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Antitumor Effects of 5′-Deoxy-5-fluorouridine in Combination with Recombinant Human Interleukin 2 on Murine Colon Carcinoma 26

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The antitumor activity of recombinant human interleukin 2 (rIL-2) in combination with 5′-deoxy-5-fluorouridine (doxifluridine; 5′-DFUR) against murine colon carcinoma 26 (Colon 26) was studied. BALB/c mice were treated daily for 15 days with 5′-DFUR, rIL-2 or both, beginning on day 7 after subcutaneous transplantation of Colon 26. While mice treated with 5′-DFUR or rIL-2 alone died of tumor growth with pulmonary metastases within 9 weeks posttransplantation, the survival time was significantly prolonged in mice treated with both 5′-DFUR and rIL-2. Most of the combination-treated animals showed the regression of local tumors and the inhibition of pulmonary metastasis. Histopathologically, many tumor cells were degenerated and necrotized, with marked infiltration of mononuclear cells including large granular lymphocytes (LGLs) with periodic acid-Schiff-positive cytoplasmic granules. The cells were positive for CD3ε, asialo GM1 and NK1.1. Spleen cells from the combination-treated mice showed high activities of natural killer (NK) cytotoxicity as well as growth inhibition of Colon 26 and Meth A fibrosarcoma in mice. The results suggest that the combination therapy of 5′-DFUR plus rIL-2 enhanced non-specific cytotoxicity of LGL/NK cells for Colon 26 in tumor-bearing mice and was effective in the inhibition of tumor growth.

Key words: Immunopathology — Recombinant interleukin 2 — 5′-Deoxy-5-fluorouridine — Combined therapy — Murine colon carcinoma 26

Advanced colorectal carcinomas are known to be highly resistant to chemotherapeutic agents.[1,2] 5-Fluorouracil (5-FU) has been a mainstay therapeutic for this cancer, despite showing gastro-intestinal toxicity and myelosuppression, and combination therapy with either leucovorin or interferon (IFN) is recommended to increase the response rate.[3–5] Interleukin 2 (IL-2) can also be used in combination with 5-FU for enhancing antitumor potentiation, including activation of natural killer (NK) cells and induction of lymphokine-activated killer (LAK) cells.[6] However, either 5-FU or IL-2 alone failed to have a significant antitumor effect in patients,[7,8] even in combination with LAK cells.[9–11]

A 5-FU derivative, 5′-deoxy-5-fluorouridine (doxifluridine; 5′-DFUR), was shown to have significant antitumor activity in experimental tumor models,[12–17] as well as in patients with gastric, colorectal and breast cancers.[18–20] In combination with IFN-α, 5′-DFUR was reported to show synergistic antitumor effects in some experimental colon cancers.[21,22] Recently, we found that 5′-DFUR in combination with IL-2 was more effective in animal colon cancer models than other chemotherapeutic agents (unpublished data).

Here we describe the therapeutic effects of 5′-DFUR in combination with IL-2 on a murine colon carcinoma 26 (Colon 26).

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MATERIALS AND METHODS

Animals Eight-week-old female BALB/c mice from Charles River Japan (Atsugi) were kept in a specific pathogen-free environment. They were used at 9 or 10 weeks of age. After subcutaneous (s.c.) tumor transplantation, the maximum and minimum axes of tumors were measured with a digimatic caliper every other day from day 7 to 28, and the body weights of tumor-bearing mice were checked twice weekly. At 4 weeks after tumor transplantation, all the mice were autopsied and spleen and s.c. tumor masses at the injection sites were weighed. The lungs were removed and the numbers of metastatic foci were counted using a dissecting microscope.

Tumor cells Colon 26 was maintained by serial s.c. passages in syngeneic BALB/c mice. For transplantation, tumor masses were minced and digested at room temparature for 60 min in Hanks' balanced salt solution (HBSS) containing 0.4% collagenase type I, 0.2% hyaluronidase and 0.05% DNase (Sigma Chemical Co., St. Louis, MO). Cell suspensions were filtered through a stainless steel mesh, and after having been washed twice in HBSS by centrifugation, cells were suspended in HBSS so as to give 1×10^6 cells/ml. The suspension (0.1 ml) was injected s.c. into the left flank of mice. Murine fibrosarcoma Meth A was maintained by serial passages by intraperitoneal inoculation into BALB/c mice. Murine lymphoma YAC-1 cells were maintained by cul-

ture in RPMI 1640 medium, pH 7.2 (Nissui, Tokyo), supplemented with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (10 m*M*) and fetal calf serum (FCS) (10%).

IL-2 and 5′-DFUR Recombinant human IL-2 (rIL-2), which had a specific activity of 1.01×10^7 Japan Reference Units (JRU)/mg-protein, was provided by the Technology Development Laboratories of this company. Dilutions in a vehicle solution (saline containing 5% normal mouse serum) were administered s.c. daily at a dose of 1, 3, 10 or 20 μg/day. 5′-DFUR purchased from Nippon Roche (Tokyo) was dissolved in saline solution and given orally at a dose of 50 or 100 mg/kg/day. Starting on day 7 posttransplantation, mice were daily treated with rIL-2, 5′-DFUR or both rIL-2 and 5′-DFUR for 2 or 3 weeks.

NK cytotoxicity assay Spleen tissues from tumor-bearing mice were immersed in cold RPMI 1640 medium supplemented with HEPES (10 m*M*) and FCS (2%), and the resulting cell suspensions were filtered through a nylon mesh and then exposed to a hypotonic shock in Gey's buffer, pH 7.2. The cells were washed three times in 10% FCS-RPMI 1640 or serum free-RPMI 1640, and suspended in the same medium. NK cell cytotoxicity was measured in a ^{51}Cr-release assay.[23] Briefly, YAC-1 cells were labeled by incubation with 100 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$; Amersham, Arlington Heights, IL) in 10% FCS-RPMI 1640 for 1 h at 37°C, and they were seeded in U-shaped, 96-well microtiter plates (Nunc, Roskilde, Denmark). Various amounts of effector cell suspensions (100 μl) were added to microtiter wells containing 1×10^4 ^{51}Cr-labeled YAC-1 cells/100 μl. After incubation for 4 h at 37°C, the plates were spun at 800 rpm for 5 min, and the supernatant (100 μl) from each well was counted for radioactivity with a gamma counter. The specific lysis (%) of target YAC-1 cells was calculated as follows:

$$\text{Specific lysis (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous and maximum releases were determined by incubating target cells in medium without or with 100 μl of 1% Triton X-100 (Sigma Chemical), respectively. All experiments were carried out in triplicate.

Tumor-neutralization (TN) assay A modification of Winn's assay[24] was performed to evaluate TN activity against tumor growth. Spleen cell suspension was prepared in serum free-RPMI 1640 so as to contain 2.5 to 5 $\times 10^6$ viable cells/ml. A mixture (0.1 ml) of the spleen cells and either Colon 26 or Meth A cells (100 : 1) was injected s.c. at the abdomen of BALB/c mice. As controls, either a mixture of normal spleen cells and tumor cells, or tumor cells only, were inoculated. Then the mice received rIL-2 (10 μg/day) for consecutive 5 days, and 10 days after the last treatment, local tumor masses were weighed.

Histopathology and immunohistology The local s.c. tumor masses, lungs, thymus, spleen and other organs were fixed with 10% neutral buffered formalin, pH 7.4 or Bouin's solution, and embedded in paraffin. Four-micrometer sections were made and stained with hematoxylin and eosin, and periodic acid-Schiff (PAS) stain. A modified avidin-biotin peroxidase complex (ABC) method was used for observation of the cell surface marker antigens. Briefly, deparaffinized sections were treated overnight at 4°C with hamster antibody to CD3ε, rat antibody to L3T4 or Ly-2, mouse antibody to NK1.1 (clone PK136) (Pharmingen, San Diego, CA) or rabbit antibody to asialo GM1 (Wako Pure Chemical Ind., Osaka). The sections were washed three times with 0.01 *M* phosphate-buffered saline, pH 7.2 (PBS), then incubated at 37°C for 60 min with a 1 : 100 dilution of biotinylated rabbit antibody to rat IgG, goat antibodies to hamster and rabbit IgG, or horse antibody to mouse IgG (Vector Labs., Burlingame, CA). They were again washed with PBS, then incubated with ABC reagent (Vector Labs.) for 30 min and further with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical). All the preparations were counterstained with hematoxylin.

Statistical analysis Tumor weights and the numbers of lung metastatic foci were expressed as means$\pm$standard deviation (mean$\pm$SD) and medians of each group, respectively. The antitumor activity was expressed as the weight ratios of tumors from treated animals to those from the controls (T/C %). The data were analyzed by the application of Dunnett's test.

RESULTS

Inhibitory effects of 5′-DFUR in combination with rIL-2 on Colon 26 growth in mice As presented in Fig. 1, the survival times were longer in the tumor-bearing mice treated with both 5′-DFUR and rIL-2 for 15 consecutive days than in non-treated controls, which had large local s.c. tumors and died with severe metastases in the lungs within 5 weeks posttransplantation. Except for 2 mice dead on day 64, the remaining combination-treated animals survived for 12 weeks or more. Those treated with either 5′-DFUR or rIL-2 alone died with lung metastases in 7 to 9 weeks. On day 28 the local s.c. tumor masses of the 5′-DFUR (50 or 100 mg/kg)- or rIL-2 (10 μg)-treated animals, however, were equivalent in size and weight to those of the non-treated controls (Fig. 2 and Table I).

In mice treated with either 100 mg/kg of 5′-DFUR or 10 μg of rIL-2 alone from days 7 to 21, the numbers of lung metastatic foci were reduced to 9 and 23% (15 and 33 foci of median), respectively, as compared with non-

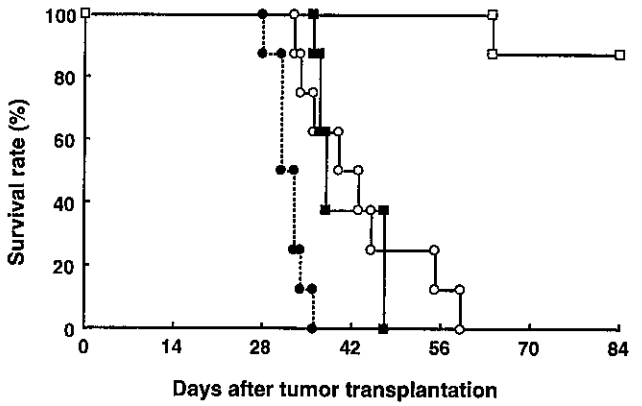

Fig. 1. Survival time of colon carcinoma 26-bearing BALB/c mice treated with 5′-DFUR (100 mg/kg/day), rIL-2 (10 μg/mouse/day), or both, daily from day 7 to 21. ●, non-treated; ■, 5′-DFUR; ○, rIL-2; □, 5′-DFUR+rIL-2.

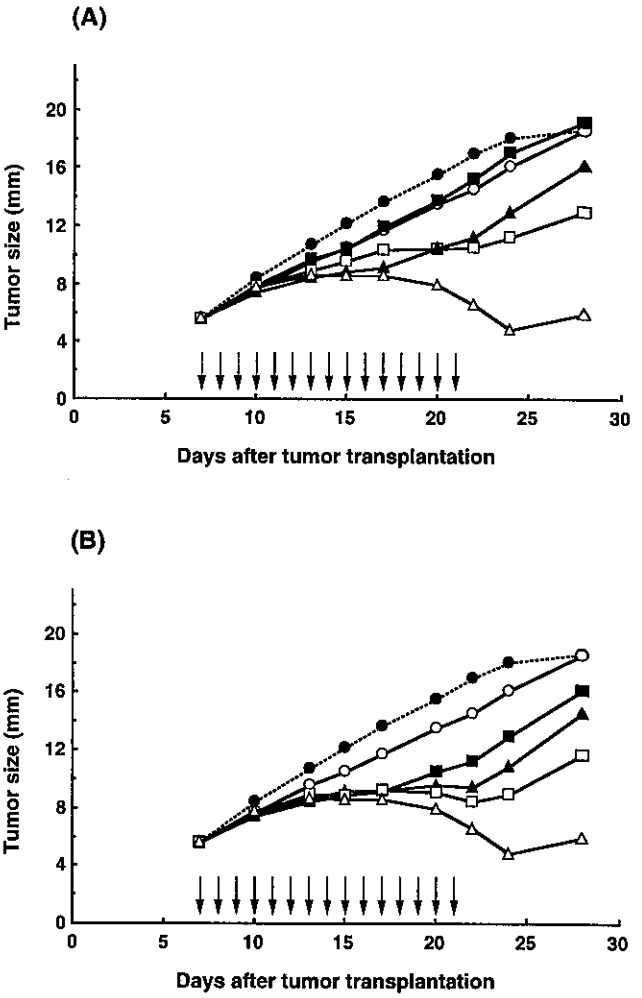

Fig. 2. Growth of colon carcinoma 26 in BALB/c mice treated with 5′-DFUR or/and rIL-2 for 15 consecutive days. A, rIL-2 (10 μg/mouse/day)+5′-DFUR. ●, non-treated; ○, rIL-2; ■, 5′-DFUR (50 mg/kg/day); □, rIL-2+5′-DFUR (50 mg/kg/day); ▲, 5′-DFUR (100 mg/kg/day); △, rIL-2 +5′-DFUR (100 mg/kg/day); B, 5′-DFUR (100 mg/kg/day)+rIL-2. ●, non-treated; ○, rIL-2 (10 μg/mouse/day); ■, 5′-DFUR; ▲, 5′-DFUR+rIL-2 (1 μg/mouse/day); □, 5′-DFUR+rIL-2 (3 μg/mouse/day); △, 5′-DFUR + rIL-2 (10 μg/mouse/day). Each point represents the mean of 5 or 10 mice.

treated controls on day 28. The combination of 50 or 100 mg/kg of 5′-DFUR with 10 μg of rIL-2 for 15 consecutive days showed a greater inhibitory effect on lung metastasis and local tumor growth. With the combination of 100 mg/kg of 5′-DFUR and 3 or 10 μg of rIL-2 for 15 consecutive days, the weights of the local s.c. tumors were 23 and 10% of those in non-treated controls, respectively, on day 28. In mice treated with a daily dose of 100 mg/kg 5′-DFUR plus 10 μg rIL-2, complete regression of the local tumor was observed without lung metastases (Table I and Fig. 2).

In the next experiment, mice were given 100 mg/kg of 5′-DFUR and 20 μg of rIL-2 daily 5 days a week for 2 or 3 weeks, or daily for 15 consecutive days. As shown in Table II, an inhibitory effect on the tumor growth was evident with the combination treatment as compared to single treatment with either 5′-DFUR or rIL-2. The effect was greatest with the combination given for 15 consecutive days, and the consecutive treatment was more effective than that with intervals.

Histopathological observations of Colon 26 in mice treated with 5′-DFUR and rIL-2 The histopathology of the s.c. tumor is summarized in Table III. On day 10 or later, the proliferation of tumor cells was marked in the s.c. tumor in non-treated mice, while a small number of mononuclear cells had infiltrated (Fig. 3a). In mice treated with either 5′-DFUR or rIL-2 alone, the proliferation of tumor cells on days 10, 14 and 17 was less than in those without treatment, showing some degeneration and necrosis (Fig. 3b). The tumor masses had some necrotized foci in cases treated with 5′-DFUR alone, and on day 10 or later, the lung metastatic foci were fewer in number in those treated with either 5′-DFUR or rIL-2 alone. In mice treated with rIL-2, a considerable number of mononuclear cells, including LGLs with PAS-positive granules, apeared in the lesions. On day 14 or later, LGL infiltration was much more intense around degenerated and necrotized foci of tumor cells (Fig. 3c).

In mice treated with both rIL-2 and 5′-DFUR, a small number of tumor cells were degenerated and necrotized as early as day 10, with infiltration of mononuclear cells including LGLs. On day 14 or later, the necrotic foci

Table I. Effect of Combination Treatment with 5′-DFUR and rIL-2 on the Growth of Colon Carcinoma 26 Transplanted in Mice (1)[a]

Group	No. of mice	Treatment		B.W. gain[b] (g)	Tissue weight (mg)			No. of lung foci	(Range)
		5′-DFUR (mg/kg, p.o.)	rIL-2 (μg/mouse, s.c.)		S.c. tumor	(%)	Spleen		
A	10	—	—	−1.0	2318±246[c]	(100)[d]	160±28[c]	182[e]	(166–208)
B	5	50	v[f]	1.4	2352±488	(101)	211±39	113	(48–165)
C	5	100	v	2.5	1164±284*	(50)	173±28	15*	(7–25)
D	5	s[g]	10	2.7	2030±268	(88)	256±44	33*	(11–86)
E	5	50	10	2.7	734±438*	(32)	204±18	0*	(0–1)
F	5	100	1	3.0	966±549*	(42)	189±27	2*	(0–9)
G	5	100	3	2.2	537±381*	(23)	163±23	0*	
H	5	100	10	1.4	227±251*	(10)	187±18	0*	

a) Colon carcinoma 26 was transplanted s.c. into the left abdominal wall of BALB/c mice on day 0, and the animals were treated with 5′-DFUR or/and rIL-2 daily from day 7 to 21. On day 28 they were killed, and the tumor and spleen were weighed and lung metastatic foci were counted.
b) Mean body weight change on day 28 as compared to that on day 7 (from 5 or 10 tumor-bearing mice).
c) Mean±SD from 5 or 10 mice.
d) (Weight of group B–group H/weight of group A)×100.
e) Median from 5 or 10 mice.
f) 5% normal mouse serum in saline, 0.1 ml, s.c.
g) 0.2 ml of saline, p.o.
* Significantly different ($P<0.01$) as compared with group A.

Table II. Effect of Combination Treatment with 5′-DFUR and rIL-2 on the Growth of Colon Carcinoma 26 Transplanted in Mice (2)[a]

Group	Treatment		B.W. gain[b] (g)	S.c. tumor weight (mg)	(%)
	5′-DFUR (100 mg/kg, p.o.)	rIL-2 (20 μg/mouse, s.c.)			
A	—	—	−1.8	2382±331[c]	(100)[d]
B	5 doses, 2w[e]	—	3.8	1900±347	(80)
C	5 doses, 3w[f]	—	3.8	1562±404*	(66)
D	—	5 doses, 3w[f]	4.0	2520±370	(106)
E	5 doses, 3w	5 doses, 3w	3.0	539±184*	(23)
F	—	16 days[g]	3.6	2324±220	(98)
G	5 doses, 2w	16 days	2.1	722±494*	(30)
H	15 days[g]	15 days	2.6	123±147*	(5)

a, b) See footnotes *a* and *b* of Table I.
c) Mean±SD from 5 mice.
d) See footnote *d* of Table I.
e) p.o. on days 7–11 and 15–19 (10 doses).
f) 5′-DFUR or rIL-2 on days 7–11, 14–18 and 21–25 (15 doses).
g) 5′-DFUR or rIL-2 daily from day 7 to 21 or 22 (15 or 16 doses).
* Significantly different ($P<0.01$) as compared with group A.

were enlarged with increased number of LGLs. On days 17 and 22, large numbers of tumor cells were degenerated and necrotized, with extensive infiltration of neutrophils, macrophages, and LGLs, and the PAS-positive granules within LGLs had increased in size and number (Fig. 4a). The LGLs were positive for CD3ε, asialo GM1 and NK1.1 (Fig. 4, b and c). There were no metastatic foci in the lungs.

NK cytotoxicity and anti-tumor growth activity of spleen cells from Colon 26-bearing mice treated with 5′-DFUR and rIL-2 On days 17 and 22 posttransplantation, the NK activity of spleen cells was significantly higher in mice treated with rIL-2 alone and in those treated with 5′-DFUR in combination with rIL-2, as shown in Fig. 5.

Spleen cells from both 5′-DFUR and rIL-2-treated mice inhibited the growth of Colon 26 cells in BALB/c

Table III. Histopathology of Mice Transplanted with Colon Carcinoma 26 and Treated with 5′-DFUR or/and rIL-2

Days post-transplantation	Treatment[a)]	Tumor cell		Infiltration	
		Proliferation	Degeneration and necrosis	LGL	Other cells
Day 10	non-treated	++	−	+	+
	5′-DFUR	+	+	−	−
	rIL-2	++	−	++	+
	5′-DFUR+rIL-2	+	+	++	+
Day 14	non-treated	+++	+	++	+
	5′-DFUR	+	+	+	+
	rIL-2	++	+	++	+
	5′-DFUR+rIL-2	+	+++	+++	+++
Day 17	non-treated	+++	+	++	+
	5′-DFUR	+	++	+	+
	rIL-2	++	+	+++	+
	5′-DFUR+rIL-2	−	+++	+++	+++
Day 22	non-treated	+++	++	++	++
	5′-DFUR	+	++	+	+
	rIL-2	++	++	++	+
	5′-DFUR+rIL-2	−	+++	+++	+++

a) Colon carcinoma 26 was transplanted s.c. into BALB/c mice on day 0, and animals were treated with 5′-DFUR (100 mg/kg, p.o.) or/and rIL-2 (10 μg/mouse, s.c.) daily from day 7 to 21. On days 10, 14, 17 and 22, animals were killed, and s.c. tumors were examined histopathologically.
+, slight; ++, moderate; +++, severe. LGL, large granular lymphocyte.

mice, after s.c. injection of a mixture of the spleen cells and the tumor cells (100 : 1). The s.c. tumor weight was 10% of that of controls on day 14 (Table IV). Additional treatments with 10 μg rIL-2 for 5 days after inoculation of the mixture enhanced the inhibitory effect, and no tumor growth was seen. No significant effect on the tumor growth was observed with spleen cells from Colon 26 tumor-bearing mice treated with either 5′-DFUR or rIL-2 alone.

In addition, spleen cells from mice treated with 5′-DFUR in combination with rIL-2 inhibited the growth of Meth A tumors in mice, and the s.c. tumor weight was 2% of that of controls on day 14.

DISCUSSION

In the present study, a synergistic effect of combined treatment with 5′-DFUR and rIL-2 was evident. This might be due to elevated cytotoxicity of 5′-DFUR, either direct or indirect, and activation of NK and LAK cells stimulated by rIL-2. No adverse effect of the treatment was observed in terms of body weight or diarrhea. There was no severe immunosuppression, such as is seen in the case of 5-FU treatment.

Consecutive treatment with either 5′-DFUR or rIL-2 alone showed insufficient suppression of Colon 26 tumor growth in mice, though pulmonary metastasis was inhibited. Systemic administration of high-dose IL-2 in mice inhibited the subcutaneous growth of tumors[25] and regressed lung and liver metastases.[26] With high-dose bolus injection or low-dose continuous infusion, IL-2 was reported to regress malignant melanoma and renal cell cancers, but colorectal carcinoma did not respond.[9–11] IL-2 is considered to exert indirect antitumor effects mediated by the host immune system, activating the cytotoxicity of monocytes, NK and LAK cells and T cells, which infiltrate into the tumor after consecutive treatments with rIL-2 and affect tumor growth and metastasis.

5-FU, singly or in combination with some modulators,[1–5] generates a slight response in patients with colorectal carcinoma, though the response rates are only 20 to 40%. Some synergistic effects of 5-FU and IL-2 were seen in a few cases of malignant colorectal carcinomas,[7, 8] but side effects were a problem. Also, the combination was not effective for the inhibition of Colon 26 tumor growth (unpublished data).

5′-DFUR, which is intracellularly converted to 5-FU, had greater therapeutic effects than 5-FU on murine tumors,[12–15] xenograft tumors,[16, 17] and human colorectal carcinomas,[18–20] showing much less toxicity than 5-FU and other derivatives in experimental[27, 28] and clinical[18, 19] situations. The activity of pyrimidine nucleoside phosphorylase (PyNPase) for conversion of 5′-DFUR to 5-FU is higher in the primary tumors of digestive organs, as well as mammary glands, than in any other tissues of

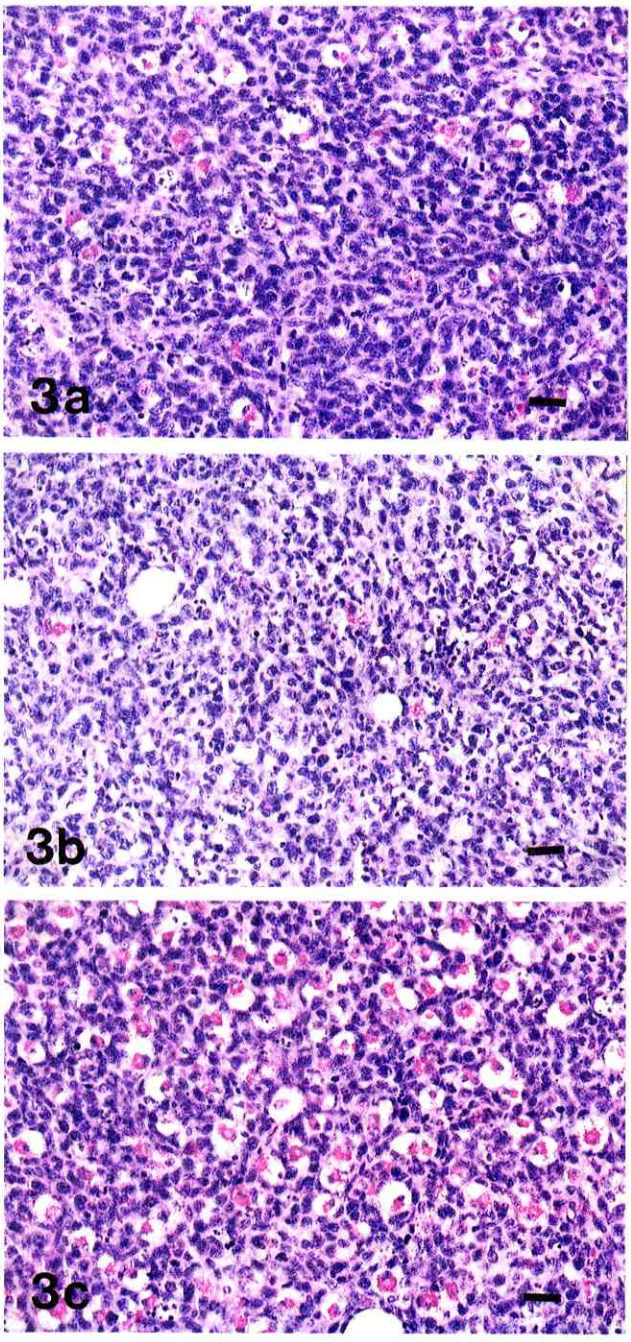

Fig. 3. Histopathology of Colon 26 tumor in BALB/c mice non-treated or treated with 5′-DFUR (100 mg/kg/day) or rIL-2 (10 μg/mouse/day), and killed on day 17. a, Proliferation of tumor cells and infiltration of some LGLs with PAS-positive granules in s.c. tumor in non-treated mouse; b, Necrotized tumor cells with a few infiltrating cells in mouse treated with 5′-DFUR (100 mg/kg/day); c, Increased infiltration of mononuclear cells and LGLs in tumor of mouse treated with rIL-2 (10 μg/mouse/day). PAS-hematoxylin, Bar = 50 μm.

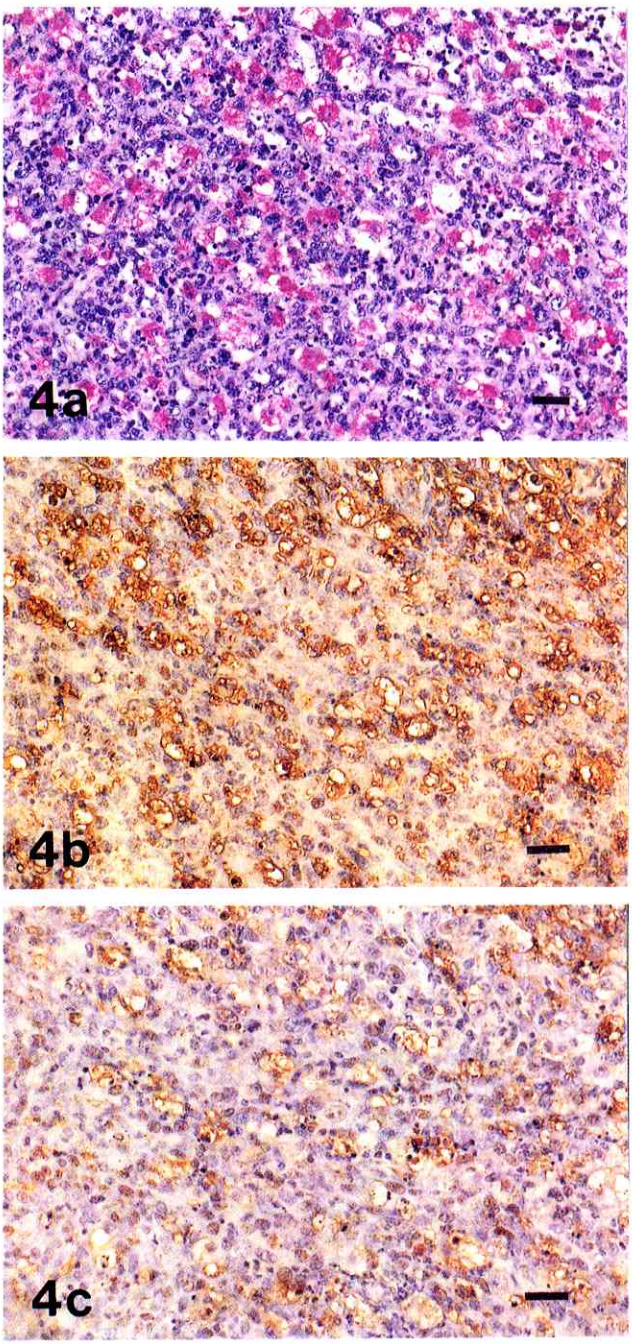

Fig. 4. Histopathology of Colon 26 tumor in BALB/c mice treated with 5′-DFUR (100 mg/kg/day) in combination with rIL-2 (10 μg/day/mouse) and killed on day 17. Degeneration and necrosis of tumor cells with severe infiltration of neutrophils, macrophages and LGLs with many PAS-positive cytoplasmic granules (a), being positive for asialo GM1 (b) and NK1.1 (c). PAS (a)- or ABC (b and c)-hematoxylin, Bar = 50 μm.

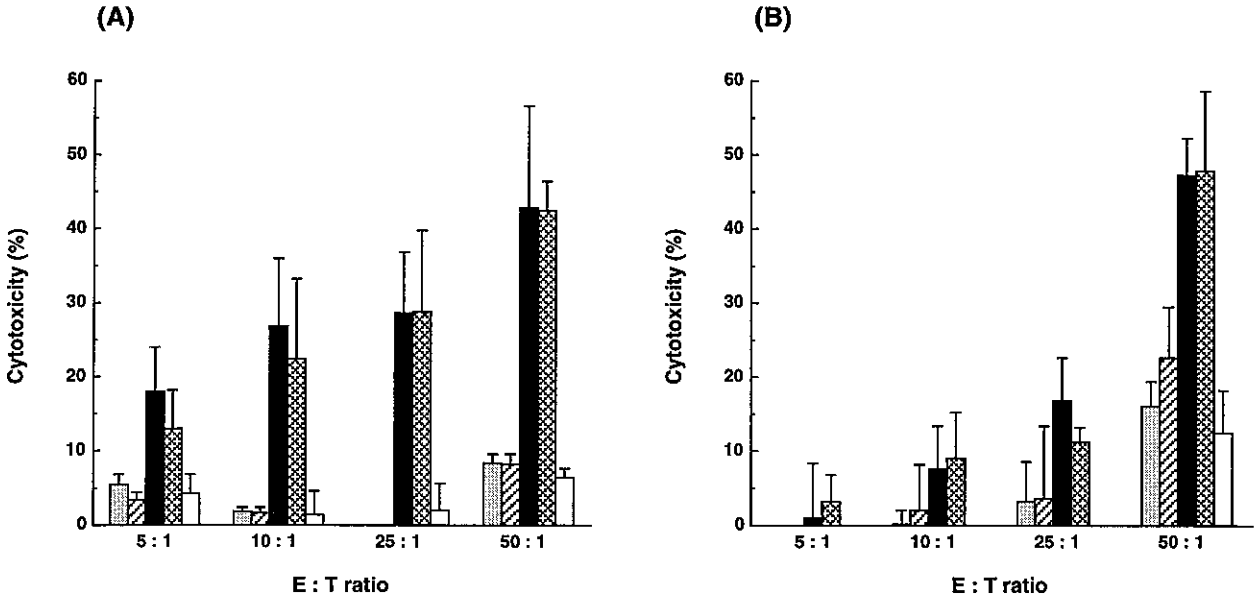

Fig. 5. NK cytotoxicity (%) of spleen cells from Colon 26-bearing BALB/c mice treated with 5′-DFUR (100 mg/kg/day) or/and rIL-2 (20 μg/mouse/day) and killed on days 17 (A) and 22 (B). Mice were transplanted with the tumor on day 0 and received treatment from day 7 to 21. Cytotoxicity was measured in terms of ^{51}Cr-release from labeled YAC-1 cells at 4 h. □, Non-treated; ▨, 5′-DFUR ; ■, rIL-2; ▩, 5′-DFUR+rIL-2; □, Non-tumor-bearing.

human and mice.[16, 29, 30] Colon 26 was reported to have a high level of PyNPase and to be relatively sensitive to 5′-DFUR.[14]

In the present study, treatment with 5′-DFUR alone resulted in delayed growth of the tumor cells with some necrosis, though 5′-DFUR was not able to regress the tumor at the later stage. Upon consecutive treatment with 5′-DFUR in combination with rIL-2, a synergistic antitumor effect on Colon 26-bearing mice was observed without significant toxicity, with regression of the local s.c. tumor and inhibition of pulmonary metastases. The effect of such combination therapy depended on the dose of rIL-2. In the most effective case, the mice were cured and survived for a long period.

Recently, Tomita *et al.*[31] reported increased susceptibility of a human renal cancer cell line to LAK cells after pretreatment with 5-FU. The target tumor cells stimulated with 5-FU were reported to be more sensitive to NK cell-mediated cytotoxicity.[23] After pretreatment with 5-FU, there was an increase in the population of 5-FU-resistant LAK precursors existing in the spleen and bone marrow of mice and rats, as well as in the total number of precursor cells and the lytic activity of IL-2 induced-LAK cells.[32–34] In this study, the combination of 5′-DFUR with IL-2 resulted in accumulation of LGL/NK cells around degenerated and necrotized tumor cells. Spleen cells from the treated mice showed high NK activity and greatly inhibited the growth of Colon 26, whereas those from mice treated with IL-2 alone did not show such an inhibitory effect. 5′-DFUR, which can be accumulated within tumor cells and converted to 5-FU, would increase the susceptibility of the cells to the IL-2-generated cytotoxic activity of NK and LAK cells.

In combination with IFN-αA/D the antitumor activity of 5′-DFUR, as well as 5-FU, for human colon carcinoma was reported to be enhanced *in vitro*[21] and *in vivo*.[22] Murine and human cells were rendered more susceptible to 5′-DFUR in combination with tumor necrosis factor-α, IL-1α and IFN-γ, showing elevated PyNPase activity in tumor cells.[35, 36] In this study, the growth inhibitory effect of 5′-DFUR on Colon 26 was enhanced by rIL-2, although it remains unclear whether IL-2 modulates PyNPase activity or not.

On the other hand, combined chemotherapy using rIL-2 is known to be effective for the recovery of immune reactivity. In the case of combination of 5-FU with rIL-2, IFN-α or both, the cytotoxic and antitumor activities of NK and LAK cells were more marked.[23, 37] In the case of combined treatment with rIL-2 and the optimal dosage of cyclophosphamide, the cytotoxicity of splenic and peripheral NK and LAK cells was significantly enhanced.[38] The present study showed that spleen cells from rIL-2-treated mice had a higher NK cytotoxic activity. In mice treated with both 5′-DFUR and rIL-2,

Table IV. Growth Inhibition of Colon Carcinoma 26 and Meth A Fibrosarcoma Injected into Mice Together with Spleen Cells from Tumor-bearing Mice with or without 5′-DFUR or/and rIL-2 Treatment[a)]

Group	Tumor cell	Spleen cell donor		rIL-2 injection in recipients[b)]	B.W. gain[c)] (g)	Weight of s.c. tumor (mg)	(%)
		Colon 26	Treatment				
1	Colon 26	—[d)]	—	—	1.5	210±38[e)]	(100)[f)]
2		—	—	+	1.5	164±22	(78)
3		non-bearer	—	—	1.3	217±32	(103)
4			—	+	1.2	218±42	(104)
5		tumor-bearer	non-treated	—	1.6	237±25	(113)
6			non-treated	+	1.5	190±25	(90)
7			5′-DFUR	—	1.5	223±31	(106)
8			5′-DFUR	+	1.6	191±35	(91)
9			rIL-2	—	1.3	228±81	(109)
10			rIL-2	+	1.6	176±52	(84)
11			5′-DFUR+rIL-2	—	0.8	20±31*	(10)
12			5′-DFUR+rIL-2	+	1.3	0*	
1	Meth A	—[d)]	—	—	2.1	225±103	(100)
2		—	—	+	2.0	185±35	(82)
3		non-bearer	—	+	1.7	225±54	(100)
4		tumor bearer	non-treated	+	1.7	113±97	(50)
5			5′-DFUR	+	1.7	195±84	(87)
6			rIL-2	+	0.8	70±61*	(31)
7			5′-DFUR+rIL-2	+	1.3	5±6**	(2)

a) Spleen cells from three tumor-bearing or normal mice were mixed with colon carcinoma 26 or Meth A fibrosarcoma cells (100 : 1). The mixture was transplanted s.c. into the left abdominal wall of female BALB/c mice (day 0). On day 14 animals were killed and the tumors were weighed.
b) Injection of rIL-2 (10 μg/mouse, s.c.) from day 0 to 4.
c) B.W. (body weight) gain on day 14 as compared to that on day 0.
d) Mice were injected with tumor cells only.
e) Mean±SD from 3 mice.
f) (Weight of each group/weight of group 1)×100.
* Significantly different at $P<0.05$ as compared to group 1.
** Significantly different at $P<0.01$ as compared to group 1.

splenic cellularity and the cytotoxicity of spleen cells towards Colon 26 were significantly increased. Flow cytometry revealed increased levels of Thy1.2$^+$ and NK1.1$^+$ cells in the spleen of tumor-bearing mice given the combined treatment, while the percentage of CD4$^+$/CD8$^-$ T cells was reduced (data not shown). NK/LAK cells seemed to be generated and their cytotoxicity was significantly augmented in mice after treatment with 5′-DFUR plus rIL-2.

5′-DFUR did not cause severe immunosuppression, such as is observed in the case of 5-FU,[18, 19, 27, 28] suggesting that cytotoxic cells and their precursors were not severely damaged by 5′-DFUR. Spleen cells from Colon 26-bearing mice treated with 5′-DFUR and rIL-2 suppressed the growth of Colon 26 tumors in the recipient mice and the effect was enhanced by additional treatment of the recipients with rIL-2. In addition, spleen cells from mice given the combined therapy also inhibited the growth of Meth A tumor, suggesting that the cytotoxic effect was non-specific.

In mice treated with 5′-DFUR in combination with rIL-2, many tumor cells were injured, with marked infiltration of LGLs, and the accumulation of these cells was greater as compared with animals treated with rIL-2 alone. NK/LAK cell precursors in mice lacked the detectable expression of asialo GM1, and the cells showed the surface antigen and granule formation after exposure to IL-2.[33] In mice treated with 5′-DFUR plus rIL-2, the LGLs were positive for CD3ε, asialo GM1 and NK1.1, and had increased PAS-positive granules. The cells generated by the combined chemotherapy might be further activated NK/LAK cells with high cytotoxicity, and could be effective in growth inhibition of colon cancer.

The present studies revealed that consecutive treatment with 5′-DFUR and rIL-2 was effective in the inhibition of tumor growth by enhancing the cytotoxic function of NK/LAK cells towards the tumor, affording improved therapeutic efficacy and a prolonged survival period of tumor-bearing mice.

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