PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 513–518 (1974)

Tilorone: Its Selective Effects on Humoral and Cell-Mediated Immunity[1] (37842)

H. MEGEL, A. RAYCHAUDHURI, S. GOLDSTEIN, C. R. KINSOLVING, I. SHEMANO, AND J. G. MICHAEL

Merrell-National Laboratories, Division of Richardson-Merrell Inc., Cincinnati, Ohio 45215, and University of Cincinnati College of Medicine, Cincinnati, Ohio 45221

Tilorone hydrochloride, 2,7-bis(diethylaminoethoxy)fluoren-9-one hydrochloride, was first described by Krueger and Mayer as an orally and parenterally active broad spectrum antiviral with activity associated with interferon induction (1, 2). In addition, antitumor activity (3, 4) and stimulation of the reticuloendothelial system (5) have been reported. Hoffman *et al.* (6) presented evidence that a single oral dose of tilorone enhanced the primary immune response to sheep red blood cells in mice as measured by the Jerne plaque technique. They also reported an increase in hemolysin titer after tilorone administration. These observations have been confirmed most recently by Diamantstein (7). Additional properties of tilorone indicating contrasting effects on humoral and cell-mediated immunity are reported in this paper.

Materials and Methods. Plaque-forming cell (PFC) assay. Adult female CF_1 mice (Carworth Farms, Portage, MI) weighing 20–25 g were used. The mice were immunized intravenously with 0.1 ml of a 20% sheep red blood cell (SRBC) suspension or with 10 μg *Escherichia coli* 0127 endotoxin (Difco, Detroit, MI). Tilorone was administered at a dose of 50 mg/kg subcutaneously 24 hr prior to immunization, and treatment was continued daily until day of sacrifice. Control mice were injected with physiologically buffered saline (PBS). The number of antibody-producing cells in spleen as measured by the modification of the Jerne technique by Mishell and Dutton (8) was used to determine the immune response to the antigens. The direct plaquing technique was used to determine 19S antibody response; 7S antibody was measured by indirect plaquing. The method used to detect antibody to *E. coli* endotoxin was that described by Veit and Michael (9). Sheep RBC were coated with *E. coli* by incubating 2 vol of 10% SRBC with 1 vol of *E. coli* 0127 lipopolysaccharide (100 μg/ml) at 37° for 1 hr. The coated erythrocytes were washed 3 times with PBS prior to incorporation into the agarose.

Reaginic (IgE-like) antibody. The method described by Mota (10) was used to evaluate the effect of tilorone on IgE-like antibody responses. Male Sprague–Dawley rats (Lab Supply, Indianapolis, IN) weighing 200–250 g were injected on Day 0 with ovalbumin (10 mg/kg) intramuscularly and killed *Bordetella pertussis* cells (2×10^{10}) were injected intraperitoneally. Booster injections of ovalbumin (1 mg/kg) and the same amount of *B. pertussis* were given on Day 6. The rats were given the drugs at doses shown in Table I starting on Day 0. The compounds were administered every other day for a total of 7 doses (to Day 12). The rats were killed by cardiac exsanguination under ether anesthesia 4 hr after the last

[1] Presented in part at the 1973 annual meeting of the Federation of the American Societies for Experimental Biology, Atlantic City, NJ.

TABLE I. Effect of Tilorone on IgE-like Antibody Titers in the Rat (48-hr PCA Response).

Treatment[a]	No. rats/ group	Mean wheal size (mm ± SD) 1:8	1:16	1:32	1:64	1:128
Saline	9	16.7 ± 2.8	13.4 ± 3.5	10.6 ± 4.8	5.1 ± 4.2	1.7 ± 2.9
Tilorone (100 mg/kg p.o.)	10	22.4 ± 6.8[b]	17.2 ± 4.6[c]	15.4 ± 4.5[b]	12.6 ± 5.7[d]	8.0 ± 7.2[d]
6-MP (25 mg/kg p.o.)	10	5.5 ± 6.2[d]	3.1 ± 3.7[d]	0.3 ± 0.9[d]	0	—
Hydrocortisone (25 mg/kg s.c.)	8	0.6 ± 1.4[d]	0	—	—	—

[a] Donor rats were given each compound on alternate days starting on day of sensitization with ovalbumin. Rats were killed 4 hr after the 7th dose on Day 12. Serum from each rat was diluted to the titers shown, and 0.1 ml of each dilution was injected intradermally into the flanks of at least 2 recipient rats. After 48 hr, the recipient rats were challenged with 15 mg ovalbumin in 0.5% Evans blue dye intracardially. Wheal diameters were measured 20 min later.

[b] $P < .05$ compared to controls.

[c] $P < .07$ compared to controls.

[d] $P < .01$ compared to controls.

dose on Day 12, and the serum obtained from each rat was diluted with PBS to the titers shown in Table I.

To test for IgE-like activity, 0.1 ml of each serum dilution was injected intradermally into the shaved flanks of at least 2 recipient rats. After 48 hr, the rats were challenged by an intracardial injection of 15 mg ovalbumin in 1 ml of 0.5% Evans blue dye in saline. The rats were killed 20 min later, and the orthogonal diameters of the PCA reaction in mm were measured and averaged.

Adjuvant arthritis (AA). A modification of the method described by Newbould (11) was used to induce AA in male Lewis rats (Simonson Laboratories, Gilroy, CA) weighing 170–320 g. Adjuvant arthritis was induced by a single subplantar injection of 0.07 ml of a sterile suspension of *Mycobacterium butyricum* (5 mg/ml) in light mineral oil into the right hind paw of rats anesthetized with ether. Swelling of the injected and noninjected paws was measured by mercury displacement with a venous pressure transducer coupled to a Hewlitt–Packard 7702 recorder.

Tilorone (100 mg/kg) or 6-mercaptopurine (6-MP) (25 mg/kg) were administered orally in volumes of 1 ml per 100 g body wt for 14 consecutive days starting 24 hr before the injection of the adjuvant. Control rats were given the vehicle (0.9% Tween 80). Paw volumes were measured prior to challenge (Day 0) and on Days 4, 10, 15, and 22 after challenge.

Experimental allergic encephalomyelitis (EAE). Experimental allergic encephalomyelitis was induced in male Lewis rats weighing 160–200 g by the method described by Rosenthale and Nagra (12) as modified by Grieg *et al.* (13). Four grams of brain and spinal cord from donor Lewis rats were homogenized in 10 ml of 0.5% aqueous phenol and blended in the cold with an equal volume of complete Freund's adjuvant (1 mg killed tubercle bacilli/ml); a thick emulsion was formed. Each rat was given subplantar injections of 0.05 ml of an isologous brain–Freund's complete adjuvant emulsion in both hind paws followed by an injection of 9×10^{10} killed *B. pertussis* organisms into the dorsum of one of the hind legs. Drug administration was initiated immediately thereafter in the dosage regimens described in Table II. Incidence of paralysis was recorded as a function of time up to 28 days after sensitization.

Delayed skin reactions to tuberculin purified protein derivative (PPD). Male Sprague–Dawley rats weighing 180–220 g were used. The procedure for inducing delayed skin reactions in rats is essentially that described by Flax and Waksman (14). Each rat was injected in one of the hind footpads with 0.05 ml of a 3 mg/ml suspension of

TABLE II. Effect of Tilorone on EAE in Lewis Rats.

Treatment	Dosage[a]			Paralysis	
	No. doses	mg/kg	route	Day 15	Day 28
Control	—	—	—	17/20	17/20
Tilorone	8 on alternate days	100	po	0/10[b]	3/10[b]
	2 at -24 hr and Day 0	100	po	0/10[b]	0/10[b]
	2 on Days 3 and 4	100	po	3/10[b]	3/10[b]
	2 on Days 6 and 7	100	po	7/10	8/10
Hydrocortisone	8 on alternate days	25	sc	2/10[b]	4/10[c]

[a] Administration of compounds was started on the day of sensitization with encephalitogen.
[b] $P < 0.01$ by chi-square test.
[c] $P < 0.05$ by chi-square test.

Mycobacterium tuberculosis (H_{37}Ra strain) in mineral oil. The compounds were given on the day of sensitization (Day 0) and continued on an alternate day basis for 8 doses (to Day 14). The rats were skin tested on Day 14 by intradermal injections of 0.1 ml of different amounts of PPD (Table III). Saline, 0.1 ml injected intradermally, served as a control. Skin reactions were read 24 hr later.

Results. PFC assay. Our results shown in Table IV indicate that tilorone significantly elevated 19S antibody-forming cells (AFC) to SRBC on Days 3 and 4 after immunization. Although 19S responses were diminished for both groups after 10 days compared to Days 3 and 4, tilorone still increased 19S antibody and, to an even greater extent, 7S antibody compared to controls. Tilorone also stimulated the 19S response to *E. coli* endotoxin, a thymus-independent antigen, on Days 3 and 4 after immunization.

Tilorone significantly increased both the 19S and 7S responses compared to the control in the secondary immune response (Table IV).

IgE-like antibody responses. Table I shows the wheal diameters for the various serum dilutions with relation to treatment. In all groups the wheal diameters decreased as a function of titer. Tilorone significantly increased IgE-like responses compared to control, whereas 6-MP decreased the responses and hydrocortisone almost obliterated them. The results of a parallel line assay using the method described in Finney (15) indicate that tilorone elevated IgE-like antibody levels 3.2 (95% confidence interval for relative potency ranged from 2.0 to 5.9) times with relation to saline control. 6-Mercaptopurine, on the other hand, reduced antibody levels to 0.12 that of controls (95% confidence interval ranged from 0.07 to 0.18).

Cell-mediated immunity. Adjuvant arthritis. The effect of tilorone on AA is shown in Fig. 1. The adjuvant produced in the injected paw the characteristic initial swelling peaking on Day 4, followed by a second inflammation beginning on Day 10 and continuing thereafter. 6-Mercaptopurine and tilorone, to a greater extent, inhibited sig-

TABLE III. Effect of Tilorone on Tuberculin Skin Reaction in the Rat.[a]

Treatment	No. rats/ group	Dosage			Wheal diameter (mm)		
		No. doses	mg/kg	route	2.5 μg PPD	5 μg PPD	10 μg PPD
Control	10	—	—	—	9.6 ± 5.3[c]	10.6 ± 4.0	11.8 ± 4.6
Hydrocortisone	10	8	25	sc	0	0	0
6-Mercaptopurine	10	8	25	po	6.7 ± 4.7	5.9 ± 5.0[c]	6.4 ± 5.5[c]
Tilorone	10	8	100	po	0	0	0

[a] Rats were injected with 0.05 ml of 3 mg/ml *M. tuberculosis* in oil in one footpad. Compounds were given on alternate days starting on the day of sensitization. The rats were skin-tested with various concentrations of PPD on Day 14, and the skin reactions were read on Day 15.
[b] Mean ± SD.
[c] Significantly different from control, $P < 0.05$.

TABLE IV. Effects of Tilorone (50 mg/kg Subcutaneously) on the Primary and Secondary Immune Response in Mice.

Antigen	No. of days after immunization	Immune response	Treatment	Type of antibodies	PFC/10^6 spleen cells mean ± SD	P
SRBC[a]	3	Primary	Control (10)[b]	(19S)	160 ± 16[e]	<0.01[f]
	3	Primary	Tilorone (10)	(19S)	390 ± 38	
SRBC	4	Primary	Control (9)	(19S)	640 ± 55	<0.01
	4	Primary	Tilorone (10)	(19S)	890 ± 55	
SRBC	10	Primary	Control (10)	(19S)	50 ± 8	<0.01
	10	Primary	Tilorone (10)	19S)	75 ± 4	
	10	Primary	Control (10)	(7S)	420 ± 35	<0.01
	10	Primary	Tilorone (10)	(7S)	910 ± 84	
E. coli[c]	3	Primary	Control (10)	(19S)	80 ± 9	<0.01
	3	Primary	Tilorone (10)	(19S)	190 ± 19	
E. coli	4	Primary	Control (10)	(19S)	210 ± 12	<0.01
	4	Primary	Tilorone (10)	(19S)	460 ± 36	
SRBC	24[d]	Secondary	Control (10)	(19S)	200 ± 12	<0.01
	24	Secondary	Tilorone (10)	(19S)	380 ± 30	
SRBC	24	Secondary	Control (15)	(7S)	710 ± 58	<0.01
	24	Secondary	Tilorone (14)	(7S)	1300 ± 98	

[a] Adult female CF_1 mice immunized with 0.1 ml of 20% SRBC intravenously.
[b] Number of mice per group.
[c] Adult female CF_1 mice immunized with 10 μg *E. coli* endotoxin intravenously.
[d] A second immunization with SRBC was given on Day 21.
[e] Mean ± SD.
[f] Level of confidence as determined by the Student's *t* test.

nificantly both the initial and secondary inflammations. In the noninjected paw, both drugs almost obliterated the secondary inflammatory response.

EAE. Eight doses of tilorone (100 mg/kg po) given on alternate days beginning on the day of sensitization completely suppressed paralysis on the day of highest cumulative incidence for the controls (Days 15) (Table II). There was little breakthrough (30%) on Day 28, when the experiment was terminated. Histologic examination of brain and

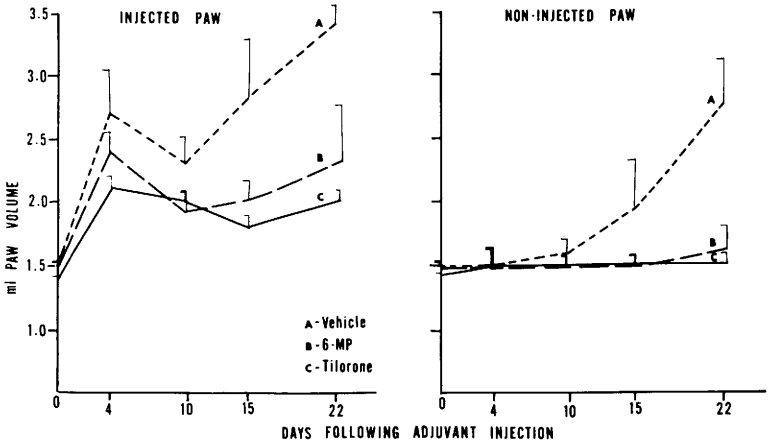

FIG. 1. Effect of tilorone and 6-MP on paw volumes as measured by mercury displacement in adjuvant arthritis model in the rat. Vehicle, 0.9% Tween 80; 6-MP, 25 mg/kg po; tilorone, 100 mg/kg po. All drugs given for 14 consecutive days starting 24 hr prior to sensitization with *M. butyricum* in light mineral oil.

spinal cord of separate groups of control and tilorone-treated rats necropsied on Days 12–15 revealed no lesions usually associated with EAE for the tilorone-treated groups, whereas perivascular cuffing and interstitial edema were marked and present in all control rats.

When tilorone was given for two consecutive doses at 24 hr before and on the day of sensitization, paralysis was completely prevented; 30% of the rats were paralyzed when the drug was given on Days 3 and 4, and 70% of the rats were paralyzed when the drug was given only on Days 6 and 7 (Table II). These data suggest that tilorone works in part during the induction of EAE. This experiment does not rule out the possibility that tilorone may affect additionally the expression of EAE.

Skin reaction to PPD. There appears to be an increase in wheal diameters as a function of PPD concentration (Table III). Tilorone (100 mg/kg po) and hydrocortisone (25 mg/kg sc) completely blocked the skin reactions when given for 8 doses on alternate days; 6-MP inhibited significantly the responses to the 5- and 10-μg PPD challenge doses.

Discussion. The results of this study confirm and extend the observations of Hoffman *et al.* (6) and Diamantstein (7) concerning the adjuvant effect of tilorone on the primary immune response. Our data indicate that daily administration of tilorone to mice resulted in approximately a twofold increase of immune responses to both thymus-dependent (SRBC) and thymus-independent (*E. coli* endotoxin) antigens. The secondary immune response to SRBC was also enhanced. In addition, the compound increased IgE-like antibody threefold. These data show that the drug serves as an adjuvant for a variety of immunoglobulin classes (IgG, IgM, and IgE antibody production). The effects on IgA responses to antigenic stimulation remain to be determined.

In contrast to the effects of tilorone on humoral antibody production, the drug suppressed cell-mediated immune responses as evidenced by the significant decrease in paralysis in the EAE model, the inhibition of the tuberculin skin reaction, and the reduction in the secondary swelling in AA.

Tilorone appears to be a unique compound in that it enhances humoral antibody while suppressing delayed hypersensitivity responses. It differs from established immunosuppressants (e.g., glucocorticoids, antimetabolites), which are capable of suppressing both. More recent reports described other synthetic compounds that also have selective effects on the immune system. Freedman *et al.* (16) and Fox *et al.* (17) have reported that oxisuran suppresses skin-graft rejections in mice, rats, and dogs but has no effect on antibody production. Renoux and Renoux (18) have shown that levamisole enhances cell-mediated immunity as evidenced by its effect on graft vs host reactions. In addition, these investigators reported antitumor activity for the drug. The results with tilorone and other synthetic compounds suggest that the immune system can be regulated selectively by pharmacologic manipulation.

The mechanisms by which tilorone exerts its influence on the immune system is unknown. The adjuvant effect of tilorone on antibody formation may be a result of B cell or macrophage stimulation. The suppression of delayed hypersensitivity reaction, on the other hand, may be a result of selective suppression of the T lymphocytes. A possibility that increased levels of circulating antibodies would have an inhibiting effect on the expression of delayed hypersensitivity cannot be excluded. Experiments are in progress to examine these possibilities.

Summary. We have shown selective activity of tilorone with regard to humoral and cell-mediated responses. The compound enhanced antibody production as shown by an increase in IgM and IgG antibody-forming cells in the primary and secondary immune responses, it enhanced reactivity to thymus-dependent and thymus-independent antigens, and it increased circulating IgE-like antibodies in the rat. Tilorone inhibits cell-mediated responses as shown by its suppression of paralysis in EAE, blocking of the tuberculin skin reactions, and its inhibition of inflammation in adjuvant arthritis.

We thank Mr. V. G. Butler, Ms. N. Munro,

and Ms. L. L. Thomas for their excellent technical assistance. We also thank Dr. J. P. Gibson for the histologic examination of tissues.

1. Krueger, R. F., and Mayer, G. D., Science **169**, 1213 (1970).

2. Mayer, G. D., and Krueger, R. F., Science **169**, 1214 (1970).

3. Adamson, R. H., Lancet **1**, 398 (1971).

4. Adamson, R. H., J. Nat. Cancer Inst. **46**, 431 (1971).

5. Regelson, W., Munson, A. E., Munson, J. A., Mayer, G. D., and Krueger, R. F., Presented at the 6th International Meeting of the Reticuloendothelial Society, Freiberg, Germany, July 29–Aug. 1, 1970.

6. Hoffman, P. F., Ritter, H. W., and Krueger, R. F., *in* "Advances in Antimicrobial and Antineoplastic Chemotherapy" (M. Hejzlar, M. Semonsky, and S. Masak, eds.), p. 217. Urban and Schwarzenberg, Munich (1972).

7. Diamantstein, T., Immunology **24**, 771 (1973).

8. Mishell, R. I., and Dutton, R. W., J. Exp. Med. **127**, 423 (1967).

9. Veit, B. C., and Michael, J. G., J. Immunol. **109**, 547 (1972).

10. Mota, I., Life Sci. **12**, 917 (1963).

11. Newbould, B. B., Brit. J. Pharmacol. **21**, 127 (1963).

12. Rosenthale, M. E., and Nagra, C. L., Proc. Soc. Exp. Biol. Med. **125**, 149 (1967).

13. Greig, M. E., Gibbons, A. J., and Elliott, G. A., J. Pharmacol. Exp. Ther. **173**, 85 (1970).

14. Flax, M. H., and Waksman, B. H., J. Immunol. **89**, 496 (1962).

15. Finney, D. J., "Statistical Method in Biological Assay," p. 99. Hafner Publishing Company, New York (1964).

16. Freedman, H. H., Fox, A. E., Shavel, J., Jr., and Morrison, G. C., Proc. Soc. Exp. Biol. Med. **139**, 909 (1972).

17. Fox, A. E., Gawlak, D. L., Ballantyne, D. L., Jr., and Freedman, H. H., Transplantation **15**, 389 (1973).

18. Renoux, G., and Renoux, M., Nature New Biol. **240**, 217 (1972).

Received July 20, 1973. P.S.E.B.M., 1974, Vol. 145.