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Therapeutic Effect of Tetrahydrofolic Acid in Midlethally X-Irradiated Mice

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Tetrahydrofolic acid exhibited a life-prolonging effect in the midlethally X-irradiated mice, when it was intraperitoneally injected in the animals after the irradiation. In the tetrahydrofolic acid-treated animals, retardation of the growth rate in the body weight was normalized in 5 days after the irradiation. Recovery in spleen and intestine weights as well as in the number of circulating blood leucocytes was more pronounced in the tetrahydrofolic acid-treated animals than in the control animals. However, it failed to increase the survival rate of the X-irradiated HeLa S_3 cells, when it was added in the culture medium after the irradiation.

In a subsequent experiment, it was clarified that folic acid was devoid of this therapeutic effect.

Some substances, such as olive oil,[3] a proteinous component of splenic tissues,[4] and nucleic acids or their related substances,[5] have been reported to possess a potency to enhance the restoration of the animals or cultured cells from radiation injury, although their effectiveness is rather weak and not always reproducible as compared with the effectiveness of a number of so-called anti-radiation chemicals which are effective, however, in the prophylactic use only. Neverthelss, the needs of the therapeutic anti radiation agents are much higher than the needs of the prophylactic agents in view of the practical usefulness in the treatment of the patients in the case of atomic industry accidents or in the case of the excessive radiotherapy of the cancer.

Recently Sonka, *et al.*[6] reported that tetrahydrofolic acid (THFA), which is known as a cofactor for the biosynthesis of purine and pyrimidine bases, afforded a life-prolonging effect, when it was injected in mice after irradiation. This was followed by the report of Schünzel, *et al.*[7] who also observed the therapeutic effect of THFA in the X-irradiated rats. Because of the importance of these findings, we decided to re-investigate the effect of THFA in the experimental animals under the conditions of radiation usually employed in our laboratory.[8]

Experimental

Animals——Male ICR-JCL mice were purchased from Japan CLEA Co. Ltd. (Tokyo). The animals were 5 weeks old and weighed 24—27 g at the time of irradiation. They were housed five per cage in a temperature-controlled room (23—25°) and fed on a laboratory chow and tap water *ad libitum* at all time. Since it has consistently been observed that the animals which survived 3 weeks after the irradiation were able to survive more than several months therafter, the observation of the irradiated animals in the present experiment was terminated in 30 days after the irradiation.

HeLa S_3 Cells——The culture medium consisted of 10% calf serum and 0.1% Difco heart infusion broth in F-10 nutrient mixture containing penicillin G (100 units/ml) and dihydrostreptomycin (100 μg/ml). The

1) On leave of absence from Yamanouchi Pharmaceut. Co. Ltd., Tokyo.
2) Location: *Anagawa 4-9-1, Chiba.*
3) J.K. Ashikawa and O.K. Anderson, *Radiation. Res.*, **13**, 99 (1960).
4) S. Katz and F. Ellinger, *Nature*, **197**, 397 (1963).
5) D. Petrovic, *Current Topics in Radiation Research*, **4**, 253 (1968).
6) J. Sonka, K. Slavik, J. Pospisil, and Z. Dienstbier, *J. Nucl. Biol. Med.* **10**, 101 (1966).
7) G. Schünzel, W. Schmidt, and A. Mouczek, *Radiobiol. Radiother.*, **10**, 685 (1969).
8) Y. Takagi, F. Sato, M. Shikita, M. Shinoda, T. Terasima, and S. Akaboshi, *Radiation Res.*, **42**, 79 (1970).

cells were plated onto the plastic petri dishes and irradiated with X-rays. Shortly after the termination of irradiation, the culture medium was replaced with a fresh medium containing 0.1 mM of THFA. After the incubation at 37° for 1 hr with this medium, the cells were rinsed three times with the fresh medium to remove the agent. Number of the cell-colonies developed was counted 2 weeks thereafter. Further details of the cell culture method were same as described previously.[8]

Irradiation——From an X-ray generator (Shimazu, Shin-ai 250-II) which was operated at 200 kVp and 20 mA, the animals received whole-body radiation of 600 R at a rate of 90 R/min. A filter of 0.5 mm Cu and 0.5 mm Al was used and the radiation dose was monitored with a Radocon inonization gauge (Model 575 A). During the irradiation, the animals were held in a plastic cabinet at the distance of 50 cm under the X-ray tube. Irradiation of HeLa cell cultures was performed in a similar way.

Tetrahydrofolic Acid——Tetrahydrofolic acid was provided from Yamanouchi Pharmaceut. Co. Ltd. (Tokyo) as a solution of pH 7.0 and of a concentration of 50 mg/ml. The solution also contained 0.5% sodium bisulfite. When this preparation was diluted 10000 folds with 1/15 M phosphate buffer (pH 7.0) containing 0.16% β-mercaptoethanol, the light absorption spectrum of the solution had a single maximum at 297 mμ with the absorbancy of 0.32. Therfore, the presence of folic acid and dihydrofolic acid in this THFA preparation is seemingly neglegible: it has been reported that the molar extinction coefficient of THFA is approximately 32000.[9] Unless otherwise noted, THFA was injected 10 min after irradiation.

Leucocyte Counting——Approximately 0.1 ml of blood was taken from the respective mouse by ophthalmic puncture with a clean glass capillary. The blood was immediately transferred into the 20-lambda self-measuring pipette (Fisher unopette). Then, the total leucocyte number was determined with the use of a Fisher autocytometer which had been calibrated by the conventional microscopic method.

Tissue Weight——Shortly after the animals were killed by dislocation of cervical vertebrae, spleen and small intestine were removed. The intestinal tract was longitudinally opened with scissors and the contents were removed by rinsing the tissue in 0.9% NaCl solution. The tissues were blotted with filter papers and weighed with a chemical balance.

Result and Discussion

Life-Prolonging Effect in Midlethally X-Irradiated Mice

Five min after termination of X-irradiation, THFA was intraperitoneally injected in the mice in the dose of 0.5 mg or 1.5 mg per animal. Control animals began dying on the 7th day of the experiment, and a half of them died from the radiation injury in 16 days after the irradiation (Fig. 1). In the THFA-treated groups, there observed a singificant delay of time before the first incidence of death of the animals occurred. Furthermore, the treatment with THFA increased the final survival rate from the control level of 50% up to 70% (0.5 mg THFA group) or up to 75% (1.5 mg THFA group).

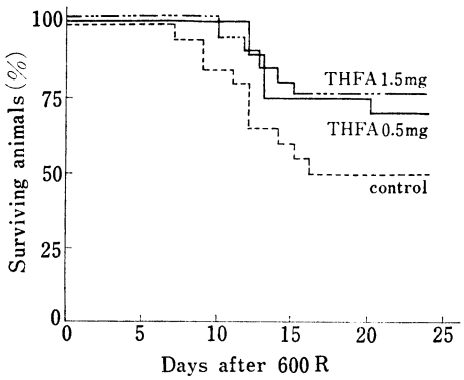

Fig. 1. Effect of the Post-Irradiation Injection of Tetrahydrofolic Acid (THFA) upon the Survival Rate of Midlethally X-Irradiated Mice

Each group consisted of 20 mice at the start of experiment.

Similar therapeutic effect of THFA was repeatedly observed in the animals in which the compound was injected with various time-intervals after or before the irradiation (Fig. 2). It seemed that THFA was most effective when it was given to the animals shortly prior to the irradiation. In the case of the post-irradiation treatment, THFA displayed larger effectiveness when it was injected 30 min after the irradiation than when it was injected 5 min after the irradiation. It is plausible that the mechanism of action of THFA which was given prior to the irradiation is different from that of THFA which was given post-irradiation. In the former

9) L.M. Prescott, R.C. Wood, and E.L. Speck, *Biochem. Biophys. Res. Commun.*, **19**, 151 (1965).

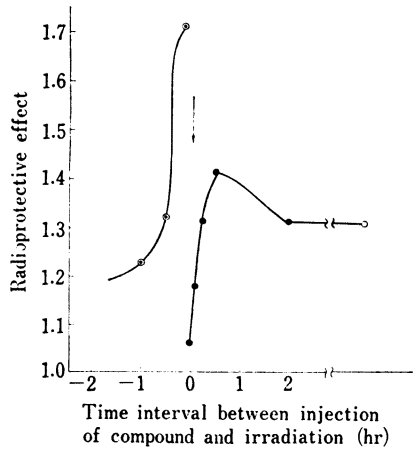

Fig. 2. Effect of the Time Interval between Injection and Irradiation upon the Radiation–Therapeutic Effect of Tetrahydrofolic Acid

The effect of the compound was expressed by the ratio of survival time of the control and the treated animals; each group consisted of 20 mice and the mean survival days of the control animals was 8.6 days.

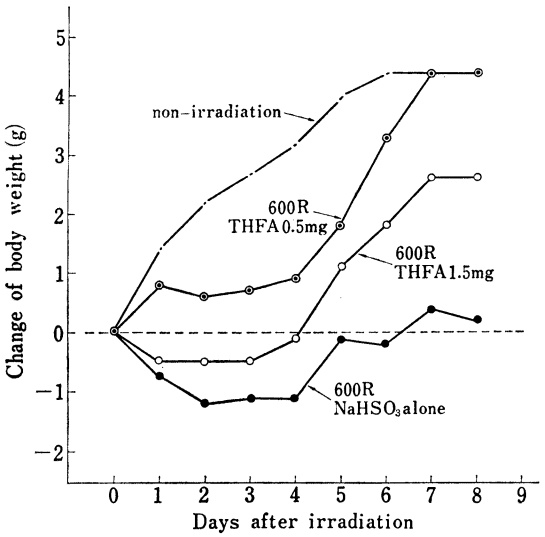

Fig. 3. Change in Body Weight of the X-Irradiated Mice and the Effect of Post-Irradiation Injection of Tetrahydrofolic Acid (THFA) thereon

The animals (20 mice in each group) were 22—26 g on the day zero.

case, the compound may be acting simply as a radical-scavenger, while in the latter case its action may be related mainly to the nucleic acid metabolism.

Effect on the Body Weight of X-Irradiated Mice

In a subsequent experiment which was performed in the same manner as above, the body weight change of the individual animals was measured daily during 8 days after the irradiation (Fig. 3). In the control $NaHSO_3$-treated animals, radiation caused a slight loss of the body weight within several days after the irradiation. Although a small recovery in the body weight occurred subsequently, this recovery process was counterbalanced by the rapid development of exhaustion which lead these animals to death. In the THFA-treated animals, on the other hand, retardation of the growth rate was normalized on the 5th day of the experiment as depicted in Fig. 3.

Change of the Weight of Intestine and Spleen

Regardless whether the animal dies or survives subsequently, the intestinal tract of the midlethally irradiated mouse recovers in weight temporarily within several days after the irradiation. The weight of the intestine often becomes larger than the initial value because of the rapid and excessive regeneration of the damaged tissues. In the THFA-treated animals, the overshoot of the intestinal recovery was seemingly more pronounced than in the control animals (Table I).

In the same manner as in the case of intestine, the weight of spleen recovers above the initial level in the sublethally irradiated animals. In this case, however, it takes about 3 weeks before a rapid recovery of the tissue weight occurs. It was observed that THFA slightly promoted the recovery overshoot of the spleen weight (Table II).

Effect on the Intestinal Cell-Division Index in the X-Irradiated Mice

Intestinal tract of the X-irradiated mice was examined histologically, and the number of dividing cells was counted in the microscopic field of four successive regions of duodenum

TABLE I. Effect of Post-Irradiation Injection of Tetrahydrofolic Acid (THFA) upon the Recovery of the Intestinal Weight of the Mice which survived 600 R X-Irradiation

Radiation (R)	Injection	Body weight of the animal (g)[a]	Intestine weight (mg)	
			Wet	Dry
Zero	none	35.8±0.6	922±38	—
600	0.5% $NaHSO_3$	29.8±0.8[b]	1190±48[b]	246±12
600	THFA 0.25 mg	29.9±0.6[b]	1209±46[b]	242±10
600	THFA 0.5 mg	29.7±0.2[b]	1279±43[b]	255± 9
600	THFA 1.0 mg	28.2±0.9[b]	1267±60[b]	257±11

a) Each group consisted of 10 mice. The animals were killed 5 days after the irradiation.
b) statistically significant against the zero R-non-injection group ($p<0.05$)

TABLE II. Effect of Post-Irradiation Injection of Tetrahydrofolic Acid (THFA) upon the Recovery of Spleen Weight in the Mice which Survived X-Irradiation of 600 R

Treatment	Number of animals	Body weight (g)	Spleen weight[a]		
			Wet (mg)	Dry (mg)	Dry/wet (%)
Zero R $NaHSO_3$	10	29.1±0.3	143± 8	32±2	22.0±0.3
600 R $NaHSO_3$	10	26.6±1.9	226±24[b]	51±5[b]	22.6±0.3
600 R THFA 0.5 mg	14	30.5±0.9	312±34[b]	66±7[b]	21.3±0.6

a) 20 days after irradiation
b) statistically significant against the zero R-$NaHSO_3$ group ($p<0.01$)

at the magnification of 300. It was shown that the treatment of the X-irradiated mice with THFA increased the number of dividing cells in the intestinal wall (Table III). This result is consistent with the aforementioned observation that the intestinal weight was increased by the treatment with THFA.

TABLE III. Intestinal Cell-Division Index in Mice which Survived X-Irradiation of 600R: Effect of Post-Irradiation Treatment of the Animals with THFA

Treatment	Cell-division index[a]	
	3 days after irradiation	6 days after irradiation
Non-irradiated	14	14
600 R, 0.5% $NaHSO_3$	10	10
600 R, THFA 0.5 mg	10	14
600 R, THFA 1.5 mg	15	15

a) Each figure in the table represents the average of 4 determinations of the number of the dividing cells.

Recovery of the Circulating Leucocyte Number

Blood leucocyte number decreases severely and rapidly after the sublethal irradiation of the animals. Within 2 weeks after the irradiation, however, a gradual recovery of the leucocyte number occurs (Fig. 4). It seemed that the treatment of the animals with THFA enhanced this recovery process of the leucocyte production.

Effect on Reproductive Integrity of X-Irradiated HeLa S_3 Cells

Post-irradiation treatment with 0.1 mM THFA failed to increase the survival rate of the X-irradiated HeLa cells (Fig. 5). When it was present in the culture medium for a long period of time, THFA of this concentration strongly inhibited the proliferation of HeLa cells. In

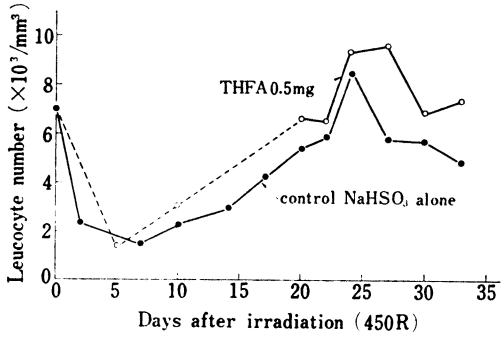

Fig. 4. Effect of the Post-Irradiation Injection of Tetrahydrofolic Acid (THFA) upon the Recovery of Blood Leucocyte Number in the X-Irradiated (450 R) Mice

Each point represents an average of duplicate determinations of the leucocyte number in ten animals.

the experiment shown in Fig. 5, however, the compound was removed from the medium after one hour's contact with the cells. Thus, the treatment with THFA, by itself, caused no change in the subsequent cell-colony development. Although we failed to demonstrate the therapeutic effect of THFA in this culture system, it is obviously early to conclude that the compound is ineffective at the cellular level. Further studies with different cell-strains or under different conditions of culture will be needed.

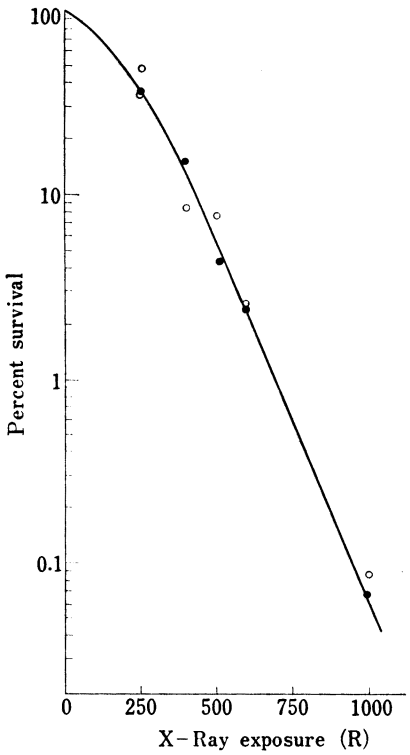

Fig. 5. Reproductive Integrity of HeLa S_3 Cells as Influenced by X-Irradiation and the Effect of the Post-Irradiation Treatment with Tetrahydrofolic Acid(THFA)

—●—: control
—○—: THFA 0.1 mM 1 hr post x-irradiation

Added in Proof (February 25, 1971)

Folic Acid *vs.* THFA: After this paper was submitted, folic acid was compared with THFA with regard to the therapentic effect in the midlethally X-irradiated mice in a subsequent experiment. The therapeutic effectiveness was specifically observed for THFA, while folic acid was powerless under the experimental conditions so far as examined below (Table IV).

TABLE IV. Life-Prolonging Effect of Tetrahydrofolic Acid (THFA) in X-Irradiated Mice as compared with the Effect of Folic Acid

Irradiation	Compound[a)]	Mean survival days	Number of survivals out of 20 mice
600 R	none	23.0±1.6	8
	folic acid 0.5 mg	21.3±2.3	11
	folic acid 1.5 mg	21.9±1.6	8
	THFA 0.5 mg	28.0±1.1[b)]	16
700 R	none	12.7±0.6	zero
	folic acid 0.5 mg	12.0±0.8	zero
	folic acid 1.5 mg	13.1±0.6	zero
	THFA 0.5 mg	15.4±1.0[b)]	1

a) injected intraperitoneally 15 min after the irradiation
b) statistically significant against the control (none) group at 5% level