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The Curative Activity of Thioridazine on Mice Infected with *Mycobacterium tuberculosis*

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Abstract. *Background: The aim of the study was to evaluate the effectiveness of thioridazine (TZ) at different dose levels on mice that had been infected intraperitoneally (i.p.) with a high dose of the Mycobacterium tuberculosis ATCC H37Rv strain. Subjects and Methods: Groups of five female BALB/C mice were infected i.p. with 10^6 colony forming units/mL. After thirty days, treatment with TZ was initiated, except for the control group. Mice were treated with TZ at equivalent concentrations to that used in the humans (1200 mg/day), ranging from 0.05 to 0.5 mg/day. Results: The results demonstrated that a daily dose of 0.5 mg/day of TZ reduced the number of colony forming units retrieved from the lungs of infected mice within one month. Conclusion: By the end of 300 days of therapy, although mycobacteria were still retained, their presence, in comparison to that of the control was 8 orders of magnitude lower.*

The therapy of antibiotic-sensitive pulmonary tuberculosis (TB) in countries that could afford the two most effective anti-TB agents, isoniazid (INH) and rifampin (RIF), reduced the rates of new cases (active disease) of pulmonary TB to the point that many predicted the total obliteration of this infection (1, 2). However, because TB is a man-made disease, that is, it is a disease whose rates of infection are affected by conditions that reduce the effectiveness of the immune system (only 5% to 10% of infections progress to active disease status), namely poverty, malnutrition, over-crowdedness, strife and war, its complete elimination is not possible (3). Nevertheless, as long as INH and RIF are readily available and their administration effective, new cases may be readily treated effectively. However, in countries where these agents are relatively costly, and their effective use limited due to the inability of the afflicted patient to purchase them as needed, treatment of the infection has been, to say the least, flawed. Because less than adequate therapy promotes the selection of mutant strains that are resistant to INH or RIF, and eventually to both, when the infection is serially transmitted and ineffective therapy is still present, the rates of new infections will increase (4, 5).

The advent of resistance to INH and RIF now termed multi-drug resistant TB (MDRTB) has meant that MDRTB infections are therapeutically problematic and hence are sources of new infections. Regardless of the strict adherence to the guidelines jointly recommended by the Center for Disease Control (CDC) and the American Thoracic Society (ATS), with the recommendation that at least four of the "first line of defence" drugs be used for the therapy of MDRTB (6, 7), mortality is very high, within one year of diagnosis, and almost 100% within two years (6, 7). Mortality takes place much sooner in MDRTB patients presenting with AIDS (8). MDRTB has hence become a global problem (9). Due to its significant mortality, as well as to its contribution to an increase of new cases of pulmonary TB, the CDC has recommended that "second line of defence" agents be used for the management of MDRTB, even though individual resistance of *M. tuberculosis* to each of these agents is higher than that for INH and RIF (6, 10). No new effective anti-TB agents have been made available for almost 40 years and none are on the horizon. The need for new and effective agents is obvious.

The phenothiazine, chlorpromazine (CPZ), has been known for many years to have *in vitro* activity against antibiotic-susceptible (3) and -resistant *M. tuberculosis* (11-13). In fact, there is much anecdotal evidence that it can cure pulmonary infections of TB (14-18). However, because

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the side-effects produced by CPZ are frequent and severe (18, 19) it has never been seriously considered for the therapy of pulmonary TB. Moreover, because *in vitro* activities take place at concentrations that are clinically irrelevant, its use seemed improbable (20, 21).

A pulmonary infection caused by *M. tuberculosis* has as its principal target the alveolar macrophage and because this macrophage has little killing activity of its own, the bacterium can exist within its intracellular trap for decades, replicating slowly. When the conditions in the environment affect the immune status of the infected individual, the organism breaks out and infects other macrophages and when expelled to the environment, it infects other individuals (22). Because CPZ was known to be concentrated by tissues rich in macrophages (23-27), Crowle *et al.* investigated whether exposure to CPZ of macrophages containing intracellular mycobacteria would promote the killing of the bacteria (28). Nevertheless, interest in the anti-tubercular potential of CPZ was assured once the agent was shown to enhance the killing of intracellular mycobacteria at concentrations in the medium which were within clinical range. The experiments conducted by Crowle *et al.* were repeated with MDRTB and CPZ; because TZ was shown to be the equal to CPZ with respect to *in vitro* activity against antibiotic-resistant *M. tuberculosis* (29, 15), this agent was also evaluated for its activity against intracellular MDRTB and shown to be as effective as CPZ (30, 31). TZ is devoid of many of the severe side-effects of CPZ, hence the use of this agent for the therapy of MDRTB has been recommended for those situations when no available agent is proven effective and the prognosis for the given MDRTB patient is poor (compassionate therapy). The need to evaluate the clinical effectiveness of TZ, nevertheless, remains, and hence, the studies described herein were conducted in order to determine whether the administration of TZ to mice infected with *M. tuberculosis* could cure the animals of this infection.

Subjects and Methods

Bacteria. M. tuberculosis ATCC H37Rv was cultured in MGIT 960 medium (Difco, Madrid, Spain) containing Tween 80 (Difco, Madrid, Spain) until an O.D. of approximately 0.6 was reached. After this, aliquots were prepared and the inoculums adjusted to 10^6 bacteria/mL in phosphate-buffered solution (PBS; Sigma-Aldrich Química SA, Madrid, Spain).

Animals. Groups of female Balb/C mice (approximately 30 g) (Harlan Iberica, Spain), each consisting of 5 controls (to be untreated) and 25 mice (to be treated) were infected intraperitoneally (*i.p.*) with 10^6 colony forming units (CFU)/mL. During and after infection, and for the duration of the experiment, the mice were housed in a P3 BioSafety Cabinet contained within a fully certifiable P3 facility (Office of Health and Safety (OHS) USA-Section VI, Recommended biosafety levels for infectious agents). The handling of animals was supervised by Dr. Marta Martins, licensed for animal handling and infectious agent studies (Course in Laboratory Animal Science, FELASA, Category C; Licensed researcher by the Direcção Geral de Veterinária (DGV), Portugal). After thirty days, treatment with TZ (Sigma-Aldrich Química SA) was initiated, except for the control groups. Each day, four groups of mice (to be treated) were injected *i.p.* with TZ with 0.05, 0.10, 0.2, and 0.5 mg/day, respectively. The maximum dose of 0.5 mg/day is equivalent, on a kg basis, to approximately twice the maximum dose a chronically treated psychotic patient receives per day. The control group was injected with saline (PBS; Sigma-Aldrich Química SA) at the same volume used to administer the agent to mice. At defined time points (monthly), blood from the animals was collected and plasma stored at –80˚C for cytokine analysis by ELISA at a later date. At the end of 30 days, and thereafter at monthly intervals, mice from each group were sacrificed by cervical dislocation and their lungs, liver and spleen removed and weighed. One lung, a lobe of the liver and a portion of the spleen were kept for histological studies. Portions of lung, liver and spleen were weighed, diced and transferred to homogenizing conical tubes containing 1 mL of 0.1N NaOH. The organs were manually homogenised and aliquots of 0.1mL transferred in triplicate to 1.0 mL of saline. From these, aliquots of 0.1 mL were serially transferred to 1.0 mL of saline and 0.1 mL of the resulting solution plated onto 7H11 agar plates (Mycobacteria 7H11 Agar; Difco, Madrid, Spain). The plates were incubated at 37˚C and after 3 to 4 weeks of incubation the CFU were counted and extrapolated to CFU/kg of tissue.

Results

The average absolute number of CFU retrieved from the lungs of mice after infection with approximately 10^6 *M. tuberculosis* ATCC H37Rv is shown in Figure 1A. Daily doses of TZ below 0.5 mg did not produce any significant change in the bacterial load as compared to the untreated control mouse group during the entire period of the study (data not shown). Thirty days after the mice were treated daily with 0.5 mg/day of TZ there was in excess of a five log reduction in the CFU/kg retrieved from the lung as compared to the control group (Figure 1B). The effects of 0.5 mg/day on the bacterial load recovered from the lung of the animals became more prominent with each passing 30 day interval, such that by the end of 300 days of treatment the difference between the treated group and its control untreated group was approximately 8 orders of magnitude (Figure 1B). The bacterial load recovered from the liver or the spleen, unlike that recovered from the lung, continued to increase regardless of therapy with TZ and was similar to that recovered from the untreated infected control mouse group (data not shown).

Discussion

Although TZ therapy appears to be very effective, after 300 days a few CFU per mg of lung tissue could still be recovered, hence the infection persisted, albeit at a very low level. The question of why mycobacteria could still be recovered 300 days

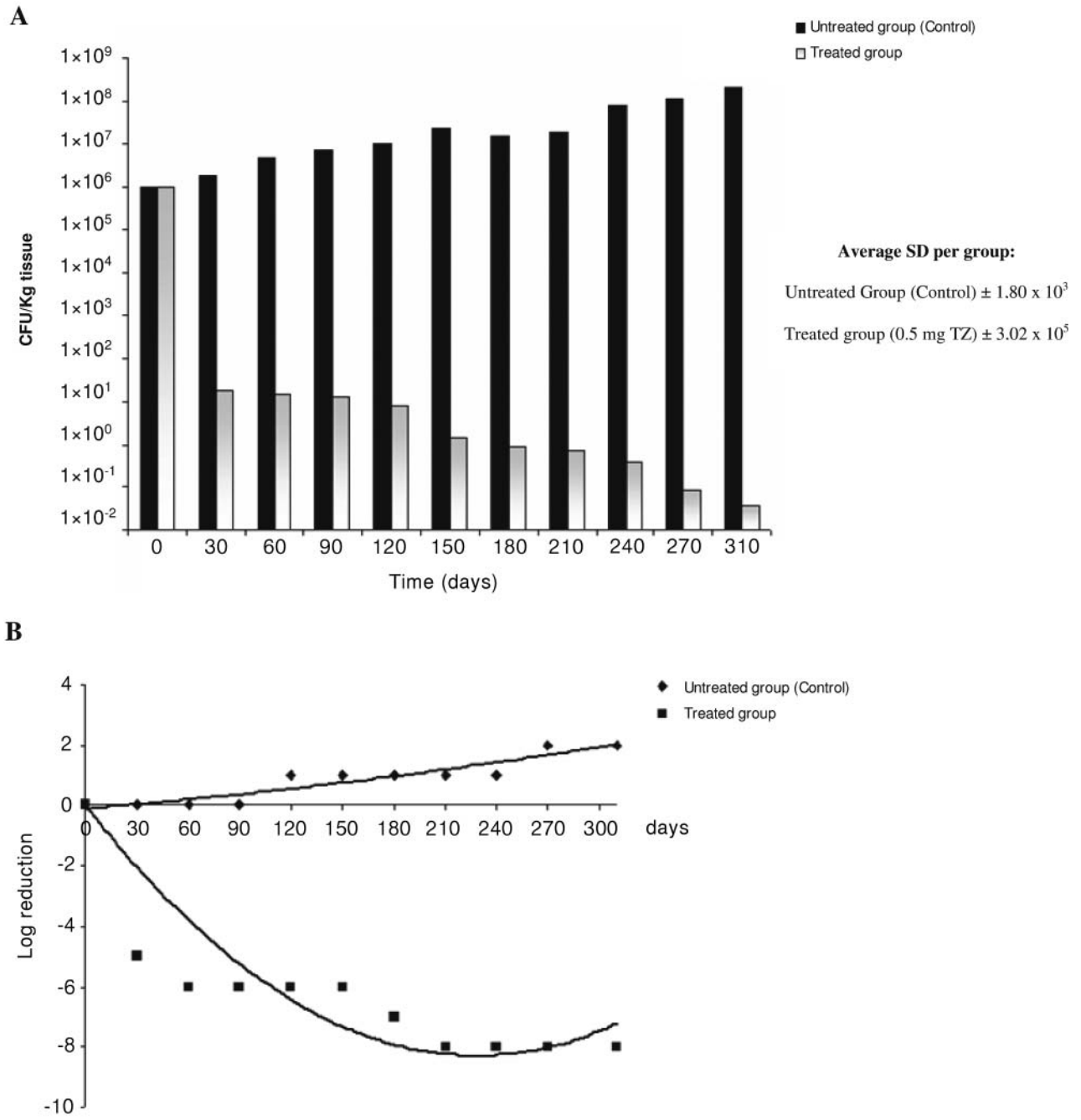

Figure 1. *Effect of daily TZ treatment of mice infected with M. tuberculosis. Mice were infected i.p. with approximately 10^6 M. tuberculosis ATCC H37Rv. After thirty days of infection, mice were treated daily with a dose equivalent in the human to 1200 mg of TZ (0.5 mg/day). A, Average absolute number of colony forming units (CFU) per kg of mouse lung retrieved from the lungs of treated and non-treated (control) infected mice. B, Log reduction of M. tuberculosis per kg of mouse lung retrieved from the lung of the treated mice as compared to the control group (no treatment).*

after daily therapy, whereas experimental data obtained from human macrophages that had been infected *ex vivo* could be completely rid of *M. tuberculosis* subsequent to exposure to as little as 0.1 mg/L of TZ (31) can be answered from the data obtained from spleens and livers of both treated and untreated mice. Daily therapy with TZ did not reduce the number of CFU of mycobacteria retrieved from the liver or the spleen (data not shown) during the time of the experiment as opposed to the decrease of mycobacteria that was evident in the lungs. The single dose of mycobacteria (10^6 CFU)

introduced *i.p.* is massive compared to that to which a human is exposed and subsequently infected with. This massive dose was necessary inasmuch as mice, even though the strain used in this study is relatively more readily infected with mycobacteria than other strains (32), are relatively resistant to infection by *M. tuberculosis*. Taking this massive dose into account, as well as the possibility that the spleen and liver were sources of mycobacteria, it was surprising to note the effectiveness of TZ therapy. The experimental protocol employed in this study does not shed any light as to whether TZ is effective against mycobacteria that are trapped in the liver and spleen given the massive *i.p.* dose administered.

Recent studies have provided evidence that phenothiazine derivatives can clear the lungs of mice that have been infected with only 200 CFU of *M. tuberculosis* (33, 34). In light of these latter studies, the significance of TZ therapy becomes even greater given its effectiveness against an infection that was 5,000 times greater. Nevertheless, we must remember that although TZ is effective for the therapy of pulmonary TB of the mouse, other than anecdotal evidence and laboratory studies that support the use of TZ (35), its effectiveness for therapy of human pulmonary TB remains to be determined.

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