ANTITUMOR AGENTS. II. EFFECT OF 5-FLUOROURACIL AND CYCLOPHOSPHAMIDE ON IMMUNOLOGICAL PARAMETERS AND LIVER MICROSOMES OF TUMOR-BEARING RATS

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Abstract—An attempt has been made to clarify immunosuppressive properties of antitumor agents by studying the effect of the agents on the thymus, the reticulo-endothelial system (RES) and hepatic drug-metabolizing enzyme activities of tumor (an ascites hepatoma, AH 130 cells)-bearing rats. A drastic decrease in the thymus weight and the total number of the lymphocytes and an enhanced activity of thymus alkaline phosphatase were detected by injecting either 5-fluorouracil (5FU) or cyclophosphamide (CP) (30 mg each/kg weight, i.p.) daily for 5 days to tumor-bearing rats. The agents, however, did not induce any conspicuous damage in microsomal mixed function oxidase system or the RES. The presence of 10-day-old tumor resulted in an extreme decrease in the weight and lymphocytes of thymus and a partial decrease in the microsomal drug metabolizing enzyme activities and the RES. Thus, these antitumor agents may lead to the decline of host-mediated immune mechanism. The multiplication of the tumor cells also appears to depress the immune functions and the host resistance.

Recent evidence indicates that immunological reactions play a significant role in host resistance to tumors. Cell-mediated immunity is often impaired both in patients with malignant tumors (1-3) and in animals bearing transplantable tumors (4). Human studies have shown that the number and function of thymus-derived cells (T cells) are frequently decreased in patients with progressively growing tumors (1), the decrease in which is much more at the advanced stage of tumor growth. In spite of the significance of host immunological responses to a tumor, many antitumor agents have immunosuppressive properties (5); administration of antitumor agents to tumor-bearing host causes a decrease in the immune functions and the host resistance, consequently the decreased immune response may result in a deterioration and transference of tumors.

It was reported by Conney et al. that an enzyme system which demethylates 3'-methyl-4-dimethylaminoazobenzene is lacking in certain hepatic tumors (6). Mascitelli-Coriandoli and Citterio described the decrease in activity of some microsomal reductases in N-fluorenyl acetamide-induced hepatic tumors (7, 8). Implantation of mice with lymphoma L5178Y ascites cells resulted in decreased levels of cytochrome P-450 (P-450) and microsomal enzyme activity (9). The activation of cyclophosphamide (CP) is catalyzed by the same hepatic microsomal mixed function oxidase system that is functional in the oxidative metabolism of many other drugs (10-12). The induction of CP-activated enzyme with phenobarbital is shown to be correlated with an enhancement of the pharmacologic effect (12). In addition,
alterations of liver drug-metabolizing enzyme activities probably affect the activity of other antitumor agents such as 5-fluorouracil (5FU), cytosine arabinoside and antitumor antibiotics.

In view of the significance of immuno-defence mechanisms and hepatic drug-metabolizing enzymes in tumor therapeutics, the immunosuppressive properties of some antitumor agents, 5FU and CP, which are representative antitumor agents, and hepatic drug-metabolizing enzyme activities of tumor-hearing rats were studied in addition to their effects on blood leucocytes.

MATERIALS AND METHODS

Materials: 5FU and CP were from Kyowa Hakko Kogyo Co., Ltd. and Shionogi Seiyaku Co., Ltd., respectively. Nicotinamide adenine dinucleotide phosphate (NADP) was 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. Aniline was used following redistillation. A carbon suspension in fish glue (Ink No. C 11/1431a, Pelikan ink) was obtained from Günter Wagner.

Animals: Male Donryu rats weighing 95–100 g, maintained on MF diets (Oriental Yeast Co., Ltd.) for 3-4 days prior to the experiment, were divided at random into 3-4 groups, each consisting of 4-6 rats, and fasted for 18 hr prior to the experiment. A) Controls, treated for 5 days from day 3 to 7 with daily i.p. injections of 0.5 ml of saline per 100 g of body weight (Control group). B) Rats were inoculated i.p. with $2.5 \times 10^6$ ascites hepatoma, AH 130 cells, on day 1 and treated for 5 days from day 3 to 7 with daily i.p. injections of saline (AH group). C) Animals, inoculated with AH 130 cells as described in B) and treated for 5 days from day 3 to 7 with daily i.p. injections of 5FU or CP (30 mg each/kg weight), dissolved in saline (AH-5FU or AH-CP groups).

The animals were decapitated 2 hr after the final administration of saline or the drugs at days 4, 7 and 10.

Preparation of liver microsomal fractions: The liver microsomal fractions were prepared according to the method of Omura and Sato (13).

Measurements of total number of thymic lymphocytes and blood leucocytes: The number of thymic lymphocytes and blood leucocytes was measured by the procedure described in the previous paper (14).

Assays of enzymes: Aniline p-hydroxylase activity was measured at 37 °C by the method of Ikeda (15). The content of P-450 was determined at room temperature with a Shimadzu MPS-50L spectrophotometer as described by Omura and Sato (13). Alkaline phosphatase activity of thymus was assayed according to the same method as described in the previous paper (14).

Granuloplectic activity of RES: Pelikan ink was centrifuged at 2700 × g for 15 min to remove all particles above 500 Å. The supernatant fluid was then analysed for carbon weight and diluted with 1 per cent gelatin so as to have preparations containing 16 mg carbon/ml. The suspension was injected into the tail vain of the rats and blood samples
(0.05 ml) were obtained at regular intervals by puncturing the leg vein with a fine capillary glass pipette previously washed with heparin. This quantity of blood was lysed in 3 ml of 0.1% Na₂CO₃ and the amount of carbon in the blood determined spectrophotometrically at 660 nm. The granulopectic index (K) was determined using the following equation (16): 

\[ K = \frac{\log C_0 - \log C_1}{T} \]

where \( C_1 \) is the concentration of carbon in the blood at the time \( T \) and \( C_0 \) the blood concentration of carbon just after the injection and before the particles are absorbed by the RES.

**Protein determination:** Protein concentration was determined by the procedure described by Lowry et al. (17) with bovine serum albumin, fraction V, as a standard.

**RESULTS**

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

As shown in Table 1, tumor-bearing rats treated with these drugs for 5 days failed to grow as compared with the controls, and the body weight of these animals was decreased at day 7 rather than that of tumor-bearing rats. The decrease in AH-5FU group was much more than in AH-CP rats. Relative liver weight (g liver/100 g body weight) was slightly increased day 7 and 10 following CP treatment when compared to that of the controls, whereas there was no significant increase in the weight during administration of 5FU and rather was only a slight decrease at day 10. The weight of tumor-bearing animals is slightly decreased at day 10, probably due to the development of the tumor.

*Effect of 5FU and CP on liver microsomal drug-metabolizing enzyme activities*

As shown in Table 2, aniline p-hydroxylase activity was decreased in AH-CP group, by 13% at day 7 and 20% at day 10, as compared with the controls, while the activity in

| TABLE 1. Effect of antitumor agents on whole body and liver weights of tumor bearing rats |
|---------------------------------|-------|------|------|------|
| Groups | 0   | 4    | 7    | 10   |
| Body weight (g) | | | | |
| Control | 102 ± 5 | 103 ± 11 | 115 ± 13 | 122 ± 11 |
| AH | 108 ± 7 | 111 ± 10 | 125 ± 11 | 116 ± 12 |
| AH-5FU | 110 ± 13 | 109 ± 12 | 102 ± 13 | 96 ± 12* |
| AH-CP | 106 ± 8 | 105 ± 11 | 110 ± 12 | 112 ± 12 |
| Relative liver weight (g liver/100 g body weight) | | | | |
| Control | — | 4.40 ± 0.40 | 4.29 ± 0.32 | 4.16 ± 0.34 |
| AH | — | 4.10 ± 0.30 | 4.18 ± 0.17 | 3.76 ± 0.47 |
| AH-5FU | — | 4.21 ± 0.36 | 4.37 ± 0.30 | 3.76 ± 0.34 |
| AH-CP | — | 4.49 ± 0.36 | 4.83 ± 0.29** | 5.55 ± 0.39*** |

Each value represents the mean of 4-6 rats ± standard error. *\( p < 0.02 \) in AH-5FU vs. control or AH. **\( p < 0.05 \) in control vs. AH-CP. ***\( p < 0.01 \) in AH vs. AH-CP. **p < 0.01 in control vs. AH-CP.
AH-5FU group was hardly affected. Although the rats treated with 5FU for 5 days showed a slight decrease in P-450 content, AH-CP group was not subjected to a significant change. The presence of 10-day-old ascites tumor resulted in aniline p-hydroxylase activity and P-450 content being reduced to 83 and 77% of the controls, respectively. Based on these results, it seems likely that the antitumor drugs did not produce severe damage to the microsomal mixed function oxidase system.

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

A significant decrease in the relative thymus weight (g thymus/100 g body weight) and

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**TABLE 2.** Effect of antitumor agents on liver microsomal drug-metabolizing enzyme activities of tumor-bearing rats

| Enzyme                  | Groups         | Days  |       |       |
|-------------------------|----------------|-------|-------|-------|
|                         |                | 4     | 7     | 10    |
| Aniline p-hydroxylase   | Control        | 0.503±0.022 | 0.517±0.032 | 0.495±0.022 |
|                         | AH             | 0.478±0.063 | 0.543±0.024 | 0.402±0.038$ |
|                         | AH-5FU         | 0.436±0.072 | 0.523±0.043 | 0.445±0.063 |
|                         | AH-CP          | 0.491±0.054 | 0.453±0.012*| 0.393±0.038* |
|                         | Control        | 0.577±0.040 | 0.626±0.039 | 0.626±0.047 |
|                         | AH             | 0.548±0.043 | 0.588±0.036 | 0.485±0.045$ |
|                         | AH-5FU         | 0.500±0.066 | 0.500±0.023 | 0.549±0.039 |
|                         | AH-CP          | 0.578±0.054 | 0.615±0.035 | 0.589±0.054 |

Each value represents the mean of 4–6 rats±standard error.  
1. The activity of enzyme is expressed as μmoles of product per min per mg of protein.  
2. P-450 content is expressed as nmoles per mg of protein.  
$p<0.05$ in control vs. AH-CP.  
$\dagger p<0.01$ in AH vs. AH-CP.  
$\ddagger p<0.05$ in AH vs. AH-5FU.

**TABLE 3.** Effect of treatment with antitumor agents on weight, lymphocytes and alkaline phosphatase activity of tumor-bearing rat thymus

| Relative thymus weight | Days     |       |       |
|------------------------|----------|-------|-------|
|                         | 4        | 7     | 10    |
| Control                | 0.262±0.030 | 0.254±0.023 | 0.276±0.020 |
| AH                     | 0.257±0.024 | 0.223±0.018 | 0.145±0.014* |
| AH-5FU                 | 0.258±0.017 | 0.100±0.004$| 0.117±0.017* |
| AH-CP                  | 0.240±0.012 | 0.082±0.010*$| 0.110±0.018* |
| Control                | 639±63   | 739±23 | 685±63 |
| Lymphocytes (×10^6/whole thymus) | Days |       |       |
| AH                     | 660±27   | 616±30 | 203±18* |
| AH-5FU                 | 568±61   | 111±14*$| 115±15*$ |
| AH-CP                  | 511±78   | 41±6*$| 21±3*$ |
| Control                | 66.1±5.8 | 60.8±2.0 | 60.5±6.6 |
| Alkaline phosphatase   | Days     |       |       |
|                         | 4        | 7     | 10    |
| AH                     | 59.8±8.2 | 63.3±2.5 | 89.2±7.3* |
| AH-5FU                 | 69.9±4.6 | 88.7±2.5*$| 101.9±7.3*$ |
| AH-CP                  | 89.6±6.0*| 148.9±7.5*$| 111.1±5.7*$ |

a), Activity is expressed as nmoles of p-nitrophenol per mg protein per 20 min.  
Each value represents the mean of 4–6 rats±standard error.  
$* p<0.01$ in control vs. AH, AH-5FU or AH-CP.  
$\dagger p<0.01$ in AH vs. AH-5FU or AH-CP.  
$\ddagger p<0.05$ in AH vs. AH-5FU.
The total number of lymphocytes was evident with a single daily dose of 5FU and CP for 5 days to tumor-bearing rats despite the remarkable loss of the ascites fluid, as shown in Table 3. The number of the lymphocytes at day 7 was decreased by 85% in AH-5FU group and 94% in AH-CP group as compared with that of untreated control rats and the decrease in the total number in these groups was much more than that in AH group. The loss of lymphocytes was much more severe than was loss of weight. The cessation of the drug treatment did not induce a restoration of the cell population and weight, suggesting that the thymus involution with ingestion of these drugs is considerable. The presence of 10-day-old ascites tumor resulted in the decrease in the thymus weight and total number of lymphocytes as compared with the controls.

The alkaline phosphatase activity in the thymus was greatly enhanced in AH-5FU and AH-CP groups, while that of tumor-bearing animals, increased with the development of tumor, was about 1.5 times the controls at the later stage (at day 10) of tumor growth.

**Effect of 5FU and CP on RES**

To investigate the granulopectic activity of cells which constitute RES, a carbon suspension in fish glue was injected into rats and the rate (granulopectic index) of clearance of carbon particles from the blood was estimated. As shown in Table 4, administration of these antitumor agents to tumor bearers for 5 days caused a decrease in the activity by 9-31%. Three days (on day 10) following the cessation of the drug treatment the restoration of the activity was not found, suggesting that the treatment by these agents leads to a slight impairment of the RES. In the tumor-bearing rats, the granulopectic activity was also decreased at day 10, although the normal activity was maintained up to day 7.

**Effect of 5FU and CP on blood leucocytes**

The effect of the antitumor agents on the blood leucocytes was tested in comparison with that of thymic lymphocytes. The result obtained is shown in Table 5. AH-5FU and AH-CP rats showed a significant decrease in the total number of the leucocytes as

**TABLE 4. Effect of antitumor agents on granulopectic activity of rats**

| Groups | Granulopectic index (K) (× 10⁻²) |
|--------|---------------------------------|
|        | Days                            |
|        | 4          | 7            | 10           |
| Control| 2.44±0.46  | 2.65±0.33    | 2.57±0.24    |
| AH     | 2.56±0.23  | 2.99±0.34    | 1.65±0.12*   |
| 5FU    | 2.44±0.23  | 2.09±0.21    | 1.82±0.17*   |
| CP     | 2.75±0.32  | 2.15±0.21    | 1.83±0.12*   |
| AH-5FU | 2.77±0.18  | 2.39±0.21    | 1.91±0.33†   |
| AH-CP  | 2.70±0.33  | 1.80±0.19‡   | 1.93±0.21‡   |

a) Animals were treated for 5 days with daily i.p. injections of drugs (30 mg/kg weight). The index (K) was calculated from a mean of the clearance 10 and 15 min after the injection of carbon (16 mg/100 g weight). Each value represents the mean of 3-6 rats±standard error. *p<0.01 in control vs. AH, 5FU or CP. †p<0.05 in control vs. AH-5FU. ‡p<0.02 in AH vs. AH-CP. All p<0.02 in control vs. AH-CP.
compared with that of the tumor-bearing rats at day 7. This indicates that the agents caused a severe impairment in the number of leucocytes, probably due to, at least partially, a drastic damage of the born marrow which produces lymphocytes. The total number in the tumor-bearing rats was also decreased to 34% of the controls at day 10 with advancement of the tumor.

**DISCUSSION**

The existence of specific antigens to tumor cells, be they transplantable or spontaneous tumors, has become increasingly evident. The tumor-bearing host can exert an immunological response and rarely an immunologically mediated rejection process. As noted earlier, the immunological reactions of the host appear to play a significant role in resistance to tumors. When antitumor agents are given to cancer patients, however, the agents seem to decrease the immuno-defence functions, since these compounds are destructive to lymphocytes, leucocytes and bone marrow cells, which multiply vigorously, rather than to tumor cells. The effect of these antitumor agents on the thymus, blood leucocytes and liver microsomes of normal rats has been already reported by our group (14).

The injection of CP to the tumor-bearing rats for 5 days (at day 7) caused only a slight decrease in aniline p-hydroxylase activity in microsomal fractions as compared with those of AH and control rats, while the content of P-450 in both AH-5FU and AH-CP groups was not significantly decreased (Table 2). This result suggests that these agents did not produce a drastic damage to the microsomal mixed function oxidase system and consequently to the activation process of CP. This is approximately consistent with the data for normal rats presented previously (14). On the other hand, P-450 content and aniline p-hydroxylase activity of the tumor-bearing rats were slightly decreased at a later stage in tumor growth, probably due to the depression of the synthesis and/or turnover of the enzymes with the constituents derived from the tumor. The result is in agreement with those of workers including Kato and Takahashi (18), Rosso et al. (19) and Reed (9). These workers have proposed that serum constituents, such as toxohormone (20), may play a role in such impairment and that surgical removal of the tumor abolishes the impairment of drug metabolism (19). In addition, the decrease in mouse liver catalase (20) and the involution of mouse thymus (21) by toxohormone has been reported. There is still the possibility that

### TABLE 5. Effect of antitumor agents on blood leucocytes of tumor-bearing rats

| Groups   | Leucocytes (×10⁵)/mm³ | Days         |
|----------|-----------------------|--------------|
|          |                       | 4            | 7            | 10           |
| Control  | 88±11                 | 84±12        | 94±8         |
| AH       | 100±5                 | 70±3         | 32±6         |
| AH-5FU   | 53±7*†                | 30±5*†       | 57±3*†       |
| AH-CP    | 54±10*†               | 12±3*†       | 36±7*        |

Each value represents the mean of 4–6 rats ± standard error. * p<0.01 in control vs. AH, AH-5FU or AH-CP. † p<0.01 in AH vs. AH-5FU or AH-CP.
the inhibitors of the drug-metabolizing systems and a deficiency of cofactors may be involved in the decrease in the activities. Adamson and Fouts, however, showed that the lack of certain drug-metabolizing enzyme activities in hepatic tumors was not caused by a lack of the cofactor, NADPH, and by inhibitors of drug-metabolizing systems (22).

Administration of both antitumor agents (The doses of 5FU and CP to rats were approx. 3 and 10 times higher than the clinically effective daily doses, respectively) to the tumor-bearing rats for 5 days was found to cause a remarkable involution of thymus and a decrease in lymphocytes in comparison with those of the controls and AH group (Table 3), suggesting that these antitumor agents induce considerable damage to the thymus and that the agents may exert a much more considerable damage to the lymphocytes than to the tumor cells. A more extensive involutionary effect was demonstrated in the thymus cortex, which plays the major role in the production of lymphocytes in the thymus, after a high total dose of CP (23). In rats bearing the tumor, a marked involution of thymus and a significant decrease in the lymphocytes were observed at a later stage in the tumor growth. It has been reported that mice bearing a methylcholanthrene-induced transplantable sarcoma contained a population of lymphoid cells capable of specifically suppressing immunity to this tumor (24, 25). These specific suppressor cells were found in both the spleen and thymus of tumor-bearing animals. Thus, a significant involution of thymus with these antitumor agents may lead to a decrease in T cells and consequently a decline of antibody production, as surgical ablation of the thymus produces a decrease in T cells and a development of tumors (26). This is strongly suggested from our data that the production of plaque-forming cells (PFC) of spleen in mice immunized with sheep red blood cells was decreased considerably with daily i.p. injections of 5FU (60 mg/kg weight) or CP (19.8 mg/kg weight) for 5 days (The doses of these agents were 6 times each the clinically effective daily doses); in 5FU-mice the clinically effective dose decreased to 2.4% and in CP-mice to 17.8% of the control value in PFC/spleen (unpublished data). As a result, 5FU more than CP seems to act as an immunosuppressive agent when the same amount of these agents in terms of the clinically effective daily doses is administered. Most, if not all, of the antitumor agents have been shown to have immunosuppressive action, and 5FU, methotrexate and 6-mercaptopurine behave as class II agents and CP as a class III agent (5). Our results also demonstrated that both agents were effective immunosuppressants. An elevation of thymic alkaline phosphatase activity observed during high doses of both antitumor agents and at the advanced stage of the tumor growth may be a characteristic change under conditions in which an involution and degeneration of the tissue develop (Table 3). This is in agreement with the reports that the activity of some enzymes in the thymus increased during involution (27).

Many investigators have been intrigued by the possibility that the RES may be involved in the host response to the neoplastic process, and numerous results can be found in the literature suggesting that the RES does exert some regulatory influence over the course of tumor development and that the functional capacity of the RES is enhanced during the early phase of various transplanted mouse tumors (28). In this study, the clearance rate of carbon from the blood of rats decreased, to some extent, at 10 days following tumor
inoculation (Table 4), indicating that the function of the RES was impaired in host with
tumor in a later stage of the growth. This result is in good agreement with the data obtained
by Old et al. (28). The granulopectic activity of tumor-bearing rats dosed with these anti-
tumor agents was slightly higher than that in tumor-bearers at day 10. Of particular interest
is that the lesser impairment of the function of the RES was shown in spite of a remarkable
involution of the thymus and lymphocytes. This can be interpreted as being the result
of a slight injury of the liver, as reported previously (14). Since this method of application
is mainly to estimate the activity of RES in liver and spleen (26, 28, 29), a slight damage of
the liver might elicit a relatively higher granulopectic activity.

Thus, these antitumor agents cause a significant involution of the thymus with a re-
markable decrease in the lymphocytes and blood leucocytes, but do impair slightly the
function of the RES, in tumor-bearing rats, indicating that these agents induce a decreased
immuno-defence mechanism. The multiplication of ascites hepatoma, AH 130 cells, also
caused a drastic loss of thymic lymphocytes, blood leucocytes and the function of the RES,
and a partial decrease in drug-metabolizing enzyme activities in liver microsomes, thereby
leading to a decline in the host-mediated immune mechanism.

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