Indian J Med Res 108, October 1998, pp 115-119

Action of metronidazole in combination with isoniazid & rifampicin on persisting organisms in experimental murine tuberculosis

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Accepted September 9, 1998

To study the activity of metronidazole on persisting tubercle bacilli, BALB/c mice were infected with *Mycobacterium tuberculosis* and, after 14 days, treated with isoniazid (H) or rifampicin (R) or isoniazid + rifampicin (HR) for 2 months. An untreated group and a group treated with metronidazole (M) alone served as controls. At the end of 2 months, M was added to the H, R, and HR regimen in half the mice, and the treatment was continued for 1 more month in all mice. At the end of treatment, no viable organisms were detected in the lung or spleen of mice treated with HR or HRM regimens. In contrast, compared to the mice treated with R alone, the $\log_{10}$ colony forming units (cfu) of mice treated with RM were lower by 1.84 and 0.52 in the lung and spleen, respectively. Similarly, compared to the H group, the $\log_{10}$ cfu were lower by 0.67 in the spleen of mice treated with HM, and no additional effect due to M was seen in the lung. Three months after stopping treatment, viable organisms were isolated from both the organs of all the groups. However, the $\log_{10}$ cfu in the lung and spleen for the groups with metronidazole were below the $\log_{10}$ cfu for the respective single or 2 drug groups, except the $\log_{10}$ cfu in the lung for the RM group. These findings suggest that metronidazole, given with bactericidal drugs such as rifampicin and isoniazid may be of value in eliminating persisting tubercle bacilli, but further studies are warranted.

Key words Dormant bacilli - metronidazole - murine model - *Mycobacterium tuberculosis* - persisters

It is well recognized that tubercle bacilli have the ability of sequestering themselves to stay in a dormant state in the host. None of the currently used antituberculosis drug regimens completely eliminate this population and the possibility of endogenous reactivation at a later date always remains. In various controlled clinical trials using short course chemotherapeutic (SCC) regimens of 6-8 months' duration, almost 90 per cent culture conversion was usually observed at the end of 2 months among patients infected with drug sensitive organisms.

But when the treatment period was shortened to 3-4 months relapse occurred in 20-25 per cent of patients with initially drug sensitive organisms suggesting that this treatment schedule perhaps produced a small population of 'dormant' or 'persisting' bacilli[1]. Mitchison[2], in his hypothesis of 'special bacterial populations', highlighted the importance of dormant bacilli and their role in the occurrence of relapse. With the recent spurt in the incidence oftuberculosis in HIV-infected individuals, there is resurgence of interest in this population[3,4].

These dormant bacteria have never been isolated and characterized. The evidence for their existence comes from animal models, and *in vitro* experiments[5-7]. In the 'Cornell model' of murine tuberculosis, mycobacterial DNA was demonstrated[8] 14 wk after stopping treatment. Although no bacteria could be

isolated from cultures of the lung or spleen by conventional methods, heavy inocula of spleen homogenates into uninfected mice resulted in the development of disease[8]. These results suggested the presence of a dormant bacterial population.

Wayne and colleagues[9,10] using an *in vitro* system, demonstrated the presence of dormant *Mycobacterium tuberculosis* in the oxygen-free layer of undisturbed cultures. These bacilli not only resisted the bactericidal effect of anaerobiosis but also exhibited partial or complete resistance to the bactericidal effect of isoniazid or rifampicin. They also showed for the first time, that metronidazole (M) could act on these bacilli[9]. More recently, Wayne and Hayes[11], using a better *in vitro* model, demonstrated a sequential downward shift into one or both of two nonreplicating stages, corresponding to microaerophilic and anaerobic persistence. We used a murine model of tuberculosis to simulate conditions as similar to the above to study the effect of metronidazole on presumably 'persisting' bacilli. Infected mice were treated with the bactericidal drugs rifampicin and isoniazid, either alone or in combination, for 2 months to generate a population of 'persisting' bacilli which were then treated for 1 month with regimens with or without metronidazole.

Material & Methods

M. tuberculosis strain : A pretreatment clinical isolate (Ts 51476) of *M. tuberculosis*, sensitive to streptomycin (S), isoniazid (H), rifampicin (R), ethambutol (EMB) and pyrazinamide (PZA), was used in this experiment. Prior to use, the minimum inhibitory concentration (MIC) for this strain to these drugs was again determined on Lowenstein-Jensen (LJ) medium using standard methods[12]. The strain was also passaged in mice before use.

Drugs : Isoniazid was obtained from Bayer Leverkusen, Germany, rifampicin from Sigma Chemical Company, and metronidazole powder from Rhone Poulenc (India) Ltd.

Infection of mice : A total of 102 BALB/c outbred female mice aged 1-2 months, obtained from the National Institute of Nutrition, Hyderabad, were infected with the *M. tuberculosis* strain (Ts 51476) by injecting through the tail vein, a suspension containing 10^6 organisms in 0.2 ml of distilled water prepared from 3 wk old growth on LJ medium'?. The number of organisms in the bacterial suspension was adjusted using a Thoma counter (Thomas Scientific, NJ, USA). The viable count (VC) set up from this suspension showed that it contained 1.9×10^6 colony forming units (cfu) in 0.2 ml. Three mice were sacrificed immediately after infection, and three others 14 days thereafter. Their spleen and lung were removed aseptically and VC was set up as follows. The whole organs were transferred to a sterile grinding tube and ground in 2 ml of sterile distilled water using a teflon-coated grinding rod. From this homogenate as the neat suspension, and from five, serial 10-fold dilutions, cultures were set up on duplicate slopes of LJ by inoculating 10 µl per slope, which were then incubated at 37°C for 4 wk and the number of colonies were counted to determine the cfu in the organs[14].

Treatment : Treatment was started on day 14 post infection. The 96 mice were divided into 8 groups of 12 animals each. The first group did not receive any treatment and served as the control. Four groups of mice were treated 6 days a week for 3 months with metronidazole (M), isoniazid (H), rifampicin (R) and isoniazid and rifampicin (HR), respectively. In the remaining 3 groups, the drugs H, R and HR respectively, were administered for the initial 2 months and M was added to this treatment schedule during the third month (MH, MR, MHR). R was always administered 30 min before the other drugs.

The drugs were administered by oral gavage in 0.2 ml of 0.1 per cent agar. The dosages were as follows : M 100 mg/kg[14], R 10 mg/kg, and H 25 mg/kg[15]. The drugs were prepared at one time point and stored at - 70° C until used.

Three mice from each group were sacrificed at the end of 1, 2 and 3 months of treatment and also at the end of 3 months after stopping treatment. Their spleen and lungs were removed aseptically and cfu in these organs was determined as described above.

Statistical methods : Significance of the data was calculated using McNemer's test.

Results

In the lung and spleen of infected mice 5.00 and 4.95 $\log_{10}$ cfu of *M. tuberculosis* respectively, were

present at the start of treatment. At the end of the second month of treatment, the $\log_{10}$ cfu of *M. tuberculosis* in the lung was the lowest in mice treated with R alone (0.97) followed by HR (1.00) and H (1.64) (Table I). In spleen, the corresponding values were 1.09, 0.67 and 2.07, respectively (Table II). Compared to the untreated control group, there was no reduction in $\log_{10}$ cfu of mice treated with M alone, either in the lung or spleen.

At the end of the third month of treatment, in the lung of mice treated with R alone the $\log_{10}$ cfu of *M. tuberculosis* increased to 1.84 from the second month count of 0.97. This was because *M. tuberculosis* was isolated from the lungs of 2 of the 3 mice sacrificed in the R group after the third month of treatment. On drug susceptibility testing of these strains, one was found to be resistant to R (MIC>128) and the other was sensitive. At this time point, *M. tuberculosis* could not be isolated from the lungs of any of the 3 mice which were treated with M in addition to R (RM group). The $\log_{10}$ cfu in the lungs of mice treated with H and HM were 0.67 and 1.04,

Table 1. $\log_{10}$ colony forming units of *M. tuberculosis* in lung
(Data are mean ± SE)

Group	Months after start of treatment			3 months after stopping treatment
	1	2	3	
Control'	7.19±1.02	5.3±0.54	3.88±0.47	5.24±0.73
M	5.34±1.45	6.44±0.88	4.48±0.41	3.71±0.34
H	3.88±0.33	1.64±0.67	0.67±0.54	3.4±1.4
R	0.98±0.80	0.97±0.79	1.84±0.79	1.09±0.89
HR	2.12±1.03	1.00±0.58	0	1.98±1.14
HM		1.64[+]±0.67	1.04**±0.85	1.83±1.50
RM		0.97[+]±0.79	0[**]	2.50±0.86
HRM		1.00[+]±0.58	0[**]	1.34±1.09

*At 2 wk after infection, the mean $\log_{10}$ cfu was 5.00
[+]Same values as for isoniazid (H), rifampicin (R) and HR respectively, since metronidazole was added only at 2 months
**1 month after start of metronidazole (M)

Table II. $\log_{10}$ colony forming units of *M. tuberculosis* in spleen
(Data are mean ± SE)

Group	Months after start of treatment			3 months after stopping treatment
	I	2	3	
Control*	7.17±0.91	4.49±0.40	3.48±0.35	3.47±0.42
M	5.98±1.15	5.73±1.21	3.78±0.28	3.13±0.35
H	4.52±0.13	2.07±0.84	1.80±0.73	3.19±0.29
R	2.39±0.23	1.09±0.89	1.49±0.62	1.44±0.59
HR	2.84±0.43	0.67±0.54	0	1.64±0.67
HM		2.07[+]±0.84	1.13**±0.92	0.98±0.80
RM		1.09[+]±0.89	0.97**±0.79	0.87±0.71
HRM		0.67[+]±0.54	0[**]	0.87±0.71

*At 2 wk after infection, the mean $\log_{10}$ cfu was 4.95
[+]Same values as for isoniazid (H), rifampicin (R) and HR respectively, since metronidazole was added only at 2 months
** 1 month after start of metronidazole (M)

respectively, at this time point. In the spleen, the $\log_{10}$ cfu of *M. tuberculosis* was lower in the RM group (0.97) compared to R group (1.49), and again in the HM group (1.13) compared to the H group (1.80). None of the above differences were statistically significant. In both lung and spleen, no detectable count was obtained at the end of 3 months in mice treated with HR, and HRM.

At 3 months after stopping treatment, *M. tuberculosis* was isolated from both the lung and spleen of all the groups, including the groups in which *M. tuberculosis* was undetectable at the end of 3 months of treatment (Tables I and II). However, in these groups, the $\log_{10}$ cfu of *M. tuberculosis* was lower in groups with M compared to the respective single or two drug groups in both lung and spleen, except in the RM in which the $\log_{10}$ cfu in the lung was 2.50 compared to 1.09 with R alone.

Discussion

The chemotherapy of tuberculosis was revolutionised by the development of the concept of SCC which utilised combinations of drugs that were effective against different populations of bacilli. However, despite effective regimens being available, relapses continue to occur in many studies. It has been suggested that the persisters, which are not susceptible to any of the regularly used antituberculous drugs, are responsible for these relapses, probably due to 'endogenous reactivation' of tuberculous disease. The currently used BCG vaccine, while possibly affording protection against fresh infection, is incapable of protecting against breakdown into disease. In this situation also, it has been suggested that the breakdown occurs when dormant bacilli find an opportune time to multiply and activate the disease process. However, despite a large body of evidence that has been generated to implicate the role of the 'dormant bacilli'[16,17], more information is required with regard to drugs or regimens which would be effective against them. It has been shown *in vitro* that metronidazole, a commonly used drug, can act on nonreplicating bacilli', but to our knowledge, ours is the first *in vivo* experiment aimed towards evaluating the activity of metronidazole in combination with other drugs on persisting tubercle bacilli.

In the present study, metronidazole showed an additive effect with rifampicin in reducing the $\log_{10}$ cfu of *M. tuberculosis* in both the lung and spleen of mice at the end of 3 months of treatment. When metronidazole was combined with isoniazid this effect was observed only in the spleen. Similar observations *in vitro* were reported by Wayne and Sramek[9]. In their study, when tubercle bacilli under anaerobic incubation were exposed to these drugs, metronidazole did not exhibit additive effect when combined with isoniazid but enhanced the bactericidal effect 10-fold when combined with rifampicin. Evidence for rifampicin activity on semidormant bacilli was also observed by Wayne and Hayes[11] in their model of sequential shift down to anaerobiosis in which only 12- 19 per cent of bacilli survived when 28 days old semidormant bacilli were exposed to rifampicin alone.

In our murine model it was observed that in mice treated with metronidazole in combination with rifampicin or rifampicin and isoniazid, *M. tuberculosis* became undetectable in the lung at the end of 3 months, while they were still present in the lung of mice treated with metronidazole and isoniazid. Treatment with metronidazole and rifampicin for the duration used in the present study, was not able to reduce the *M. tuberculosis* counts in the spleen to an undetectable level even though they were lower than those in the spleen of mice treated with rifampicin alone. A longer duration of treatment with this combination may be required to sterilise the organs.

Our findings that metronidazole exhibited an additive effect against tubercle bacilli when combined with rifampicin, suggests that metronidazole may have value in eliminating persisters and thus in reducing relapse rates. The period at which metronidazole is to be added to isoniazid and rifampicin requires to be determined. Considering the limited number of animals tested at each time point, the small differences observed and the limited duration of treatment with metronidazole in the present study, further studies on larger numbers of animals at each time point and a longer follow up are necessary to fully assess the activity of metronidazole with other anti-tuberculosis drugs at different concentrations and at different &rations of treatment.

The use of metronidazole may be beneficial in individuals who are HIV positive and who need to receive antituberculosis therapy as chemoprophylaxis to prevent endogenous reactivation of any dormant tubercle bacilli[4]. Besides, metronidazole would also help them by containing concomitant opportunistic parasitic and other anaerobic infections. Further studies are necessary to evaluate this novel approach of management of tuberculosis infection in this special group.

Acknowledgment

The authors thank Dr P. Venkatesan, for help in statistical analysis and Sh. P.R. Somasundaram, for useful comments and assistance in the preparation of this paper.

References

1. Mitchison DA. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months [letter]. *Am Rev Respir Dis* 1993; *147* : 1062-3.
2. Mitchison DA. Basic mechanisms of chemotherapy. *Chest* 1979; 76 : 6 Suppl. 771-81.
3. Narain JP, Raviglione MC, Kochi A. HIV-associated tuberculosis in developing countries : epidemiology and strategies for prevention. *Tuber Lung Dis* 1992; *73* : 311-21.
4. Raviglione MC, Narain JP, Kochi A. HIV-associated tuberculosis in developing countries : clinical features, diagnosis and treatment. *Bull World Health Organ* 1992; *70* : 515-26.
5. McCune RM Jr, Tompsett R, McDermott W. The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. *J* Exp *Med 1956; 104 :* 763-802.
6. McCune RM, Feldmann FM, Lambert HP, McDermott W. Microbial persistence. 1. The capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med* 1966; *123* : 445-68.
7. McCune RM, Feldmann FM, McDermott W. Microbial persistence. Il. Characteristics of the sterile state of tubercle bacilli. *J Exp Med* 1966; *123* : 469-86.
8. de Wit D, Wootton M, Dhillon J, Mitchison DA. The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. *Tuberc Lung Dis* 1995; *76* : 555-62.
9. Wayne LG, Sramek HA. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis. Antimicrob Agents Chemother* 1994; *38* : 2054-8.
10. Wayne LG. Dynamics of submerged growth of *Mycobacterium tuberculosis* under aerobic and microaerophilic conditions. *Am Rev Respir Dis* 1976; *114* : 807-11.
11. Wayne LG, Hayes LG. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* 1996; *64* : 2062-9.
12. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, *et al*. Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity tests in tuberculosis control programmes. *Bull World Health Organ* 1969; *41* : 21-43.
13. Dickinson JM, Mitchison DA. Efficacy of intermittent pyrazinamide in experimental murine tuberculosis. *Tubercle* 1991; *72* : 110-4.
14. Taylor JA Jr, Migliardi JR, Wittenau MS. Tinidazole and metronidazole pharmacokinetics in man and mouse. *Antimicrob Agents Chemother* 1969 : 267-70.
15. Grosset J, Truffot-Pernot C, Bismuth R, Lecoeur H. Les donnecs recentes de la chimiotherapie de la tuberculose experimentale de la souris. *Bull Int Union Tuberc* 1983; *58* : 90-6.
16. Mitchison DA. The Garrod Lecture. Understanding the chemotherapy of tuberculosis-current problems. *J Antimicrob Chemother* 1992; *29* : 477-93.
17. Dickinson JM, Mitchison DA. Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. *Am Rev Respir Dis* 1981; *123* : 367-71.

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