ANTITUMOR AGENTS. I. EFFECT OF 5-FLUOROURACIL AND CYCLOPHOSPHAMIDE ON LIVER MICROSOMES AND THYMUS OF RAT

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Abstract—Effects of antitumor agents on rat liver microsomal drug-metabolizing enzyme activities and thymus lymphocytes were studied in male Wistar rats. High doses of 5-fluorouracil (5-FU) and cyclophosphamide (CP) given parenterally for 6 days caused a partial decrease in whole body weight and the microsomal enzyme content such as cytochrome P-450 and cytochrome b5. Aniline p-hydroxylase and aminopyrine N-demethylase activities also decreased in rats given CP for 6 days. The Michaelis constant for aniline p-hydroxylase of rats dosed for 5 days decreased compared with the control. Both compounds in the high concentrations produced spectral change of "modified type II". However, the magnitude of the spectral changes observed was independent of the concentration of substrate added. The addition of NADPH to the microsomes-substrate mixture modified the spectral change. Both drugs caused a considerable decrease in thymus weight and the number of thymus lymphocytes, while the alkaline phosphatase activity was enhanced in 5-FU group, indicating that the agents cause a significant involution of the thymus. Decrease in the total number of the lymphocytes was greater than that in the blood leucocytes.

5-Fluorouracil (5-FU) and cyclophosphamide (CP) are representative antitumor agents and widely used in cancer therapy. The former, which was first synthesized by Duschinsky et al. (1), inhibits DNA and RNA syntheses (2) as the competitive agent, and the latter, which is non-toxic to cancer cells in vitro, is considered to be first converted, presumably by the microsomal mixed-function oxidases, into 4-hydroxycyclophosphamide which may then break down to yield phosphoramide mustard (3), an active and potent antitumor agent (4). 5-FU has many adverse reactions such as hematopoietic depression, diarrhea, weight loss and hemorrhages in the lungs and intestines (5), while CP with a low toxicity (6) causes a decrease in leucocytes (7).

One of our objectives herein was to establish the effects of the antitumor agents on the thymus which plays an important role in immuno-defense mechanisms. When antitumor agents are given to cancer patients, the activity of the reticulo-endothelial system generally decreases, consequently the decreased immuno-response may result in a deterioration and development of tumors (8). It is thus of interest to elucidate the influence of antitumor agents on the thymus in terms of drug therapy of a tumor. The thymus in the neonate exerts an essential role in the development of lymphoid tissues and the establishment of immune functions (9, 10), and thymectomy of neonatal animals leads to a decrease in immune functions (11, 12). In adult animals, the thymus is related to the establishment and restoration of the immune functions, in particular the production and development of lymphocytes (13).
Thus, the thymus provides resistance against the tumorigenesis and development of tumor in animals. Although antitumor agents do have side effects as described above, damage to the liver is relatively slight in the clinically effective doses (14, 15). The second objective was to clarify the mechanism when even a slight lesion was evident in the liver after administration of these antitumor drugs.

Alterations of the drug-metabolizing enzyme activities, thymus alkaline phosphatase activity and the total number of thymus lymphocytes in comparison with blood leucocytes were investigated after high doses of these drugs.

MATERIALS AND METHODS

**Materials:** 5-FU and CP were a gift from Kyowa Hakko Co., Ltd. and Shionogi Seiyaku Co., Ltd., respectively. Nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH) and cytochrome c were purchased from Sigma Chemical Co. Glucose 6-phosphate (G-6-P) dehydrogenase [EC 1.1.1.49] from Oriental Yeast Co., Ltd. and G-6-P from Boehringer Mannheim were also used in this experiment. Acetylacetone, aniline, phenol and aminopyrine were used following purification by redistillation or recrystallization.

**Animals:** Male Wistar rats weighing about 100 g, maintained on MF diets (Oriental Yeast Co., Ltd.) for 3-4 days prior to the experiment, were divided at random into 3 groups of 4-8 rats each and fasted for 12 hr prior to the experiment: A) controls, treated for 6 days with daily i.p. injections of 0.5 ml of saline per 100 g of body weight (Control group). B) Animals treated for 6 days with daily i.p. injections of 5-FU or CP (30 mg each/kg body weight) solution, dissolved in saline (5-FU or CP groups).

The animals were decapitated 2 hr after the final administration of saline or the drugs at 3, 6 and 10 days. The doses of 5-FU and CP to rats were approx. 3 and 10 times higher than the clinically effective daily doses, respectively.

**Preparation of liver microsomal fractions:** The liver microsomal fractions were prepared according to the method of Omura and Sato (16).

**Measurements of the total number of blood leucocytes and thymus lymphocytes:** The blood of male Wistar rats was collected from the abdominal aorta into heparin-containing tubes (100 units per ml blood). The whole thymus, which was washed once with saline, was gently homogenized in saline with a glass homogenizer. The total number of the leucocytes and lymphocytes was counted using a Bürker Türk counting chamber.

**Protein determination:** Protein concentrations of microsomal preparation and the thymus suspension were estimated by the method of Lowry et al. (17).

**Assays of enzymes:** NADPH-cytochrome c reductase activity was assayed at 20°C by observing the change in absorbance at 550 nm resulting from cytochrome reduction, according to the procedure described by Slater and Sawyer (18). Aniline p-hydroxylase activity was estimated at 37°C according to the method of Ikeda (19). The demethylation of aminopyrine was assayed at 37°C by the method of Ariyoshi and Takabatake (20), and
formaldehyde formed was determined by the method of Nash (21). The concentration of cytochrome P-450 (P-450) and cytochrome b$_5$ (b$_5$) was determined at room temperature with a Shimadzu MPS-50L spectrophotometer as described by Omura and Sato (16). Alkaline phosphatase activity was determined in rat thymus homogenized in saline. A mixture consisting of 1 ml of p-nitrophenylphosphate (0.015 M in the reaction medium) and 1 ml of 0.09 M glycine buffer (pH 10.0) was preincubated at 37°C for 10 min and then 0.2 ml of thymus suspension was added. After incubation at 37°C for 20 min, the reaction was terminated with the addition of 1 ml of 0.1 M NaOH and the amount of the released p-nitrophenol was determined by measuring the absorbance at 410 nm with a Hitachi spectrophotometer Model-101.

**Difference spectrophotometry:** The difference spectra were measured at a room temperature of 20–25°C with a Shimadzu MPS-50L spectrophotometer after addition of an excess of drugs dissolved in 0.05 M tris-HCl buffer (pH 7.5) to freshly prepared microsomes. The drug concentrations of 5-FU and CP in the medium were $2 \times 10^{-5} - 5 \times 10^{-2}$ M. Part of the measurement was done in the presence of 0.33 mM NADPH.

**RESULTS**

**Effect of 5-FU and CP on body and liver weights**

As shown in Table 1, animals injected with these drugs failed to grow, whereas there was a normal increase in the body weight of control animals, and the weight of animals treated with these drugs decreased at day 6 rather than that on the first day. The cessation of the drug treatment, however, caused a partial increase of the body weight in 5-FU-injected rats at 10 days, while a slight decrease of the weight was observed in CP group which was given the drug in a dose ten times the clinically effective daily dose. The dose was three-fold in the 5-FU group. Relative liver weight (g liver/100 g body weight) was increased slightly after CP treatment but there was no significant increase in the weight of the 5-FU group, as compared with the controls.

| TABLE 1. Whole body and liver weight changes after parenteral antitumor agents |
|---|---|---|---|---|
| | Groups | 0 | 3 | 6 | 10 |
| **Body weight (g)** | Control | 121±11 | 130±18 | 138±13 | 142±10 |
|  | 5-FU 12 | 126±8 | 128±7 | 112±11* | 121±5† |
|  | CP | 119±7 | 121±7 | 108±5* | 100±7* |
| **Relative liver weight** | Control | -- | 4.2±0.2 | 4.3±0.5 | 4.6±0.4 |
|  | 5-FU | -- | 4.3±0.3 | 4.6±0.5 | 4.6±0.3 |
|  | CP | -- | 4.2±0.4 | 4.9±0.3* | 5.1±0.5* |

Each value represents the mean of five rats ± standard error.

* p≤0.001 in Control vs. 5-FU or CP
† p<0.05 in Control vs. 5-FU
Effect of 5-FU and CP on liver microsomal drug-metabolizing enzyme activities and the components

To clarify the effect of antitumor agents on the activity of the microsomal drug-metabolizing system, enzyme activities of the microsomes from the groups on the drugs for 3 or 6 days were measured. As shown in Table 2, NADPH-cytochrome c reductase activity was not significantly altered and it would appear that the enzyme is little affected by these drugs or the metabolites. The slight increase in P-450 and b5 levels attributed to the enzyme induction was observed in both groups administered the drugs for 3 days, whereas at 10 days the content of P-450 had significantly decreased, by 26% in 5-FU group and 48% in CP group, as compared with that of the control group, respectively. The b5 level of CP group was also decreased at 10 days. The decrease in these pigment contents may be due to the decreased syntheses and/or the increased degradation with accumulation of the drug and the toxic metabolites in situ. Aniline p-hydroxylase activity was also decreased 43%, after administration of CP for 6 days in contrast with the slight decrease in rats dosed 5-FU. The decreased activity in CP group was restored to 75% of the control value after cessation of administration, at 10 days. The decrease in aminopyrine N-demethylase activity was also shown in CP-treated rats at 10 days, however, there was only a partial change of this activity during administration of these antitumor agents.

**Table 2. Effects of antitumor agents on microsomal drug-metabolizing enzyme activities and components in rat liver**

| Enzymes                        | Groups         | Days       |
|--------------------------------|----------------|------------|
|                                |                | 3          | 6          | 10          |
| NADPH-cytochrome c reductase   | Control        | 24.6±1.0(5)| 21.9±1.4(5)| 23.6±2.4(5) |
|                                | 5-FU           | 23.0±2.8(6)| 22.9±2.2(6)| 21.7±2.2(6) |
|                                | CP             | 21.8±2.4(6)| 22.8±1.4(6)| 23.7±3.6(4) |
| Aminopyline p-hydroxylase     | Control        | 7.84±0.84(8)| 8.31±0.89(4)| 8.13±0.62(6) |
| N-demethylase                  | 5-FU           | 7.75±0.61(8)| 9.00±0.88(7)| 8.26±0.93(4) |
|                                | CP             | 9.08±0.98(6)| 9.75±0.82(5)| 6.54±0.60(7)*|
| Aniline p-hydroxylase         | Control        | 0.21±0.04(7)| 0.21±0.02(5)| 0.24±0.03(5) |
|                                | 5-FU           | 0.23±0.04(6)| 0.18±0.04(5)| 0.21±0.03(4) |
|                                | CP             | 0.23±0.04(7)| 0.12±0.04(6)**| 0.18±0.01(4)*|
| Cytochrome P-450               | Control        | 0.81±0.13(6)| 0.83±0.10(7)| 0.82±0.10(4) |
|                                | 5-FU           | 0.96±0.09(5)| 0.94±0.13(7)| 0.61±0.04(4)*|
|                                | CP             | 0.96±0.08(5)| 0.74±0.07(5)| 0.42±0.10(8)*|
| Cytochrome b5                  | Control        | 0.41±0.05(8)| 0.43±0.05(6)| 0.42±0.06(8) |
|                                | 5-FU           | 0.46±0.06(8)| 0.50±0.08(7)| 0.40±0.07(7) |
|                                | CP             | 0.53±0.04(8)*| 0.44±0.08(8)| 0.25±0.07(8)*|

Each value represents the mean± standard error with the number of animals in parentheses.
1): The activities of enzymes are expressed as µmoles of product per min per mg of protein.
2): Contents of cytochrome P-450 and b5 are expressed as µmoles per mg of protein.
* p<0.01 in Control vs. 5-FU or CP  ** p<0.001 in Control vs. CP
The Michaelis constant (Km) for aniline p-hydroxylase was measured after dosing of the drugs for 5 days. As shown in Fig. 1, the Km values were decreased to about one half or one third (3.31 x 10^{-4} M for 5-FU group, 2.62 x 10^{-4} M for CP group and 7.46 x 10^{-4} M for control group) as compared with the value in control rats. The affinity of the drug for microsomes appears to be enhanced by administration of these antitumor agents and the decrease in the enzyme activity, as shown in Table 1, may be due to the decline in the enzyme levels.

**Spectral studies of drug interaction with hepatic microsomal cytochrome**

The addition of various substrates of microsomal mixed-function oxidase system to aerobic liver microsomes causes two types of spectral changes (22). One class (termed type I) of spectral change is characterized by the appearance of a trough at 420 nm and an absorption peak at 385–390 nm as shown for barbiturates, aminopyrine and chlorpromazine. The second class (termed type II) of spectral change is characterized by an absorption peak at about 430 nm and a trough at about 390 nm. The spectral changes produced with 5-FU and CP were similar to those obtained with acetanilide and rotenone (termed modified type II) (22), which had a peak at 420 nm and a trough at about 390 nm, as shown in Fig. 2, indicating that these antitumor agents produce a modified type II spectrum. The magnitude of the spectral changes observed was not dependent upon the concentrations of the substrates added to the microsomal suspension in the range from 10^{-3} to 5 x 10^{-2} M. In addition, relatively large concentrations of the substrates were required for the reaction of the agents with the cytochrome of the liver microsomes as shown in Fig. 2, suggesting that the binding force of the substrates to microsomes is fairly weak. The addition of NADPH to the microsomal suspension pretreated with 5-FU or CP resulted in a modification of the spectral change, i.e. a pronounced increase in the absorption peak and a shift in the location of the maximum to 430 nm and the minimum to 408 nm, as shown in Fig. 2, indicating an increase in their binding.

A plot of the change of the absorbance at fixed wavelength (420 nm) as a function of 5-FU or CP concentrations was made, and the reciprocals of these data were plotted as illustrated in Fig. 3.
FIG. 2. Spectral changes after addition of antitumor agents and NADPH to rat liver microsomes. Four ml of microsomal suspension (1.8 and 3.6 mg of protein per ml in the presence and absence of NADPH, respectively) was divided into two cuvettes and a baseline of equal light absorbance was recorded, and after the addition of the different agents (0.5 ml) to one cuvette the difference spectra were recorded. Spectra were obtained at a room temperature of 20–25 °C.

- - - : 2.5 × 10⁻² M 5-FU  - - - : 5 × 10⁻² M CP
- - - - : 1 × 10⁻² M 5-FU: 0.33 mM NADPH
- - - - : 1 × 10⁻² M CP: 0.33 mM NADPH  - - - : 2.5 × 10⁻⁸ M aniline

FIG. 3. Effect of concentration of the substrate on the magnitude of spectral changes. The microsomal protein concentration was 3 mg per ml. The drug concentration was 10⁻⁴ to 5 × 10⁻² M. The difference spectra was recorded using the same method described for Fig. 2. •: 5-FU  •: CP

Effect of 5-FU and CP on weight, lymphocytes and alkaline phosphatase activity of thymus

We found that a single daily dose of 5-FU and CP for 6 days produced a remarkable decrease in the thymus weight and the total number of lymphocytes. After termination of administration, the decreased weight and lymphocytes in CP group remained unchanged, in contrast to the partial increase in the 5-FU group at 10 days, as shown in Table 3. However, the greater effect of CP on the thymus is no doubt due to the higher dose of this drug. A further increase (50 mg per kg body weight) of a dose of these drugs was found to induce a greater decrease in the thymus weight and the number of the lymphocytes, followed by increase in the lethal rate at 6 days (30% death).
TABLE 3. Effect of treatment with antitumor agents on weight, lymphocytes and alkaline phosphatase activity of rat thymus

| Groups     | Days       | 3            | 6            | 10           |
|------------|------------|--------------|--------------|--------------|
| Relative thymus weight (g/100 g body wt) | Control | 0.327±0.05(4) | 0.282±0.05(4) | 0.287±0.05(4) |
| 5-FU      | 0.206±0.03(4)* | 0.103±0.02(4)* | 0.151±0.07(4) |
| CP        | 0.148±0.03(4)* | 0.059±0.01(4)* | 0.064±0.03(4)* |
| Lymphocytes (×10⁶/whole thymus) | Control | 543±75(4) | 500±165(4) | 573±109(4) |
| 5-FU      | 198±53(4)* | 43±26(4)* | 64±25(3)* |
| CP        | 220±39(4)* | 13±4(4)* | 12±9(4)* |
| Alkaline phosphatase (nmoles p-nitrophenol per mg protein per 20 min) | Control | 1.98±0.20(7) | 1.99±0.20(6) | 1.83±0.33(6) |
| 5-FU      | 2.06±0.67(5) | 3.29±1.10(6)† | 2.12±0.23(3) |
| CP        | 1.61±0.17(6)* | 2.07±0.52(4) | 1.73±0.45(4) |

1) Activity is expressed as 10⁻⁶ moles of p-nitrophenol per mg protein per 20 min.
* p<0.01 in Control vs. 5-FU or CP   † p<0.05 in Control vs. 5-FU
Each value represents the mean±standard error with the number of animals in parentheses.

Following treatment with 5-FU, the alkaline phosphatase activity in the thymus was greatly increased at 6 days, while the activity in the CP group was only slightly increased at the same day. The increased activity returned to the control level after termination of administration at 10 days. CP given for 3 days induced a decrease in the activity, suggesting that the effect of CP on the thymus may be different from that of 5-FU.

Effect of 5-FU and CP on blood leucocytes

The effect of 5-FU and CP on the blood leucocytes was tested in comparison with that of thymus lymphocytes. The administration of these drugs for 6 days decreased significantly the total number of the leucocytes, although the decreased leucocytes partially recovered after termination of administration, as shown in Table 4. Decrease in the total number of the leucocytes was much less than that seen in lymphocytes.

TABLE 4. Effects of antitumor agents on rat blood leucocytes

| Groups | Leucocytes (×10⁹)/mm³ |
|--------|-----------------------|
|        | 3 days | 6 days | 10 days |
| Control | 77±12 | 78±16 | 80±13 |
| 5-FU | 36±9* | 27±11* | 34±9* |
| CP | 20±7* | 11±10* | 30±4* |

Each value represents the mean of eight rats±standard error.
* p < 0.001 in Control vs. 5-FU or CP

DISCUSSION

High doses of both antitumor agents for 6 days were found to cause a significant involution of the thymus and a decrease in lymphocytes as compared with those of control
rats, however, a slight increase in thymus weight and lymphocytes in 5-FU group occurred with cessation of treatment, while in CP group an increase was not seen, probably due to the higher dose of CP given. The thymus involution was observed immediately after administration, suggesting alteration of the tissue based on the inhibition of DNA and RNA syntheses (Table 3). On the other hand, the thymus alkaline phosphatase activity was significantly enhanced despite the extreme involution of the thymus following the drug treatment. Kahri et al. reported that a high total dose of CP (30 mg per kg body weight, every second day during a period of 10 days) resulted in a cessation of the growth of rats, an almost complete thymolytic reaction and a clearly raised activity of succinate reductase and acid phosphatase of the thymus (23). An elevation of acid phosphatase (24), adenosintriphosphatase and 5-nucleotidase (25) have also been demonstrated biochemically in the rat thymus following X-irradiation. Further, it is known that the activities of β-glucuronidase (26), cathepsin and arylsulfatase (27) increase during involution. The activity enhanced by the antitumor agent treatment may be a characteristic change under conditions in which an involution and degeneration of tissue develop.

The inductive formation of the drug metabolizing enzymes of rat liver by administration of these drugs was not significant, although a small increase in P-450 and b5 levels was seen at 3 days. Inversely, administration of the drugs, every day on six occasions, caused a significant decrease in aniline p-hydroxylase at 6 days and in P-450 and b5, at 10 days (Table 2). This decrease is ascribed to the inhibition and degradation of the enzymes by the metabolites, fluoroacetate from 5-FU (28) and phosphoramide mustard from CP (3), which are known to be cytotoxic agents, and to an increased inhibition of protein synthesis due to accumulation of the drugs in situ. It has been reported that toxicity of CP is significantly enhanced by increased accumulation (7). The decreased P-450 content found after 10 days dosing is attributed, at least partially to damage of the electron transport pathway of microsomal mixed function oxidase system, and consequently in a decrease of aniline p-hydroxylase and aminopyrine N-demethylase activities. The fact that no significant effect was found on NADPH-cytochrome c reductase suggests a special property of the enzyme. This assumption is strengthened by previous data that the activity was not inhibited by free radicals and lipid peroxides in carbon tetrachloride intoxication. The observation that these drugs in a higher concentration cause modified type II spectrum as a result of substrate interaction with a hepatic microsomal cytochrome and that the magnitude of the spectral changes is little dependent upon the concentration of substrate added to the microsome suspension suggests that the binding force of the drugs to the microsomes, probably P-450 (22), is fragile (Figs. 2 and 3). The possible explanation for this phenomenon may lie in the highly polar character (CP and 5-FU) or low lipid solubility (5-FU) and may explain the lack of damage to the liver during these drug treatments. The extreme NADPH dependence of the binding of these substrates to microsomes is interesting since reduction of the P-450-substrate complex by NADPH has been suggested as the rate limiting step in the microsomal oxidation of some substrates, such as ethylmorphine (29) and cyclohexane (30).

Thus these antitumor agents cause significant involution of the thymus and decrease
in the lymphocytes. The administration of the agents for 6 days produced a partial decrease in liver microsomal enzyme activities and such was attributed to the metabolites and the increased accumulation of the drugs. Binding of the drugs to the microsomes was fragile and was strongly affected by addition of NADPH.

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