COMPARATIVE STUDIES ON CYTOLOGICAL DIFFERENCES BETWEEN ORIGINAL AND LUNG-PASSAGED SUBLINE OF EHRLICH CARCINOMA

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The prevention of metastasis is one of important projects in the field of cancer chemotherapy. Intravenous injection of tumor cells into experimental animals has been widely used as a model system for tumor metastasis.1,2,3,7)

When Ehrlich carcinoma cells were inoculated intravenously into ddY mice, survival time of the hosts fluctuated, so that it was almost impossible to estimate the exact effect of drugs on life-span prolongation of the host animals. We attempted to isolate a subline from the original Ehrlich carcinoma cells which would prefer to grow in the lung and show 100% of lethal take to the hosts within a relatively short period.

In the present work, we established such a subline from the original Ehrlich carcinoma cells which would prefer to grow in the lung and show 100% of lethal take to the hosts within a relatively short period.

In the present work, we established such a subline from the original cell line by our technique of repeated cycle-passages of 12 times from lung tumor foci to ascites form. This tumor was called lung-passaged subline (LP-12), and cytological characteristics of the tumor cells were compared with those of the original cell line.

MATERIALS AND METHODS

Animals and Tumor Cells The animals used throughout this experiment were mostly ddY male mice and, in part, C3H/He female and ICR-JCL male mice, weighing 20–30 g. They were fed with semi-synthetic diet CE-2 (CLEA Japan Inc., Tokyo) and given water ad libitum. The ddY and ICR-JCL mice were supplied from an animal farm in Shizuoka Prefecture and CLEA Japan Inc. in Tokyo, respectively. The C3H/He strain was an inbred colony supplied from the Kyorin Chemical Laboratory, Tokyo.

Ascitic Ehrlich carcinoma cells were used as the original cell line, which were maintained by serial intraperitoneal transplantation into ddY male mice.

Cycle-passages of Cells from the Lung to the Ascites Heparinized Glucosol (NaHCO₃-free Tyrode) solution (2 ml) was injected intraperitoneally to a tumor-bearing animal 10 days after the transplantation. The tumor ascites was withdrawn in the same syringe and number of tumor cells were adjusted to 3 × 10⁷ cells/ml of Glucosol solution. The cells in 0.2 ml (6 × 10⁶ cells) were slowly inoculated intravenously from the tail vein of each mouse. On the 12th day after the inoculation, the mouse was sacrificed and tumor foci growing in the lung were removed. Tumor cell suspension was prepared through a stainless wire sieve of 200 mesh to remove abundant lung cells and added with a few drops of penicillin and streptomycin solution. The cells were then inoculated intraperitoneally into normal mice. Ten-day-old tumor

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ascites was offered again as a material for the next intravenous transplantation into other mice. The same procedure was repeated 12 times, alternatively, intravenous and intraperitoneal transplantation. The subline, LP-12, thus established has been maintained by further transfer generations of intraperitoneal transplantation into ddY mice.

Chromosomal and Histological Examination Chromosome preparations of the cells in each cell line were made principally according to the technique described by Makino and Sasaki, or by Isaka et al. Four-day-old ascites tumor was pretreated with 1 µg of colchicine 3 hr previously and exposed to hypotonic Glucosol solution at 37°C for 10 min. Tumor cells collected by centrifugation were fixed and stained with 2% acetic-orcein solution. On squashed preparations, 100 cells of well-spread metaphase were examined, photographed, and karyotyped.

For the histological examination, various organs or tissues of the tumor-bearing animals were fixed in 10% Formalin and supplied for making preparations in a conventional way.

RESULTS

Establishment of LP-12 Cell Line The LP-12 cell line was established from the cells on the 12th cycle-passage. Fig. 1 shows survival curves of the hosts when ddY mice were transplanted intravenously with $6 \times 10^6$ tumor cells from their tail vein. In case of the original line, the survival curves were different with every experimental group; sometimes more than 50% of the animals survived for 30 days after the transplantation (B in Fig. 1). All the animals which survived more than 30 days showed tumor foci in the lung when they were sacrificed. The LP-12 cells, however, were more malignant and 100% of the animals were killed within 20 days after transplantation. The grade of malignancy of this cell line, as far as survival time of the animals was concerned, has not changed during over 80 transfer generations (A—D in Fig. 1), from December, 1967, to June, 1969.

Autopsy and Histological Findings of Hosts Intravenously inoculated with Each Cell Line When the LP-12 cells were

![Fig. 1. Survival curves of hosts transplanted intravenously with $6 \times 10^6$ cells of the original and LP-12 cell line of Ehrlich carcinoma](image)
intravenously inoculated from the tail vein of ddY mice, the tumor foci in the lung were easily detected at autopsy, although sometimes the lung of mice which died after 8-10 days showed only swelling of the tissue. The hemato-hydrothorax was observed in almost all the animals. Histological examinations were carried out on various organs, such as lung, heart, brain, kidney, adrenal, spleen, liver, and testis. As shown in Table I (see also the survival experiment, C in Fig. 1), infiltration of the tumor cells into heart or brain was more prominent in mice transplanted with the LP-12 and was 70% or 35%, respectively, while in mice transplanted with the original cell line, it was only 50% or 5%. Any metastatic foci could not be found in liver or testis. There were only a few tumor foci in the lung tissues of the hosts which survived for 30 days after the original Ehrlich tumor cells were inoculated into mice in other experiments.

**Transplantation of Each Cell Line to ddY or Other Strains of Mice** Table II summarizes the transplantability and survival time of hosts or tumor weight when 3 or $6 \times 10^6$ cells of each cell line were inoculated by various routes into ddY, C3H/He, or ICR-JCL mice. For this experiment, LP-12 cells of the 49th-51st generation were used.

When $6 \times 10^6$ cells of each cell line were transplanted intravenously into ddY or ICR-JCL male mice, all the animals showed tumor take whereas 50% survival time of the ddY mice, transplanted with the LP-12 cells, was significantly shorter than that of other mice. In the case of intraperitoneal inoculation, however, differences in tumor take as well as survival time of the hosts were not detected in any of the groups. Tumor growth following subcutaneous inoculation of $3 \times 10^6$ cells of each cell line indicated that tumor could develop more rapidly in the ICR-JCL mice.
Fig. 2. Distribution of chromosome number of the original and LP-12 cell line of Ehrlich carcinoma

Fig. 3. Metaphase chromosomes of the original and LP-12 cell line of Ehrlich carcinoma. Marker chromosomes are shown in black. A pair of satellited submetacentric chromosomes is observed only in karyotype of original cell line (arrow).
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although there was no significant difference between the original and LP-12 cell line.

**Chromosomal Constitution** Chromosome counts and karyotype analysis are summarized in Figs. 2 and 3. Modal number of chromosomes in the original and the LP-12 cell line was found to be 72 (27%) and 73 (43%), respectively. The incidence of polyploid cells falls to 10.8% for the original and to 5.2% for the LP-12 line. As shown in Fig. 3, marker chromosomes including one large submetacentric and 2 meta-centric chromosomes, and a pair of small submetacentric chromosomes with satellite were detected in the original cell line. On the other hand, these markers were not detected in the karyotype of LP-12 cell line, except one large submetacentric and one large metacentric chromosomes.

**DISCUSSION**

Increasing interest in chemotherapeutic effect on tumor metastasis has led to the development of a technique for artificial metastasis in experimental animals. Following intravenous inoculation, tumor cells would first be caught in the lung tissue, where they form multiple tumor foci. The incidence or distribution of these foci, however, could vary by the biological nature of tumor cells and by a difference in histocompatibility between the tumor and the host animals.

When a limited number of the original Ehrlich carcinoma cells were intravenously inoculated into ddY mice, formation of tumor foci in the lung was not always uniform, so that the mean survival time of the hosts were widely spread even in the same group of experimental system. This could partly be explained by the general heterogeneity of ddY mice. However, our present experiment suggested that there would be a possibility of isolating a subline like LP-12, which acquired new characteristics such as relatively high affinity to the lung or more progressive growth in the ddY mice.

The cytological characteristic of the LP-12 subline seems to be inherent and has not changed during transfer generations in ddY mice for over 1.5 years. As far as we have observed, the LP-12 subline did not show any significant difference from the original cell line in other biological characteristics such as transplantability in different strains of mice or susceptibility to some well-known antitumor drugs. It might be considered that any difference initially present was lost by reselection during intraperitoneal passages of 12 cycle-passages although we did not carry out such a comparative study on the earlier stage of transfer generations.

Koch\(^5\) demonstrated that Ehrlich carcinoma cells gradually acquired a characteristic which provoked rather high incidence of lymphatic metastasis when the cells which have very similar biological characteristics to the LP-12 cells appeared on the 6th cycle-passage, but not on the 2nd or 3rd. It could be said, therefore, that the LP-12 cells were derived as a result of gradual selection from the cell population at least after the 6th cycle-passage.

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