

## STUDIES ON ANTI-TUMOR AGENTS. IV

Effect of TC-17 on Tumor Cells

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## INTRODUCTION

In the first paper of this series<sup>1)</sup>, it has been shown that TC-17 has very marked inhibitory activity on solid tumors in mice and rats but little effect on the ascites forms of the same tumor when the treatment is given parenterally prior to tumor inoculation.

From these previous findings, it may be assumed that the cytotoxic activity of TC-17 on tumor cells is mild.

The present paper reports the results of experiments performed in order to elucidate the direct action of TC-17 on tumor cells.

## MATERIALS AND METHODS

## Cytolytic Activity:

The observations for morphological changes of various ascites tumor cells infused with various concentrations of TC-17 at 37°C were carried out as compared with the activities of the compound on rabbit erythrocyte (designated as RBC).

TC-17 saline solutions with various concentrations were prepared (A)\*.

The tumor cells of EHRLICH carcinoma, Sarcoma 180 and YOSHIDA sarcoma were harvested separately from mice and rats bearing 7-day-old ascites tumors. The milky fluids were centrifuged at the speed of 1,000 rpm for 5 minutes. The sedimented tumor cells were washed 3 times with chilled physiological saline and resuspended in HANK'S solution and adjusted the final concentration of tumor cells to contain  $2 \times 10^7$  cells per ml (B)\*.

Blood (20 ml) was taken by a syringe directly from the heart of rabbit. After the fibrin was removed from the whole blood by a glass-bead and filtered with a wet mesh gauze. The filtrate was centrifuged at the speed of 2,000 rpm for 5 minutes. The precipitated RBC was washed 3 times with physiological saline and resuspended in the amount

of 20 ml of physiological saline (C)\*.

Mixtures of 1.0 ml of (A) with 1.0 ml of (B); and 1.0 ml of (A) with 1.0 ml of (C) were incubated at 37°C. The morphological changes of tumor cells or RBC in the incubated mixture were observed visually and microscopically at 5, 10, 15, 30, 45, 60, 120, 180 and 300 minutes later.

## Cytocidal Activity:

If the tumor cells were dead after the contact with drug, the treated tumor cells inoculated mice should be prolonged the survival time.

Cytocidal activity of TC-17 on tumor cells was estimated by the prolongation of the life span of the mice inoculated intraperitoneally the tumor cells which were incubated with TC-17.

EHRLICH ascites tumor cells infused with TC-17 (final concentration, 0.5%) at 37°C for the intervals of 15, 60 and 180 minutes were inoculated intraperitoneally into mice (ICR-JCL, male 6 weeks old) (A).

A control group was set up with intraperitoneal inoculation of EHRLICH ascites tumor cells incubated with vehicle only, with the same experimental conditions of (A)-(B).

The mean survival days of (A) and (B) groups were calculated. Observation period was 35 days. Mice still alive on 35 days after the inoculation were sacrificed and autopsied.

Activity of TC-17 on respirations and glycolysis of EHRLICH ascites tumor cells were determined according to the method of KUN *et al*<sup>2)</sup>.

EHRLICH ascites tumor cells were harvested from mice bearing 7-day-old ascites tumors. The tumor cells were washed 3 times with a chilled KREBS-RINGER phosphate solution at the speed of 1,000 rpm for 5 minutes and resuspended in the KREBS-RINGER phosphate solution and adjusted the final concentration of tumor cells to contain  $5 \times 10^7$  cells per ml or  $2.5 \times 10^7$  cells per ml.

In the measurements of respirations, 1.0 ml of the

\*: (A), (B) and (C) are used as the designation of each prepared solution in this paper.

cell suspension ( $5 \times 10^7$  cells/ml), 1.0 ml of KREBS-RINGER phosphate, and 0.7 ml of water were placed by pipette in the main compartment of Warburg flask with 0.2 ml of 10% KOH in the center well and 0.3 ml of the sample solution was placed in the side arm. The temperature of water bath was 37°C. The gas phase was air. The respiratory quotient was determined by the direct method.

Anaerobic glycolysis was determined manometrically in KREBS-RINGER bicarbonate solution with 95% N<sub>2</sub> and 5% CO<sub>2</sub> as the gas phase. One ml of the cell suspension ( $2.5 \times 10^7$  cells/ml), 1.0 ml of KREBS-RINGER bicarbonate solution, 0.5 ml of water, and 0.2 ml of 1.5% glucose were placed in the main compartment of Warburg flask and 0.3 ml of the sample solution was placed in the one side arm (A) and 0.2 ml of 5N-H<sub>2</sub>SO<sub>4</sub> in the other sidearm (B).

Determinations of DNA, RNA, and Protein:

Activity of TC-17 on nucleic acids and protein synthesis was determined using HeLa cells. HeLa cells grown in YLE culture medium containing 20% calf serum were washed 3 times with the fresh medium and harvested. The precipitate was resuspended in the culture medium ( $2 \times 10^5$  cells/ml) and 4.0 ml of the cell suspension was placed in a culture flask, and incubated at the temperature of 37°C. After 24 hours the supernatant fluid was removed, and the fresh culture medium containing various concentrations of the drug was added and incubated to 37°C.

Quantitative changes of nucleic acids and protein in HeLa cells were measured in 24, 48 and 96 hours after the addition of the drug.

Fractionation of cellular components was carried out according to the method of SCHNEIDER<sup>3)</sup>. The tumor cells in the culture flask were washed with physiological saline 3 times, and raked up with pipette and then harvested in the amount of 10 ml of 80% ethanol. The precipitate was washed with ether-ethanol (3:1) 2 times and then with the amount of 2.5 ml of cold 10% trichloroacetic acid 2 times. The nucleic acid fraction was obtained by the extraction with the amount of 4.0 ml of 5% trichloroacetic acid at 90°C for 20 minutes.

The protein was measured by the FOLIN method<sup>4)</sup>. The hot trichloroacetic acid-insoluble fraction was dissolved in 1.0 ml of 2.8% NH<sub>4</sub>OH, and added to 5.0 ml of LOWRY reagent and 0.5 ml of phenol reagent (2.6 times dilution by water). And absorption intensity of the solution was measured at the wave length of 660 m $\mu$  after left to stand for 60 minutes.

The DNA was measured by the orcinol method<sup>5)</sup>. One ml of the hot acid-soluble fraction was added to 3.0 ml of orcinol reagent and heated for 20 minutes in a boiling water. Absorption intensity of the solution was measured at the wave length of 660 m $\mu$ .

The RNA was measured by the indole method<sup>6)</sup>. One ml of concentrated HCl and 1.0 ml of indole reagent (0.04%) were added to 2.0 ml of the hot acid-soluble fraction and boiled for 10 minutes.

Table 1. Effects of TC-17 on tumor cell and rabbit erythrocyte

mint.	TC-17 conc. (%)															
	0.01				0.25				0.25				0.5			
	E	S	Y	RBC	E	S	Y	RBC	E	S	Y	RBC	E	S	Y	RBC
5	-	-	-	-	+	+	+	-	+	+	+	-	++	+	++	-
10	-	+	+	-	+	+	++	-	++	++	+++	-	+++	+	+++	-
15	+	+	+	-	++	++	+++	-	+++	+++		-		+++		-
30	+	+	+	-	+++	+++		-				-				-
45	+	+	+	-				-				-				+
60	++	++	+	-				-				+				+
120		++	+	-				-				+				+
180			++	-				-				+				++
300				-				-				++				+++

E: Ehrlich ascites tumor cell

S: S 180 ascites tumor cell

Y: YOSHIDA ascites tumor cell

RBC: Erythrocyte (Rabbit)

Cytolysis

- : No effect    + : Slight effect    ++ : Moderate effect    +++ : Marked effect

Hemolysis

- : No effect    + : Slight effect    ++ : Moderate effect    +++ : Marked effect

After cooling, the colored solution was washed with chloroform 3 times, and the absorption intensity of the supernatant was measured at the wave length of 490 m $\mu$ .

**Tumor Cell Staining by TC-17 :**

The smears of YOSHIDA sarcoma ascites cells were immediately fixed in a mixture of 100 ml of 95% ethanol and ether (1:1) for 20 minutes and dried.

Five ml of a stock solution of 0.2% TC-17 in distilled water was separately diluted 20 times with 95 ml of 1/10 M phosphate buffer solutions of pH 6.0, pH 7.0 and 1/10 M acetate buffer solutions of pH 4.0 and pH 5.0. The smears were stained for 20 minutes in above prepared 0.01% TC-17 solu-

tions.

After staining, the slides were washed sufficiently with phosphate buffer or acetate buffer until all surplus dye was removed and observed under fluorescence microscopy equipment(Nihon Kogaku K.K.).

In an effort to determine the staining for living tumor cells, 0.02% saline solution(1.0 ml) of TC-17 was mixed with YOSHIDA sarcoma ascites cell suspension (1.0 ml) and incubated for 30 minutes at 37°C. Then a mixture was centrifuged at the speed of 1,000 rpm for 5 minutes and the sedimented tumor cells were washed sufficiently with chilled physiological saline, and observed under the fluorescence microscope.

**RESULTS**

**Cytolytic and Hemolytic Activities :**

Cytolytic and hemolytic activities of TC-17 are shown in Table 1. Tumor cells infused with high concentration (0.25~0.5%) of TC-17 produced predominantly marked erosion and disintegration within 15 minutes, although some intact cells surrounded by disintegrated cells were observed microscopically.

A low concentration of 0.01% caused only a swelling of tumor cells. In concentrations which showed no predominating damage to RBC, TC-17 caused considerable morphological changes to tumor cells.

**Cytocidal Activity:**

The tumor cells incubated with TC-17 0.5% for 15 minutes or more, in spite of its marked disintegration showed high growth in abdominal cavity of mouse as shown in Fig.1.

It was suggested that some durable tumor cells without marked damage were proliferated rapidly. The result shows that TC-17 has only a mild cytocidal activity.

**Activity on Respirations and Anaerobic Glycolysis:**

Figure 2 shows the activity of TC-17 in a concentration of 10<sup>-2</sup>, 5 $\times$ 10<sup>-3</sup> and 10<sup>-3</sup> M on oxygen uptake and anaerobic glycolysis of EIRLICH ascites tumor cells. TC-17 showed inhibitory activity in a higher concentration of 10<sup>-2</sup> M and little activity in 5 $\times$ 10<sup>-3</sup> M on both res-

Fig.1 Transplantation of Ehrlich ascites tumor cells after contact with TC-17 (0.5%)

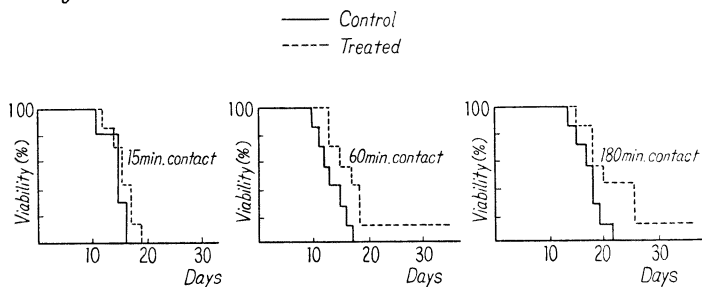


Fig.2 Effect of TC-17 on enzymic activities of Ehrlich ascites tumor cells

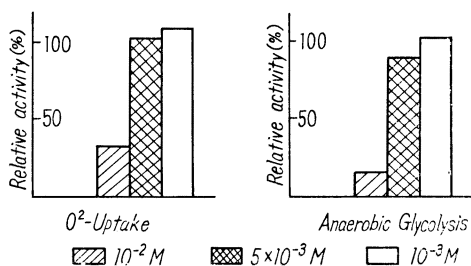


Fig.3 Effect of TC-17 on protein and nucleic acids synthesis of HeLa cell

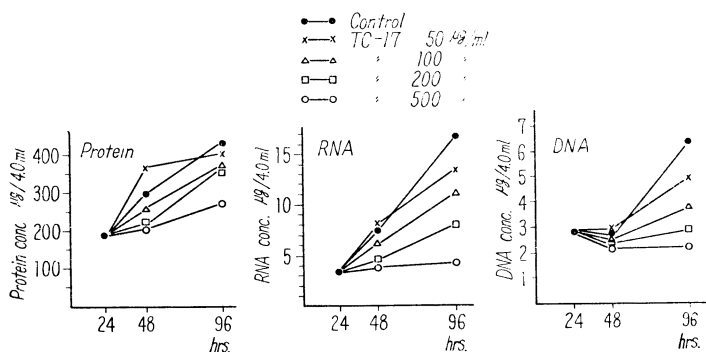
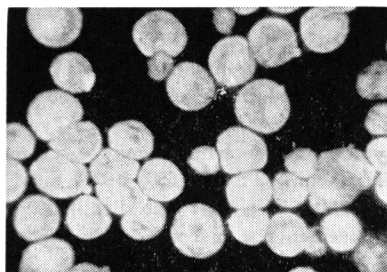
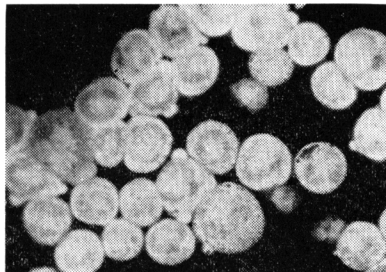


Fig. 4 Fluorescent staining of YOSHIDA sarcoma ascites cells with TC-17 ( $\times 600$ )

pH 6.0



pH 5.0

pirations and anaerobic glycolysis.

Activity on Protein and Nucleic Acids Synthesis:

Activity of TC-17 on protein and nucleic acids synthesis in HeLa cells is shown in Fig. 3. TC-17 showed a moderate inhibition in 200  $\mu\text{g}/\text{ml}$  and a marked inhibition in 500  $\mu\text{g}/\text{ml}$  on the protein synthesis.

The inhibitory activity on the nucleic acids synthesis increased gradually with increasing a concentration of TC-17 and the synthesis was completely inhibited at the higher concentration of 500  $\mu\text{g}/\text{ml}$ .

There was little evidence of any selectivity for inhibition of DNA, RNA or protein synthesis.

Staining by TC-17:

The fixative tumor cells stained with TC-17 produce a strong white blue fluorescence when activated by ultra-violet light (Fig. 4). Brilliance of color was very good in the range of pH 5.0 to pH 6.0 and cell components showed slight differences in their affinity for TC-17.

As shown in Fig. 4, the fluorescence of nucleolus and cytoplasm were more strong than that of nucleus at pH 5.0 and pH 6.0.

TC-17 staining of the living tumor cells produced a weak fluorescence by ultra-violet light, but in this case, the differential behavior of the compound towards the cellular rebo-senucleoproteins and desoxyrebo-senucleoproteins could not be observed under the fluorescence microscope.

#### COMMENTS

It has been found that TC-17 showed a marked cytolytic activity on various ascites tumor cells (EHRlich carcinoma, Sarcoma 180, YOSHIDA sarcoma) and inhibited completely the nucleic acids synthesis in HeLa cells with a concentration of 0.05%. The inhibitory activity of TC-17 on respirations and anaerobic glycolysis of EHRlich ascites tumor cells was very weak even at the higher con-

centration of 0.59% ( $5 \times 1.0^{-3} \text{M}$ ), and the same cells infused with 0.5% concentration of TC-17 for 3 hours have still maintained a high proliferating activity.

From these findings, it was concluded that the tumor cell damage by TC-17 was very insufficient in regard to cytotoxic activity. Therefore, the facts<sup>1)</sup> which TC-17 showed only moderate activity on solid tumors and little or no effect on ascites tumors when the post-treatment was performed be supported by the results reported herein. While, the marked inhibiting activity<sup>1)</sup> of TC-17 with pretreatment on solid tumors is not found to be associated with the direct tumor cell damaging actions of the compound.

It seems reasonable to assume that animals treated TC-17 prior to tumor inoculation have got some defence mechanisms against proliferation of inoculated tumor cells.

#### SUMMARY

Direct action of a potential anti-tumor agents, TC-17 on tumor cells was investigated.

1. The compound showed a marked damaging activity on ascites tumor cells and inhibition of nucleic acids synthesis in HeLa cells with a concentration of 0.05%.

2. The cytolytic activity on tumor cells was more strong and selective than the hemolytic activity on rabbit RBC.

3. Inhibitory activities on the respirations and anaerobic glycolysis of EHRlich ascites tumor cells were very weak even at the high concentration of  $5 \times 10^{-3} \text{M}$  (0.59%).

4. The tumor cell damaging activity of the compound was very insufficient in regard to cytotoxic activity.

5. Fluorescent staining of tumor cells with TC-17 showed that the compound had a strong affinity for intra and extra nuclear nucleoproteins.

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