Jpn. J. Cancer Res. (Gann), 79, 1147–1154 ; October, 1988

Effects of a Combination of Cyclophosphamide and Human Recombinant Interleukin 2 on Pulmonary Metastasis after the Surgical Removal of a 3-Methylcholanthrene-induced Primary Tumor in Autochthonous Mice

Masuo HOSOKAWA,[*1] Takashi YABIKU,[*2] Jun-ichi IKEDA,[*2] Yutaka SAWAMURA,[*3] Futoshi OKADA,[*1] Masashi KOMATSUMOTO,[*1, *2] Tatsuzo TANABE[*2] and Hiroshi KOBAYASHI[*1]

*[1]*Laboratory of Pathology, Cancer Institute,* *[2]*Second Surgery Department and* *[3]*Department of Neurosurgery, Hokkaido University School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060*

We have investigated the therapeutic effects of a combination of cyclophosphamide (CY, 150 mg/kg, iv) and human recombinant interleukin 2 (IL-2, 5×10^4 JU/day, ip for 5 days) on autochthonous tumors induced in mice by 3-methylcholanthrene. The initial treatment was carried out when the tumor had reached 8 to 10 mm in diameter. Twenty-eight out of 35 mice (80%) died of local recurrence and pulmonary metastasis of tumor cells within 53±40 days (mean survival time, MST±SD) after the surgical removal of the primary tumor. When these mice were treated with both CY and IL-2 following the operation (Op), only 10 out of 20 mice (50%) died of recurrence and metastasis. The survival rate, however, was not improved by CY chemotherapy alone or IL-2 immunotherapy alone, although each provided a prolongation of the MST. Natural killer cell and LAK precursor cell activities in the spleen cells from the treated mice were found to be restored by IL-2 alone or CY+IL-2, whereas they were suppressed by CY alone. These findings reveal that the restoration of the antitumor activity of spleen cells does not provide an improved therapeutic effect by itself and that IL-2 immunotherapy requires the associated effect of CY chemotherapy to achieve an improved therapeutic effect.

Key words: Autochthonous tumor — Interleukin 2 — Cyclophosphamide — Pulmonary metastasis

We have previously observed that local recurrence and pulmonary metastasis of 3LL lung carcinoma after cyclophosphamide (CY) chemotherapy are inhibited by its combination with interleukin 2 (IL-2) immunotherapy.[1] The administration of IL-2 was found to be insufficient by itself and the associated effect of CY chemotherapy was required for a satisfactory therapeutic effect on 3LL tumors. We found evidence that LAK cells which have been activated by IL-2 are able to accumulate more densely in the tumor tissue of mice which have previously been treated with CY than in the tissue of untreated mice. The ehnanced accumulation of LAK cells in CY-treated tumors was able to bring about a complete cure of 3LL-bearing mice. In the case of the transplanted 3LL tumor, an appropriately timed combination of CY and IL-2 appeared to be effective. We have been considering, however, whether the experimental immunotherapy should also be evaluated on autochthonous tumors in order to find out if it will be relevant for the clinical treatment of cancer.

Frequent local recurrence and pulmonary metastasis were observed in autochthonous mice treated by the surgical removal of a primary tumor which had been induced by 3-methylcholanthrene (MCA).[2,3] This study was carried out in order to attempt a preclinical evaluation of the effectiveness of a combination of CY chemotherapy and IL-2 immunotherapy on the locally recurrent and metastatic tumors after surgical removal of the primary tumor in autochthonous hosts.

MATERIALS AND METHODS

Mice Female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu).

Tumors Tumors were induced by the injection of 1 mg of 3-methylcholanthrene (MCA, Sigma Chem. Co., St Louis, Mo.) in 0.1 ml of olive oil into the distal muscle of the right hindlimb of the mice as reported previously.[2,3] All mice were examined to determine the difference of thickness between the

right and left limbs (the estimated tumor size) every week from 8 weeks after the MCA injection. When the estimated tumor size had reached 8 to 10 mm, the initial treatment was carried out.

Surgical Removal of the Tumor The primary tumor was removed by the amputation of the right hindlimb at the hip joint under ethyl ether anesthesia. The mice were divided into 4 groups as shown in Table I. There was no significant difference among these 4 groups of mice in the day of operation after the MCA injection or the mean weights of tumors removed.

Administration of Agents Cyclophosphamide (CY; Shionogi Pharm. Co., Osaka) was administered iv at a dose of 150 mg/kg as chemotherapy. Immunotherapy was carried out by the administration of interleukin 2 (IL-2). Human recombinant IL-2 was supplied by Shionogi Pharm. Co.[4] IL-2 was given systemically by 5 serial ip injections at a dose of 5×10^4 Jurkat units (JU)/day; this unit is approximately 5 times larger than an international unit. The IL-2 administration was started 5 days after the surgical treatment or the CY chemotherapy in accordance with the result of experiments in which we have already demonstrated the effect of a combination of CY and IL-2 on transplanted 3LL lung carcinoma in C57BL/6 mice.[1]

Observation of Treated Mice All the mice were kept under observation until they died of either the primary tumor, a local recurrence of the tumor or a pulmonary metastatic tumor. The survival time in days for each mouse after the initial treatment was recorded to obtain the mean survival time (MST) and the survival curve of mice in each treated group. When mice survived until 150 days after the removal of the primary tumor without local recurrence, they were recorded as survivors. The mice which died were autopsied for an examination of recurrent and metastatic tumors.

Antitumor Activity of Spleen Cells The spleen cells of mice in each group were examined to determine their cytotoxicity against YAC-1 cells and, after a 3-day incubation with IL-2 (1000 JU/ml), against P815 cells by a 6 hr chromium release test. The former was taken as natural killer (NK) cell activity and the latter was taken as lymphokine-activated killer (LAK) precursor cell activity. We used 3 or 4 spleens for each experiment. Spleens were removed aseptically and teased with loose-fitting glass homogenizers. Single cell suspensions were obtained by a passage through 4 layers of gauze. Cell suspensions were washed twice with cold RPMI 1640 (Nissui Pharm. Co. Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS). The number of viable cells was determined by a trypan blue exclusion test.

Chromium Release Test A chromium release test was carried out for the detection of the cytotoxicity of spleen cells by the method described previously.[1] Target cells: YAC-1 or P815 cells were mixed with sodium chromate (^{51}Cr) at 100 μCi/10^6 cells/ml. The mixture was incubated for 1 hr at 37° in a CO_2 incubator. The ^{51}Cr-labeled target cells (1×10^4/well) were incubated with effector lymphocytes (5×10^5/well) for 6 hr in a 96-well microplate. The radioactivity of 0.1 ml of supernatant in each well was counted, and the percentage of cytolysis was assessed by means of the formula below, in which a is the percentage of release cpm with effector cells, b is the percentage of maximum release cpm by 1N HCl and c is the percentage of spontaneous cpm with medium alone.

$$\text{Cytolysis } (\%)=\frac{a-c}{b-c}\times100$$

We expressed the level of antitumor activity of tumor-bearing spleen cells as a percentage of the activity of spleen cells obtained from normal C57BL/6 mice, examined at the same time.

Statistical Analysis The incidences of local recurrence, of pulmonary metastasis and of dead mice were compared by means of the χ^2-test between the two treated groups. A significant difference in the MST and survival curves of treated mice from those of the control mice was established by using Student's t-test and the generalized Wilcoxon test, respectively.

Table I. Condition of Primary Tumors in Autochthonous C57BL/6 Mice at the Time of Operation of Each Treated Group

Group of treatment	No. of mice	Operation: Days after MCA injection	Mean weight (g) of tumors removed
Op alone	35	127±15	2.64±0.67
Op+CY	27	126±18	2.94±0.60
Op+IL-2	24	127±11	2.46±0.60
Op+CY+IL-2	20	130±17	3.02±0.81
Total	106	127±16	2.75±0.67

Results

Effects of Combination Treatment with CY and IL-2 on MCA-induced Tumor in Autochthonous C57BL/6 Mice without Surgery Tumors developed between 80 to 100 days after the MCA injection. A number of the autochthonous tumor-bearing mice were treated with CY or/and IL-2 when the tumor had reached 8 to 10 mm in diameter without the surgical removal of the primary tumor. The MSTs of mice were found to be prolonged in the groups of mice given the CY chemotherapy alone or CY plus IL-2, although no mouse survived after the treatment (Table II). We observed macroscopical pulmonary metastases in 6 out of 20 untreated mice (30%) after the mice had died. The incidence of pulmonary metastasis was decreased in mice treated with the combination of CY and IL-2, while the incidences did not change in mice treated with either CY or IL-2 alone.

Effects of Combination Treatment with CY and IL-2 on Local Recurrence and Pulmonary Metastasis after Surgery Twenty-eight out of 35 mice (80%) died of locally recurrent tumor and/or pulmonary metastasis in the group treated with surgery alone. The incidence of dead mice decreased in the groups

Table II. Effects of Combination Treatment with Cyclophosphamide (CY) and Human Recombinant Interleukin-2 (IL-2) on Methylcholanthrene-induced Primary Tumors in Autochthonous C57BL/6 mice without Operation

Treated[a] with	Died/treated (%)	MST±SD[b] in days	Positive[c] pulmonary metastasis (%)
None	20/20 (100)	19±9	6/20 (30)
CY alone[d]	10/10 (100)	30±10*	6/10 (60)
IL-2 alone[e]	10/10 (100)	25±8	5/10 (50)
CY+IL-2[f]	12/12 (100)	34±10*	1/12** (8)

a) Methylcholanthrene in oil (1 mg/0.1 ml) was injected im into the right hindlimb of mice and the initial treatment with either CY or IL-2 was carried out when tumor had reached 8 to 10 mm in diameter.
b) Mean survival time in days and standard deviation after the initial treatment.
c) Macroscopic metastasis was evaluated by autopsy when the mice had died.
d) CY (150 mg/kg) was administered iv.
e) IL-2 (5×10^4 JU/day) was administered ip for 5 days.
f) IL-2 was initiated 5 days after CY.
* Statistically significant difference as compared with the untreated group ($P<0.05$).
** Statistically significant difference as compared with the group of mice treated with CY alone or IL-2 alone ($P<0.05$).

Table III. Effects of the Combination Treatment with Cyclophosphamide (CY) and Human Recombinant Interleukin-2 (IL-2) on Local Recurrence and Pulmonary Metastasis after the Surgical Removal (Op) of Methylcholanthrene-induced Tumors in Autochthonous C57BL/6 Mice

Treated[a] with	Recurrence/treated (%)	Metastasis/without recurrence (%)	Died/treated (%)	MST±SD[b] in days
Op alone	14/35 (40)	14/21 (67)	28/35 (80)	53±40
Op+CY[c]	13/27 (48)	9/14 (64)	22/27 (82)	66±39
Op+IL-2[d]	10/24 (42)	7/14 (50)	17/24 (71)	58±24
Op+CY+IL-2	6/20 (30)	4/14 (29)*	10/20 (50)**	65±30

a) Methylcholanthrene in oil (1 mg/0.1 ml) was injected im into the right hindlimb of mice and the primary tumor was surgically removed when it had reached 8 to 10 mm in diameter.
b) Mean survival time and standard deviation of dead mice.
c) CY (150 mg/kg) was administered iv 5 days after the operation.
d) IL-2 (5×10^4 JU/day) was administered ip for 5 days from 10 days after the operation.
*, ** Statistically significant difference as compared with the operation alone group (* $P<0.05$, ** $P<0.025$).

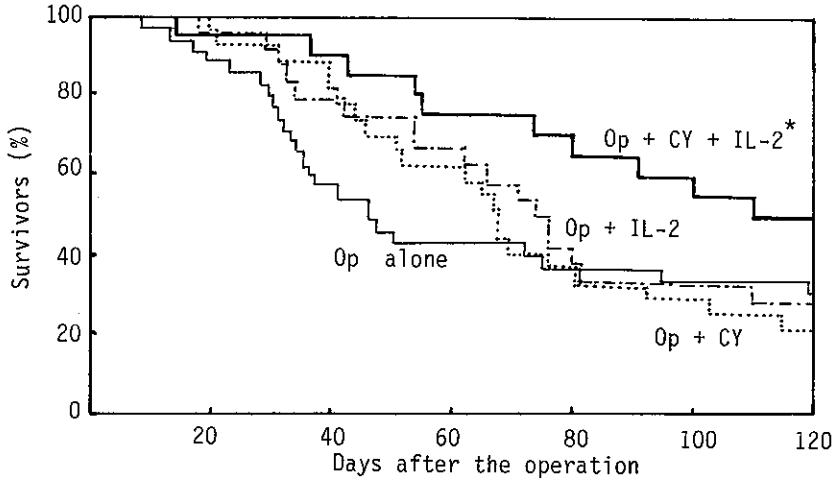

Fig. 1. Survival curves of C57BL/6 mice treated with a combination of cyclophosphamide (CY) and/or interleukin 2 (IL-2) after surgical removal (Op) of autochthonous tumors induced by methylcholanthrene (MCA). MCA in olive oil (1 mg/0.1 ml) was injected im into the right hindlimb of C57BL/6 mice and the primary tumor was surgically removed when it had reached 8 to 10 mm in diameter. Then mice were treated with CY (150 mg/kg, iv) 5 days after the operation or/and with IL-2 (5×10^4 JU/day, ip) for 5 days from 10 to 14 days after the operation. *, Statistically significant difference from Op alone group by the generalized Wilcoxon test ($P<0.01$).

Table IV. Effects of the Combination Treatment with Cyclophosphamide (CY) and Human Recombinant Interleukin 2 (IL-2) on Local Recurrence and Pulmonary Metastasis after the Surgical Removal (Op) of Methylcholanthrene-induced Tumors in Autochthonous BALB/c Mice

Treated[a] with	Recurrence/treated (%)	Metastasis/without recurrence (%)	Died/treated (%)	MST±SD[b] in days
Op alone	8/10 (80)	1/2 (50)	9/10 (90)	56±25
Op+CY	5/8 (60)	2/3 (67)	7/8 (88)	57±21
Op+IL-2	6/9 (67)	2/3 (67)	8/9 (89)	57±12
Op+CY+IL-2	3/9 (33)*	0/6 (0)	3/9 (33)**	48±11

a) Methylcholanthrene in oil (1 mg/0.1 ml) was injected im into the right hindlimb of mice and the primary tumor was surgically removed when it had reached 8 to 10 mm in diameter.
b) Mean survival time and standard deviation of dead mice.
c) CY (150 mg/kg) was administered iv 5 days after the operation.
d) IL-2 (5×10^4 JU/day) was administered ip for 5 days from 10 days after the operation.
*, ** Statistically significant difference as compared with the operation alone group (* $P<0.05$, ** $P<0.02$).

treated with the combination of CY and IL-2 (only 10 out of 20 mice (50%) died), while the incidence did not decrease significantly in the groups of mice treated with CY alone or IL-2 alone (Table III). The improved therapeutic effects seem to be due to the decreased incidence of pulmonary metastasis, since the frequency of mice that died of metastasis was significantly suppressed as compared with that in the group of mice treated with surgery alone (29% vs. 67%). A single treatment with either CY or IL-2, however, tended only to prolong the survival time of mice which ultimately died. The survival curves in Fig. 1 indicate that CY chemotherapy or IL-2 immunotherapy after the surgery inhibits the

Table V. Extent of Pulmonary Metastasis in Dead C57BL/6 Mice

Treated[a] with	Total		Died of recurrence		Died of metastasis	
	Positive met./ died (%)	MNM[b] (MWL)	Positive met./ with rec. (%)	MNM[b] (MWL)	No. of mice	MNM[b] (MWL)
Op alone	19/28 (68)	12.1±16.6 (445±435)	5/14 (35)	4.7±13.2 (257±211)	14	19.6±16.6 (634±522)
Op+CY[c]	14/22 (64)	7.8±10.6 (309±135)	5/13 (39)	3.5±6.0 (247±104)	9	13.1±13.5 (399±129)
Op+IL-2[d]	10/17 (59)	8.9±16.2 (412±347)	3/10 (30)	0.6±1.1 (223±90)	7	20.7±20.5 (682±407)
Op+CY[c]+IL-2[d]	7/10 (70)	15.7±21.8 (535±459)	3/6 (50)	2.2±2.5 (280±163)	4	36.0±22.4 (918±513)

a) Methylcholanthrene in oil (1 mg/0.1 ml) was injected im into the right hindlimb of mice and the primary tumor was surgically removed when it had reached 8 to 10 mm in diameter.
b) Mean number of metastatic nodules in lung of dead mice. The value in parenthesis is the mean weight (mg) of lung.
c) CY (150 mg/kg) was administered iv 5 days after the operation.
d) IL-2 (5×10^4 JU/day) was administered ip for 5 days from 10 days after the operation.

early death of mice after surgery, although the generalized Wilcoxon test established a significant difference in the survival curve compared with that of the control group only in the group treated with the combination ($P<0.01$). We have observed the same grade of therapeutic effect of the combination of CY and IL-2 in an experiment in which we used BALB/c mice (Table IV), although the number of mice was not large enough to obtain a precise evaluation.

We examined pulmonary metastasis after the mice had died (Table V). There is no difference in the frequency of metastasis among the groups of dead mice. The extent of metastasis was estimated in terms of the mean number of nodules on the surface of the lung and the mean lung weight of dead mice. We observed that the extent of metastasis was smaller in mice which died of local recurrence of tumors than in mice wihtout local recurrence of the tumor. The extent was greater, however, in mice treated with the combination of CY and IL-2 than in mice of other treated groups when the dead mice were compared. This finding indicates that there are heterogeneous populations of metastatic tumor cells, a number of which are sensitive to the combination treatment of CY and IL-2, while others are strongly resistant to the treatment.

Chronological Changes of Antitumor Cytotoxicity of Spleen Cells Obtained from Treated Mice The cytotoxicity of fresh spleen cells obtained from the treated mice was examined against YAC-1 cells by means of a chromium release test. The estimated NK activity was inhibited in untreated tumor-bearing mice to about half the level in normal mice. The activity of spleen cells from mice treated by surgery alone did not change. The NK activity of spleen cells was suppressed further by CY chemotherapy following surgical treatment (Fig. 2). The suppression of NK cell activity following surgery and the CY chemotherapy was apparently restored by the administration of IL-2. The LAK precursor cells were also restored by the administration of IL-2 in mice which had been treated by surgery alone or by surgery plus CY (Fig. 3). The level of the LAK precursor activity in the spleens obtained from untreated tumor-bearing mice was lower than in that from normal mice. The activity was suppressed 3 days after the surgical removal of the tumor, although it tended to recover thereafter without any further treatment. The LAK precursor activity was strongly suppressed, however, in mice treated with CY 5 days after the operation. It was noteworthy that the suppressed activity of LAK precursor was already restored one day after the administra-

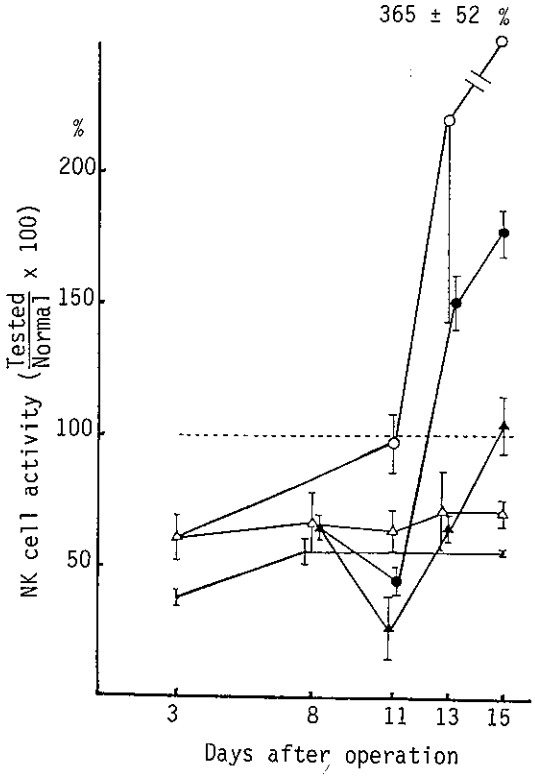

Fig. 2. Chronological changes of cytotoxic activity against YAC-1 cells of fresh spleen cells obtained from mice treated by various regimens. The cytotoxicity was examined by a ^{51}Cr-release test. The activity is expressed as a percentage, based on the activity of normal spleen cells as 100%. ○, Op+IL-2; ●, Op+CY+IL-2; ▲, Op+CY; △, Op alone; —, without Op. Each point is the mean of 3 or 4 values and bars indicate the standard deviation.

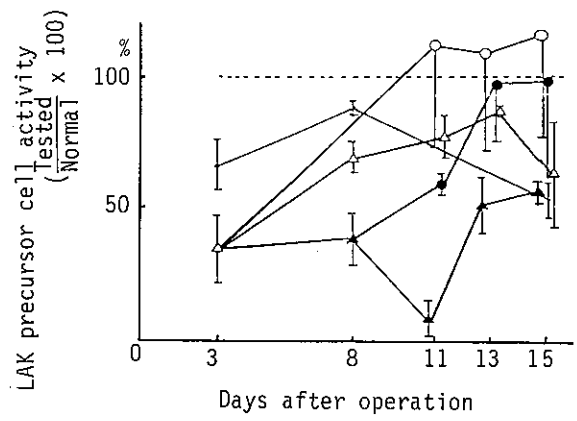

Fig. 3. Chronological changes of cytotoxic activity against P815 cells of IL-2 activated spleen cells obtained from mice treated by various regimens. Spleen cells were cultured for 3 days with RPMI 1640 supplemented with 10% FCS and IL-2 (1000 JU/ml) prior cytotoxicity was examined by a ^{51}Cr-release test. The activity is expressed as a percentage based on the activity of normal spleen cells as 100%. ○, Op+IL-2; ●, Op+CY+IL-2; ▲, Op+CY; △, Op alone; —, without Op. Each point is the mean of 3 or 4 values and bars indicate the standard deviation.

tion of IL-2 in the groups of Op+IL-2 and Op+CY+IL-2 as compared with that in the groups of Op alone and Op+CY, respectively. The activation of NK cells and LAK precursor cells in spleen cells is, therefore, not sufficient in itself for the inhibition of growth of metastatic tumor cells, although a combination of CY and IL-2 brought about the activation of antitumor killer cells as well as showing therapeutic efficacy.

Discussion

The results observed in this study reveal that a combination of CY chemotherapy and IL-2 immunotherapy is effective on autochthonous mice in which tumors have been induced by methylcholanthrene (MCA). The mice died of a high incidence of local recurrence and pulmonary metastasis of tumor cells following the surgical removal of the primary tumors. This tumor recurrence and the metastasis model were initially reported by Wexler and Rosenberg.[5] We have previously observed effects of immunotherapy with an immunostimulatory protein-bound polysaccharide (PSK) on local recurrent and metastatic tumors in the autochthonous hosts.[2,3] Only a prolongation of the survival time but no improvement of survival rate among the mice was obtained by the PSK immunotherapy. The combination of CY and IL-2, however, did improve the survival rate of mice as well as prolonging the survival time through the inhibition of pulmonary metastasis following the operation. The inhibition of pulmonary metastasis by the combination of CY and IL-2 was also observed in mice whose primary tumors were not removed (Table II). As we

have discussed in our previous paper,[1] the effects of the combination treatment of CY and IL-2 are thought to be brought about both by the direct cytotoxic action on tumor cells of CY and by its enhancing action on LAK cell accumulation in tumor tissue. The IL-2 immunotherapy alone showed only a slight therapeutic effect on the tumors in autochthonous mice, although the kinetics of antitumor effector activities indicate that NK and LAK precursor activities are strongly enhanced by the administration of IL-2 alone. The CY chemotherapy seems, therefore, to prepare conditions in which the IL-2 immunotherapy has a significant effect on pulmonary metastasis in autochthonous hosts. The benefit of CY chemotherapy in combination with IL-2 immunotherapy has also been reported by others.[6,7] Recently, Mitchell *et al.* have reported similar attempts to use a combination of CY and IL-2 on patients with melanoma.[8] With regard to the timing of the IL-2 administration, it needs to be initiated 5 days after CY chemotherapy, by which time the LAK precursor activity suppressed by CY has been restored.

The combination of CY and IL-2 brought about a cure in only 50% of the autochthonous mice whose primary tumor had been surgically removed, while it brought about complete cure in almost all mice which had been implanted with 3LL tumors, as we have reported previously.[1] The therapeutic effects of the combination of CY and IL-2 are, therefore, still not sufficient in themselves to deal with autochthonous tumors as compared with their effect on transplanted tumors. We can consider the difference of the therapeutic effects on tumors, difference between autochthonous and transplanted tumors as follows.

First, we must take account of the tumor-host relationship in experimental tumor models.[2,3] The tumor-host relationship in autochthonous tumors is established during a long period of tumor development in the host, so that the tumor-host relationship is thought to be "natural." On the other hand, this relationship is artificially established in transplanted tumors by an abrupt transplantation of a large number of tumor cells. It is likely that hosts are able to respond to such abruptly transplanted tumor cells more strongly than to tumor cells which have been growing for a long period in the autochthonous hosts.

Next, we have to consider the heterogeneity of tumor cells.[9–13] We speculate that a primary tumor in the autochthonous host consists of a heterogeneous population of tumor cells, while a transplantable tumor consists of relatively homogeneous tumor cells. For instance, 3LL tumors show a similar response to CY chemotherapy and to IL-2 immunotherapy in individual mice, whereas the autochthonous tumors studied in this work showed a wide range of response among the individuals. We believe that there is no single treatment which is effective against all lines of transplantable tumors. One may fortuitously select a tumor line which happens to be sensitive to a certain therapeutic regimen. It does not necessarily follow, therefore, that a result of experimental therapy obtained by using such a selected tumor line can be used to predict the result of clinical treatment of cancer. Further, since autochthonous tumors consist of heterogenous populations of tumor cells, tumor cells insensitive to the therapy eventually kill the hosts after the treatment, although the treatment can prolong the survival time of the hosts.

In view of these aspects of experimental therapy, it is recommended to use transplantable tumor lines for the initial screening of a therapeutic regimen for cancer treatment. Several reports of experimental treatments of autochthonous tumors in mice[2,3,13–17] and rats[18,19] have been published. It has been believed that the treatment of an autochthonous tumor is more difficult than that of transplanted tumors in syngeneic hosts. Reevaluation of the therapeutics of autochthonous tumors seems to be desirable before any clinical application of an experimental treatment is made. As we have already mentioned, there is a difference in efficacy between the treatment of transplanted and autochthonous tumors. The difference may provide valuable information about possible therapeutic efficacy in clinical trials.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare and from the Ministry of Education, Science and Culture.

(Received July 18, 1988/Accepted Sept. 14, 1988)

REFERENCES

1) Hosokawa, M., Sawamura, Y., Morikage, T., Okada, F., Xu, Z-Y., Morikawa, K., Itoh, K. and Kobayashi, H. Improved therapeutic effects of interleukin 2 after the accumulation of lymphokine activated killer cells in tumor tissue of mice previously treated with cyclophosphamide. *Cancer Immunol. Immunother.*, **26**, 250–256 (1988).
2) Hosokawa, M., Mizukoshi, T., Sugawara, M. and Kobayashi, H. Therapeutic effects of PS-K and busulfan on the recurrent and metastatic diseases after the surgical removal of 3-methylcholanthrene-induced autochthonous tumors in C57BL/6 mice. *Jpn. J. Cancer Res. (Gann)*, **76**, 61–67 (1985).
3) Hosokawa, M., Mizukoshi, T., Morikawa, K., Xu, Z-Y. and Kobayashi, H. The therapeutic effects of an immunopotentiator, PS-K, on 3-methylcholanthrene-induced autochthonous tumors in C57BL/6 mice in combination with the surgical removal of primary sites. *Cancer Immunol. Immunother.*, **22** 181–185 (1986).
4) Devos, R., Plaelinck, G., Cheroute, H., Simons, G., Degrave, W., Tayernier, J., Remaut, E. and Fiers, W. Molecular cloning of human interleukin 2 cDNA and its expression in *E. coli*. *Nucleic Acids Res.*, **11**, 4307–4323 (1983).
5) Wexler, H. and Rosenberg, S. A. Pulmonary metastasis from autochthonous 3-methylcholanthrene-induced murine tumors. *J. Natl. Cancer Inst.*, **63**, 1393–1395 (1976).
6) Nishimura, T., Togashi, Y., Goto, M., Yagi, H., Uchiyama, Y. and Hashimoto, Y. Augmentation of the therapeutic efficacy of adoptive tumor immunotherapy by *in vivo* administration of slowly released recombinant interleukin 2. *Cancer Immunol. Immunother.*, **21**, 12–18 (1986).
7) Papa, M. Z., Yang, J. C., Vetto, J. T., Shiloni, E., Eisenthal, A. and Rosenberg, S. A. Combination effects of chemotherapy and interleukin 2 in the therapy of mice with advanced pulmonary tumors. *Cancer Res.*, **48**, 122–129 (1988).
8) Mitchell, M. S., Kempf, R. A., Harel, W., Shau, H., Boswell, W. D., Lind, S. and Bradley, E. C. Effectiveness and tolerability of low-dose cyclophosphamide and low-dose interleukin-2 in disseminated melanoma. *J. Clin. Oncol.*, **6**, 409–424 (1988).
9) Heppner, G. H., Dexter, D. L. and Miller, F. R. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. *Cancer Res.*, **38**, 3758–3763 (1978).
10) Fidler, I. J. and Kripke, M. L. Tumor cell antigenicity, host immunity, and cancer metastasis. *Cancer Immunol. Immunother.*, **7**, 201–205 (1980).
11) Kerbel, R. S. Implications of immunological heterogeneity of tumors. *Nature*, **280**, 358–360 (1976).
12) Schirrmacher, V. Shift in tumor cell phenotypes induced by signals from the microenvironment. Relevance for the immunobiology of cancer metastasis. *Immunobiology*, **157**, 89–98 (1980).
13) Ishidate, M., Jr. Antigenic specificity of different cell lines of rat ascites hepatoma originating in the same individual. *Gann Monogr. Cancer Res.*, **16**, 195–210 (1974).
14) Kidera, Y. and Baba, T. Blood flow-interrupting hyperthermic chemotherapy on established autochthonous mouse sarcoma induced by 3-methylcholanthrene. *Cancer Res.*, **38**, 556–559 (1987).
15) Tokuzen, R., Okabe, M., Nakahara, W., Azuma, I. and Yamamura, Y. Suppression of autochthonous tumors by mixed implantation with a *Nocardia rubra* cells-wall skeleton and related bacterial fractions. *Gann*, **69**, 19–24 (1978).
16) Ichinose, Y., Yasumoto, K., Tanaka, K., Yaita, H. and Nomoto, K. Combined treatment of autochthonous 3-methylcholanthrene-induced murine tumors by immunotherapy and radiotherapy. *Gann*, **74**, 143–147 (1983).
17) Suga, T., Shino, T., Maeda, Y. Y. and Chihara, G. Antitumor activity of lentinan in murine syngeneic and autochthonous hosts and its suppressive effect on 3-methylcholanthrene-induced carcinogenesis. *Cancer Res.*, **44**, 5132–5137 (1984).
18) Hayakawa, K., Yamashita, T., Suzuki, K., Tomita, K., Hosokawa, M., Kodama, T. and Kobayashi, H. Comparative immunological studies in rats following cryosurgery and surgical excision of 3-methylcholanthrene-induced primary autochthonous tumors. *Gann*, **73**, 462–469 (1982).
19) Nagano, N., Yasumoto, K., Tanaka, K., Ichinose, Y., Yaita, H. and Nomoto, K. Successful regional immunotherapy with cell-wall skeletons of BCG and *Nocardia rubra* against autochthonous rat tumors. *Gann*, **73**, 613–617 (1982).