Agents and Actions, vol. 15, 3/4 (1984)

0065-4299/84/030398-05$2.50

A dual effect of tilorone on multiplication of *Mycobacterium leprae* in mice

LOUIS LEVY[2] and FANNY AIZER

Department of Comparative Medicine, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Abstract

Tilorone, a synthetic inducer of interferon found earlier to inhibit multiplication of *Mycobacterium leprae* in the foot pad of the mouse while it enhanced infections of mice by *M. lepraemurium* and *M. marinum*, has been shown to exert a dual effect on *M. leprae* infection of the mouse. When administered continuously, incorporated into the mouse diet in a concentration of 0.015 g per 100 g diet, the drug was usually immunosuppressive, permitting enhanced multiplication of the organisms. When administered in a 3-fold larger concentration beginning during the lag phase or early during logarithmic multiplication, tilorone was antimicrobial; however, when administered in the larger concentration beginning after logarithmic multiplication had been well established, the drug was immunosuppressive. The antimicrobial action of tilorone against *M. leprae* appears to be a direct action that is weak and slow in onset. The mechanism of the immunosuppressive action remains to be elucidated.

Introduction

Tilorone (2,7-bis[2-diethylaminoethoxy]fluorene-9-one dihydrochloride) is a synthetic inducer of interferon that also exerts immunosuppressive effects. In an earlier report [1], we described enhancement of infections by *Mycobacterium lepraemurium* and *M. marinum* in BALB/c mice administered tilorone, whereas, in mice of the same strain administered tilorone in the same dosage, multiplication of *M. leprae* was inhibited. Because the mice infected by *M. leprae* were undoubtedly immunosuppressed to the same degree that were those infected by *M. lepraemurium* and *M. marinum*, we concluded that tilorone exerted a direct, antimicrobial effect on *M. leprae*, that outweighed the immunosuppression.

More recently, we have carried out experiments in tilorone-treated CBA mice, in which multiplication of *M. leprae* was sometimes inhibited and sometimes enhanced. This paper represents a preliminary report of this work.

Materials and methods

Tilorone was generously supplied by Merrell-Dow Pharmaceuticals, Inc., Cincinnati, OH, USA. *M. leprae* of the strain employed in the earlier experiments [1] were supplied by the Leprosy Research Unit, Public Health Service Hospital, San Francisco, CA, USA, and carried subsequently in serial mouse-passage. The mice were locally-bred male weanlings of the CBA/CaHN strain.

Inoculation of mice with *M. leprae* in both hind foot pads and harvest and enumeration of acid-fast bacilli (AFB) from the infected tissues were performed at intervals by established methods [2, 3]. Each harvest was performed from a pool of four foot pads. Tilorone was incorporated into the ground mouse pellets by means of a liquid-solid twin-shell blender (Patterson-Kelly Co., East Stroudsburg, PA, USA).

Statistical analysis of mouse-foot-pad harvest data was performed by multiplying together the exact probability [4] of each observed arrangement of results, in the manner recommended by SHEPARD [5]. If the numbers of AFB recovered in single harvests from tilorone-treated mice exceeded (or were smaller than) all of the numbers of AFB recovered in four simultaneous harvests from untreated control mice at two consecutive intervals, the probability that the difference of the results in treated mice from those in control mice had occurred by chance is smaller than 0.05 ($p = 0.2 \times 0.2 = 0.04$).

Results

In the first experiment, groups of 15 mice were inoculated, each mouse in both hind foot pads, with $10^{3.7}$ *M. leprae* per foot pad. Begin-

[1] Supported in part by grants from les Amis du Pere Damien, Brussels; Nederlandse Stichting voor Leprabestrijding, Amsterdam; and le Secours aux Lepreux, Montreal.

[2] To whom requests for reprints should be sent.

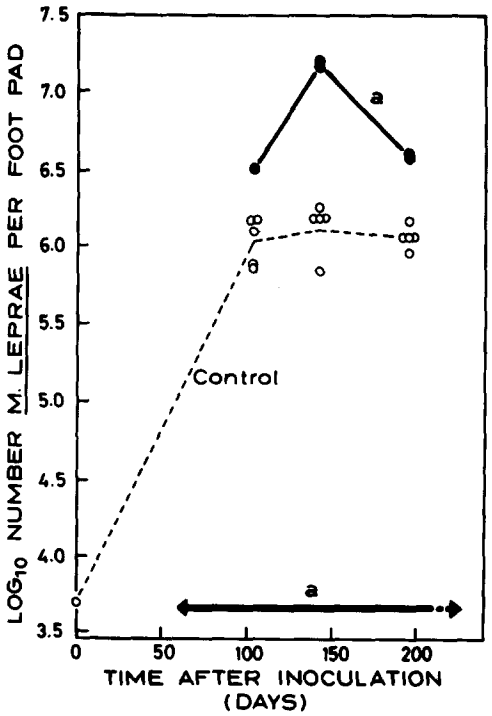

Figure 1
Log_{10} number of *M. leprae* per foot pad as a function of time after inoculation. Tilorone was administered in a concentration of 0.05 g per 100 g during the period represented by the solid line drawn parallel to the abscissa. The closed circles represent the results of harvests of *M. leprae* from tilorone-treated mice.

ning 60 days after inoculation, tilorone was administered to two groups of mice in a concentration of 0.05 g per 100 g mouse meal, whereas four groups of mice were held as untreated controls. As shown in Fig. 1, five harvests of *M. leprae* performed from control mice 103 days after inoculation yielded $10^{5.85}$–$10^{6.15}$ AFB per foot pad. A harvest of *M. leprae* performed on the same day from the tilorone-treated mice yielded $10^{6.50}$ AFB per foot pad. No further increase of the number of *M. leprae* in control mice was found as the result of harvests performed 142 and 195 days after inoculation. On the other hand, multiplication of *M. leprae* in tilorone-treated mice reached a maximum of $10^{7.18}$ AFB per foot pad, the mean of two harvests performed on day 142. Two harvests of *M. leprae* from tilorone-treated mice 195 days after inoculation yielded a mean of $10^{6.57}$ AFB per foot pad. Because the yields of harvests from tilorone-treated mice performed on days 142 and 195 were greater than the yields of all of five control harvests on each of the two days, it may be concluded that the infection in tilorone-treated mice was enhanced ($p = 0.167 \times 0.048 \times 0.048 = 0.0038$).

It appeared difficult to reconcile the enhanced multiplication of *M. leprae* demonstrated in this experiment with the inhibition of multiplication observed in the earlier experiment [1]. In addition to the fact that BALB/c mice had been employed in the first experiment, whereas CBA mice were employed in the present study, tilorone had been administered in the same concentration but by different treatment schedules in the two experiments. In the earlier experiment, administration of tilorone was begun either on the day of inoculation, or 70 days later, at which time the *M. leprae* had multiplied to the level of only $10^{4.6}$ per foot pad. In the present experiment, as shown in Fig. 1 by the growth curve of *M. leprae* in control mice, multiplication of *M. leprae* had achieved the level of 10^5 per foot pad on day 60, when the administration of tilorone was begun.

Therefore, a second experiment was performed, like the first in all respects, except that administration of tilorone was begun either on the day of inoculation or 60 days later; drug-administration was stopped on day 159 to the mice beginning treatment on the day of inoculation. In addition, tilorone was administered to one group of mice in a concentration of 0.015 g per 100 g mouse meal, beginning on day 60.

As may be observed in Fig. 2, multiplication of *M. leprae* was inhibited in both groups of mice administered tilorone in the concentration of 0.05 g per 100 g. Harvests of *M. leprae* performed on days 155 and 188 from the mice beginning treatment on the day of inoculation yielded, respectively, $10^{4.43}$ and $10^{5.29}$ AFB per foot pad. These results are lower than those of all four of the harvests from control mice performed on those days, which yielded, respectively, $10^{5.91}$–$10^{6.20}$ and $10^{5.86}$–$10^{6.05}$ AFB per foot pad. On day 215, a harvest from tilorone-treated mice yielded $10^{5.64}$ AFB per foot pad, a value lower than all but one of the five control harvests performed at the same time (these yielded $10^{5.49}$–$10^{6.38}$ AFB per foot pad). In contrast to the results of the preceding experiment, tilorone treatment of the mice in this experiment inhibited

the multiplication of *M. leprae* ($p = 0.2 \times 0.2 \times 0.33 = 0.0132$).

In the mice administered tilorone in the larger concentration beginning 60 days after inoculation, *M. leprae* had multiplied to $10^{4.98}$ per foot pad on day 155, and to $10^{5.32}$ per foot pad on day 188; both of these yields are smaller than all of the corresponding results from control mice ($p = 0.2 \times 0.2 \times 0.04$). By 215 days after inoculation, despite continued administration of tilorone, *M. leprae* had multiplied to a level of $10^{6.12}$ per foot pad, a value indistinguishable from the corresponding control values. Similarly, tilorone administered in a concentration of 0.015 g per 100 g meal continuously from day 60 had no effect.

Again, it appeared difficult to reconcile the results of this experiment with those of the two preceding experiments. That the drug exerted an antimicrobial effect appeared to exclude a difference between mouse strains as the source of the discrepant results obtained from the first two experiments. On the other hand, scrutiny of the growth curve of *M. leprae* in the control mice of this experiment (Fig. 2) shows that growth of the organisms was slower in this than in the preceding experiments (Fig. 1 and Ref. [1]). By day 60, multiplication of *M. leprae* had reached only to the level of about $10^{4.2}$ per foot pad, and multiplication to the level of 10^5 per foot pad occurred only after day 100. Thus, it appeared that, in terms of the state of the organisms at the time of beginning administration of tilorone, there was little difference between the two schedules of drug-administration in this experiment; when drug administration was begun on the day of inoculation, the organisms were in lag phase, whereas by day 60, they were probably very early in the phase of logarithmic multiplication.

A third experiment was carried out to examine in greater detail the influence of tilorone concentration and timing. Except in two respects, this experiment was identical to the two previous experiments. Administration of tilorone in two concentrations—0.05 and 0.015 g per 100 g—was begun on the day of inoculation, and to

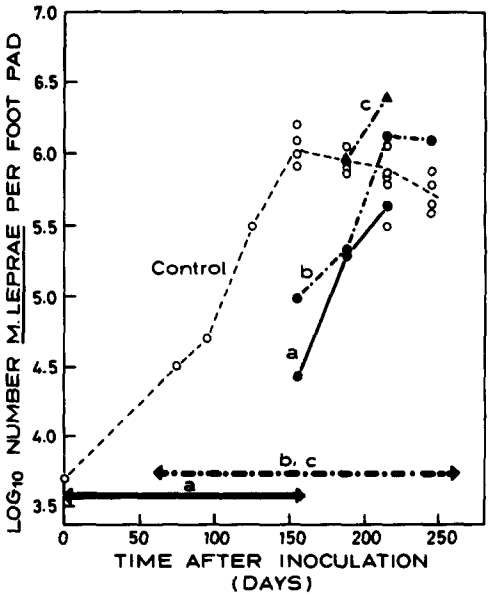

Figure 2
Log_{10} number of *M. leprae* per foot pad as a function of time after inoculation. Tilorone was administered in a concentration of 0.05 g per 100 g during the period represented by the solid line drawn parallel to the abscissa, and in a concentration of 0.05 (●) or 0.015 (▲) g per 100 g during the period represented by the broken line that parallels the abscissa.

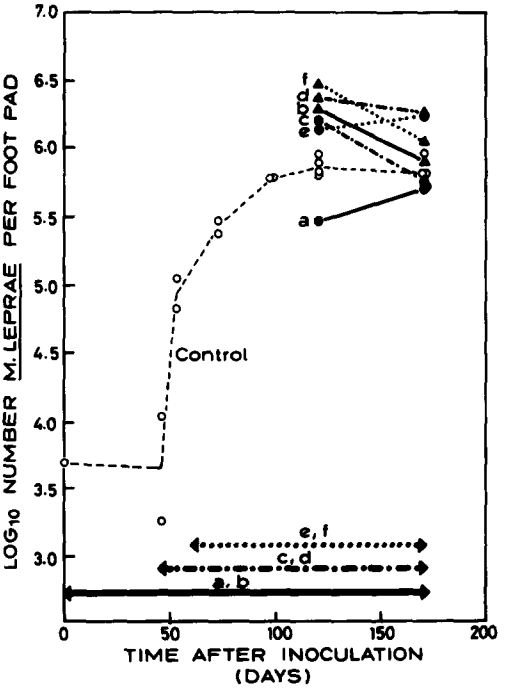

Figure 3
Log_{10} number of *M. leprae* per foot pad as a function of time after inoculation. Tilorone was administered in a concentration of 0.05 (●) or 0.015 (▲) g per 100 g for the periods represented by the lines drawn parallel to the abscissa.

additional groups of mice 45 days later. Administration of tilorone to yet additional groups of mice was delayed until the results of early harvests showed that logarithmic multiplication of *M. leprae* was well established. When two harvests from control mice on day 53 yielded values of $10^{4.83}$ and $10^{5.05}$ AFB per foot pad (see Fig. 3), it was decided to begin tilorone administration on day 60.

Subsequent control harvests revealed that multiplication of the organisms reached a maximum, $10^{5.71}$–$10^{5.95}$ AFB per foot pad, about 100 days after inoculation. Later harvests from both control and treated mice revealed that tilorone, administered in the larger concentration from the day of inoculation, had inhibited multiplication of *M. leprae* ($p = 0.04$), whereas, administered in the smaller concentration by this schedule, the drug was without effect. When administration of tilorone was begun 45 days after inoculation, the larger concentration produced no effect, whereas the smaller concentration was immunosuppressive ($p = 0.04$). Both concentrations of the drug produced enhancement of the infection when administration was begun 60 days after inoculation ($p = 0.04$ in each case).

Discussion

Infection with *M. leprae* of the hind foot pad of the immunologically normal mouse is characterized by an apparent lag phase of variable duration, depending upon the proportion of viable organisms in the standard inoculum ($10^{3.7}$–$10^{4.0}$ AFB per foot pad) [2]. Thereafter, logarithmic multiplication proceeds, with an apparent doubling time of 11–12 days [6], until a maximum of $10^{6.0}$–$10^{6.3}$ AFB per foot pad is reached [2]. The number of *M. leprae* per foot pad changes only slightly after the maximum of multiplication had been attained [2].

On the other hand, in mice immunosuppressed to a degree that threatens survival in a conventional environment, as by adult thymectomy followed by whole-body irradiation [7] and in congenitally athymic mice [8], the maximum of multiplication may be raised by as much as 100- to 1000-fold. This and other evidence suggests that multiplication of *M. leprae* in the foot pad of the immunologically normal mouse is limited by a cell-mediated immune response. Thus, multiplication of *M. leprae* in some tilorone-treated mice to levels not ordinarily encountered in normal mice may be attributed to an immunosuppressive effect of the drug. That the tilorone-treated mice appeared healthy, and that no unusual mortality was observed suggests the operation of a different mechanism of immunosuppression.

A problem encountered in work on *M. leprae*-infection of mice is that one cannot measure the quality of a given inoculum except by inoculating mice. In general, the proportion of viable organisms can be optimized by harvesting them from passage mice at the time that multiplication is maximal or shortly before this time. Once multiplication has reached its maximum, the proportion of viable *M. leprae* decreases exponentially [9]. However, the behaviour even of a given 'fast' [10] strain of *M. leprae*, which has been carried through many passages and employed in many similar experiments, cannot always be predicted. This fact, which appears to explain the variation of the bacterial growth curve in untreated mice from experiment to experiment, led to the unexpected observations reported in this paper.

Administered to mice in the smaller concentration (0.015 g per 100 g meal), tilorone appeared incapable of exerting an antimicrobial effect against *M. leprae*. However, when the drug was administered in a 3-fold larger concentration, the antimicrobial effect was observed when administration was begun early enough during logarithmic multiplication. These observations suggest that tilorone exerts only a weak antimicrobial effect that is slow in onset. When administration of the drug is begun in mid-logarithmic multiplication, not enough time remains for the drug to exert an observable antimicrobial effect. The usual maximum of multiplication would ordinarily be reached within 3.3 to 4.3 doublings (5–7) weeks after *M. leprae* had multiplied to the level of 10^5 per foot pad. So slow an onset of an antimicrobial effect may appear unlikely; however, a delay of onset equivalent to 3 to 5 doublings (5–9 weeks) was occasionally recognized when BALB/c mice infected by this strain of *M. leprae* were treated with dapsone in small but effective concentrations [11]. Thus, when tilorone administration is begun late, the antimicrobial effect might be recognized only by failure of multiplication of the organisms to be enhanced.

It thus appears that which of the two effects of tilorone on multiplication of *M. lepae* in mice is predominant is determined by the concentration

in which the drug is administered, and by the timing of its administration. The immunosuppressive effect of tilorone which results in enhanced multiplication of the organisms is exerted by both concentrations of the drug. And because this effect is exerted on the host, it should be present, although perhaps not measurable, whatever the timing of drug administration.

The antimicrobial effect, weak though it is, is of some interest. Earlier work with another inducer of interferon – polyinosinic-polycytidylic acid – suggested that the activity of that compound in inhibiting multiplication of *M. leprae* was independent of the induction of interferon [12]. On the other hand, COLLINS [13] has reported minimal inhibitory concentrations, measured *in vitro*, of tilorone for BCG (1.2×10^{-4} *M*), *Listeria monocytogenes* (5.3×10^{-4} *M*), and *Salmonella enteritidis* (1.06×10^{-3} *M*). And work in progress in our laboratory has identified strains of several cultivable mycobacterial species against which the minimal inhibitory concentration of tilorone is smaller than 10^{-4} *M*. Although the antimicrobial potency of tilorone against *M. leprae* is too small for the drug to be useful, several analogues, also provided by Merrell-Dow Pharmaceuticals, Inc., are presently being screened against *M. leprae* in mice, in the hope that one or more might be shown to be more potent than tilorone, and to provide the information necessary for analysis of structure-activity relationships.

The immunosuppressive effect of tilorone is perhaps of greater interest. There has accumulated a large literature on the mechanism of immunosuppression by tilorone (see Refs [1] and [14] for reviews). Two recent papers appear of particular interest. As the result of a study of the enhancement by tilorone of several intracellular infections of mice, COLLINS [15] concluded that the drug interfered with the production or deployment of activated macrophages. On the other hand, LEVINE and his coworkers [16] attributed the immunosuppressive effects of tilorone on the development of experimental allergic encephalomyelitis in the rat primarily to selective depletion of T-lymphocytes from peripheral lymphoid tissue, to impairment of the ability of effector lymphocytes to transfer the process, and to alteration or destruction of the non-specific reactive cells that comprise the perivascular inflammatory infiltrates characteristic of this process. These workers were unable to find evidence of a mechanism involving activation of suppressor cells.

Studies of the mechanism of the immunosuppressive effect of tilorone on *M. leprae* infection of the mouse are in progress.

Received 18 July 1983

References

[1] L. LEVY, F. AIZER, H. NG and T.M. WELCH, *The effects of tilorone on mycobacterial infections of mice*, Lepr. Rev. *49*, 215–222 (1978).

[2] C.C. SHEPARD, *The experimental disease that follows the injection of human leprosy bacilli into footpads of mice*, J. exp. Med. *112*, 445–454 (1960).

[3] C.C. SHEPARD and D.H. MCRAE, *A method for counting acid-fast bacteria*, Int. J. Lepr. *36*, 78–82 (1968).

[4] S. SIEGEL, *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill, New York 1958.

[5] C.C. SHEPARD, *Statistical analysis of results obtained by two methods for testing drug activity against* Mycobacterium leprae, Int. J. Lepr. *50*, 96–101 (1982).

[6] L. LEVY, *Studies of the mouse foot pad technique for cultivation of* Mycobacterium leprae. *3. Doubling time during logarithmic multiplication*, Lepr. Rev. *47*, 103–106 (1976).

[7] R.J.W. REES, *Enhanced susceptibility of thymectomized and irradiated mice to infection with* Mycobacterium leprae, Nature, Lond. *211*, 657–658 (1966).

[8] M.J. COLSTON and K. KOHSAKA, The nude mouse in studies of leprosy. In *The Nude Mouse in Experimental and Clinical Research*, vol. 2, pp. 247–266 (Ed. J. FOGH and B.C. GIOVANELLA). Academic Press, New York 1982.

[9] T.M. WELCH, R.H. GELBER, L.P. MURRAY, H. NG, S.M. O'NEILL and L. LEVY, *Viability of* Mycobacterium leprae *after multiplication in mice*, Infect. Immun. *30*, 325–328 (1980).

[10] C.C. SHEPARD and D.H. MCRAE, *Hereditary characteristic that varies among isolates of* Mycobacterium leprae, Infect. Immun. *3*, 121–126 (1971).

[11] L. LEVY and J.H. PETERS, *Some characteristics of the action of dapsone on multiplication of* Mycobacterium leprae *in the mouse*, Lepr. Rev. *48*, 237–245 (1977).

[12] L. LEVY and T.C. MERIGAN, *Inhibition of multiplication of* Mycobacterium leprae *by polyinosinic-polycytidylic acid*, Antimicrob. Ag. Chemother. *11*, 122–125 (1977).

[13] F.M. COLLINS, *Effect of tilorone treatment on intracellular microbial infections in specific-pathogen-free mice*, Antimicrob. Ag. Chemother. *7*, 447–452 (1975).

[14] S. LEVINE, T-cell depletion and other effects of tilorone. In *Modulation of Host Immune Resistance in the Prevention or Treatment of Induced Neoplasia*, pp. 89–96 (Ed. M.A. CHIRIGOS). Fogarty International Center Proceedings No. 28, U.S. Government Printing Office, Washington 1977.

[15] F.M. COLLINS, *Mechanism of cellular suppression induced by oral tilorone treatment of mice*, Infect. Immun. *30*, 289–296 (1980).

[16] S. LEVINE, R. SOWINSKI and S.L. ABREU, *Suppression of experimental allergic encephalomyelitis by tilorone: cell transfer and interferon studies*, Immunopharmac., *6*, 23–30 (1983).