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Protective effect of an acidic glycoprotein obtained from culture of *Chlorella vulgaris* against myelosuppression by 5-fluorouracil

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Abstract An acidic glycoprotein prepared from a culture of *Chlorella vulgaris* (CVS) was examined for its protective effect on 5-fluorouracil(5FU)-induced myelosuppression and indigenous infection in mice. Subcutaneous administration of CVS greatly reduced the mortality of non-tumor-bearing mice given a high dose of 5FU, and could increase the LD_{50} value of 5FU for these mice. After 5FU treatment, indigenous infection developed probably as a result of the impairment of the host defense system. CVS reduced the incidence of indigenous infections and this effect was attributable to the acceleration of recovery from 5FU-induced myelosuppression. Early recovery of hematopoietic stem cells, or cells responding to interleukin-3 or granulocyte/macrophage-colony-stimulating factor, was especially observed in the bone marrow of CVS-treated mice on days 4–9 after the injection of 5FU. When tumor-bearing mice were given CVS during treatment with 5FU, CVS prolonged the survival of mice without affecting the antitumor activity of 5FU. In addition, CVS was itself shown to exert an antitumor effect. These results suggested that CVS may be beneficial for the alleviation of side-effects in cancer chemotherapy without affecting the antitumor activity of the chemotherapeutic agent.

Key words 5FU · *Chlorella* · Antitumor effect · Hematopoiesis · Indigenous infection

Introduction

Chemotherapeutic agents for cancer are sometimes of limited use, though quite effective for tumor cells, because of side-effects. One of the critical side-effects appears to be leukopenia induced by their suppressive effect on bone marrow hemopoiesis, which results in opportunistic infections, therefore prevention of bone marrow suppression or restoration of impaired hemopoiesis is required to obtain a satisfactory outcome in the cancer chemotherapy.

A hot-water extract (glycoprotein-rich fraction) of *Chlorella vulgaris* (CVE) and the whole cells have been shown to exhibit various immunostimulant activities. The extract exerts an indirect antitumor effect [13, 22, 24] and a protective effect on bacterial [6, 23] and viral [8, 10] infections in murine systems. When the extract was given subcutaneously to mice [14] or orally to rats [7] that had been rendered neutropenic by cyclophosphamide treatment, an accelerated recovery of neutrophils and a restoration of protection against infection with *E. coli* could be observed. These results suggested that a hot-water extract not only activates mature leukocytes but also stimulates hemopoietic stem cells in the bone marrow.

Recently, we have found that a glycoprotein with antitumor activity is released into the culture supernatant of *C. vulgaris* strain CK-22. This active substance, designated as CVS, is an acidic glycoprotein with an approximate molecular mass of 218 kDa and consists of galactose-rich carbohydrate (67.2%) and protein (33.5%). The precise nature and the structure of CVS showing antitumor activity will be reported elsewhere [18].

In the present study, we have examined the protective effect of CVS against myelosuppression induced by 5FU in normal as well as in tumor-bearing mice.

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Materials and methods

Animals

Specific-pathogen-free 7-week-old female mice, CDF1 and BALB/c, were purchased from SLC Inc., Shizuoka, Japan. The mice were housed in polypropylene cages (CLEA Japan Inc., Tokyo) under barrier-sustained conditions with automatically controlled conditions of temperature (23.0 ± 0.5 °C), humidity (55 ± 5%), and light (12 h light:12 h dark).

Tumor

MethA, a methylcholanthrene-induced fibrosarcoma, was maintained in the peritoneal cavity of BALB/c mice. Tumor cells (5×10^6) were injected subcutaneously into the right flank of mice for experiments. Tumor growth was expressed as the product of the longest diameter and the shortest diameter.

Agents

Fluorouracil (5FU; Kyowa Hakko Inc., Tokyo, Japan) was injected intraperitoneally into mice at a lethal dose (500 mg/kg or 550 mg/kg) or a sublethal dose (250 mg/kg).

Preparation of CVS

CVS was prepared from the culture fluid of *Chlorella vulgaris* CK-22 by centrifugation ($6200\times g$ for 30 min), ultrafiltration (10 kDa) and column (100×150 mm) chromatography with Q-Sepharose (Pharmacia Japan, Tokyo) eluted with a linear NaCl gradient (0–400 mM) in 50 mM acetate buffer (pH 5.3) at a flow rate of 150 ml/min. The approximate molecular mass was estimated to be 218 kDa by gel filtration on Sephacryl S-300HR (10×470 mm column, Pharmacia Japan) by comparison with protein standards (Pharmacia Japan). The agent was subcutaneously injected once every other day for a total of six injections during a period of 2 weeks. A dose of 50 mg/kg CVS was used as the standard in most of the experiments.

Bacteria

Mice were anesthetized with nembutal sodium solution, and blood was obtained from the heart. Their livers were removed, and homogenized with Teflon homogenizers. The blood and liver homogenates were diluted tenfold and spread on Bromothymol blue lactose/agar (Nissui, Tokyo, Japan) plates and brain/heart infusion agar (Nissui) plates to detect Enterobacteriaceae and total bacteria respectively.

Assay of hematopoietic progenitor cells

To determine the numbers of viable cells (colony-forming units, cfu) responding to interleukin-3 (IL-3) or granulocyte/macrophage-colony-stimulating factor (GM-CSF), a modification of a previously described in vitro soft-agar culture technique was used [4]. Briefly, 5×10^5 unfractionated bone marrow cells were suspended in 5 ml minimal essential alpha medium (Gibco BRL, Grand Island, N.Y.) containing 0.3% agar (Agar noble; Difco), 10% fetal bovine serum and 20 units of mouse recombinant IL-3 (Genzyme, Boston, Mass.) or mouse recombinant GM-CSF (PharMingen, San Diego, Calif.) as CSF sources, and 1 ml cell suspension was plated into a 35-mm petri dish. On the 5th or 7th day after the culture, colonies (more than 40 cells) were classified as viable cells (cfu) responding to GM-CSF or IL-3 respectively.

Assay of CSF activity

For the assay of CSF activity in the test serum, we measured the soft-agar colony formation as shown above, and test serum was added to 5% in place of IL-3 or GM-CSF. After 7 days of culture at 37 °C in 5% CO_2, the numbers of colonies consisting of more than 40 cells were counted under microscope.

Statistical analysis

The statistical significance of data was determined by Student's *t*-test and the χ^2-test. All *P* values less than 0.05 were taken as significant.

Table 1 Protective effect of *C. vulgaris* glycoprotein (*CVS*) on 5FU-treated mice. CDF1 mice were injected with CVS (50 mg/kg, six times, s.c.) before or after treatment with 5FU (500 mg/kg, i.p.) on day 0. *PBS* phosphate-buffered saline

Treatment	Mortality (dead/tested)	Survival (%)
5FU		
No addition	24/25	4
+ PBS	8/8	0
+ CVS × 6 (days –21 to –7)	13/25*	48
+ CVS × 6 (days –14 to –1)	8/24*	68
+ CVS × 6 (days +1 to +14)	10/10	0

* $P < 0.05$ versus the group given 5FU alone or 5FU and PBS

Results

Protective effect by administration of CVS on 5FU-induced lethality in normal CDF1 mice

Most of the normal CDF1 mice died between 11 and 17 days after administration of a lethal dose of 5FU, the mean survival time being 13.3 days. When mice were subcutaneously given 50 mg/kg CVS before 5FU treatment, a significant increase was observed in the survival rate (Table 1). The percentage survival in the group injected up to 7 days before and the survival of another group injected up to 1 day before were 48% and 75% respectively. This effect was not observed if CVS was administered after the mice had been treated with 5FU (500 mg/kg). No effect was observed in mice injected with phosphate-buffered saline (PBS) six times when compared to nontreated mice, therefore control mice were not injected with PBS in the following experiments.

In the experiment to determine the dose-dependence of the effect of CVS, 550 mg/kg 5FU was employed in order to obtain 100% mortality of the control mice. CVS exerted a dose-dependent effect on the survival of 5FU(550 mg/kg)-treated mice within a range of 50–500 mg/kg (Table 2). Compared with control mice without 5FU treatment, mice treated with 5FU showed a significant weight loss. CVS was also shown to be effective dose-dependently in the

Table 2 Protective effect of the different doses of CVS against the toxicity of 5FU. CDF1 mice were injected with CVS (5–500 mg/kg, six times, s.c.) on days –14 to –1, and treated with 5FU (550 mg/kg, i.p.) on day 0. Mice were observed for 20 days following the 5FU treatment. Body weights are means ± SD

Treatment	Body weight (g) at day 13	Mortality (dead/tested)	Survival (%)
5FU	16.7±0.1**	8/8	0
+ CVS 5 mg/kg	17.7±0.5*, **	6/8	25
+ CVS 50 mg/kg	18.1±2.8**	6/8	25
+ CVS 150 mg/kg	20.1±2.0*	4/8	50
+ CVS 500 mg/kg	21.5±2.3*	0/7	100
None	22.1±1.5		

* $P < 0.005$ versus the group given 5FU alone
** $P < 0.005$ versus the untreated control

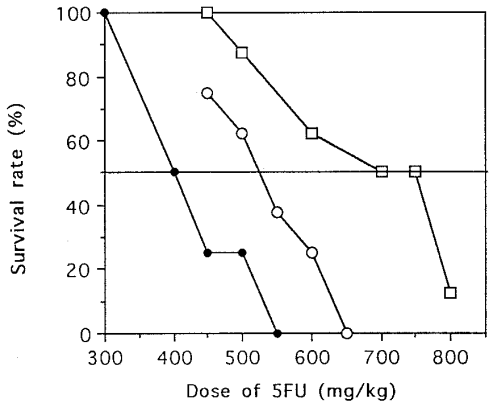

Fig. 1 Effect of *C. vulgaris* glycoprotein (*CVS*) on LD_{50} of 5-fluorouracil (*5FU*). Mice were injected with CVS: 50 mg/kg (❍) or 500 mg/kg (❑) or none (●), and were treated with 5FU at the doses indicated. Survival was recorded daily for 20 days

prevention of 5FU-induced weight loss (Table 2). We found that the survival of mice treated with 50 mg/kg CVS (25%) was lower than that observed in a similar experiment shown in Table 1 (75%). This difference appeared to be attributable to the increase of 5FU dose. In this study, we injected 5FU intraperitoneally to determine the effect of CVS. We also showed that CVS was similarly effective in mice given 5FU via an intravenous route (data not shown).

In order to discover to what extent CVS protects against 5FU-induced lethality, mice were treated with various doses of 5FU after 50 mg/kg or 500 mg/kg CVS had been administered. The 50% lethal dose (LD_{50}) of 5FU increased from 400 mg/kg in a group not given CVS to 520 mg/kg and 700 mg/kg in groups given 50 mg/kg and 500 mg/kg of CVS respectively (Fig. 1).

Protective effect of CVS against indigenous infection

The cause of the lethal effect of irradiation or 5FU treatment is reported to be indigenous bacterial infection [3, 19]. In the 5FU treatment protocol used, we determined the emergence of bacteria in liver and peripheral blood. On day 5 after treatment with 5FU, cultures of liver homogenate and blood did not show bacterial growth. Dissemination of bacteria became evident in the liver on day 8 and in both the liver and blood of all 5FU-treated mice on day 11 (Table 3). In contrast, the emergence of bacteria in the systemic circulation was significantly inhibited (4/8 compared to 8/8) in mice treated with CVS before administration of 5FU. Moreover, the number of bacteria in cases showing positive cultures was much lower in the CVS-treated group compared with the group without CVS treatment. This result indicates that CVS is protective against indigenous infection induced by 5FU treatment.

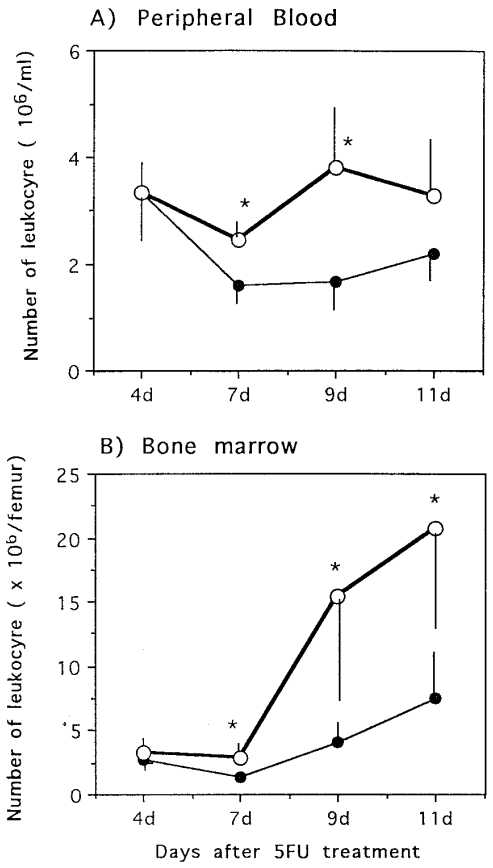

Fig. 2A,B Effect of CVS on the number of leukocytes in peripheral blood (**A**) and bone marrow (**B**) of 5FU-treated mice. CDF1 mice were injected with CVS (50 mg/kg, six times, s.c.) on days −14 to day −1, and were treated with 5FU (250 mg/kg, i.p.) on day 0. ● 5FU-treated mice, ❍ 5FU- and CVS-treated mice. * Significant difference ($P<0.05$) from the group receiving 5FU alone

Effect of CVS on 5FU-induced myelosuppression

5FU impairs hematopoietic stem cells resulting in peripheral leukopenia. The indigenous infection observed in 5FU-treated mice appears to be attributable to leukopenia-related impairment in the host defense system.

Table 3 Protective effect of CVS against 5FU-induced indigenous infection. CDF1 mice were injected with CVS (50 mg/kg, sixtimes, s.c.) on days –14 to –1, and treated with 5FU (550 mg/kg, i.p.) on day 0. Bacterial counts are means ± SD

Treatment	Day 8		Day 11	
	Detected/ tested	Bacterial no. (log_{10})	Detected/ tested	Bacterial no. (log_{10})
Liver				
5FU	8/8	8.31 ± 0.47	8/8	9.68 ± 0.35
+ CVS	3/8	< 3.69 ± 3.23**	7/8	< 6.84 ± 3.12*
Peripheral blood				
5FU	5/8	< 1.99 ± 1.44	8/8	3.20 ± 0.48
+ CVS	3/8	< 1.29 ± 0.57	4/8	< 1.78 ± 1.00**

* P < 0.05 and ** P < 0.01 versus the group given 5FU alone

Table 4 Restorative effect of CVS on progenitor cells in the bone marrow of 5FU-treated mice. CDF1 mice were injected with CVS (50 mg/kg, six times, s.c.) on days –14 to –1, and were treated with 5FU (250 mg/kg, i.p.) on day 0. Results are means ± SD. *IL-3* interleukin-3, *GM-CSF* granulocyte/macrophage-colony-stimulating factor, *NT* not tested

5FU	Day examined	Viable cells (cfu) responding to IL-3/femur		Viable cells (cfu) responding to GM-CSF/femur	
		–CVS	+CVS	–CVS	+CVS
–		4188 ± 541	NT	7378 ± 953	NT
+	4	24 ± 7	83 ± 27**	24 ± 6	50 ± 16*
+	7	235 ± 45	1385 ± 47***	47 ± 9	785 ± 266**
+	9	684 ± 165	3565 ± 2328*	259 ± 6	5805 ± 3790*
+	11	3463 ± 2039	4077 ± 1940	6953 ± 4094	21455 ± 10213*

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus the group given 5FU alone

We have examined the kinetic change of peripheral leukocytes in mice after 5FU treatment. The total number of leukocytes decreased, reaching the lowest level on day 7, after which a gradual recovery was observed (Fig. 2A). The overall kinetics was almost the same in the CVS control group; however, the leukopenia on day 4 was milder and a rapid recovery was observed. No change was observed when CVS was administered to normal mice without 5FU either in the total leukocyte count or in the differential counts (data not shown).

The restorative effect of CVS on hemopoiesis was evident in the bone marrow. Again, the decrease in the number of total marrow cells was less prominent than in the control, and the recovery was more rapid (Fig. 2B).

Restorative effect of CVS on the progenitor cells in the bone marrow of 5FU-treated mice

The preceding results suggest that CVS exhibits a restorative effect on hemopoiesis at the level of progenitor stem cells. Using a colony-forming assay in vitro, we have examined the change of hematopoietic stem cells in the bone marrow of 5FU-treated mice that had or had not received CVS. The number of cells responding to IL-3 by colony formation was decreased by 5FU to 1/200 of the normal level on day 4, but showed a gradual recovery by day 11 (Table 4). The number of colonies responding to IL-3 in CVS-treated mice was 3.4-fold higher on day 4, and a complete recovery was observed as early as day 9. GM-CSF-responding colonies also showed an accelerated recovery following administration of CVS. A recovery of viable cells (cfu) was also observed on day 9 in the spleen of mice pretreated with CVS (data not shown).

The fact that a CVS-mediated early recovery of the number of viable colonies was observed especially on days 4–9 after 5FU treatment implied that this effect was significant in the protection of mice against indigenous infection occurring after day 5 of 5FU treatment.

CSF activity in the sera of mice pretreated with CVS

It is assumed that some factor(s) mediating hemopoiesis is produced in mice treated with CVS. In order to confirm this, CSF activity in the sera of mice was assessed using in vitro soft-agar colony formation by bone marrow cells. A significant level of CSF activity was detected in the sera obtained a few hours after the final injection of 50 mg/kg of CVS (Fig. 3). Though the precise nature of this CSF activity is not known and remains to be determined, this result suggested the induction by CVS of a factor contributing to the accelerated hematopoiesis and recovery after treatment with 5FU.

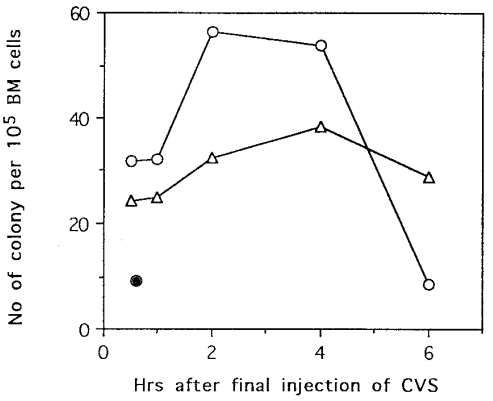

Fig. 3 Colony-stimulating factor activity in serum of CVS-injected mice. CDF1 mice were injected with CVS (50 mg/kg, three or six times), and serum was obtained 0–6 h after the final injection. ● Nontreated mice, △ mice injected three times with CVS, ❍ mice injected six times with CVS

Protective effect of CVS against 5FU-induced side-effects in tumor-bearing mice

It became clear that CVS is highly protective against side-effects of 5FU in normal mice. Since 5FU is not applied to normal hosts but is administered to tumor-bearing hosts as cancer chemotherapy, we next examined whether the observed effect is exerted even in mice bearing the MethA tumor.

When tumor-bearing mice were treated i.p. with 5FU alone on day 14, a significant weight loss was observed (Fig. 4A), which may, in part, be due to 5FU. Subcutaneous

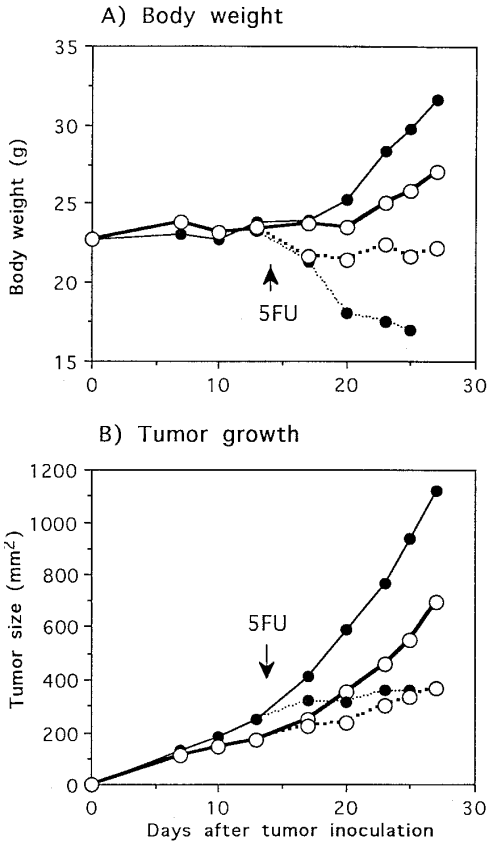

Fig. 4A,B Effect of a combination of CVS and 5FU on body weight (**A**) and tumor growth (**B**) in tumor-bearing mice. MethA cells (5×10^6) were implanted s.c. on day 0. CVS was injected s.c. near the tumor on days 1, 3, 6, 8, 11 and 13. 5FU was injected i.p. at a dose of 250 mg/kg on day 14. –●– Nontreated tumor-bearing mice, –●– 5FU-treated mice, –○– mice given CVS, –○– mice injected with 5FU and CVS. Each group consisted of 12 mice

injections of CVS near the tumor showed a beneficial effect on weight loss after the tumor had been treated with 5FU.

With respect to the antitumor effect (determined by tumor growth) 5FU was superior to CVS (Fig. 4B). The combination of 5FU plus CVS appears to be better than or comparable to the effect of 5FU alone.

An intraperitoneal injection of 5FU at a dose of 250 mg/kg is not lethal in normal mice, but 100% mortality was recorded within 2 weeks after 5FU injection in mice bearing MethA tumor (Fig. 5). The mean survival time in a group of MethA-bearing mice receiving 5FU (23.1 ± 1.2 days) was significantly shorter than that of untreated tumor-bearing mice (29.0 ± 3.5 days). When tumor-bearing mice were given CVS without 5FU treatment, their survival time was prolonged (37.6 ± 6.2 days). Moreover, the combination of CVS and 5FU was highly effective, leading to the longest survival after transfer of MethA tumor (48.5 ± 8.7 days, $P<0.001$).

These results indicate that the injection of CVS is highly effective in promoting the antitumor effects of a chemotherapeutic agent and in reducing its side-effects.

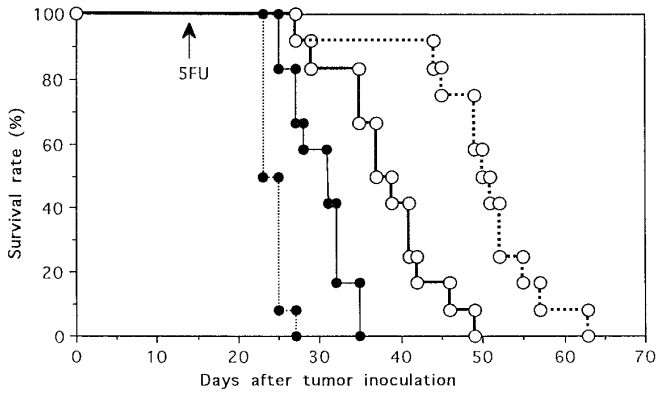

Fig. 5 Effect of combination of CVS and 5FU on the survival in mice bearing MethA tumor. The procedure for the treatment of mice was the same as that described in Fig. 4. Each group showed a significant difference ($P<0.005$) from each of the other group

Discussion

A hot-water extract of *C. vulgaris* has been extensively examined for its action on the number and function of leukocytes and is shown to enhance the protection of rodents against bacterial infection [6–8, 14, 23].

In the present study, we have employed CVS, an acidic galactose-rich glycoprotein prepared and semi-purified from the culture supernatant of *C. vulgaris* CK-22. CVS was shown to reduce the side-effects caused by 5FU not only in normal mice but also in tumor-bearing mice. The protective effects were exhibited against lethality and weight loss resulting in a significant prolongation of survival of the host (Tables 1, 2; Fig. 5).

The indigenous infection following bacterial translocation from the gut appears to be responsible for 5FU-induced lethality [3, 19]. 5FU treatment may cause some damage to a barrier effect of the gut enabling intestinal flora to invade across the intestinal wall. Besides, 5FU-induced suppression of hematopoietic function of the bone marrow should result in a rapid impairment of the host defense system, which depends upon bone-marrow-derived leukocytes. Once intestinal bacteria enter the circulation, leukopenic hosts are no longer capable of coping with the invading bacteria and bacteremia or septicemia will develop. Therefore, it seems critically important to develop any means to avoid bacterial translocation and suppress the growth of invading bacteria.

CVS was effective in reducing the incidence of bacteremia after 5FU treatment and the growth of invading bacteria was also significantly suppressed. Though the mechanism for preventing bacterial translocation in normal gut is not fully understood, various factors have been implicated in the control of bacterial translocation, including enzymatic activity in the gut [1] and the action of stroma-derived growth factor IL-11 on mucosal cells [5]. It is not clear at present what kind of mechanism is operating

in the gut of CVS-treated mice and this remains to be determined.

There is no doubt that CVS-induced accelerated recovery of hematopoiesis plays a crucial role in the prevention of indigenous infection, since it is generally accepted that prevention of marrow dysfunction and recovery of hemopoiesis overcome the development of a septic state after bacterial translocation [2, 20]. The observations of accelerated recovery of stem-cell hemopoiesis (Table 4) and protection against peripheral leukopenia (Fig. 2) suggested that CVS contributed to the prevention of indigenous infection mainly through these effects.

5FU is considered to be a cell-cycle-specific agent in that proliferating cells exhibit greatly enhanced susceptibility in comparison to non-proliferating cells. Bone marrow stem cells are known to be susceptible to 5FU but pluripotent stem cells are not [9]. Pluripotent stem cells are capable of responding to the presence of several factors together: colony-stimulating factor 1 (CSF-1) + IL-3 + stem cell factor (SCF) or CSF-1 + IL-1 + (IL-6 or SCF or GM-CSF) [15], or SCF + (IL-3 or GM-CSF) [25]. In our experiment, the numbers of colony-forming cells responding to IL-3 or GM-CSF only were increased in the bone marrow 4 days after CVS treatment (Table 4). It was possible that CVS accelerated the differentiation of stem cells into the 5FU-resistant stage.

There are many immunostimulants that prevent host damage induced by irradiation or 5FU; i.e., poly(I) poly(C), an interferon inducer [11, 21], lipopolysaccharide-induced IL-1 [17], GM-CSF [16, 25], G-CSF [2, 12, 20], and so on. These reports suggest that some factors or cytokines are involved in the mechanism of action of CVS. So far, in our preliminary study, inflammatory cytokines including IL-1 and TNF have not been detected. Instead, CSF activity was detected in the sera of mice treated with CVS (Fig. 3). Though the precise nature of this CSF activity is not known and remains to be determined, this result suggested that CVS induced the production of factors contributing to the accelerated hematopoiesis and recovery after treatment with 5FU. Since inflammatory cytokines are known to mediate septic shock, the absence of activity to induce these detrimental cytokines might be regarded as being advantageous to the host.

The effect of CVS was expressed in tumor-bearing mice without altering the effect of 5FU used as the therapeutic agent. In addition to CVS preventing the side-effects of 5FU, a direct antitumor activity could also be observed (Fig. 4A). We are convinced that this is not due to a direct cytotoxicity but is mediated by host T cells (unpublished results). In the protocol used in this study, complete tumor regression was not obtained; however, the antitumor activity of CVS may contribute to a complete regression if the timing or dose of CVS administration along with 5FU are changed. The clinical application of CVS may soon be possible to prevent side-effects and support cancer chemotherapy.

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