PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 1044–1047 (1975)

Enhancement of Interferon Production *in vitro*: A Property of Tilorone-Poly rI:rC/DEAE-Dextran (38685)

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Tilorone hydrochloride, 2,7-bis[2-(diethylamino)ethoxy]-9H-fluoren-9-one dihydrochloride, is an orally active antiviral agent and interferon inducer in mice (1–4) and rats (5, 6). This compound has been reported neither to induce interferon in cell cultures (3, 4) nor to enhance the interferon-inducing potential of the polynucleotide duplex poly rI:poly rC (rI:rC) in human skin fibroblast cultures (3). In contrast, interferon was found in human lymphocyte suspensions in the presence of tilorone (7). We now report enhanced interferon production in mouse L929 cells after treatment with mixtures of tilorone and rI:rC/diethylaminoethyl (DEAE)-dextran, as well as differences in the capacity of other cells to respond in a similar manner. Furthermore, treatment of mouse embryo fibroblasts with tilorone alone stimulated the production of an antiviral component that was found in the cell supernates.

Materials and Methods. The appropriate concentrations of tilorone hydrochloride (Merrell-National Laboratories, Cincinnati, OH), rI:rC (Bipolymers, Inc., Dover, NJ), and DEAE-dextran (mol wt 2×10^6, Pharmacia, Uppsala, Sweden) were prepared in Eagle's basal medium with Earle's salts (EBME) containing fetal calf serum (2%), sodium bicarbonate (0.1%), and antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml, 50 μg neomycin/ml, and 0.25 μg fungizone/ml). Two ml each of rI:rC and DEAE-dextran were first mixed and an equal volume of EBME with or without tilorone was then added. The concentration ratio of rI:rC to DEAE-dextran was 1:4 throughout these studies.

Confluent L929 cells in 75 cm^2 disposable tissue culture flasks (Falcon Plastics, Oxnard, CA) were exposed to 8 ml of freshly prepared mixtures for 2.5 hr at 37°. The compounds were then removed, and the cells washed three times with Hanks' salt solution. After an additional 24-hr incubation period with 10 ml EBME (serum free) the supernates, if not assayed immediately, were frozen (−70°) until assayed. L929 cells were exposed to dilutions of supernates (1 ml vol) for 24 hr at 37° in a humidified 5% CO_2 incubator. The cells were then challenged with 100 $TCID_{50}$ of vesicular stomatitis (VS) virus (1 tissue culture infective $dose_{50}$ destroyed 50% of the cells) and reincubated for 48 hr. Titers represent the reciprocal of the highest dilution of supernate that protected 50% of the cells from the cytopathic effect of the virus and are reported as untis/ml. The NIH mouse serum interferon standard containing 2.5×10^4 units/ml titered 3.0×10^4 units/ml in our assay system.

Results. The ability of the compounds, individually and in various combinations, to induce an antiviral substance in L929 cells is shown in Table I. Antiviral activity was detected in the supernates of cells treated with mixtures of rI:rC and DEAE-dextran but not in supernates after treatment with rI:rC alone or in combination with tilorone. This requirement of DEAE-dextran for the induction of interferon in L929 cells by rI:rC has been reported previously (8). DEAE-dextran and tilorone alone or in combination did not evoke antiviral activity. Less activity resulted after the cells were treated with lower concentrations of rI:rC and DEAE-dextran. However, with tilorone these lower

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TABLE I. STIMULATION OF AN ANTIVIRAL SUBSTANCE IN L929[a] CELLS AFTER EXPOSURE TO MIXTURES OF POLY rI:POLY rC, DEAE-DEXTRAN, AND TILORONE.

Compounds (μg/ml)			
Poly rI:Poly rC	DEAE-dextran	Tilorone	Interferon titer[b]
100	0	0	<25
0	400	0	<25
0	0	50	<25
100	0	50	<25
0	400	50	<25
100	400	0	4800
100	400	50	9600
15	60	0	300
15	60	50	6400
10	40	0	50
10	40	50	2400

[a] L929 cells exposed to compounds for 2.5 hr (37°), washed, and then EBME (serum-free) added.

[b] Supernates, collected 24 hr after removal of compounds, expressed as units/ml.

concentrations of rI:rC and DEAE-dextran stimulated levels of activity comparable to those obtained with 100 μg rI:rC/ml and DEAE-dextran. In contrast, the activity obtained with 100 μg rI:rC/ml and DEAE-dextran was not significantly enhanced by tilorone. Similar results were obtained with rI:rC from another source (Miles Laboratories, Elkhart, IN); exposure of L929 cells to Miles rI:rC (10 μg/ml) and DEAE-dextran (40 μg/ml) with or without tilorone (50 μg/ml) resulted in antiviral titers of 2400 and 37.5, respectively.

The antiviral substance induced in mouse L929 cells by rI:rC/DEAE-dextran, with or without tilorone, had properties characteristic of interferon. Activity was abolished upon treatment with trypsin (0.1%) for 24 hr; the antiviral substance was nondialyzable and nonsedimentable (149,000 $g \times 2$ hr) and did not inactivate virus directly; it did not protect monkey vero cells or human skin fibroblasts against VS virus cytopathology; primary mouse embryo fibroblasts were protected.

To determine the concentration of tilorone necessary for maximum enhancement of interferon production, L929 cells were

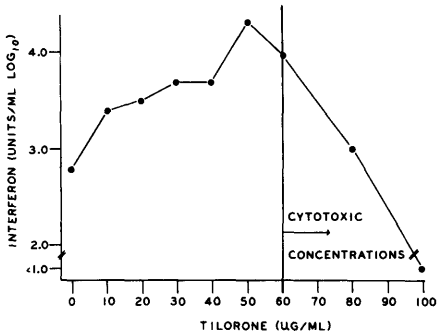

FIG. 1. Effect of tilorone concentrations on the induction of interferon by poly rI:poly rC/DEAE-dextran in L929 cells. Tilorone in EBME (2% fetal calf serum) combined with poly rI:poly rC (15 μg/ml) and DEAE-dextran (60 μg/ml) and added to cells for 2.5 hr. Supernates collected 24 hr after removal of compounds.

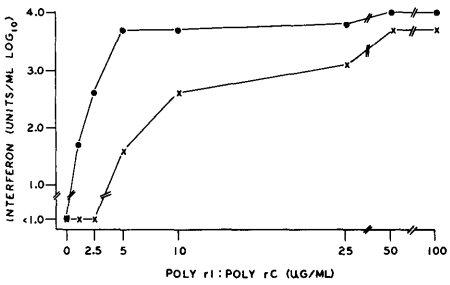

FIG. 2. Effect of various concentrations of poly rI:poly rC/DEAE-dextran (1:4) with constant tilorone concentratinons on interferon induction in L929 cells; poly rI:poly rC/DEAE-dextran in EBME (2% fetal calf serum) combined with 0 (×) or 50 (●) μg tilorone/ml and added to cells for 2.5 hr. Supernates collected 24 hr after removal of compounds.

exposed to various concentrations of the compound in combination with rI:rC (15 μg/ml) and DEAE-dextran. Increasingly higher interferon yields were obtained with tilorone concentrations up to 50 μg/ml (Fig. 1). Treatment with concentrations of tilorone greater than 60 μg/ml for 2.5 hr resulted in cellular toxicity within 24 hr and a concomitant reduction in interferon titers.

Concentrations of rI:rC (1 and 2.5 μg/ml)/DEAE-dextran that were inadequate to stimulate interferon production did so when tilorone was added (Fig. 2). Tilorone combined with rI:rC (5 μg/ml) and DEAE-dextran elicited a 128-fold increase in inter-

feron production in comparison to non-tilorone treated controls. The ability of tilorone to enhance interferon production in L929 cells decreased with progressively greater concentrations of rI:rC/DEAE-dextran.

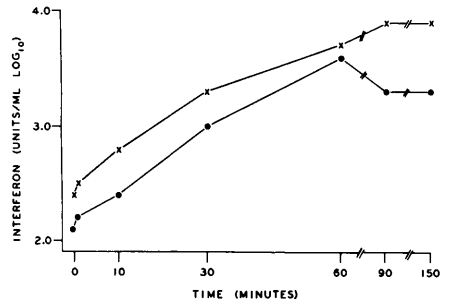

FIG. 3. Effect of exposure to tilorone before or after poly rI:poly rC/DEAE-dextran treatment of cells. Confluent L929 cells in 75 cm² tissue culture flasks exposed to tilorone (50 μg/ml) for indicated time before (×) or after (●) treatment with poly rI:poly rC (10 μg/ml)/DEAE-dextran (40 μg/ml) for 2.5 hr. Cells washed after each treatment interval and then 10 ml EBME (serum free) added. Supernates collected 24 hr after removal of final compounds.

Elevated interferon levels were also obtained without simultaneous treatment of cells with rI:rC/DEAE-dextran and tilorone. Greater than 20-fold increases in interferon titers resulted after L929 cells were first treated with tilorone (50 μg/ml) for 60, 90, or 150 minutes and then exposed to rI:rC/DEAE-dextran for 2.5 hr (Fig. 3). Reversal of the order of exposure to compounds gave similar results.

Primary mouse embryo fibroblasts (MEF) and human skin fibroblasts (HSF, a gift from Dr. Thomas C. Merigan) were also used to test for increased levels of antiviral activity after treatment of the cells with rI:rC/DEAE-dextran and tilorone. Cells were grown to confluence in 6-well (9.6 cm²) tissue culture trays (Linbro Chemical Co., New Haven, CT). Each well, containing 2 ml media with compounds, was incubated for 2.5 hr, thoroughly washed, and overlaid with 1 ml EBME (2% serum) for 24 hr.

TABLE II. ANTIVIRAL RESPONSES OF MEF AND HSF[a] CELLS AFTER EXPOSURE TO MIXTURES OF POLY rI:POLY rC, DEAE-DEXTRAN, AND TILORONE.

Cell	Compound (μg/ml)			Antiviral titer[b]
	Poly rI:Poly rC	DEAE-dextran	Tilorone	
MEF	0	0	0	<5
	0	0	25	15
	0	40	25	7.5
	1	4	0	<5
	1	4	25	7.5
	5	20	0	5
	5	20	25	80
	10	0	25	15
	10	40	0	300
	10	40	25	600
	25	0	0	<5
	25	100	0	300
	25	100	25	300
HSF	0	0	10	<5
	1	4	0	60
	1	4	10	40
	10	40	0	80
	10	40	10	30
	50	0	0	15
	50	0	10	<5
	50	200	0	240

[a] Confluent MEF and HSF cells in tissue culture trays exposed to mixtures for 2.5 hr (37°) washed, and then EBME (2% fetal calf serum) added.

[b] In supernates of two pooled wells collected 24 hr after removal of compounds, expressed as units/ml.

Pooled supernates from 2 wells were assayed as previously mentioned on either L929 cells or HSF. In order to treat these cells for 2.5 hr with tilorone, the concentration of the compound had to be decreased to avoid cytotoxicity.

Increased levels of antiviral activity were produced in MEF cells treated with rI:rC/DEAE-dextran and tilorone (25 μg/ml) (Table II). As with L929 cells, DEAE-dextran was required for the stimulation of antiviral activity by rI:rC. Slight antiviral activity was detected in the supernates of MEF treated with tilorone alone. Unlike the mouse cells, the antiviral activity elicited in HSF cells by rI:rC/DEAE-dextran could not be potentiated with concurrent tilorone treatment (Table II). No antiviral activity was found in the supernates of tilorone-treated L929 cells reassayed at a 0.2 dilution.

Discussion. In mouse cell cultures, concentrations of rI:rC/DEAE-dextran that induced low levels of interferon displayed markedly enhanced responses in combination with tilorone. When concentrations of rI:rC/DEAE-dextran that induced high levels of activity were used, the addition of tilorone had little or no effect. Tilorone did not enhance the antiviral response of HSF cells. If tilorone acted on the rI:rC/DEAE-dextran then this species barrier would not exist and simultaneous treatment with tilorone and rI:rC/DEAE-dextran, or at least a pre-treatment of rI:rC/DEAE-dextran with tilorone, would be required. Action of tilorone at the cellular level rather than on the interferon inducer (rI:rC/DEAE-dextran) is indicated by the disparity in interferon enhancement with cell cultures from different species utilizing the same stimulus and the lack of a requirement for simultaneous treatment with rI:rC/DEAE-dextran and tilorone. It is interesting to speculate that interferon induction by tilorone may lie in the ability of this compound to bind to native nucleic acid of specific base sequences (9) found in some, but not all, cell species.

Summary. Tilorone and Poly rI:rC, in the presence of DEAE-dextran, were found to exhibit a marked synergism with respect to the induction of interferon in L929 and primary mouse embryo fibroblasts, but not human foreskin fibroblasts, in cell cultures. The degree of synergism was proportional to the concentrations of tilorone and Poly rI:rC and was influenced by the times of addition of the compounds relative to each other.

The authors wish to acknowledge the excellent technical assistance of Carroll Hull and Charles Meiser, and to thank Katherine Ludwig for the art work.

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Received October 18, 1974. P.S.E.B.M. 1975, Vol. 148.