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The Anti-inflammatory Actions of Tilorone Hydrochloride[1] (38749)

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Tilorone hydrochloride, 2,7,-*bis* (diethylaminoethoxy) fluoren-9-one hydrochloride, in addition to its antiviral activities (1, 2), has the unusual properties of stimulating humoral immune responses and inhibiting immune responses associated with cell-mediated immunity (3). In the adjuvant arthritis model in rats, tilorone not only inhibited the secondary rat paw edema considered to be immunologic in origin, but also significantly inhibited the acute inflammation in the injected paw, suggesting that the compound may have anti-inflammatory properties as well. In this study tilorone was further evaluated as an anti-inflammatory agent. Its effect was investigated in the carrageenan-induced paw edema and abscess models in rats and in the direct passive Arthus model, an immunologically mediated inflammatory model in rats in which complement plays a role (4, 5).

Materials and Methods. Carrageenan-induced paw edema. The method of Winter *et al.* (6) was used to induce paw edema in adult female Sprague–Dawley rats (Charles River Breeding Lab., Wilmington, MA) weighing 100–150 g. The left hind paw of each rat was injected with 0.05 ml of a 1% sterile aqueous solution of carrageenan (Marine Colloids, Inc., Springfield, NJ) and the increase in paw volume was measured 3 hr later by mercury displacement. Tilorone, phenylbutazone and indomethacin were given orally in graded doses in a volume of 0.5 ml per 100 g body wt at 24 hr and again at 1 hr before the carrageenan challenge. The controls were given water only.

To determine the possible role of the pituitary-adrenal axis in the action of tilorone, rats were adrenalectomized and maintained on 1% saline as drinking water and tested 1 and 5 days after surgery in the carrageenan paw edema model. Sham-operated rats were also prepared. Both tilorone and phenylbutazone were administered orally at doses of 100 mg/kg at 24 hr and at 1 hr before the carrageenan injection. Control groups were given saline at the same time-periods. Increases in paw volume were measured as previously described and compared statistically to the controls using the Student's *t* test.

Carrageenan-induced abscess formation. Tilorone was further evaluated in the carrageenan-induced abscess model as described by Goldstein and Schnall (7). Adult female rats of the Sprague-Dawley strain were injected subcutaneously in the sacral region with 0.5 ml of a 2% sterile solution of carrageenan. The abscesses were removed 24 hr later and the wet abscess weights of the drug-treated and control rats were compared statistically using the Student's *t* test.

Direct passive Arthus reaction (DPAR). The method described by Denk *et al.* (4, 5) was used to evaluate tilorone in this model. Female Sprague–Dawley rats, 80–100 g, were injected in the tail vein with 0.25 ml of an antiserum produced in rabbits against bovine serum albumin (anti-BSA). The antiserum in lyophilized form (Cappel Lab., Downington, PA) was reconstituted to half the original volume. Thirty minutes after sensitization each rat was given a subplantar injection (0.025 mg/0.1 ml) of bovine serum albumin (BSA). Paw volumes were measured by mercury displacement before the antigen was injected and 3 hr later when swelling was maximal. Phenylbutazone, indomethacin, aspirin, and the complement-depleting compound, cobra venom factor (CVF) (Cordis Lab., Miami, FL) (8) were given as single doses 30 min before sensitization (equivalent to 1 hr prior to the antigen challenge). Tilorone and hy-

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drocortisone were given 24 hr and 0.5 hr before sensitization. The increases in paw volume were compared statistically to those of sensitized controls given water but no drug and the results are reported as a percent of control.

Serum hemolytic complement. Preliminary studies were carried out to assess the effect of tilorone on serum hemolytic complement. Female Sprague–Dawley rats were given tilorone at a dose of 100 mg/kg orally and a second dose was given 24 hr later. Control rats were given water at a dose of 0.5 ml per 100 g body wt. Groups of rats, treated and control, were sacrificed for serum complement analysis 24 hr after a single dose and other groups were killed at 4, 8, and 24 hr after the second dose. Total serum hemolytic complement levels were measured by the method described by Mayer (9).

Results. Carrageenan-induced paw edema. The effects of graded doses of tilorone, phenylbutazone, and indomethacin are shown in Fig. 1. The data indicate a dose-related anti-inflammatory action of tilorone and indomethacin. Those doses of tilorone and indomethacin that reduced paw edema to 70% of carrageenan injected control levels (generally a statistically significant reduction by the Student's *t* test in our experience) were estimated to be 82.0 and 3.2 mg/kg, respectively, as calculated from the parallel line assay. Phenylbutazone inhibited paw edema (73–79% of control levels) at all doses tested but no dose-response relationship was observed in this experiment.

With regard to the role of the pituitary–adrenal axis, the results shown in Table I indicate that tilorone markedly inhibited paw edema both in the adrenalectomized and sham-operated groups. The effect of phenylbutazone also was not influenced by adrenalectomy.

Carrageenan-induced abscess. Tilorone initially was found to be inactive when the drug was administered at the time of carrageenan injection and again 4 hr later. The possible influence of priming 24 hr before the insult as in the carrageenan paw

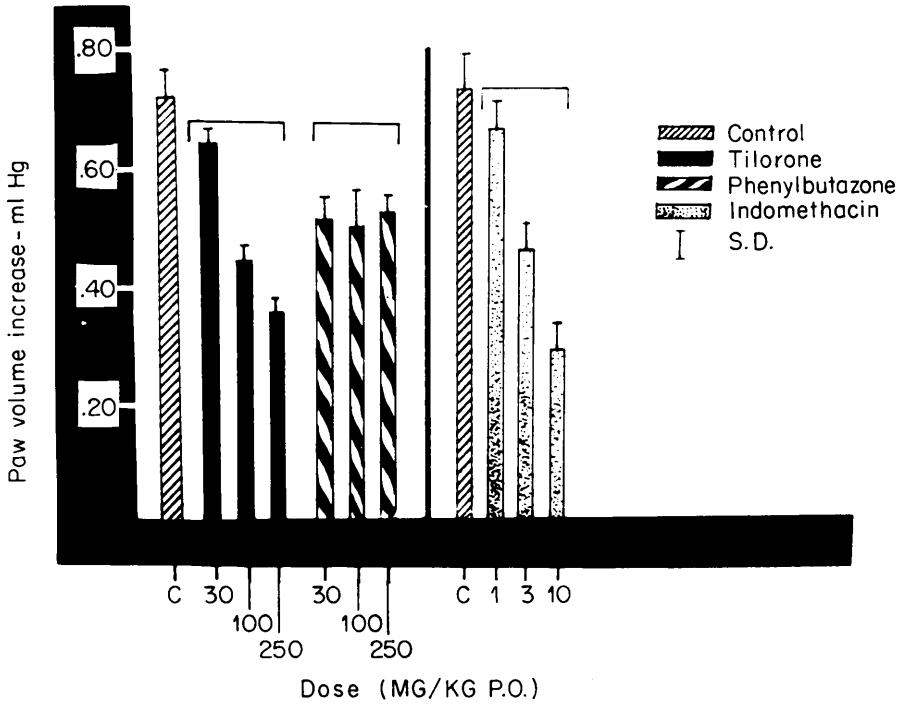

FIG. 1. Effect of tilorone, phenylbutazone, and indomethacin on paw volumes as measured by mercury displacement in the carrageenan-induced paw edema test. The compounds were administered at the indicated doses 24 hr and 1 hr prior to the carrageenan insult. Paw volumes were measured 3 hr later.

edema study prompted us to compare the effect of different dosage regimens. In regimen no. 1, tilorone and phenylbutazone were given at the time of carrageenan injection and 4 hr later on the same day. In regimen no. 2, the compounds were given 24 hr and again shortly before the carrageenan injection. The data show that priming with tilorone 24 hr prior to the injection was necessary to demonstrate its anti-inflammatory action in the abscess test (Table II). Similar priming with phenylbutazone 24 hr before the carrageenan challenge reduced its anti-inflammatory effectiveness.

When the dose response of tilorone and phenylbutazone were compared in the abscess test in which the more optimal dosage regimens were used for each drug, both compounds had marked anti-inflammatory effect (Table III). Phenylbutazone appeared to be somewhat more potent than tilorone in this test.

Direct passive Arthus reaction (DPAR). Tilorone was tested in this model to determine its effect on an immunologically mediated inflammation in which complement is involved. The results shown in Table IV indicate that of the compounds tested, CVF, aspirin, and tilorone were the only ones that significantly inhibited DPAR. The minimal effective dose of tilorone was 25 mg/kg orally. Hydrocortisone, phenylbutazone, and indomethacin were not active. The results of a separate experiment indicated that a single oral dose of tilorone (100 mg/kg) 24 hr before sensitization inhibited the DPAR but the same dose given 30 min prior to sensitization was inactive. This finding is consistent with those previous carrageenan experiments, which indicated that priming with compound 24 hr prior to the inflammatory insult was required to demonstrate efficacy.

Serum hemolytic complement. The effect of tilorone on total serum hemolytic complement was surprising in that tilorone induced a two- to threefold increase in

TABLE I. EFFECTS ON CARRAGEENAN-INDUCED PAW EDEMA IN RATS.

Treatment[a]	Paw volume increase % of Control Postsurgery: Intact (sham-operated) 1 day	Intact (sham-operated) 5 days	Adrenalectomized 1 day	Adrenalectomized 5 days
Tilorone 100 mg/kg p.o.	37[b] (8)[c]	42[b] (6)	59[b] (8)	51[b] (9)
Phenylbutazone 100 mg/kg p.o.	88 (8)	78[d] (10)	68[b] (8)	78[b] (10)

[a] Adrenalectomized and sham-operated rats were used 1 day and 5 days after surgery. Tilorone and phenylbutazone were given 24 hr and 1 hr prior to the subplantar injection of carrageenan. Paw edema was measured by mercury displacement 3 hr after the carrageenan challenge and the results are reported as a percent of control.

[b] $P < 0.01$.

[c] () = Number of rats per group.

[d] $P < 0.05$.

TABLE II. CARRAGEENAN-INDUCED ABSCESS IN FEMALE RATS—EFFECT OF DOSAGE REGIMEN.

	Treatment[a]	Regimen No. 1 0 Hr and 4 Hr: Abscess wt (g) ± SD	Regimen No. 1 0 Hr and 4 Hr: % Control	Regimen No. 2 −24 Hr and −1 Hr: Abscess wt (g) ± SD	Regimen No. 2 −24 Hr and −1 Hr: % Control
Control (7)[b]	None	2.93 ± 0.50	—	3.14 ± 0.51	—
Tilorone (5)	2 × 100 mg/kg p.o.	2.59 ± 0.62	88	2.00 ± 0.57[c]	64
Phenylbutazone (5)	2 × 50 mg/kg p.o.	1.62 ± 0.18[c]	55	2.65 ± 0.36	84

[a] Adult female Sprague–Dawley rats were given the compounds at each of the two dosage regimens shown above with relation to the subcutaneous injection of carrageenan. Abscesses were removed 24 hr after the carrageenan injection and the abscess wet weights of the drug-treated group were compared statistically to those of the control.

[b] () = Number of rats per group.

[c] $P < 0.01$.

serum complement over control levels (Fig. 2). In separate experiments, CVF at a dose of 200 units/kg intraperitoneally induced a striking depletion of complement within 30 min, which persisted for 24 hr.

Discussion. We have reported previously that in the adjuvant arthritis model in rats, tilorone given prophylactically not only inhibited the secondary paw edema considered by many investigators (10–12) to be immunologic in origin but also inhibited the acute non-immune inflammation in the injected paw (3). In this same model, the prophylactic administration of tilorone completely inhibited the tuberculin skin reaction (3). Subsequently we have found that tilorone (100 mg/kg) administered therapeutically to arthritic rats with established tuberculin skin reactions reduced the paw edema and virtually abolished the skin reaction after 14 daily doses (unpublished results).

TABLE III. CARRAGEENAN-INDUCED ABSCESS IN FEMALE RATS[a]

Dose mg/kg p.o.	Abscess wts (% of Control) Tilorone (−24 Hr and −1 Hr)	Phenylbutazone (0 Hr and 4 Hr)
2 × 25 (5)[b]	—	52[c]
2 × 50 (5)	61[c]	50[c]
2 × 100 (5)	56[c]	46[c]
2 × 250 (5)	44[c]	—

[a] Adult female Sprague–Dawley rats were given tilorone or phenylbutazone at the doses and times shown above with relation to the subcutaneous injection of carrageenan. Abscesses were harvested 24 hr later and the wet weights compared statistically to those of the control rats given no compound. The results are reported as a percent of control.

[b] () = Number of rats per group.

[c] $P < 0.01$.

The results of this study demonstrate that tilorone suppressed inflammation induced by immune (DPAR) as well as non-immune (carrageenan) insults. Complement has been implicated in both reactions (4, 5, 13–15). Of interest was that tilorone appeared to be superior to the other anti-inflammatory agents in the immunologically-induced DPAR model. The marked increase in serum complement levels following tilorone was totally unexpected and appears to rule out activity of the CVF-type, in which decomplementation prior to the immunologic challenge results in an understandable inhibition of paw edema. The several possible explanations for a rise in total serum complement are: (a) increased synthesis of complement components, (b) a decreased utilization or degradation of complement, or (c) a combination of both. Since the second possibility is consistent with the observed suppression of

TABLE IV. EFFECTS ON DIRECT ARTHUS REACTION IN FEMALE RATS.

Treatment[a]	No. of rats/group	Dose and route	Time of admin.	% Control
Tilorone	(4)	2 × 15 mg/kg p.o.	−24 hrs and −30 min	83
	(4)	2 × 25 mg/kg p.o.		60[b]
	(13)	2 × 50 mg/kg p.o.		32[c]
	(10)	2 × 100 mg/kg p.o.		8[c]
Hydrocortisone	(4)	2 × 10 mg/kg s.c.		136
Cobra venom factor	(12)	1 × 200 U/kg i.p.	−30 min	21[c]
Phenylbutazone	(12)	1 × 100 mg/kg p.o.		82
Indomethacin	(9)	1 × 10 mg/kg p.o.		73
Aspirin	(5)	1 × 250 mg/kg p.o.		42[b]

[a] The direct passive Arthus reaction was induced in rats by the intravenous administration of anti-BSA followed in 30 min by a subplantar injection of BSA. Increases in paw volume as measured by mercury displacement were recorded 3 hr after the antigen challenge. The compounds used and times of administration for each of the compounds with relation to the anti-BSA administration are shown above. The increases in paw volume were compared statistically to those of control. The results are reported as a percent of control.

[b] $P < 0.05$.

[c] $P < 0.01$.

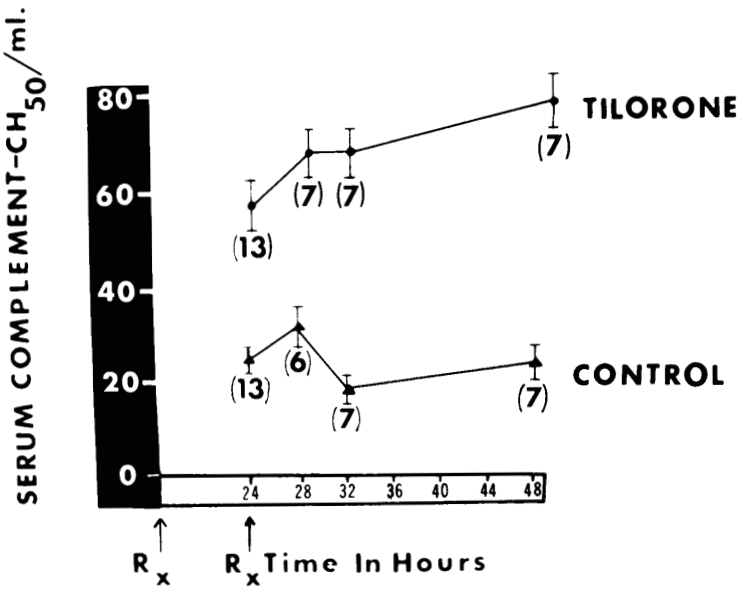

FIG. 2. Effect of tilorone (100 mg/kg p.o.) on serum complement levels in the rat. Total serum hemolytic complement levels were measured 24 hr after a single dose and at 4, 8, and 24 hr after a second dose. The arithmetic mean ± standard error are shown for each of the time periods. The number in ( ) indicates the number of rats per group.

the Arthus and carrageenan-induced inflammatory reactions, our present working hypothesis is that tilorone somehow interferes with the consumption of complement via the classical pathway by immune complexes or via the activation of the alternate pathway. Such an effect could conceivably explain, in part, both the anti-inflammatory actions of tilorone and its ability to induce a rise in serum complement.

Summary. Tilorone suppressed inflammation induced by immune (direct passive Arthus reaction) as well as by non-immune agents (carrageenan-induced paw edema and abscess formation), if the compound is given 24 hr prior to the proinflammatory agonists. The non-immune anti-inflammatory effect is independent of the adrenals. A surprising finding was that total serum hemolytic complement is markedly elevated 24 hr after a single dose of tilorone.

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