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The Effects of Tilorone on Mycobacterial Infections of Mice

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Tilorone (2,7-bis[2-diethylaminoethoxy]fluoren-9-one dihydrochloride), administered in a concentration of 0.05 g per 100 g in the mouse chow, was found to inhibit multiplication of *Mycobacterium leprae* in the mouse footpad. Infection was enhanced in mice inoculated with *M. marinum* or *M. lepraemurium* to which the drug was administered in the same dosage. Tilorone appears to have exerted an antimicrobial effect on *M. leprae* that outweighed the immunosuppressive effect of the drug on the mouse host.

Introduction

We have recently demonstrated that intensive treatment of mice infected with *Mycobacterium leprae* with the interferon inducer polyinosinic:polycytidylic acid (polyI:C) inhibits multiplication of the organisms in the mouse footpad (Levy and Merigan, 1977). These results led us to study the effects on *M. leprae* infection of treatment of mice with another synthetic interferon inducer, tilorone (2,7-bis[2-diethylaminoethoxy]fluoren-9-one dihydrochloride). Treatment with tilorone was accompanied by inhibition of multiplication of *M. leprae* in the mouse footpad, but the character of the inhibition appeared more consistent with that produced by an antimicrobial agent than with an effect that could be attributed to enhancement of host resistance. Therefore, we also studied the effects of tilorone treatment of mice infected with *M. marinum* and *M. lepraemurium.* The results of our studies suggest that tilorone inhibited multiplication of *M. leprae* in the mouse footpad by a direct antimicrobial action rather than by non-specific enhancement of host resistance.

Materials and Methods

Tilorone was generously supplied by Merrell-National Laboratories, Cincinnati, Ohio. The *M. leprae* were of a strain that had been originally isolated from a lesion of a patient with previously untreated lepromatous

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leprosy by C. C. Shepard, Center for Disease Control, Atlanta, Georgia, and subsequently carried in mouse passage. Inocula of *M. leprae* were prepared from organisms recovered from the footpad tissues of mice inoculated earlier in the hind footpads. The *M. lepraemurium,* of the Hawaiian strain, were supplied by B. H. Tepper, Leonard Wood Memorial Leprosy Research Laboratory, the Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland, and subsequently carried in mouse passage. Inocula of *M. lepraemurium* were prepared from organisms recovered from the omental fat of mice inoculated intraperitoneally (i.p.) earlier. The culture of *M. marinum* was a gift of A. Back, City-County Health Department, San Francisco, California. The mice were locally bred BALB/c mice. Drug was incorporated into the mouse chow by means of a liquid-solid twin-shell blender (Patterson-Kelly Co., East Stroudsburg, Pennsylvania).

Inoculation of mice in the footpad with *M. leprae* or *M. marinum* and harvest of acid-fast bacteria (AFB) from the infected tissues were performed by published methods (Ng *et al.,* 1973; Shepard, 1960; Shepard and McRae, 1968). In the case of *M. leprae*-infected mice, the harvested AFB were only enumerated. In the case of mice inoculated in the footpad with *M. marinum,* the harvested AFB were enumerated by microscopic examination, and the numbers of colony-forming units (CFU) were counted in aliquots of the same suspensions. Footpad swelling was measured by means of a "Schnelltaster" caliper ("Quick-test", Dyer Co., Inc., Lancaster, Pennsylvania). Mice were also inoculated intravenously (i.v.) in a tail vein with *M. marinum* or i.p. with *M. lepraemurium.* These mice were observed at short intervals, deaths were recorded, and the "survival index" of Smith and Westgarth (1957) was calculated.

Results

M. LEPRAE INFECTION

Groups of 15 mice each were inoculated with $10^{3.7}$ *M. leprae* in each hind footpad. Several groups served as untreated controls. To one group of mice, tilorone was administered incorporated in the mouse chow in a concentration of 0.05 g per 100 g, the maximal tolerated dosage, for a period of 30 days, beginning 70 days after inoculation. Tilorone was administered in the same dosage to the mice of another group for 150 days, beginning on the day of inoculation. The results of treatment of *M. leprae*-infected mice with tilorone are shown in Fig. 1, in which each point represents the results of harvest of *M. leprae* from the pooled tissues of 4 footpads. The 95% confidence limits around the results of such a harvest are -50% and +100% when the yield of AFB per footpad is near 10^6 (Krushat *et al.,* 1976). The broken line represents the linear regression of the $\log_{10}$ number of *M. leprae* per footpad on the number of days after inoculation of untreated control mice during logarithmic multiplication. The slope of the regression line yields an estimated doubling time of 12.4 days. The harvest of *M. leprae* performed 147 days after inoculation yielded $10^{5.39}$AFB per footpad of mice treated between days 70

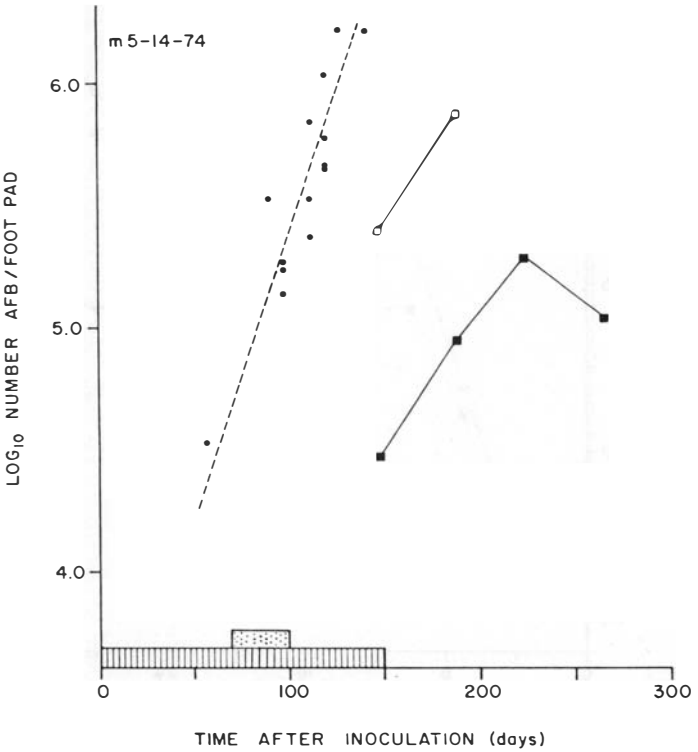

Fig. 1. Multiplication of *M. leprae* in the footpads of mice as a function of time after inoculation. The broken line is the regression of the $\log_{10}$ number of AFB per footpad in untreated mice on the number of days after inoculation. (●) Results of harvests of *M. leprae* from the footpads of untreated mice; (■) results of harvests from mice treated with tilorone for 150 days from day of inoculation; (□) results of harvests from mice treated with tilorone for 30 days from day 70 after inoculation. The shaded bars along the abscissa represent the periods of drug administration.

and 100 after inoculation, and $10^{4 \cdot 48}$ AFB per footpad of mice treated from the day of inoculation. Subsequent harvests demonstrated that, after termination of treatment, the organisms multiplied in both groups of mice. After the mice had been treated with tilorone for 30 days, the *M. leprae* multiplied almost to the maximal level of $10^{6 \cdot 0}-10^{6 \cdot 3}$ at a rate not greatly different from that in the untreated mice. After the 150-day period of treatment with tilorone, the organisms multiplied at the same rate but to a somewhat lower maximum, plateauing at the level of $10^{5 \cdot 03}-10^{5 \cdot 28}$. These results demonstrate that multiplication of *M. leprae* was modestly inhibited in mice treated with tilorone administered for 30 days during logarithmic multiplication of the organisms. Multiplication of *M. leprae* was inhibited more strongly in mice treated with tilorone for 150 days, beginning on the day of inoculation.

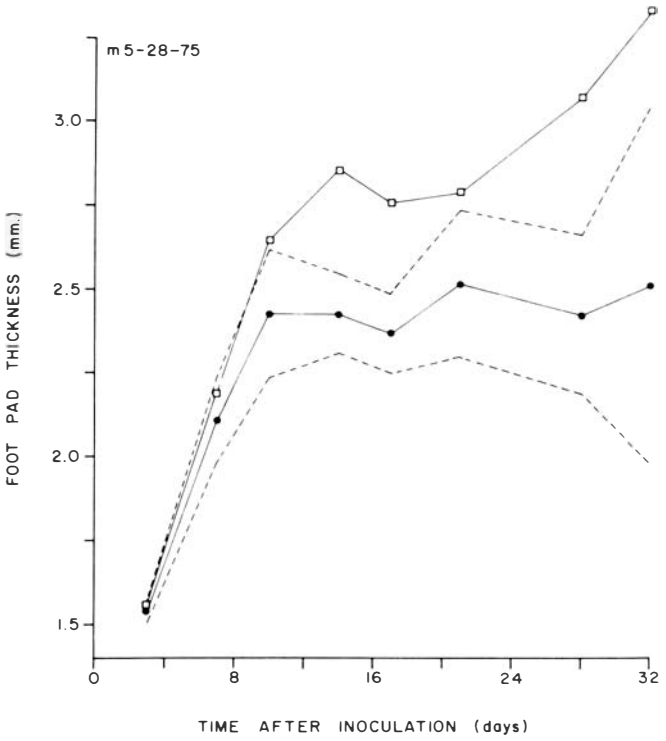

Fig. 2. Footpad thickness of mice as a function of time after inoculation with *M. marinum*. (●) Results of measurements in untreated mice; (□) results of measurements in tilorone-treated mice. (— — —) 95% confidence limits around measurements of footpad thickness in untreated mice. The points at 28 and 32 days represent measurements made on 6 feet (3 mice); all other points were based on measurements of 10 feet (5 mice).

INFECTION WITH *M. MARINUM* AND *M. LEPRAEMURIUM*

The effects of tilorone treatment of mice infected with *M. marinum* and *M. lepraemurium* were also studied. In one experiment, tilorone was administered continuously in a concentration of 0.05 g per 100 g of diet to 10 mice inoculated with $10^{3 \cdot 7}$ *M. marinum* in both hind footpads, beginning one week before inoculation. Ten untreated mice similarly inoculated were observed as controls. As shown in Fig. 2, footpad swelling was greater in the tilorone-treated mice than in the untreated control mice. The results of harvests of *M. marinum* from the infected footpad tissues of some of the same control and tilorone-treated mice are summarized in Table 1. In the control mice, the numbers of AFB and of CFU appeared to reach maximal values 14–21 days after inoculation, after which the numbers diminished. Maximal numbers of AFB and CFU were achieved in tilorone-treated mice at about the same time as in control mice; however, the maxima were at least one order of magnitude

TABLE 1

Results of harvests of M. marinum *from untreated and tilorone-treated mice after footpad inoculation*

Treatment	Time after inoculation (days)	AFB No. per footpad ($\times 10^5$)	CFU No. per footpad ($\times 10^5$)
None	7	8.42(4.21–16.8)*	1.43(1.32–1.54)
	14	11.4(5.70–22.8)	1.25(1.03–1.47)
	21	10.2(5.10–20.4)	4.24(2.95–5.53)
	49	1.47(0.74–2.94)†	0.10(0.07–0.13)†
Tilorone 0.05 g/100 g	7	7.88(3.94–15.8)	1.34(1.24–1.44)
	14	64.2(32.1–128)	80.2(79.7–80.7)
	21	82.0(41.0–164)	94.0(93.0–95.0)
	49	114(57.0–288)†	112(104–120)†

*Mean (95% confidence limits).
†Each value for the number of AFB or CFU per footpad was derived from the pooled footpad tissues of 2 mice (4 footpads), except for the 49-day values, which were derived from harvests from the pooled tissues of 4 mice (8 footpads).

greater in the treated mice, and the numbers did not decrease during the period of observation. In two subsequent experiments, mice were challenged i.v. with $10^{6 \cdot 6}$ or $10^{7 \cdot 6}$ *M. marinum* suspended in Hanks' balanced salt solution (BSS); control mice were left untreated, whereas 0.05 g per 100 g tilorone was administered to other groups of mice continuously, beginning 1 week before challenge. As shown in Table 2, the survival of tilorone-treated mice was much shortened compared to that of untreated mice.

In a final experiment, untreated mice and mice to which the administration of 0.05 g per 100 g tilorone was begun on the day of inoculation were challenged i.p. with 10^7 or 10^8 *M. lepraemurium* suspended in BSS. As shown in the lower portion of Table 2, the survival of tilorone-treated mice after challenge with *M. lepraemurium* was much shortened compared to that of control mice. Although the intake of tilorone by *M. marinum*- and *M. lepraemurium*-infected mice was not measured, it undoubtedly decreased as the animals became ill. Nevertheless, it appears certain that the mice died of infection and not of drug toxicity. The *M. leprae*-infected mice administered tilorone in the same dosage for 150 days showed no evidence of toxicity; as demonstrated by the data of Table 2, no *M. marinum*- or *M. lepraemurium*-infected mouse treated with tilorone survived longer than 118 days.

Discussion

Poly I:C has been demonstrated to protect mice against infection with a wide variety of bacterial, fungal, and protozoal pathogens (Merigan, 1973), and we have recently demonstrated that intensive treatment of mice with poly I:C for a short time during logarithmic growth inhibits multiplication of *M.*

TABLE 2
Survival of untreated and tilorone-treated mice after intravenous challenge with M. marinum *or intraperitoneal challenge with* M. lepraemurium

Experiment no.	Challenge	Survival	
		Control	Tilorone-treated
1	*M. marinum* $10^{7.6}$	25.4* [20, 26, 30, 33(7), 41(9),† 44, 48(3), 56(4), 57, 59(2)]	51.3 [12, 16, 20(27), 23]
2	*M. marinum* $10^{7.6}$	18.7 [30, 35(2), 38(2), 41, 46(5), 59, 83,(4), 84, 93, 132, 135]	41.7 [18(5), 21(2), 23(4), 25(5), 46(4)]
	$10^{6.6}$	<7.37 [41, 46, 65, 83(2), 155, 187, 260(13)‡]	29.0 [21(2), 23, 28(4), 30(3), 46(10)]
3	*M. lepraemurium* 10^{8}	7.02 [106, 113(3), 125(3), 128(3), 149(2), 160, 167, 222, 229, 238(2)]	11.0 [83, 86, 90(4), 93(2), 97(2)]
	10^{7}	5.27 [149(2), 167, 170(2), 173(2), 183, 187, 194, 201, 204(2), 208, 211, 222(4), 225]	9.25 [97(2), 100, 111(2), 113(2), 118(2)]

*Survival index of Smith and Westgarth (1957).
† [Number of days from inoculation to death (number dead on day indicated)]. If the number dead is not specified, only one mouse was found dead.
‡The number of mice indicated within the parenthesis survived on the day indicated.

leprae by a mechanism that does not depend upon interferon induction (Levy and Merigan, 1977). The purpose of this study was to examine the possibility that tilorone, another synthetic inducer of interferon, might also non-specifically enhance the resistance of mice to infection with *M. leprae*.

The distinction between a direct antimicrobial effect of a drug and action of a drug to enhance host resistance may not be easily made. In the case of poly I:C, the inference that the effect was exerted on the host and not on *M. leprae* rested primarily on the extremely broad spectrum of drug action (Levy and Merigan, 1977; Merigan, 1973). When tilorone was administered in an oral dosage of 0.05 g per 100 g (50–100 mg per kg body weight) to *M. leprae*-infected mice, multiplication of the organisms was inhibited. Consistent with a direct antimicrobial effect was the apparent resumption of multiplication of *M. leprae* virtually immediately after cessation of drug administration at a rate not very different from that in untreated mice. Failure of the organisms to multiply to the level of 10^6 per footpad after the end of the 150-day period of tilorone administration may be taken as evidence of enhanced resistance of the mice. However, it appears equally likely that the *M. leprae* infection was eradicated

in the footpads of some of the treated mice, whereas organisms survived treatment and were able to multiply to the normal maximum in the footpads of other mice once treatment had stopped; because the harvests were made from the pooled tissues of 4 footpads, tissues devoid of *M. leprae* diluted the organisms present in the other footpad tissues in the pool, yielding mean numbers of *M. leprae* per footpad significantly smaller than the normal maximum.

The results of the studies in *M. marinum*- and *M. lepraemurium*-infected mice appear clearly to demonstrate that the resistance of the mice to these pathogens is decreased as a consequence of tilorone treatment.

This dual effect of tilorone on mycobacterial infections of mice should, perhaps, have been anticipated. Before this work was begun, it had already been reported that, although tilorone demonstrated antiviral effects both *in vivo* and *in vitro,* including some effects possibly not mediated by the mechanism of interferon induction, the drug prolonged survival of skin allografts in mice (Mobraaten *et al.,* 1973), both stimulated and inhibited the resistance of mice to challenge with allogeneic leukemia cells (Friedlander *et al.,* 1974), and selectively depleted the lymphocytes of thymus-dependent areas of the lymphoid tissues of mice and rats (Levine *et al.,* 1974). More recently, the drug has been shown to exhibit anti-inflammatory properties in rats (Megel *et al.,* 1975), the survival of allografts in rats has been shown to be prolonged by tilorone (Wildstein *et al.,* 1976), and inhibition of T-lymphocyte function has been confirmed (Gibson *et al.,* 1976; Megel *et al.,* 1974, 1976).

Particularly pertinent are the results of studies by Collins (1975) and by Gruenewald and Levine (1976). Collins found that oral administration of tilorone at dosages of 10 or 100 mg per kg body weight on alternate days to mice inoculated with *Listeria monocytogenes, M. bovis* (BCG), *M. tuberculosis* H37Rv, or *Salmonella enteritidis* was accompanied by enhanced multiplication of the organisms, and reduction of the response to tuberculin in the case of the mycobacterial infections. The minimal inhibitory concentrations of tilorone were 60 μg per ml for BCG, 250 μg per ml for *L. monocytogenes,* and 500 μg per ml for *S. enteritidis.* Grunewald and Levine found that tilorone, administered in a single subcutaneous dose of 50 mg per kg, increased susceptibility of mice to intravenous challenge with *L. monocytogenes.*

Thus, it appears likely that, in the case of *M. leprae*-infected mice, tilorone exerted the same immunosuppressive effect demonstrated by our studies of *M. marinum*- and *M. lepraemurium*-infected mice, and by the studies of Collins (1975) and Gruenewald and Levine (1976) of mice infected with *L. monocytogenes, M. tuberculosis,* and BCG. That multiplication of *M. leprae* was inhibited during the period of tilorone treatment of the mice suggests, therefore, that *M. leprae* are much more susceptible to the antimicrobial effect of tilorone than either of the other two mycobacterial species studied.

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