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Bactericidal action of pulsed exposure to rifampicin, ethambutol, isoniazid & pyrazinamide on *Mycobacterium tuberculosis in vitro*

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The bactericidal action of pulsed exposure to rifampicin (R), ethambutol (Emb), isoniazid (I) and pyrazinamide (Z) togcthcr on alternate days (REmbIZ) and as REmb and IZ separately on alternate days (REmb/IZ) on *M.tuberculosis* H_{37}Rv, two isolates of *M.tuberculosis* sensitive to these drugs, as well as four isolates resistant to one or more drugs, was studied using an *in vitro* method. The experimental duration was 6 days. REmbIZ and REmb/IZ appeared to have equally good bactericidal action on *M.tuberculosis* strains in the *in vitro* system. The results suggest that splitting REmbIZ into REmb and IZ on alternate days in short course chemotherapy regimens for tuberculosis may not affect the bactericidal action of the regimens.

Several highly effective short course chemotherapy (SCC) regimens of 6-8 months' duration have been evolved for the treatment of sputum-positive pulmonary tuberculosis. In most of these regimens, four drugs, *viz.*, rifampicin (R), isoniazid (I), pyrazinamide (Z) and streptomycin (S) or ethambutol (Emb) are given together, usually for two months initially, in a single dose either daily or intermittently. The bulk of the drugs to be consumed in a single dose is therefore large and may affect patient compliance. Further, the incidence of adverse reactions such as arthralgia[1] and jaundice[2] is more when the drugs are given daily than if given less frequently. One way of overcoming these difficulties would be to split the four-drug regimen into two, 2-drug combinations (REmb & IZ) and to give them in an alternating regimen in which each 2-drug combination is taken on successive days. No information is, however, available as to whether such an alternating regimen would be as effective as when all 4 drugs are given together, 3 times a week.

In vitro studies can be used to examine the bactericidal action of such pulsed exposure to drugs on *Mycobacterium tuberculosis* strains[3,4]. In the present study, using an *in vitro* system, we investigated the bactericidal action of pulsed exposure to combinations of REmb and IZ on cultures of *M. tuberculosis* H_{37}Rv, isolates of *M.tuberculosis* sensitive to these drugs, as well as isolates resistant to one or more drugs.

Material & Methods

M.tubercu1osis strains: Seven strains of *M.tuberculosis* including *M. tuberculosis* H_{37}Rv, 2 clinical isolates sensitive to S, I, R and Emb, 3 isolates resistant to I, IR and SIR, respectively, and 1 isolate resistant to SIREmb by minimum inhibitory concentration (MIC) method on Lowenstein-Jensen (LJ) slopes were used in the study.

Chemotherapeutic drugs: Ethambutol (Sigma, USA), isoniazid (Bayer, Germany) and pyrazinamide (Sigma, USA) were dissolved in sterile distilled water. Rifampicin (Sigma, USA) solution was prepared fresh each day by dissolving in minimum quantity of N/10 HCl and immediately diluting to the required concentration with sterile distilled water. All drug solutions were sterilised by filtration through 0.22

µm membrane filters (Millipore). R, Emb, I and Z were added to universal containers with 10 ml log phase cultures of the *M.tuberculosis* strains in Middlebrook's 7H9 liquid medium (Difco, USA) with 0.05 per cent Tween 80 (Sigma, USA) and albumin/dextrose enrichment. The final concentrations of R, Emb, I and Z in 7H9 medium were 1, 4, 1 and 25 µg/ml, respectively. These concentrations were not meant to be analogous to the peak concentrations that will be attained in patients' sera with the doses normally employed[5,6] because such concentrations are not maintained throughout a 24h exposure in patients. The concentrations used were meant to be as low as possible without them being close to the minimal inhibitory concentrations (Prof. D.A. Mitchison, personal communication).

Log phase cultures of M. tuberculosis: The *M.tuberculosis* strains were first grown in 50 ml of 7H9 medium for 7 days at 37°C, spun down at 3000 rpm for 30 min and resuspended in 10 ml of 7H9. The total number of bacilli/ml in this suspension was counted using the Thoma bacteriological counting chamber (Gallenkamp, London) and appropriate volume was inoculated into 100 ml of fresh 7H9 medium to give 10^5 bacilli/ml. These cultures were incubated at 37°C for 3 days to bring them to log phase[7] and distributed into 10 ml aliquots in universal containers (day 0).

Pulsed exposure to REmb/IZ and REmbIZ: Duplicate 10 ml aliquots were treated with pulsed exposure to REmb on one day and 12 on the next day (REmb/IZ) or REmbIZ together on alternate days (REmbIZ) using a filtration and resuspension procedure.

In the 'REmbIZ' cultures, all the 4 drugs were added on day 0. On day 2 and day 4 old medium with the drugs was replaced with fresh medium by filtering the cultures through 0.45 µm, 25 mm diameter membrane filter (Millipore HAWP 02500) and holder assembly (Millipore Swinnex SX 0002500) with a syringe, following through twice with an equal volume of sterile distilled water and then resuspending the bacilli which were now deposited on the membrane filter by removing and placing the membrane in a universal container with two 5 mm sterile glass beads and 10 ml fresh 7H9 medium without drugs and shaking for 1h. The 4 drugs were then added again.

In the 'REmb/IZ' cultures, R and Emb were added on day 0. Using the filtration and resuspension method as above, this was replaced with fresh medium and IZ on days 1, 3 and 5, and REmb on days 2 and 4.

Cultures which were not treated with any drugs and left undisturbed (undisturbed controls) as well as cultures which were not treated with any drugs but were subjected to the same filtration and resuspension procedure (filtered controls) as the cultures treated with drugs, were also included as controls.

Viable counts: Viable counts were set up from all the cultures on days 0, 2, 4 and 6, before the addition of drugs, by inoculating 10 µl of the neat cultures and five serial 10-fold dilutions on duplicate slopes of LJ. The LJ slopes were incubated at 37°C for 4 wk. From the number of colonies appearing on the LJ slopes and the dilution factor, colony forming units/ml (cfu/ml) was calculated. When there were no detectable colonies on any of the slopes, to facilitate handling of data, the cfu/ml was recorded as 0.25.

Centrifugation vs filtration for removal of drugs: During the course of the pulsed exposure experiment, the drugs in the culture medium have to be removed at intervals when viable counts are set up and for pulsing. To select and standardise a suitable method for this, in addition to the filtration method, centrifugation for 30 min at 3000 rpm in universal containers following which the supernatant was discarded before resuspending the pellet in fresh medium, was also tested in an experiment using *M. tuberculosis* $H_{37}RV$.

Analysis of data : The area under the curve (AUC) was calculated by applying the trapezoidal rule for the log cfu/ml in the filtered control (FC), and in the cultures treated with REmbIZ and REmb/IZ, taking (the strains of *M. tuberculosis* individually. For this, the cut off point was taken as 1.4 because that was the minimum detectable log cfu/ml in the system employed. The mean AUCs for the log cfu/ml of the 3 strains which were sensitive to S, I and R and for the other 3 strains which were resistant to one or more of these drugs were compared separately using paired t-test (one-tail).

Table I. Serial viable counts of *M.tuberculosis* $H_{37}Rv$ subjected to filtration/centrifugation and resuspension

Initial total count/ml	Treatment	Log cfu/ml on day						
		0	1	2	3	4	5	6
	Undisturbed	6.66	6.74	7.08	7.18	7.38	8.14	8.20
10^6	Filtered	6.66	6.18	6.48	6.30	6.89	6.85	6.11
	Centrifuged	6.66	5.86	5.30	4.65	4.30	3.78	3.74
	Undisturbed	8.07	8.36	8.25	8.05	8.65	8.62	8.67
10^7	Filtered	8.07	6.94	6.28	7.02	7.22	6.97	6.41
	Centrifuged	8.07	7.43	7.10	6.60	5.85	6.40	4.04
	Undisturbed	8.40	8.26	8.62	8.50	8.59	8.60	8.72
10^8	Filtered	8.40	8.09	6.27	7.08	6.81	6.80	5.83
	Centrifuged	8.40	7.36	6.52	5.81	5.44	5.30	6.41

Results

The experiment to compare centrifugation against filtration for removal of drugs was done using *M.tuberculosis* $H_{37}Rv$ at three different initial concentrations (6.66, 8.07 and 8.40 log cfu/ml) in duplicate. The results are presented in Table 1. Among the cultures left undisturbed, the greatest increase in the log cfu/ml over the six day period was seen in the culture with initial count of 6.66 log cfu/ml. In the cultures with higher initial counts (8.07 and 8.40 log cfu/ml), the net increase over the six day period was small. Again, in the cultures subjected to filtration and resuspension every day, there was a net reduction in the log viable counts over the six day period, and this reduction was smallest in the cultures with initial count of 6.6 log cfu/ml. These results indicated that to examine the effect of pulsed exposures on log phase cultures, in experimental systems similar to the one used in the present study, initial counts closer to 6 log cfu/ml would be more suitable. In the cultures subjected to centrifugation and resuspension also there was loss of bacilli every day resulting finally in a greater net loss over the six day period.

The results of the experiment on pulsed exposure to REmb/IZ and REmbIZ for the 7 strains of *M. tuberculosis* are presented in Table 11 and the Fig. The undisturbed controls (UC) of all the 7 strains showed more than 1 log increase in the log cfu/ml over the 6 days, indicating that the cultures were in a state of active growth. One of these strains (PE7) was resistant to S, I, R and Emb. Pulsed exposure to REmb/IZ as well as REmbIZ did not have any bactericidal activity on this strain, with the log cfu/ml on day 6 being 6.57 and 6.68 in the REmb/IZ and REmbIZ treated cultures, respectively, compared to 5.49 on day 0.

With all the other strains, a greater fall in the log cfu/ml was observed in both the REmb/IZ and REmbIZ treated cultures compared to the filtered control (FC) cultures over the 6 day period. In the drug sensitive strains (PE1, PE2 and PE3), the fall in the log cfu/ml was 5.08, >4.45 and >3.65 respectively in the REmb/IZ treated cultures and 5.56, 4.45 and > 3.65 respectively in the REmbIZ treated cultures, compared to only 0.96, 0.72 and 1.34 respectively in the filtered control cultures. On calculating and comparing the mean AUCs, in the manner described here, for the log cfu/ml in the cultures of these drug sensitive strains subjected to different treatment procedures, it was found that the differences observed between the cultures treated with pulsed exposure to REmb/IZ and REmbIZ were not significant ($P > 0.05$), while those between the filtered controls and the cultures treated with REmb/IZ and REmbIZ were significant ($P < 0.02$). Similarly, in the 3 strains, PE4, PE5 and PE6, which were resistant to one or more drugs, the fall in the log cfu/ml over the 6 days was > 4.12, > 4.07 and >3.88 respectively in the REmb/IZ treated cultures and > 4.12, 3.59 and >3.88 respectively in the REmbIZ treated cultures, compared to only 0.82, 0.17 and 2.71 respectively in the filtered control cultures. The differences observed in the AUCs between the REmb/IZ and REmbIZ treated cultures were not significant ($P > 0.1$), while that between the filtered controls and the cultures treated with the drugs were significant ($P < 0.05$).

These results suggested that REmbIZ together or separately as REmb and IZ on alternate days would have similar bactericidal action on *M.tuberculosis* strains.

Table II. Serial viable counts of each strain of *M. tuberculosis* after pulsed exposure to REmb/IZ or REmbIZ

Strain no.	Drug sensitivity	Treatment	log cfu/ml on day 0	2	4	6
Strains sensitive to S, I and R :						
PE1	SIR sensitive	UC	6.96	6.97	8.14	8.47
		FC	6.96	6.44	6.63	6.00
		REmb/IZ	6.96	4.24	2.44	1.88
		REmbIZ	6.96	3.18	2.00	1.40
PE2	SIR sensitive	UC	5.85	6.20	6.94	8.31
		FC	5.85	5.60	4.99	5.13
		REmb/IZ	5.85	4.07	2.24	<1.40*
		REmbIZ	5.85	3.88	2.10	1.40
PE3	SIR sensitive	UC	5.05	5.81	5.40	6.09
		FC	5.05	4.81	3.41	3.71
		REmb/IZ	5.05	2.40	<1.40*	<1.40*
		REmbIZ	5.05	<1.40*	<1.40*	<1.40*
Strains resistant to one or more drugs among S, I rind R :						
PE4	I resistant	UC	5.52	6.24	6.99	7.97
		FC	5.52	5.63	5.44	4.70
		REmb/IZ	5.52	3.35	<1.40*	<1.40*
		REmbIZ	5.52	3.10	1.70	<1.40*
PE5	IR resistant	UC	5.47	6.21	7.16	7.98
		FC	5.47	5.11	5.40	5.30
		REmb/IZ	5.47	4.48	3.40	<1.40*
		REmbIZ	5.47	4.60	3.18	1.88
PE6	SIR resistant	UC	5.28	6.08	6.11	7.78
		FC	5.28	4.68	3.88	2.57
		REmb/IZ	5.28	3.57	2.18	<1.40*
		REmbIZ	5.28	4.01	2.78	<1.40*
SIREmb resistant strain :						
PE7	SIREmb resistant	UC	5.49	6.24	6.99	7.97
		FC	5.49	4.72	5.51	5.48
		REmb/IZ	5.49	6.01	6.51	6.57
		REmbIZ	5.49	5.85	6.48	6.68

UC = undisturbed control; FC, filtered control; REma/IZ, REmb and IZ on alternate days; REmbIZ, REmbIZ together on alternate days; *, 1.40 was the minimum detectable log cfu/ml R. rifampicin; Emb, ethambutol; I, isoniazid; Z, pyrazinamide; S, streptomycin

Discussion

In vitro systems have been used earlier by Dickinson and Mitchison[3,4,8] to examine the choice of drugs for intermittent chemotherapy of tuberculosis and the suitability of new rifamycins for intermittent chemotherapy. The bactericidal effect observed in the present study may be an indirect measure of the early bactericidal action of these drugs on the population of actively growing tubercle bacilli in cavity walls. It is unlikely to measure the ultimate sterilising activity of the drugs, as measured by the proportion of negative sputum cultures at 2 months and the relapse rate after stopping chemotherapy[9].

In the filtration and resuspension method used in the present study, loss of bacilli occurred during the 6 daily pulses even in the control cultures subjected to the procedure but not treated with drugs. This loss of bacilli might have been due to adherence of the organisms on the filter membranes. However, in the cultures with drugs, the viable counts fell at a greater rate over the 6 day period allowing an estimation of the bactericidal action of the drugs. Preliminary experiments suggested that the loss of bacilli in filtration could be minimised by using a low initial count, washing the filter membranes with sterile water and inclusion of glass beads for resuspension. Even greater losses of bacilli occurred if drugs were removed by centrifugation and resuspension.

The overall results obtained with the sensitive and resistant strains, analysed together or separately, indicate that under *in vitro* conditions, pulsed exposure to REmb/IZ will have bactericidal action comparaable to that of all 4 drugs together. Both REmb/IZ and REmbIZ treatments had equally good bactericidal action *in vitro* on *M. tuberculosis* strains sensitive to S, I and R and also on strains resistant to one or more of these drugs. So, splitting up REmbiZ into REmb and IZ separately on alternative days in SCC rcgimcns may not affect the bactericidal action of the regimens.

The limitations such as loss of bacilli during filtration and resuspension, and the possible lack of activity of pyrazinamide in the *in vitro* model used in the present study may be overcome by using mouse peritoneal macrophage or human macrophage system[10-15]. In the macrophage system, it is easy to pulse with drugs as the cells are stuck to the wells, and

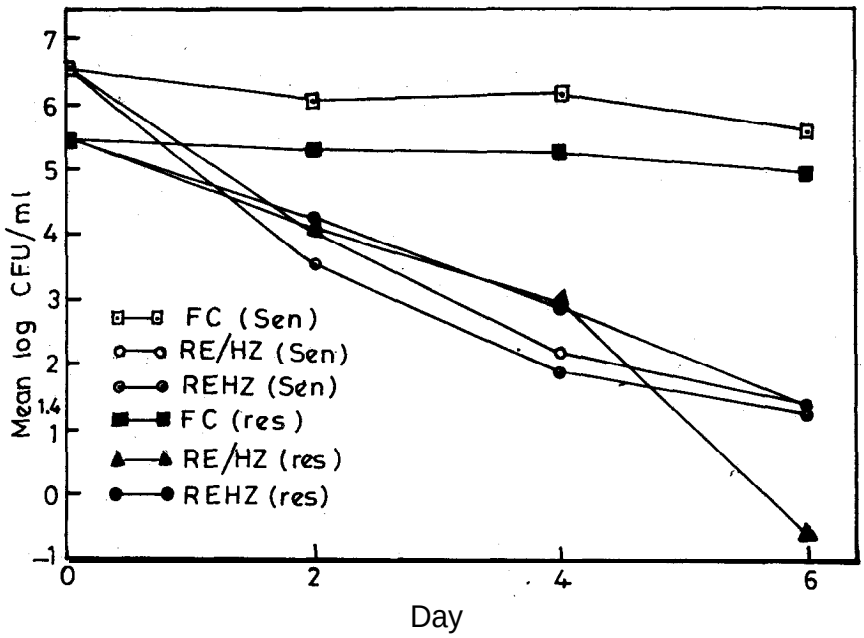

Fig. Serial viable counts of drug sensitive (n=3) and drug resistant isolates (n=3) of *M. tuberculosis* treated with pulsed exposure to REmbIZ or REmb and IZ. R, rifampicin; Emb, ethambutol; I, isoniazid; Z, pyrazinamide; FC, filtered control; REmb/IZ, treated with REmb and IZ on alternate days; REmbIZ, treated with REmbIZ together on alternate days; (sen), *M. tuberculosis* isolates sensitive to S, I and R; (res), *M. tuberculosis* isolates resistant to one or more drugs among S, I and R.

pyrazinamide as well as the other drugs are all active together. The experimental murine tuberculosis model of Grosset and associates[16] is another system which could be useful. Finally, the relevance of the findings from these models would depend on pharmacokinetic data as well as assessments of bactericidal activity in tuberculosis patients by serial sputum viable count examinations[9].

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