Modulation of the Acquisition and Expression of Immunity by Tilorone: I. Delayed-type Hypersensitivity Responses

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Abstract: The chronic administration of Tilorone [(2,7-bis(diethylamino)ethoxy]fluoren-9-one) with antigen seems to prevent the generation of DTH effector cells. The administration of Tilorone proximal to DTH challenge blocks the expression of DTH. By local transfer of DTH effector cells, this latter effect can be shown to be due to the lack of nonspecific inflammatory cells. By the use of both systemic and local transfer systems, it is also shown that specific DTH effector cells from animals treated with Tilorone are unable to circulate in the normal manner. Overall, these experiments suggest that Tilorone does not block the generation of DTH effector cells, but it does greatly alter the expression of DTH. The relation between these effects and the known interferon induction capacity on Tilorone remains unclear.

Key Words: Tilorone; Delayed-type hypersensitivity.

INTRODUCTION

Tilorone has been shown to exhibit antitumor and antiviral activities in animal models (Adamson, 1971a, 1971b). The activities have been associated with the interferon inducing capacity of these low molecular weight compounds in rodents (Mayer and Kreuger, 1970; DeClercq and Merigan, 1971). Tilorone has also been shown to have a paradoxical effect on immune responsiveness showing an adjuvant-like effect on humoral immunity and an ability to suppress cellular immunity (Diamantstein, 1973; Megel et al., 1974; Munson et al., 1972; Friedlaender et al., 1974) perhaps associated with a temporary leucocytopenia which seems restricted to T lymphocytes (Raychaudhuri and Megel, 1976; Megel et al., 1977). This investigation seeks to establish the mode of action of Tilorone on the acquisition and expression on delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) in inbred mice, a system which is becoming well characterized. Tilorone is shown here to have at least two modes of affecting DTH reactions, one associated with the ability of Tilorone to alter the ability of DTH effector cells to circulate.

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METHODS

Tilorone was dissolved in saline 10 mg/ml and administered as 0.1 ml subcutaneously in the flank at the indicated time. All mice used were [C57BL/6 × DBA/2]F_1 (BDF_1) 8–12 weeks old obtained from Jackson Memorial Labs, Bar Harbor, Maine. SRBC and CRBC (chicken red blood cells) were obtained as blood in Alsevers from Colorado Serum Company, Denver, Co. RBC were washed three times in balanced salt solution (BSS) prior to immunization and used as antigen in footpad challenge. Footpad swelling was determined as described by Halliday and Webb (1969). Transfer of spleen cells was as described by Kettman and Mathews (1975). Optimal sensitization as described by Kettman (1972) was used.

RESULTS

Effect of Tilorone on Acquisition of Delayed-type Hypersensitivity

BDF_1 mice which receive small amounts of SRBC intravenously develop hypersensitivity of the delayed type to challenges of SRBC placed in the footpad (Kettman, 1972). This reaction is characterized by showing a maximum swelling reaction between 24 to 48 hr after challenge. These footpads show on sectioning and staining, a massive infiltration of mononuclear cells into the site of antigen depot. This reaction is a function of thymus-derived cells. It has been reported that Tilorone prevents delayed-type skin reactions in the rat (Megel et al., 1974). To confirm and extend this finding, groups of mice received an intravenous sensitization with low doses of SRBC and were also given a milligram of Tilorone per mouse throughout the period when sensitivity is acquired. The results of footpad swelling reactions after challenge with SRBC is indicated in Table 1. Mice which did not receive a sensitizing dose of SRBC failed to respond to challenge in the footpad with SRBC. Mice which received only Tilorone treatment also failed to show responsiveness. Mice given small (10^5) amounts of SRBC showed good swelling responses while high doses of SRBC which give rise to a good antiSRBC PFC

Table 1 *Effect of Tilorone treatment on generation of sensitivity to show delayed hypersensitivity reactions*

SRBC-sensitizing dose	*Tilorone treatment*	*Footpad swelling reaction (Δmm at 24 hr)*		
		A	*B*	*C*[b]
0	–	0.16[a] (0.21)	—	
	+	0.40[a] (0.08)	0.40[a] (0.10)	0.32 (0.02)
10^5	–	1.78[c] (0.09)	2.02[c] (0.16)	1.20 (0.10)
	+	0.68 (0.20)	0.92 (0.19)	0.83 (0.17)
10^8	–	0.28[b] (0.14)	—	—
	+	0.22[b] (0.22)	—	—

[a] Not statistically significant.

[b] Mice received only 1 mg of Tilorone/mouse 2 hr prior to sensitization with SRBC.

[c] Tilorone depressed the response, $P < 0.01$.

Groups of 4–5 mice received the indicated dose of SRBC on Day 0. On that and subsequent days, each mouse in the indicated group also received 1 mg of Tilorone in the flank subcutaneously. On Day 4 mice were challenged in one footpad with 20 μl of 10% SRBC. The contralateral footpad received 20 μl BSS. The footpads were remeasured after 24 hr. The data shown is the mean difference between contralateral footpads. The SEM is also indicated. A, B, and C are individual experiments.

response in the spleen, largely fail to show delayed responsiveness after footpad challenge (Kettman, 1972; Kettman and Lubet, 1976). Mice receiving both Tilorone and a large dose of SRBC show higher PFC responses than mice receiving only SRBC but there is no greater ability to show delayed-type hypersensitivity responses (Table 1). Mice which received lower SRBC priming doses showed good footpad swelling responses, but sensitization with both Tilorone and SRBC yielded mice which demonstrated lower footpad swelling responses, but higher splenic PFC responses (Table 1) and (Diamantstein, 1973).

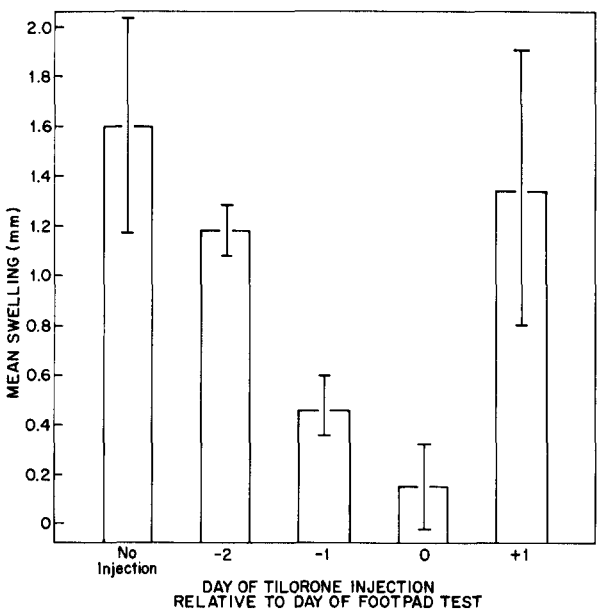
In one experiment, a group of mice were given an optimal sensitizing dose of SRBC and a single dose of Tilorone 2 hr prior to the intravenous SRBC. This group of mice showed similar DTH reactions to mice receiving SRBC only (Table 1).

The Effect of Tilorone on the Expression of DTH

This latter observation suggested that there might be an important relationship between the time of Tilorone injection and the time of footpad challenge. To investigate this relationship, groups of mice were sensitized with an appropriate dose of SRBC and given single injections of Tilorone at various times relative to footpad challenge. The results shown in Figure 1, suggest that Tilorone given at the time of footpad measurement, 24 hr after footpad challenge, causes no perceptable change. However, Tilorone injections in the preceeding 24–48 hr severely depress the ability of sensitized mice to show delayed-type hypersensitivity reactions.

Some footpads of mice used in the previous experiments were fixed, imbedded, sectioned, and stained. Examination showed that mice which had depressed swelling responses also failed to show mononuclear cell infiltrate at the site of antigen depot. Mice which had received Tilorone 72 hr prior to footpad challenge showed the normal infiltration pattern.

Figure 1 *The effect of Tilorone on the footpad response of SRBC sensitized mice. Groups of nine mice were sensitized with 0.2 ml of 0.01% SRBC i.v. Groups then received 1 mg Tilorone subcutaneously in the flank at different times relative to footpad challenge which took place 4 days after SRBC i.v. injection. The value shown is the mean footpad swelling reaction 24 hr after footpad challenge. The bar indicates 2 SEM.*



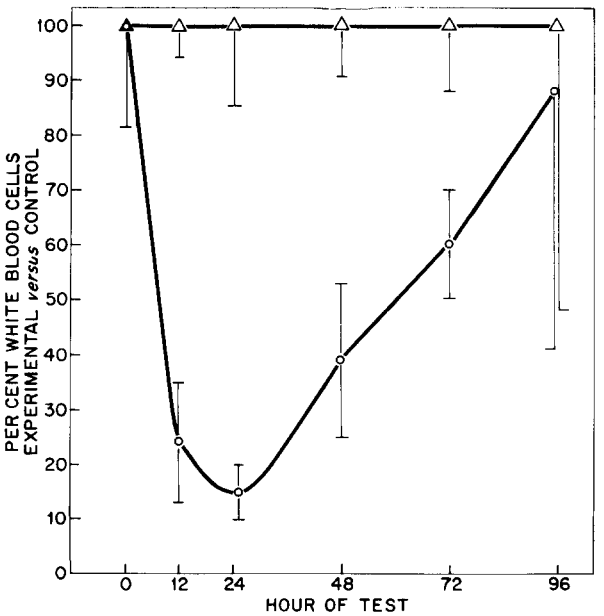


Figure 2 *Effect of Tilorone in blood leucocytes. Two groups of 3 mice were given 1 mg of Tilorone subcutaneously in the flank while 2 other groups received an equivalent amount of BSS. One group of treated and control mice was bled from the tail at 0, 12, 24, and 48 hr. The second set was bled at 48, 72, and 96 hr. From each bleeding, 25 μl of blood was lysed with Zap-isoton and then counted with the Coulter Counter, Model B. At the same time, samples of blood were smeared, fixed and stained. Smears were counted and percent polymorphonuclear of total nucleated cells was determined by counting a minimum of 200 nucleated cells on each smear. The data shown is the % Coulter Counter cell count of Tilorone treated cells compared to controls. The bars show 2 SEM for Tilorone treated and 1 SEM for controls. In each case, control mean was set at 100.*

Effect of Tilorone on the Expression of Delayed Hypersensitivity

The depression of delayed-type hypersensitivity reactions in mice could conceivably be caused by interference with the ability to recirculate DTH effector cells or by an alteration of the ability to recirculate of the mononuclear cells that infiltrate the site of antigen depot. To test the effect of Tilorone on the host contribution to delayed reactions, groups of mice were given Tilorone subcutaneously and then bled sequentially. The number of blood leukocytes was determined and smears were prepared so that the type of blood leukocytes could also be monitored. The results are shown in Figure 2. Soon after Tilorone injection, a loss of blood leukocytes was observed. Polymorphonuclear cells, monocytes or small lymphocytes were uniformly lost. However, by 24–48 hr there was a strong replenishment of blood leukocytes with levels in the normal range returning after 72 to 96 hr (Megel et al., 1977).

This transitory effect of Tilorone could explain the inability of Tilorone treated animals to exhibit delayed hypersensitivity reactions. The recovery of primed cells after Tilorone injection to show delayed-type responsiveness suggests that the specific thymus derived cells responsible for the swelling reaction *also* return during recovery from Tilorone-induced leucocytopenia.

Intravenous Transfer of DTH to Tilorone-treated Normal Hosts

To further extend these observations, spleen cells of sensitized mice were transferred intravenously to a group of normal mice, a portion of whom received Tilorone subcutaneously in the previous 4 hr. These mice as well as normals not receiving a spleen cell inoculum were

all challenged in the footpad with the sensitizing antigen, SRBC. The results of this experiment are shown in Table 2. Hosts which received no spleen cell inoculum, failed to show a delayed-type footpad swelling response while normal mice receiving spleen cells from primed mice did. However, hosts treated with Tilorone failed to show delayed-type hypersensitivity reactions. This result suggests that Tilorone altered the circulation of primed inoculum in the host or that the host's component in the footpad infiltrate had been depressed. These experiments might substantiate the idea that recently Tilorone-treated mice cannot express DTH because of the lack of circulating effector cells. The possibility exists that Tilorone-treated primed spleen cells also are subject to the effect of Tilorone which results in leucocytopenia.

Ability of Sensitized and Tilorone-treated Spleen Cells to Transfer Delayed Hypersensitivity

The above experiments showed that delayed-type reactions could be induced in normal mice after the intravenous transfer of between 10^7 and 3×10^7 spleen cells from sensitized mice. A dose of spleen cells was chosen so that a change in the response of the spleen inoculum could be detected by the response of the recipients. The results of Table 3 show rather clearly that spleen cells of sensitized mice treated with Tilorone in the previous 24 hr were not able to confer delayed hypersensitivity to the recipients. The result suggests that cells responsible for delayed hypersensitivity in the inoculum were missing or failed to behave in the same manner as the cells from sensitized but untreated mice.

Local Transfer of DTH

The problem of the ability of Tilorone to either remove DTH effector cells or alter their pattern of circulation could be solved by the use of local transfer procedures for DTH. The use of this procedure eliminates the requirement for circulation for the expression of DTH (Kettman and

Table 2 *Ability of spleen cells of SRBC-sensitized mice to transfer delayed hypersensitivity into Tilorone-treated mice*

Spleen cells transferred	*Tilorone treatment of host*	*Footpad swelling (Δmm, SEM)*	
None	−	0.03 (0.07)[a]	
1.5×10^7	−	0.70 (0.06)[b]	$P < 0.01$
1.5×10^7	+	−0.07 (0.09)[a]	
3×10^7	−	0.65 (0.15)[b]	$P < 0.05$
3×10^7	+	0.10 (0.10)[a]	

[a] Swelling not significant.

[b] Swelling significant, $P < 0.05$, Student's paired data t-test.

A pool of spleen cells of mice injected 4 days previously with 0.2 ml of 0.01% (v/v) SRBC was injected i.v. into groups of four normal BDF_1 mice. The indicated groups were given 1 mg of Tilorone subcutaneously in the flank 4 hr before spleen cell transfer. After intravenous transfer of spleen cells, all mice including a group not receiving either Tilorone or transfer of spleen cells, were challenged in one footpad with 20 μl in 10% SRBC. The contralateral footpad received 20 μl or 10% CRBC. Footpads were measured at zero time, after 4 and 24 hr. Significant reactions were found only at 24 hr. The value presented is mean difference and SEM between the two injected footpads, e.g., $\Delta mm = F_{pSRBC} - F_{pCRBC}$. No significant swelling differences were found at 4 hr.

Table 3 *Efficiency of transfer of delayed-type hypersensitivity from SRBC primed mice recently treated with Tilorone*

	Delayed (24 hr) footpad swelling reaction (Δmm, SEM)	
Donor	*A*	*B*
Tilorone treated	0.16 (0.06)[a]	0.26 (0.16)[a]
Control treated	0.72 (0.06)[b]	0.84 (0.14)[b]

[a] Swelling is not significant (Student's *t*-test).

[b] Swelling significant, $P < 0.01$, Student's *t*-test.

Groups of 3 mice were sensitized with SRBC by i.v. injection of 0.2 ml of 0.01% SRBC (v/v), Day −5. On Day −1, one group received 1 mg of Tilorone in the flank while the control group received an injection of BSS. On Day 0, the mice were killed, spleens teased and pooled. Into groups of five normal BDF_1 mice was transferred i.v. 5×10^6 of either pool of spleen cells. At the same time one footpad was injected with 20 μl of 20% SRBC and the contralateral footpad received 20 μl of BSS. No significant swelling was found after 4 hr, while significant swelling did occur at 24 hr after spleen cell transfer and footpad challenge.

Table 4 *Effect of Tilorone treatment on the local transfer of DTH responsiveness*

Donor: SRBC primed	*Recipient: Normal*	*DTH reaction (Δmm 24 hr, SEM) (mean)*	
Tilorone treatment at −4 hr	*Tilorone treatment at transfer time*		*Group*
−	−	1.05 (0.11)	A
−	+	−0.03 (0.09)	B
+	−	1.15 (0.14)	C
+	+	−0.10 (0.06)	D
Test for Significance	A vs B	$P < 0.0005$	
	C vs D	$P < 0.0005$	
	A vs C	ns	

Donor mice were primed 5 days prior to transfer by 0.2 ml 0.01% SRBC injected intravenously. In the Tilorone treated group, mice received 1 mg Tilorone 4 hr prior to sacrifice and removal of spleen cells. Control group received same volume of BSS in the flank. The recipient mice received 10^7 primed spleen cells in 0.03 ml 10% SRBC or 10% CRBC (control). The control group received 0.1 ml BSS in the flank 24 hr prior to receipt of transferred cells. The Tilorone treated group received 1 mg Tilorone in the flank 24 hr prior to transfer. DTH was measured 24 hr post-transfer.

Mathews, 1975). The results of this experiment are shown in Table 4. Mice were sensitized with SRBC and treated or *not* treated with Tilorone 4 hr prior to transfer. The recipients of these sensitized cells were mice which received *or did not* receive Tilorone 24 hr previously. The results clearly show two points. First, Tilorone treated mice do not respond to sensitized locally transferred spleen cells while untreated controls do. This confirms the inability of Tilorone treated mice to support DTH effector cells. The second major point is that sensitized spleen cells from a recently Tilorone-treated mouse *can* transfer DTH. This strongly supports the notion that Tilorone does not depress or eliminate the generation of DTH effector cells but from the experiment shown in Table 3, the ability of these cells to circulate is impaired. The actual organ of residence of the DTH effector cells remain to be determined.

DISCUSSION

The experiments presented here confirm the notion that Tilorone interferes rather dramatically with the ability to show DTH reactivity. Part of this effect, especially the short-term effects, can clearly be shown to be due to the short term profound general leucocytopenia induced by Tilorone in mice. However, this loss of effector cells will not completely account for the loss of DTH activity. The data presented here clearly shows that DTH effector cells are affected by short-term Tilorone administration. This effect is seen as an altered ability to circulate of DTH effector cells in normal animals. The actual disposition of these cells was not ascertained. It is clear that when introduced into the site of the antigen depot, the Tilorone-treated effector cells express DTH activity, but when placed in circulation, they do not express their activity at the site of antigen localization, i.e., the cells are not eliminated by Tilorone but their ability to physiologically express activity is altered. These results are broadly similar to the effects of irradiation on DTH effector cells in that irradiated cells are unable to transfer DTH into normal recipients when injected intravenously but are able to transfer DTH when injected locally into the footpad with the antigen (Kettman and Mathews, 1975).

The results obtained here are inconsistent with the generation of a suppressor cell as described by Friedlaender et al., (1974) that affects DTH effector cells. How these observations relate to the known ability of Tilorone to induce interferon is not established and remains a point for further investigation.

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