THE EFFECTS OF THE CARCINOSTATIC AGENTS ON THE CELL CYCLE OF EHRLICH ASCITES CARCINOMA CELLS

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ABSTRACT

The influence of tumor cell aging upon growth inhibition by carcinostatic agents was investigated with Ehrlich tumor in the form of ascitic cells.

In the experiment on tumor growth, the rate of growth decreased with the lapse of time following tumor inoculation. The generation time of the 2-day old tumor was approximately 12 hours; that of the 6-day old tumor was approximately 42 hours. There were also significant differences in the DNA labeling index and mitotic index between the 2-day old and the 6-day old tumors, which were correlated with their generation times.

Mitomycin-C affected more markedly the growth of the 2-day old fast growing tumor than that of the 6-day old tumor. Enhanced antitumor activity of the carcinostatic agent was seen when the drug was used during the rapidly growing stage of the Ehrlich ascites tumor.

INTRODUCTION

The effect of cancer chemotherapy on the kinetics of tumor cell population has been the subject of intensive investigation[1,2,3,4]. Recently, Bruce, *et al.*[5,6] reported that difference in the action of chemotherapeutic agents was observed between dividing murine lymphoma cells and predominantly nondividing normal bone marrow cells. Maruo and Modoc-Jones[7] observed that carcinostatic agents have some relationship between cell generation cycle and their lethal effect. The correlation between the effect of chemotherapeutic agents and generation time of tumor, however, is not yet fully understood. The latter subject is clearly pertinent in cancer chemotherapy, and it is necessary to ascertain whether any relationship exists between the rate of growth of tumor cells and the effect of chemotherapeutic agents.

MATERIAL AND METHOD

Experimental Tumor

Ehrlich ascites tumor used in this experiment was a hyperdiploid subline

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grown in Swiss mice. The tumor cells were maintained in the form of ascites in the abdominal cavity of a Swiss mouse by successive implantation of 500×10^4 cells every 6 days.

Experimental Mice

The Swiss mice were derived from an inbred colony which had been maintained for over 10 years in this laboratory by strict brother-sister mating. The animals were fed on laboratory chow of commercial source, and with water. The weight of the mice used in this experiment ranged from 21 gm to 25 gm.

Measurement of Tumor Growth

Of 30 mice inoculated with 250×10^4 cells derived from the one mouse, groups of 3 mice each served for preparing a mean growth curve at proposed intervals. Immediately after the mice were killed by ether inhalation, the cells were harvested from the peritoneum of each animal in a given volume (50-100 ml) of phosphate bufferd saline solution (P.B.S.) by repeated washing with the same solution. Ehrlich cells were identified and counted with a hemocytometer. The viability was measured by staining with 0.5% trypan blue solution.

Measurement of Cell Cycle Time with Autoradiograph

Mice bearing tumor cells were injected with tritiated thymidine (Daiichi Chemical Co.; specific activity 12 mC/m mol) which had been diluted in physiological saline to 100 μC/ml. H^3-thymidine was injected intraperitoneally in a dose of 10 μC per mouse for mice bearing the 2-day old tumor and 40 μC for mice bearing the 6-day old tumor. Ehrlich cells were withdrawn into 2 ml of P.B.S. solution immediately after the death of the mice which were killed at appropriate times after a single injection of H^3-thymidine. Tumor cell smears were made on clean slides, dried and fixed in absolute alcohol for 15 minutes. The slides were dipped in Sakura NRM 2 emulsion, exposed and developed in a Konidol developer. Exposure times ranged from 2 weeks to 3 weeks. The fraction of labeled mitosis was plotted against the time course for determination of cell cycle time[8)9)]. The fraction of labeled nuclei was counted for determination of lebeled index. Labeled cells in 1000 total cells were counted. Labeled mitotic cells were also counted in 100 total mitotic cells. Microscopic fields were chosen from all parts of the smear to prevent selective counting of large or small cells in the smears.

Measurement of H^3-Thymidine Incorporation

Each mouse bearing 2-day old tumor was injected with 10 μC of H^3-thymidine at appropriate intervals after the injection of 1/10 LD_{50} dose of Mitomycin-C (0.52 mg/kg), while the mice bearing 6-day old tumor were injected with

30 μC of H^3-thymidine. Tumor cells were withdrawn into 2 ml of ice cold P.B.S. solution and homogenized with a Potter Elvehjem homogenizer, and DNA extraction was performed by the Ogur-Rosen's method[10]. After 0.1 ml of extracted DNA solution was mixed in 10 ml of Dioxan scintillator[11] (Naphthalin 100 g : DPO 10 g : POPOP 250 mg : Dioxan 1 1), radioactivity was counted with Ten liquid scintillation counter. Quantity of DNA was measured by Burton's method[12]. Specific activity was calculated by the ratio of DNA counts (dpm) to quantity of DNA (μg). Inhibition index was expressed as a percentage of the control.

Effect of Treatment with Mitomycin-C and Cyclophosphamide on the Survival Time

Mice bearing the 2-day old and the 6-day old Ehrlich ascites tumor were treated with a single administration of 0.2 ml of P.B.S. solution containing 1/10 LD$_{50}$ dose of Mitomycin-C or cyclophosphamide, while mice bearing tumor given only 0.2 ml of P.B.S. solution served as control. Four hours after the treatment, Ehrlich ascites cells were harvested from each group and adjusted to 250×10^4 cells/0.2 ml, respectively. In order to oberve the mean survival time, 0.2 ml of the cell suspensions were inoculated into the abdominal cavity of each group of 10 mice.

RESULTS

Cell growth of the Ehrlich tumor following the cell inoculation is shown in Fig. 1. From 2 to 4 days after the inoculation, the cell number increased exponentially with a doubling time of approximately 12 hours; after 6 to 9 days the growth rate began to slow down with a doubling time of approximately 48 hours.

The results of pulse labeling index and mitotic index are shown in Fig. 2. In contrast to the growth curve, these two indices gradually decreased following inoculation. It was noted that the population of Ehrlich ascites tumor with S phase decreased remarkably, from 70% to 27% with lapse of day following tumor inoculation. Since the doubling time and the labeling index was remarkably different between the 2-day old and the 6-day old tumor, further experiments were performed to determine their cell cycle times with the labeled mitosis curve, as shown in Fig. 3 and Photo. 1, and the duration[13][14] of the phases of cell cycle was determined as shown in Table 1. The generation time obtained from these mitosis curves for the 2-day old and the 6-day old tumor were approximately 12 hours and 42 hours, respectivelv. The data presented in Table 1 indicated that there were also marked difference in each cell cycle phase.
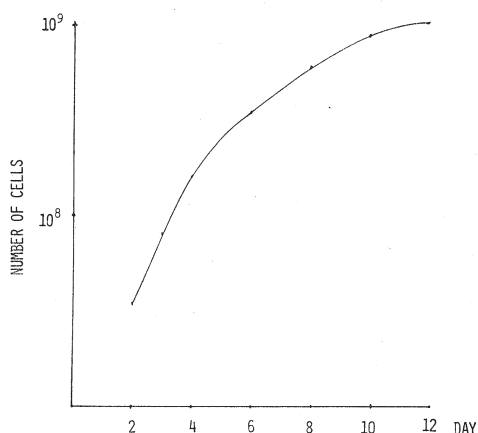
FIG. 1. Growth curve of Ehrlich ascites tumor cells following inoculation of 250×10^4 cells into the peritoneal cavity of Swiss mice.
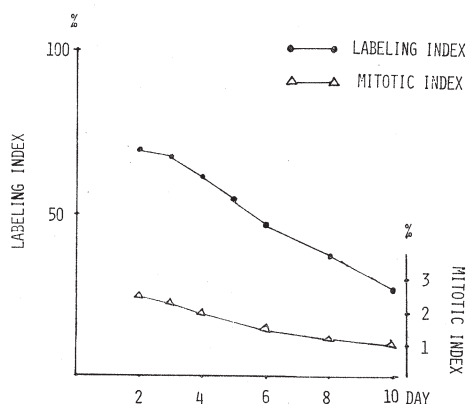

FIG. 2. Labeling index and mitotic index of Ehrlich ascites tumor following inoculation of 250×10^4 cells into the peritoneal cavity of Swiss mice.
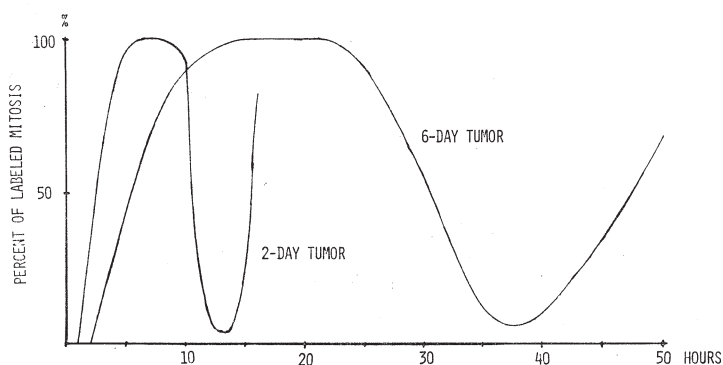

FIG. 3. Cell cycle curve of the 2-day old and 6-day old Ehrlich ascites tumor following inoculation of 250×10^4 cells into Swiss mice.

Effect of Mitomycin-C on both fast growing tumor and slow growing tumor was estimated by the inhibition index of DNA synthesis. As shown in Fig. 4, the inhibition index obtained from the fast growing cells was much greater than that from the slow growing cells. The curve of the fast growing cells rapidly fell to 30% within 1 hour after the administration of Mitomycin-C and the value of 25% was sustained thereafter. In contrast, the curve of the slow growing cells decreased gradually from 80% to 60% and the value was much higher than that of the fast growing cells throughout the course. As shown in Fig. 5, the result of the dose-DNA inhibition curves showed the same trend as that of the time course experiment. As shown in Fig. 6, the survival time
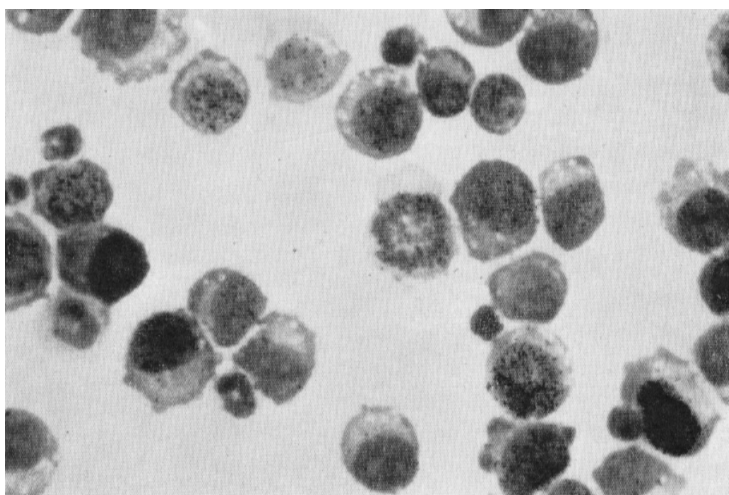
PHOTO. 1. Labeled mitotic nucleus is shown. ($\times 400$)

TABLE 1. Cell Cycle Phase of the 2-day old and the
6-day old Ehrlich Ascites Tumor

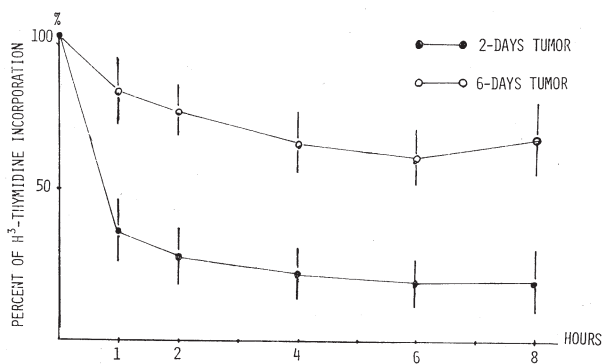|	2-day old tumor (hr.)	6-day old tumor (hr.)
Cell cycle time (hr.)	12	42
G_1	2.0	14.5
S	8.0	20
G_2	1.7	7.0
M	0.3	0.5

FIG. 4. Time course on inhibition of H^3-thymidine
incorporation by the 2-day old and 6-day old Ehrlich
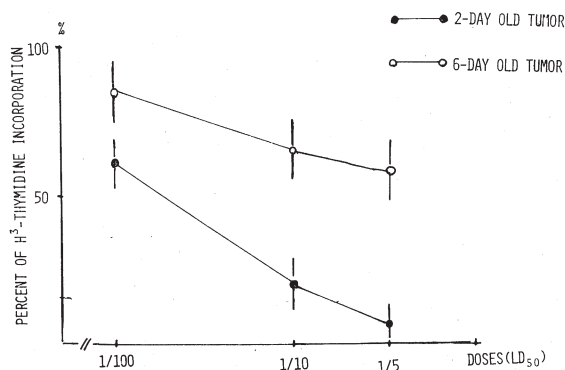asites tumor treated with 1/10 LD_{50} dose of MMC.

FIG. 5.   Dose-DNA inhibition curve for the 2-day
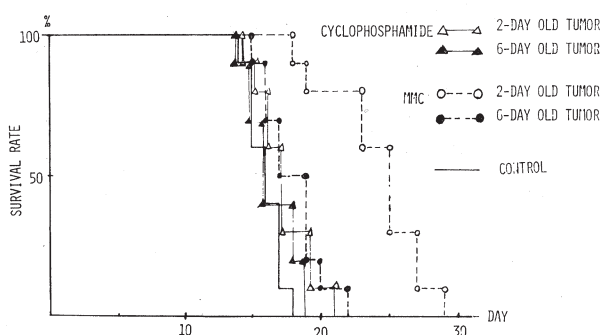old and 6-day old Ehrlich ascites tumor treated with
MMC.

FIG. 6.   Survival time of Swiss mice inoculated with
250×10^4 cells of the 2-day old and 6-day old Ehrlich ascites
tumor which had been contacted with either $1/10$ LD_{50} doses
of MMC or cyclophoshamide for 4 hours.

of mice inoculated with the 2-day old cells which had been contacted with
Mitomycin-C was longer than that of mice inoculated with the 6-day old cells.
In the case of cyclophosphamide administration, the survival time of mice
which were inoculated with the 2-day old tumor cells was the same as that
of mice inoculated with the 6-day old cells.   No prolongation of survival time
was observed in both tumors.

DISCUSSION

From the results obtained, it is evident that Mitomycin-C has a greater
antitumor effect on the 2-day old fast growing tumor than on the 6-day old
tumor, whereas cyclophosphamide shows no appreciable difference in the effects
on the two tumors.   These differences may be due to either the different bio-

logical action of the antitumor agents or to their cell cycle times. Recently, Maruo and Modoc-Jones[7] reported that a wide variety of drugs known to have different biological action exhibited well-defined, distinctive age response through the generation cycle. From the point of biological action, Mitomycin-C[15)16)17] which is known to be an ihibitor of DNA synthesis affects the S phase of the cell cycle. Accordingly, it is reasonable to suppose that Mitomycin-C has more marked effect on the 2-day old tumor with 70 percent in the S Phase than on the 6-day old tumor with 40 percent in the S phase. The fact that cyclophos-phamide was ineffective on both tumors is apparently due to the narrow cell cycle phases sensitive to cyclophosphamide presented under the conditions in this experiment. De Wye[18] reported that cyclophosphamide effected both the G_2 phase and M phase of the cell cycle. The data presented in this experi-ment indicated that the effects of carcinostatic agents show a signigicant cor-relation with the rate of growth of Ehrlich ascites tumor. These findings should, theoretically, yield important applications in cancer chemotherapy. For example, when either simple or combined administration is made, a choice should be considered of the possibility of maximizing the lethal effect on differ-ent phases of the division cycle. Of course, such theoretical considerations are difficult to apply if the cell cycle parameters of the tumor cells of the cell renewal systems are unknown. However, the effect of chemotherapy on the kinetics of tumor cell population should be more rationally considered in cancer chemotherapy[19].

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