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Murine Liver Metastasis Model Using L5178Y-ML Lymphoma and the Effect of Antitumor Agents on the Metastasis

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A reproducible tumor model for liver metastasis has been developed from murine L5178Y lymphoma line by sequential cycles of subcutaneous inoculation of liver tumor cells, that were originally generated in livers of female (BALB/c × DBA/2)F_1 mice by injecting the parental cells into the tail vein. This variant (L5178Y-ML) metastasized predominantly to the liver after intravenous or subcutaneous injection. The livers of the animals killed 9 days after intravenous implantation of 5×10^5 tumor cells were about 3 times the weight of control livers. All tumor-bearing mice died 10 to 12 days after inoculation. Subcutaneous implantation of L5178Y-ML in the side flank of mice induced metastatic nodules spontaneously in the livers. The tumor cells proliferated more in livers than in the implanted sites, compared with the parental L5178Y cells. The effects of 5-fluorouracil, mitomycin C, *cis*-platinum and doxorubicin on the liver metastasis of L5178Y-ML were examined at subtoxic doses; 5-fluorouracil was the most effective in both inhibiting the tumor growth in livers and prolonging the survival period of mice. This model provides a useful tool for the experimental therapy of hepatic tumors in mice.

Key words: Murine liver tumor — Liver metastasis — L5178Y lymphoma

Metastasis, the spread of malignant cells from a primary site to distant organs, presents a problem in the pathogenesis and treatment of cancer. The liver, as well as the lungs, is one of the organs most frequently involved in metastatic deposits from primary tumors.[1-3] Consequently, experimental tumor systems inducing liver metastases are essential.

In order to develop therapies effective against hepatic metastases, several experimental models have been presented. Among them the models utilizing intraportal injection,[4] intracecal injection,[5] or intrasplenic injection followed by splenectomy[1, 6] seem to be satisfactory in terms of the organ selectivity. However, both intraportal and intracecal injections with tumor cells are time-consuming and require considerable technical expertise. Moreover, immune responses in asplenic animals are not the same as in normal animals; that is a problem in studies on the treatment of cancer because immune mechanisms play an important role in the host-mediated prevention of metastasis. There have been a few studies of liver metastasis models utilizing conventional implantation methods such as intravenous or subcutaneous injection.[7-10] Among them ESb is a metastatic variant of L5178Y that arose spontaneously in DBA/2 mice.[7, 8] Many studies using this line have been accumulated by Shirrmacher and his coworkers.[11-16] On the other hand, our data show that intravenously injected L5178Y cells tend to propagate in the liver more than in other organs. Thus, an attempt to develop a variant specifically metastatic to the liver was undertaken.

This paper is concerned with a new conventional model system for liver metastasis by injection of L5178Y-ML cells into the tail vein or subcutis of mice, and the effects of 5-fluorouracil, mitomycin C, *cis*-platinum and doxorubicin in this model.

MATERIALS AND METHODS

Animals Female BALB/c×DBA/2 (CDF_1)[*4] mice were used throughout the study. They were purchased from Shizuoka Laboratory Animal Center (Shizuoka), housed in a barrier system, and fed standard mouse chow and given water *ad*

[*3] To whom correspondence should be addressed.
[*4] Abbreviations used: CDF_1, (BALB/c×DBA/2)F_1; MMC, mitomycin C; 5-FU, 5-fluorouracil; DXR, doxorubicin; CDDP, *cis*-platinum.

libitum. They were 6 to 8 weeks old at the beginning of each experiment.

Tumors L5178Y, a murine T-lymphoma cell line derived from methylcholanthrene-induced lymphoma in DBA/2 mice, was passed in 1975 from Dr. S. Okada (School of Medicine, University of Tokyo) to Dr. N. Tanaka (Institute of Applied Microbiology, University of Tokyo). In 1986 we obtained this line from Dr. Tanaka and maintained it *in vitro* using RPMI-1640 medium supplemented with 10% calf serum and antibiotics.

L5178Y-ML cells were developed as described below and kept *in vivo* and *in vitro*. In the case of *in vivo* passage, liver metastasis taken from mice subcutaneously injected with the tumor cells 2 weeks before, was minced with scissors and 5 or 10 cubes of 1 mm^3 were subcutaneously implanted in the side flank of the recipient mice. Tumor cells generated in the liver were transferred to *in vitro* culture after physical dispersion. By repeating *in vitro* passage 3 times, coexisting hepatocytes were discarded. The medium used for *in vitro* culture was the same as that for parental L5178Y. It was not necessary to add β-mercaptoethanol.[11]

Development of Metastatic Cell Line Parental L5178Y cells (1×10^6 cells) were injected into the tail vein of CDF_1 mice. After 7 days, the liver with metastatic nodules was taken from the mice and 10 cubes (1 mm^3) of minced liver were implanted subcutaneously into the side flank of recipient mice. Subcutaneous implantation of tumors generated in livers was repeated 15 times. The variant thus selected was named L5178Y-ML.

Drugs Mitomycin C (MMC) and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Doxorubicin (DXR) and *cis*-platinum (CDDP) were from Sigma Chemical Co. (St. Louis, Mo.) and Nippon Kayaku Co., Ltd. (Tokyo), respectively.

Drug Sensitivity Test To estimate the *in vitro* drug sensitivity of L5178Y (parental) and L5178Y-ML cells, the cells plated into wells of 48-well test plates at 5×10^4 cells/ml were cultured with the medium containing serial dilutions of antitumor drugs. After incubation of the cells for 72 hr at 37°, cytotoxicity was determined by cell counting using a Coulter counter.

In vivo evaluation was performed by the use of CDF_1 mice as hosts. Drugs were administered intraperitoneally. In the case of intravenous implantation, mice were inoculated with 4×10^5 L5178Y-ML cells. The effects of drugs were evaluated by measuring survival period and liver weight increase. The increase of liver weight was calculated by subtracting the mean liver weight of tumor-free mice in each group (counterpart) from the individual liver weights of tumor-bearing mice given drugs. When the tumor cells (1×10^6 cells/mouse) were subcutaneously injected into the side flank of mice, in addition to the survival period, tumor growth in the implanted site was monitored with the use of calipers 14 days after implantation. Tumor size was calculated from $(L\times W^2)/2$, where L=length (mm) and W=width (mm).

Results

Organ Selectivity of Metastases CDF_1 mice were implanted with 5×10^5 cells of L5178Y-ML or its parental line intravenously and

Table I. Organ Selectivity of Metastases[a]

Tumor	Implantation route	Mean weight±SD (g)							Weight ratio of liver to tumor[b]
		Whole body	Subcutaneous tumor	Liver	Heart	Lung	Spleen	Kidney	
Expt. 1									
None	—	19.2±0.5	—	0.83±0.03	0.11±0.01	0.25±0.06	0.07±0.01	0.23±0.01	
L5178Y (parental)	iv	20.2±1.3	—	0.94±0.07[c]	0.10±0.01	0.25±0.07	0.06±0.01	0.23±0.02	
L5178Y-ML	iv	20.9±0.6[d]	—	2.53±0.29[d,e]	0.13±0.03	0.30±0.08	0.13±0.02[d,e]	0.24±0.04	
Expt. 2									
None	—	20.7±1.0	—	1.12±0.11	0.12±0.02	0.16±0.01	0.09±0.01	0.34±0.02	
L5178Y (parental)	sc	22.7±1.2[c]	2.02±1.10	1.42±0.08[d]	0.11±0.01	0.16±0.02	0.14±0.07	0.29±0.03[d]	0.59±0.17
L5178Y-ML	sc	22.6±1.7[c]	1.55±0.54	2.73±0.94[d]	0.14±0.04	0.23±0.10	0.17±0.06	0.32±0.04	1.87±0.75[e]

a) CDF_1 mice (6 weeks old: n=6) were sacrificed 9 days after the intravenous injection of 5×10^5 cells (Expt. 1) or 19 days after the subcutaneous injection of 1×10^6 cells (Expt. 2).
b) The mean±SD of the ratio in individual animals.
c) $P<0.05$ against normal in each experiment by the Mann-Whitney U-test.
d) $P<0.01$ against normal in each experiment by the Mann-Whitney U-test.
e) $P<0.05$ against parental by the Mann-Whitney U-test.

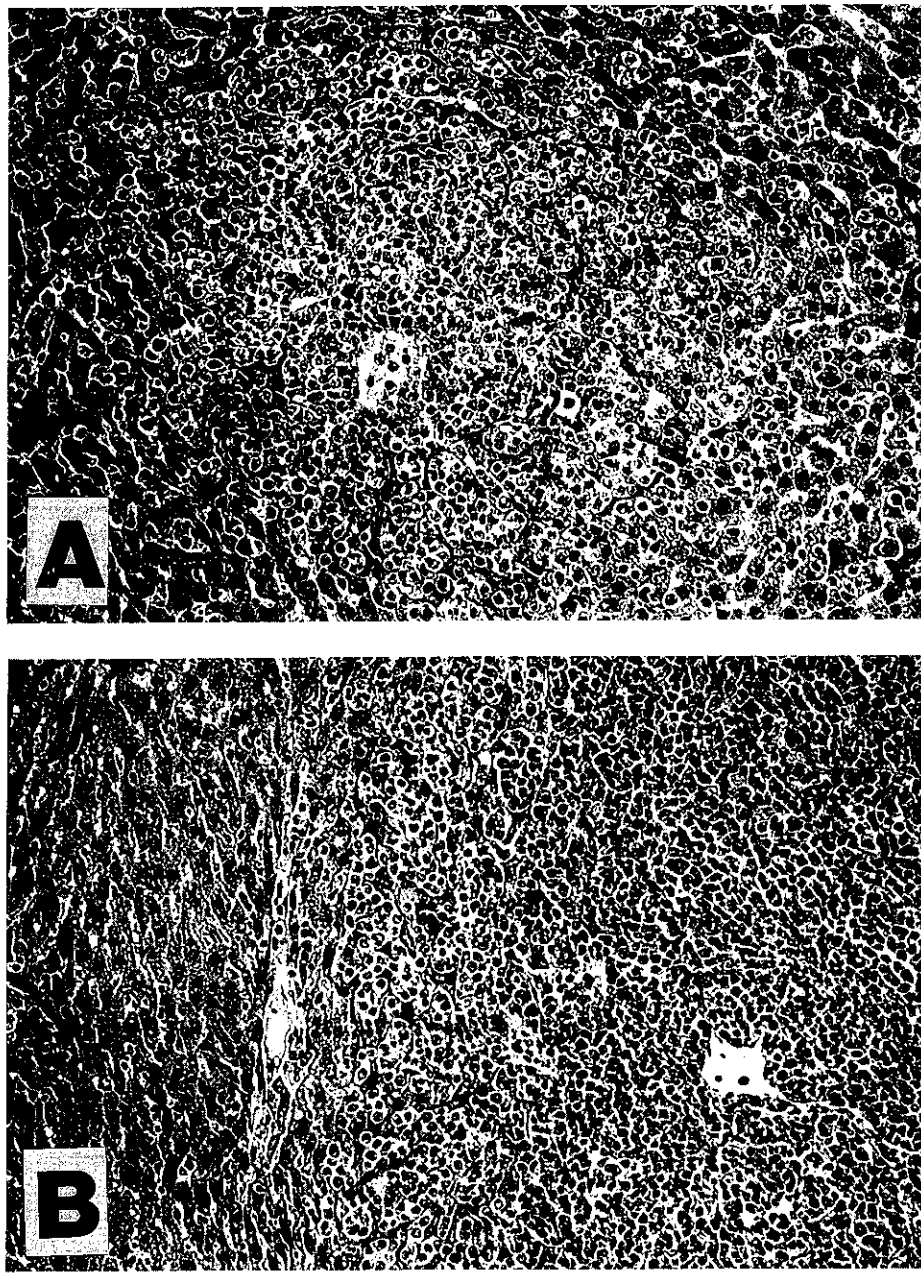

Fig. 1. Liver metastases of L5178Y-ML. Focal-type organ infiltration (A, B) ×100. Hematoxylin-eosin staining.

sacrificed to weigh their organs 9 days after the inoculation. The organ weight in mice injected with L5178Y parental cells intravenously was almost the same as that in healthy mice, while L5178Y-ML cells infiltrated diffusely and propagated very markedly in the liver (Table I). Increase of spleen weight was also observed in one experiment.

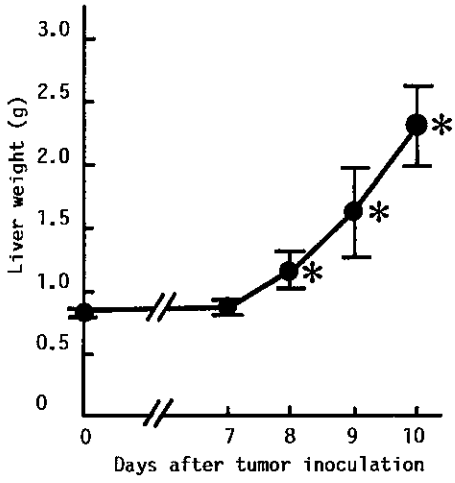

Fig. 2. Liver weight increase after intravenous inoculation of L5178Y-ML cells. CDF_1 mice (6 weeks old) were inoculated with 5×10^5 L5178Y-ML cells into their tail vein. Bars show SD. *: $P<0.05$ against normal (Mann-Whitney U-test).

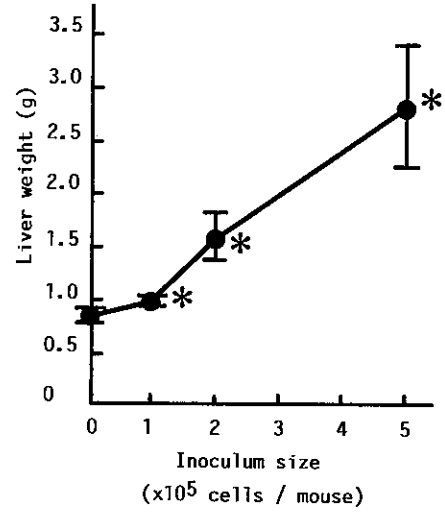

Fig. 3. Correlation between inoculum size and liver weight increase. CDF_1 mice (6 weeks old) were inoculated with 1×10^5–5×10^5 L5178Y-ML cells intravenously. The liver weight was measured 9 days after the implantation. Bars show SD. *: $P<0.05$ against normal (Mann-Whitney U-test).

In the case of subcutaneous implantation, L5178Y-ML cells propagated more in livers than in the implanted sites; the average value of the individual ratio of the liver weight to the subcutaneous tumor weight in mice with the metastatic tumor cells was 1.87 ± 0.75, which was significantly different ($P<0.05$ by Mann-Whitney U-test) from that (0.59 ± 0.17) in mice with the parental tumor cells. Infiltration of L5178Y-ML cells into a liver was mostly focal, but diffused tumor cells were also observed (Fig. 1A). In the case of developed tumor nodules, the border between the nodule and liver tissue was clear (Fig. 1 B). Hepatocytes were not found in the tumor nodule. Histologically, micrometastases were not found in lungs or kidneys but were noted in some spleens.

Growth of L5178Y-ML Cells in the Liver

The liver weight of mice given an intravenous injection of 5×10^5 L5178Y-ML cells (day 0) was evaluated. The liver weight did not change until day 7, after which time it increased very rapidly (Fig. 2). Mice injected with 5×10^5 cells intravenously died between day 10 and day 12 (mean$\pm$SD, 11.3 ± 0.8). Moreover, the liver weight and the survival time of mice implanted with the tumor cor-

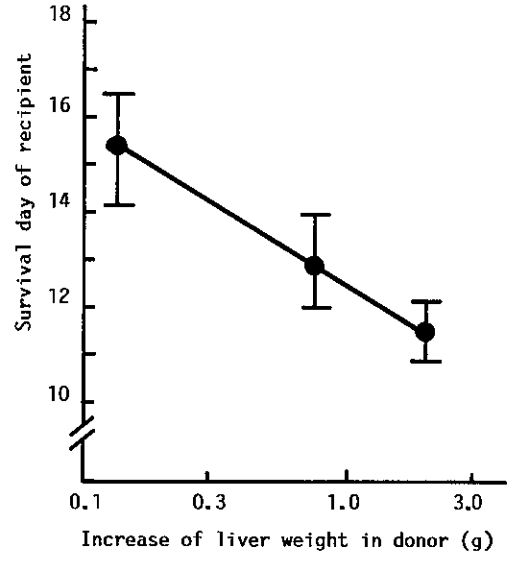

Fig. 4. Correlation between the increase of liver weight in mice (donors) implanted with L5178Y-ML cells and the survival time of mice (recipients) injected with aliquots of dispersed liver from the donor mice. Methods are described in the text. Bars show SD.

related with the number of tumor cells implanted (Fig. 3 and Fig. 4). The correlation coefficient between liver weight and inoculum size was calculated to be 0.93.

Correlation between Liver Weight Increase and Tumor Growth in the Liver CDF_1 mice were implanted intravenously with 1×10^5, 2×10^5, or 5×10^5 L5178Y-ML cells. After 9 days, livers were taken from these mice, weighed, and then dispersed physically. Aliquots of dispersed liver (1/840 of whole liver) were intraperitoneally injected into other mice. The survival time of the recipient mice correlated highly with the increase of liver weight in donor mice (correlation coefficient, 0.84; Fig. 5). The liver weight increase was calculated by subtracting the mean liver weight of age- and body weight-matched normal mice from the individual liver weights of the tumor-bearing mice. Moreover, by converting the survival time into the number of implanted tumor cells using a standard survival curve (Fig. 5), it was revealed that the increase of the liver weight correlated with the number of tumor cells in the liver (Fig. 6). The correlation coefficient was 0.86.

Effect of Antitumor Drugs on the *in vitro* Growth of L5178Y-ML Cells The doubling times of L5178Y (parental) and L5178Y-ML cells were 11.0 and 12.5 hr, respectively. MMC, DXR, 5-FU, and CDDP strongly inhibited the cell growth (Table II). Among them, 5-FU was the most effective; IC_{50} of 5-FU against the growth of the variant cells was 14 ng/ml, which was less than a half of the others.

Effect of Antitumor Drugs on the Metastases of L5178Y-ML Mice implanted intravenously with the tumor cells (day 0) were given drugs intraperitoneally for 5 days from day 1 through day 5. They were killed and the

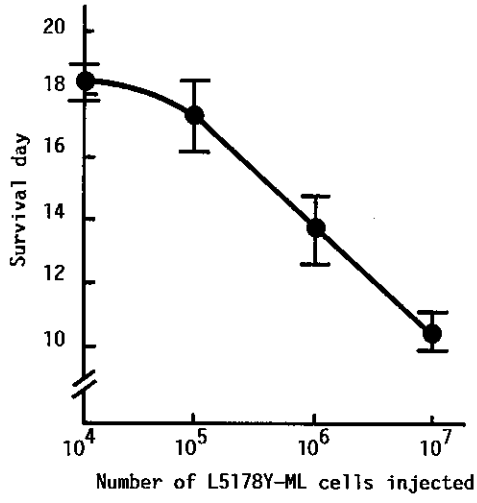

Fig. 5. Standard survival curve of mice injected intraperitoneally with L5178Y-ML cells. CDF_1 mice (6 weeks old) were inoculated with 1×10^4–1×10^7 L5178Y-ML cells intraperitoneally. Bars show SD.

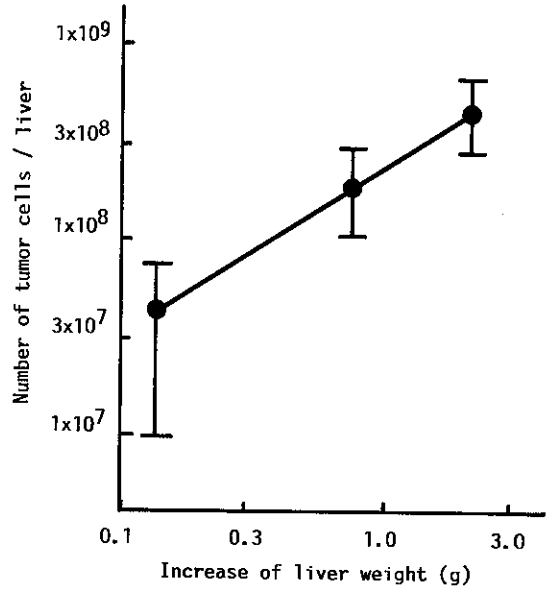

Fig. 6. Correlation between increase of liver weight in mice injected with L5178Y-ML and number of tumor cells in the liver. Methods are described in the text. Bars show SD.

Table II. *In vitro* Drug Sensitivity of L5178Y-ML Cells[a)]

Cell	IC_{50} (ng/ml)			
	Mitomycin C	Doxorubicin	5-Fluorouracil	*cis*-Platinum
L5178Y (parental)	52	110	9	120
L5178Y-ML	36	56	14	64

a) Cells (5×10^4 cells/ml) were cultured in the presence of drugs for 72 hr.

Table III. Effect of Antitumor Drugs on the Growth of L5178Y-ML Cells in Liver[a]

Drug (ip)	Dose (mg/kg/day)	Mean liver weight (g)		
		tumor (+)	tumor (−)	Δ[b]
Control	—	2.81	0.84	1.97
Mitomycin C	0.25	2.33	0.86	1.46[c]
	1.00	1.35	0.87	0.48[d]
Doxorubicin	0.50	2.80	0.86	1.93
	2.00	1.90	0.86	1.04[d]
5-Fluorouracil	5	2.55	0.86	1.69[c]
	20	1.08	0.84	0.24[d]
cis-Platinum	0.25	2.61	0.84	1.77
	1.00	1.28	0.84	0.44[d]

a) CDF_1 mice (7 weeks old: n=8) were implanted intravenously with 4×10^5 L5178Y-ML cells on day 0, and given drugs once daily for 5 days from day 1. Liver weight was examined on day 9.
b) Increase of liver weight (Δ) was calculated by subtracting the mean liver weight of tumor-free mice in each group from the individual liver weights of tumor-bearing mice.
c) $P<0.05$ by the Mann-Whitney U-test.
d) $P<0.01$ by the Mann-Whitney U-test.

Table IV. Life-span-prolonging Effect of Antitumor Drugs in Mice Intravenously Implanted with L5178Y-ML Cells[a]

Drug (ip)	Dose (mg/kg/day)	Schedule	Survival time (day) Mean±SD
Control	—	Days 1–5	9.9±0.5
Mitomycin C	0.25	〃	9.4±0.5
	1.00	〃	10.4±0.5
Doxorubicin	0.50	〃	9.3±0.5
	2.00	〃	9.6±0.7
5-Fluorouracil	5	〃	10.4±0.5
	20	〃	13.1±1.2[b]
cis-Platinum	0.25	〃	10.6±1.8
	1.00	〃	11.5±0.5[b]
Control	—	Days 5–9	9.8±0.4
Mitomycin C	0.25	〃	9.9±1.0
	1.00	〃	10.8±1.3
Doxorubicin	0.50	〃	9.8±0.9
	2.00	〃	10.0±0.9
5-Fluorouracil	5	〃	12.5±0.5[b]
	20	〃	14.0±0.5[b]
cis-Platinum	0.25	〃	10.0±0.8
	1.00	〃	11.4±1.4

a) CDF_1 mice (8 weeks old: n=8) were implanted with 5×10^5 L5178Y-ML cells intravenously on day 0, and given drugs once daily for 5 days.
b) $P<0.01$ by the Mann-Whitney U-test.

livers were weighed on day 9. The increase in liver weight, which reflected tumor growth in the liver, was significantly suppressed by 1 mg /kg/day MMC, 2 mg/kg/day DXR, 20 mg/kg/day 5-FU, or 1 mg/kg/day CDDP (Table III). However, at the same administration schedule, only 5-FU prolonged the survival period of mice intravenously inoculated with tumor cells (Table IV). Since liver weight in mice given intravenous injection of the tumor

Table V. Effect of Antitumor Drugs in Mice with Subcutaneous L5178Y-ML Tumors[a]

Drug (ip)	Dose (mg/kg/day)	Primary tumor size (mm^3)[b]		Survival time (day)	
		Mean±SD	n[c]	Mean±SD	Range
Control	—	2272±799	7	17.4±4.4	13–26
Mitomycin C	0.25	1847±579	7	15.9±2.0	14–20
	1.00	1118±580[d]	8	21.3±4.5[d]	16–29
Doxorubicin	0.50	1290±770	3	16.3±3.8	13–23
	2.00	1628±439	6	17.1±3.6	14–24
5-Fluorouracil	5	1440±702	8	21.1±3.1[d]	17–26
	20	121±115[e]	8	22.3±0.5[e]	22–23
cis-Platinum	0.25	1168±215[d]	5	15.9±3.5	13–23
	1.00	1616±587	8	18.9±2.0	15–20

a) CDF_1 mice (7 weeks old: cont, n=12; test, n=8) were implanted subcutaneously with 1×10^6 L5178Y-ML cells on day 0, and given drugs once daily for 10 days from day 5 through day 14.
b) Tumor size was determined on day 14 and calculated from $(L\times W^2)/2$; L=length (mm) and W=width (mm).
c) Number of mice alive on day 14.
d) $P<0.05$ by the Mann-Whitney U-test.
e) $P<0.01$ by the Mann-Whitney U-test.

cells increased very rapidly after day 7 (Fig. 2), treatment beginning from day 5 was also tested. As Table IV shows, mice administered 5-FU from day 5 to day 9 survived longer than the controls.

The antitumor effects of these drugs in mice with subcutaneous tumors were examined. Based on the result that the mean survival time of mice implanted subcutaneously with the tumors was around 17 days, mice with subcutaneous tumors were given drugs for 10 days from day 5 through day 14. In addition to survival time, the size of subcutaneous tumors in mice alive on day 14 was monitored. As Table V shows, 20 mg/kg/day 5-FU inhibited the tumor growth in the implanted site by 95%. MMC was also effective at a dose of 1 mg/kg/day. Although 5 or 3 mice out of 8, which were treated with 0.5 mg/kg/day DXR or 0.25 mg/kg/day CDDP, had died by the day of tumor size determination, the mean tumor size in groups given these drugs was smaller than that of the controls. The effect of 5-FU (20 mg/kg/day) on the growth of primary tumors was also apparent 10 days after the implantation; the inhibition was 93% ($P<0.01$ by Mann-Whitney U-test). However, the other drugs were ineffective (not shown). MMC at 1 mg/kg/day and 5-FU at 5 and 20 mg/kg/day were effective in prolonging the survival period of mice with subcutaneous tumors.

Discussion

Tumor lines reported to induce preferentially hepatic metastases are mostly lymphoid tumor lines such as RAW117-H10,[9] MDAY-D2,[17] and ESb.[7] The organ-specific nature of metastatic spread is well recognized,[3, 18, 19] and the selective metastases of these lymphoid tumor cells to the liver might depend on host and tumor cell properties. Although the liver is rich in Kupffer cells, playing an effector role against tumor cells,[1, 20, 21] liver metastatic lymphoid tumor cells might be resistant to the Kupffer cells as well as natural killer cells. Both L5178Y-ML and ESb are metastatic variants of L5178Y origin, but the properties of these cell lines seemed to be not identical. Infiltration of ESb was reported to be mostly diffuse,[7] while L5178Y-ML generated tumor foci in livers. This difference might explain why L5178Y-ML cells were not histologically found in lung or kidney; it was reported that ESb cells occasionally metastasized to these organs in addition to intestine, thymus, heart and brain. It would be intriguing to compare the biochemical and immunological properties of these two lines, and such a comparison may provide

novel information about the mechanism of cancer metastases.

In the case of experimental metastasis by intravenous injection with L5178Y-ML cells, the deviation of the tumor growth and the survival period of tumor-bearing mice are very small. Although the growth of hepatic tumors induced by subcutaneous injection with L5178Y-ML cells fluctuates, this is a spontaneous metastasis system including all the metastatic processes, and does not require splenectomy. This spontaneous metastasis should be useful in evaluating antimetastatic agents and biological response modifiers in addition to cytocidal anticancer agents.

MMC, 5-FU, DXR and CDDP are usually effective in inhibiting the growth of various ascitic or subcutaneous tumors in experimental animals at the dosages employed in the present study.[22] According to our previous experiments, administration of MMC at doses effective in the treatment of Lewis lung carcinoma grown in the implanted site (side flank) inhibits pulmonary metastases.[23] MMC also prevents the development of B16 melanoma in the lungs.[23] As described in "Results," these four drugs also inhibited the growth of L5178Y-ML cells in the livers of mice intravenously injected with the tumor cells, except that the effect of DXR was controversial compared with those of the other three drugs. The effect of the four drugs on the survival period of the tumor-bearing mice appeared to reflect the inhibitory effect on the tumor growth in the livers, and thus essentially reflected the sensitivity of L5178Y-ML cells to each drug.

The most important result was that the life-span-increasing effect of the antitumor drugs in mice inoculated with L5178-ML cells was insufficient, compared with the inhibitory effect on the tumor growth in livers. Provided that the major cause of death of mice inoculated with L5178Y-ML cells was the metastasis to the liver, to prolong the life-span sufficiently, the release of L5178Y-ML cells into the circulation should be prevented almost completely. Agents that markedly increase the survival period of mice with the tumor would be very potent antitumor agents.

Consequently the hepatic tumor system utilizing L5178Y-ML cells seems to be an advantageous system for *in vivo* screening for agents effective against tumor growth in the liver, though L5178Y-ML is not a hepatoma but a lymphoma. Furthermore, the developmental method employed (intravenous injection of parental cells — subcutaneous implantation of liver tumors — repeat the subcutaneous passage of liver tumors) might be also an efficient way to establish metastatic variants of other cell lines, in addition to L5178Y.

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