 2-by-4 chi-square test, between the treatment groups in the proportions of spleens that yielded a positive culture either at 26 or at 35 weeks or for the combined results for both periods. However, considering the results for all spleens, the proportions with positive cultures were 74% in the R group, 63% in the RH group, 65% in the RZ group, and 53% in the RHZ group; the trend toward decreasing positivity in these four groups is just significant (trend $\chi^2 = 4.2$; $P = 0.04$) when scores of 1, 2, 2, and 3 were given to the R, RH, RZ, and RHZ groups, respectively, on the basis of the number of drugs in the combination. In the lung cultures at 26 weeks, a positive result was obtained for only 1 mouse in the RH group (this mouse also had a positive spleen culture), whereas 6 to 11 positive cultures were obtained for mice in each of the other three groups (by the two-tail exact test, $P = 0.005$). This low yield from lungs in the RH group was not evident in the 35-week results, but nevertheless, it caused the pooled lung culture results at 26 and 35 weeks for the RH group (20% positive) to be positive significantly less often than the results for the other three groups (30 to 49% positive). In the pooled results for 26 and 35 weeks, positive lung cultures were obtained when the spleen cultures were negative for three mice in the R group and eight mice in the RHZ group but for none of the mice in the RH and RZ groups, suggesting that although the RHZ treatment may have been more effective in sterilizing the spleens, this superiority was not evident for the lung cultures; no significant differences in the proportions of mice with either organ positive were found between any of the groups.

DISCUSSION

Several pharmacokinetic studies in humans have failed to show any decrease in the absorption of oral RMP when it is given simultaneously with INH, *p*-aminosalicylic acid, or EMB (1, 2, 10). A single study has shown a lower area under the concentration-time curve when INH and PZA were administered with RMP, but the difference from the curve for RMP alone was very small (11). In the mouse, the interference with RMP absorption occurred with all drug combinations, although it was greatest when it was given simultaneously with PZA or with PZA and other drugs (6). Thus, there appears to be considerably greater drug interaction in mice than in humans, possibly because the volumes of gavage doses in mice are much greater than the volumes of tablets in humans relative to the respective sizes of their stomachs and duo-

TABLE 2. Mouse organ cultures positive for *M. tuberculosis*

Wk	Organ	No. (%) positive cultures for the following groups:				χ^2	*P*
		R	RH	RZ	RHZ		
26	Spleen	12 (86)	8 (62)	7 (54)	10 (67)	3.4	0.3
	Lungs	11 (79)	1 (8)	6 (46)	6 (40)	13.9	<0.01
	Total	14 (100)	13 (100)	13 (100)	15 (100)		
35	Spleen	20 (69)	18 (64)	21 (70)	14 (47)	4.5	0.2
	Lungs	10 (34)	7 (25)	7 (23)	12 (40)	2.6	0.4
	Total	29 (100)	28 (100)	30 (100)	30 (100)		
All (26 + 35 wk)	Spleen	32 (74)	26 (63)	28 (65)	24 (53)	4.3	0.2
	Lungs	21 (49)	8 (20)	13 (30)	18 (40)	8.9	0.03
	Either organ	35 (81)	26 (63)	28 (65)	32 (71)	4.0	0.3
	Total	43 (100)	41 (100)	43 (100)	45 (100)		

denums. To make the mouse model realistic for application of the results to humans, it was essential to prevent the interactions. This was achieved in the mouse by allowing an interval of 10 to 45 min between the gavage with RMP and the subsequent gavage with the other drugs used in the treatment groups.

Although the differences in the proportions of positive spleen cultures for the four treatment groups were small and a multiple chi-square test was not significant, a trend chi-square test suggested, but did not prove, that the proportions probably decreased as either INH or PZA was added to RMP and decreased even further when both were added. There is no obvious explanation for the discrepancy in the 26-week lung cultures in which positive results were obtained in only 1 (8%) of the mice in the RH group but in 23 (48%) of the mice in the other three groups. One can therefore consider it to be due to extreme experimental variation. In this case, it is reasonable to pool the 26- and 35-week results when the low proportion of positive lung cultures in the RH group (20%) is just significantly lower than the corresponding proportions in the remaining three groups (30 to 49%), and the pooled results for cultures of either spleens or lungs showed no difference between the regimens (and no trend toward fewer positive cultures as the number of drugs in the regimen was increased); positive results were obtained for 81% of the mice in the R group, 63% of the mice in the RH group, 65% of the mice in the RZ group, and 71% of the mice in the RHZ group. This is the preferred conclusion. However, an alternative explanation for the 26-week lung culture results is that sterilizing activity in the lungs was greater in the RH group than in the other three groups. This explanation seems unlikely since there is no reason from other comparisons of drug activity that the RH regimen should have this uniquely greater activity (4), nor is it shown by the results at 35 weeks, when there were no significant differences between the positivity of lung cultures for the four regimens. If it is nevertheless felt that sterilization of lungs and spleens was through different mechanisms, the pooling of the results for both organs is unjustified, and it would then be concluded that, in terms of spleen cultures only, efficacy was marginally associated with the number of drugs in the regimen, while in terms of lung cultures only, the RH regimen was more effective than either of the other three regimens.

Our findings support the view that some of the differences in the preventive activities of RMP-containing regimens in the French study were due to pharmacokinetic effects which could be prevented by suitable spacing of the gavage doses of RMP and the other drugs in the regimen. However, we failed to confirm their findings of an apparent superiority of the regimen of RMP-PZA over RMP alone. They used a model of chronic tuberculosis closely similar to the model explored by Rees and Hart (17), except that immunity at the time of infection with the challenge organisms was conferred by BCG vaccination rather than by growth of a small inoculum of virulent organisms. Rees and Hart (17) and Wallace (18) showed that the CFU counts in the lungs of their mice were stable because the organisms were in a stationary state rather than a mixture of dying and replicating bacilli. However, the amount of bacillary metabolism could still be appreciable. On the other hand, the amount of metabolic activity among residual viable bacilli in the Cornell mouse model during effective, sterilizing chemotherapy is likely to be negligible. In pulmonary tuberculosis, PZA is an excellent sterilizing drug during the first 2 months of treatment, but is not bactericidal thereafter (8). Thus, its sterilizing activity is associated with recent bacillary multiplication, and it seems reasonable to expect it to be more bactericidal in the chronic model of mouse tuberculosis than in the Cornell mouse model, which corresponds well with the continuation phase of treatment in human disease.

Irrespective of the views one may have about the relevance of the two mouse models to preventive treatment in humans, the models provide quite different assessments of the efficacy of the regimen of RMP-PZA. In the chronic tuberculosis model, this regimen was shown to be superior to RMP and was not tested against RMP-INH, while its superiority over RMP-INH-PZA might have been due to a purely pharmacokinetic effect on the absorption of RMP. In the Cornell model, RMP alone, RMP-INH, and RMP-PZA all had similar efficacies. The relevance of these differences between the two mouse models to preventive treatment in humans can only be assessed by comparative trials in humans. It is unfortunate that an overenthusiastic response to the original report of the chronic tuberculosis model (12) and the associated editorial (9) led to the setting up of clinical studies in Haiti (7) and Zambia (16) in which the regimen of RMP-PZA has been tested for its preventive activity without a comparison with another RMP-containing regimen of the same duration such as RMP alone, RMP-INH, or RMP-INH-PZA. These studies may well need to be repeated if effective RMP-containing preventive regimens are to be developed.

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