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Enhancement of Antitumor Activity of Cyclophosphamide with Imipramine[1]

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Potentiation of cyclophosphamide activity by non antitumor agents which might affect the liver microsomal enzymes, was examined with Sarcoma 180. That activity of low dose, only 24% inhibition in tumor growth, was enhanced to 94% by imipramine. But any potentiation was not shown by phenobarbital, pentobarbital, isoniazid, meprobamate or glutethimide.

The cyclophosphamide activating enzymes in liver were enhanced by phenobarbital but not imipramine treatment, besides both concentrations of the activated and intact cyclophosphamide in blood were not elevated and not maintained by the latter.

Nigrosine stainability of the tumor cell was stimulated by less than 1 mg/ml of imipramine *in vitro*, that was a different phenomenon from the surface activity of Tween 80.

One of the possible couses for enhancement in antitumor activity of cyclophosphamide by imipramine was considered to be the stimulation of membrane permeability.

Cyclophosphamide has been reported to be inactive *in vitro* unlike other alkylating agents but active *in vivo*,[3] which was explained as being due to the alteration of the drug into active form by the microsomal drug metabolizing enzymes in liver.[4,5] On the other hand, phenobarbital and other related compounds have been known to stimulate the activities of these enzymes.[6]

The present experiments were performed to see if there is a correlation between the potentiation of antitumor activity of cyclophosphamide and the activation of enzyme activities by phenobarbital and other agents.

Materials and Methods

Animals and Tumor System——Female ddN mice weighing 18—22 g were used and the commercial chow diet (CA-1, CLEA Japan, Inc., Tokyo) and water were offered ad libitum. Sevenday-old Sarcoma 180 ascites was inoculated intraperitoneally in 0.05 ml (3×10^7 cells) doses.

Administration of Cyclophoshpamide and Agents——About ED_{20} of cyclophosphamide (20 mg/kg/day) and phenobarbital (55 mg/kg/day), meprobamate (200 mg/kg/day), pentobarbital (40 mg/kg/day), glutethimide (100 mg/kg/day), isoniazid (45 mg/kg/day) and imipramine (40 mg/kg/day) were used. Quantities of the above agents administered are one-third of each LD_{50} except one-fifth of phenobarbital due to its high toxicity. Meprobamate and glutethimide were suspended in 0.5% carboxymethylcellulose (CMC)-saline solution, and other agents were dissolved in saline solution. Animals were divided into 4 groups (A, B, C and D) of 12 each. They were treated as follows: A, control group, was injected with physiological saline only; B with cyclophosphamide alone at 10.00 a.m. from day 1 to 7 except day 5 after transplantation; C with one of the imipramines alone at 4.00 p.m. from day 0 to 6 except day 5; D, combination group, with cyclophospha-

1) Presented in part at the 26th Annual Meeting of the Japanese Cancer Association, Nagoya, Oct. 1967, and the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, Apr. 1968.
2) Location: *Tsukiji 5-chome, Chuo-ku, Tokyo.*
3) G.E. Foley, O.M. Fredman and B.P. Drolet, *Cancer Res.*, **21**, 57 (1961).
4) N. Brock and H.J. Hohorst, *Cancer*, **20**, 900 (1967).
5) H.M. Rauen and K.P. Kramer, *Arzneim. Forsch.*, **14**, 1066 (1964).
6) R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pahrmacol.*, **13**, 69 (1964).

mide and one of the imipramines each in the same procedures as groups B and C. They were killed at day 8, and ascites was withdrawn and measured. The total packed cell volume (TPCV) of the tumor was measured by Sassenrath' smethod and the respective inhibition ratio of groups B, C and D against group A was calculated.[7]

Assay of Enzyme Activity——The cyclophosphamide activating enzymes in liver were determined by Hart's method[8] on day 2,4,6 and 8 after transplantation. Liver was homogenized in a glass homogenizer with 3 volumes of cold isotonic KCl solution (1.15%) and centrifuged at $9000 \times g$ for 10 min. The substrate and cofactors were cyclpohosphamide (0.5 μmole), nicotinamide (50 μmol), triphosphopyridinenucleotide (1.38 μmole), glucose-6-phosphate (12.5 μmole) and $MgSO_4$ (12.5 μmole). 0.5 ml of liver supernatant fraction and 2 ml of the substrate and cofactors in 0.1M phosphate buffer (pH 7.4) were mixed and incubated with shaking for 60 min at 37°. After that equal volume of 4% $HClO_4$ solution was added to the mixture, shaken well and centrifuged at 3000 rpm for 10 min. Total and activated cyclophosphamide quantities were determined by Morita's method.[9]

Determination of Cyclophosphamide in Blood——Cyclophosphamide of 500 μmol/kg were intraperitoneally injected 18 hrs after the phenobarbital (55 mg/kg) or imipramine (40 mg/kg) administration. Blood samples were drawn from vena cava at 5, 15, 30 and 60 min each after the final injection under anesthetizing with ether. Then both types of cyclophosphamide were assayed as described above.

Nigrosine Stainability of Tumor Cells——Each of imipramine in 0.1, 0.2, 0.4 and 0.8 mg/ml amount was added to the 10% tumor cell suspension (7 day-old-cells) in Krebs-Ringer phosphate buffer and each mixture was incubated for 5, 15, 30, 60 and 90 min at 37°. 10 μl/ml of Tween 80 was used for comparing under the same condition. Then, the cell suspension was mixed with 4 volumes of 0.3% nigrosine solution, allowed to stand for 5 min, and stained cells were counted microscopically (nigrosine stainability = number of stained cells/number of total counted cells $\times$ 100%).

Results

Effect of Agents on Antitumor Activity of Cyclophosphamide

As shown in Table I, the highest inhibition (94%) was found in the combination group (group D) of imipramine, whereas it was non active on the administration of agent alone (group C). Glutethimide did not inhibit but rather stimulated, but cyclophosphamide activity was not depressed by the combination. Phenobarbital and other agents had no effect in combination.

Table I. Effect of Cyclophosphamide and Other Agents on Sarcoma 180

Compound	Dose (mg/kg/day)	Growth inhibition (%)	Compound	Dose (mg/kg/day)	Crowth inhibition(%)
Cyclophosphamide	20	41	Cyclophosphamide	20	24
Phenobarbital	55	37	Imipramine	40	36
Combination	20+55	66	Combination	20+40	94
Cyclophosphamide	20	41	Cyclophosphamide	20	16
Pentobarbital	40	23	Glutethimide	100	− 8
Combination	20+40	59	Combination	20+100	18
Cyclophosphamide	20	24	Cyclophosphamide	20	28
Isoniazid	45	42	Meprobamate	200	13
Combination	20+45	45	Combination	20+200	41

Activation of Cyclophosphamide by Liver Enzymes

The liver enzyme activities per unit weight and per mouse under tumor bearing are summarized in Table II. Gradual decreases of the activity were noted not only per unit weight but also per animal along the time after tumor transplantation in the control. The

7) E.N. Sassenrath, *Ann. N.Y. Acad. Sci.*, **76**, 601 (1968).
8) L.G. Hart, R.H. Adamson, H.P. Morris and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **149**, 7 (1965).
9) M. Morita, the 24th Annual Meeting of the Japanease Cancer Association, Fukuoka, Oct. 1965.

enzyme activity was only slightly increased after the imipramine administration, while, it was significantly elevated after the phenobarbital administration especially on day 4.

Table II. Effect of Imipramine and Phenobarbital on Cycolophosphamide Activation by the Liver of Tumor Bearing Mouse

Group	Time after transplantation (day)	Liver weight (g)	Enzyme activity[a] (Activated cyclophosphamide) (μmol/g liver/60 min) mean ±S.E.	(μmol/mouse/60min) mean ±S.E.
Control	0	1.1	3.30±0.06	3.76±0.07
	2	1.2	2.55±0.17	3.08±0.27
	4	1.5	1.99±0.24	2.98±0.38
	6	1.3	1.48±0.17	1.97±0.27
	8	1.3	1.26±0.27	1.64±0.17
Phenobarbital	2	1.4	3.62±0.23	5.17±0.53
	4	1.6	3.68±0.13	5.88±0.33
	6	1.5	2.50±0.39	3.64±0.58
	8	1.5	2.38±0.09	3.56±0.09
Imipramine	2	1.2	3.37±0.14	4.09±0.14
	4	1.3	2.78±0.45	3.58±0.53
	6	1.1	3.02±0.43	3.40±0.42
	8	1.1	2.26±0.21	2.58±0.27

a) 5 animals per each group

Cyclophosphamide Concentration in Blood

Both total and activated cyclophosphamide concentrations in blood were determined after the treatment with imipramine or phenobarbital. As shown in Fig. 1, imipramine treatment did not elevate and did not maintain the concentrations of activated and intact cyclophosphamide in blood at any time, whereas the activated form in phenobarbital treatment group was significantly higher at early stage after cyclophosphamide injection. In view of the above facts, the enhancement of cyclophosphamide activity by imipramine would be due to neither the elevation of enzyme activity in liver nor the maintenance of activated cyclophosphamide concentration in blood.

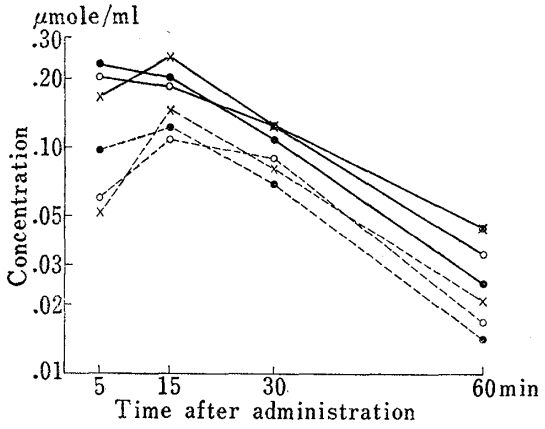

Fig. 1. Effect of the Drug Pretreatment on Cyclophosphamide Concentration in Blood after 500 μmol/kg Injection in the Mouse

total —×—: control
—○—: pretreated with imipramine
—●—: pretreated with phenobarbital
activated ---×---: control
---○---: pretreated with imipramine
---●---: pretreated with phenobarbital

Membrane Permeability of Tumor Cell

The cell permeability was determined by nigrosine stainability measurement. As shown in Fig. 2, the nigrosine stainability of tumor cells was increased by even low concentration of imipramine and tumor cell destruction was found in over 1 mg/ml of imipramine. When the time courses of imipramine and Tween 80 effects were compared, the maximum effect of the former was found within 15 min, in which almost 80% of tumor cells were stained. But that of the latter was very weak at early period and only 50% of tumor cells were stained even 60 min after (Fig. 3). It was suggested that cell permeability was stimulated by imipramine which may have different mechanism from Tween 80.

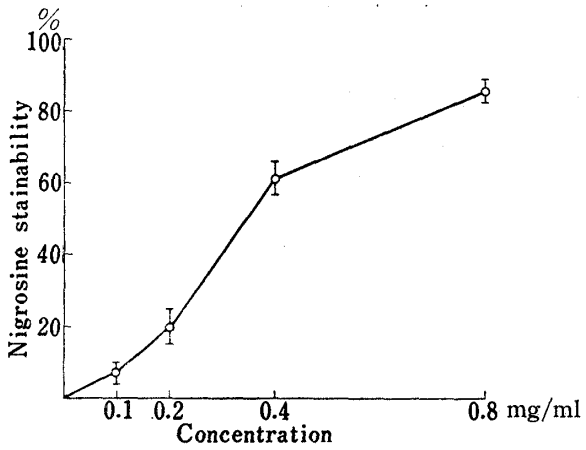

Fig. 2. Effect of Imipramine Concentration on Nigrosine Stainability of Tumor Cells after 30 min Incubation

Results represent the mean and standard error of 5 specimens.

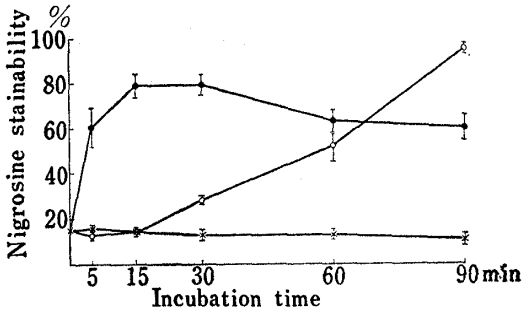

Fig. 3. Effect of Imipramine and Tween 80 on Nigrosine Stainability of Tumor Cells

—×—: control
—●—: incubated with imipramine (0.8 mg/ml)
—○—: incubated with Tween-80 (10 μl/ml)
⊤●⊥ : mean and standard error of 5 specimens

Discussion

Cyclophosphamide is known to be activated *in vivo* by the liver enzymes[3,4] which are stimulated by phenobarbital and other drugs.[6] When the rat is pretreated with phenobarbital, the activated cyclophosphamide in blood is also elevated.[5] However, in the present experiments, the antitumor activity of cyclophosphamide was not enhanced by phenobarbital, while imipramine, which did not stimulate the activity of the drug metabolizing enzymes in liver, clearly enhanced the antitumor activity of cyclophosphamide. Further, when the cyclophosphamide concentrations of either activated and intact forms in blood were compared between imipramine and phenobarbital treatments, both forms in the latter case were rather higher than in the former. These facts contradict the present results that imipramine potentiated the tumor inhibiting effect of cyclophosphamide and on the contrary phenobarbital did not show any effect. Therefore, the stimulating effect on enzyme activities can not explain the enhancement of cyclophosphamide activity by imipramine.

On the other hand, as imipramine was thought to have the direct action on tumor cells apart from the above facts, the stimulation of the cell permeability was examined *in vitro* as compared with Tween 80. And it was shown that imipramine had direct action on tumor cells and was more potent than Tween 80, notwithstanding that the surface activity of imipramine is one-hundredth of that of detergent.[10] The mechanism of increasing permeability by this agent may differ from the action of Tween 80 as detergent.

From these results, a possibility is suggested that the enhancing function of imipramine may be caused by the changes in the membrane permeability of tumor cell.

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10) P.M. Seeman and H.S. Bialy, *Biochem. Pharmacol.*, **12**, 1181 (1963).