

Prevention by Chitosan of Myelotoxicity, Gastrointestinal Toxicity and Immunocompetent Organic Toxicity Induced by 5-Fluorouracil without Loss of Antitumor Activity in Mice

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We examined the antitumor activity and side effects (myelotoxicity, immunocompetent organic toxicity and gastrointestinal toxicity) of combined treatment with the cancer chemotherapy drug 5-fluorouracil (5-FU) and dietary fiber chitosan in sarcoma 180-bearing mice. 5-FU (12.5 mg/kg×2/day) plus chitosan (150, 375 and 750 mg/kg×2/day) inhibited the tumor growth as well as 5-FU alone. Chitosan (150 and 750 mg/kg×2/day) blocked the reduction of blood leukocyte number caused by 5-FU administration, and it prevented the injury of the small intestinal mucosa membrane and delayed the onset of diarrhea induced by 5-FU. Furthermore, chitosan (750 mg/kg×2/day) prevented the reduction of spleen weight induced by 5-FU in sarcoma 180-bearing mice, and the reduction of lymphocyte and CD8⁺ T cell numbers induced by 5-FU was also prevented by the oral administration of chitosan (750 mg/kg×2/day) in C57BL/6 mice. Chitosan (150 and/or 750 mg/kg×2/day) reduced the 5-FU incorporation into RNA fractions of small intestine and spleen without affecting the 5-FU incorporation into the tumor in sarcoma 180-bearing mice. These findings suggest that prevention of the 5-FU side effects by chitosan might be partly due to the selective inhibition of 5-FU uptake into the small intestine and spleen, resulting in the reduction of immune function toxicity, myelotoxicity and gastrointestinal toxicity of 5-FU. Therefore, it is concluded that the combination of chitosan and 5-FU might be useful for the prevention of side effects such as gastrointestinal toxicity, immunotoxicity and myelotoxicity caused by 5-FU.

Key words: 5-Fluorouracil — Chitosan — Antitumor activity — Prevention of side effects

Chitin and chitosan are polymers containing more than 5,000 acetylglucosamine and glucosamine units, respectively, and their molecular weights are around one million daltons. Although chitin is widely distributed in natural products such as the protective cuticles of crustaceans and insects, and cell walls of some fungi and microorganisms, it is usually prepared from shells of crabs and shrimp. Alkaline hydrolysis (45% NaOH, 100°C) of chitin converts it to chitosan. Previously, we reported that chitosan reduced the blood pressure elevation caused by NaCl intake, augmented cytolytic activity of mouse lymphocytes and prevented the hyperlipidemia and fatty liver induced by a high-fat diet.^{1–3} Though it has recently been suggested that chitosan might enhance the antitumor activity of cancer chemotherapy drugs and prevent the side effects induced by cancer chemotherapy drugs, these effects are as yet unproven.

5-Fluorouracil (5-FU), which was first synthesized by Duschinsky *et al.*,⁴ has been used extensively in the treatment of certain types of cancer.^{5–8} However, gastrointestinal toxicity and myelotoxicity are induced by the

administration of 5-FU through its phosphorylation in the digestive tract,⁹ and bone marrow tissue.¹⁰ Therefore, to clarify whether chitosan enhances the antitumor activity of 5-FU and prevents the side effects induced by 5-FU, we examined the antitumor activity and side effects, such as myelotoxicity, immunocompetent organic toxicity and gastrointestinal toxicity of combined treatment with chitosan and 5-FU in sarcoma 180-bearing mice.

MATERIALS AND METHODS

Materials Chitosan supplied by Fuji Bio Co. (Shizuoka) was converted to the chloride salt (intrinsic viscosity about 100 cP). The average molecular weight was about 100 to 200 kDa based on the viscosity, and the degree of acetylation was 14%. Chitosan was suspended in distilled water for use in this study. 5-FU was purchased from Wako Pure Chemical Inc. (Osaka). [6-³H]5-FU (specific activity; 462.5 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Mouse lymphocyte separation medium (Lymphocytes-Mouse) was purchased from Dainippon Pharmacy Co., Ltd. (Osaka). Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD8 and phycoerythrin (PE)-labeled anti-mouse NK1.1 were purchased from

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Serotec Ltd. (Oxford, England). Other chemicals were of reagent grade. Sarcoma 180 was maintained in the laboratory of the 2nd Department of Medical Biochemistry, School of Medicine, Ehime University, and used in this study.

Animals Male ICR strain mice (6 weeks old) and male C57BL/6 strain mice (5 weeks old) were obtained from Clea Japan (Osaka) and Charles River Japan (Tokyo). ICR and C57BL/6 mice were housed for 1 week in a room maintained at $25 \pm 1^\circ\text{C}$ with 60% relative humidity and given free access to food and water. The room was illuminated for 12 h per day starting at 7:00 a.m.

Measurement of antitumor activity and side effects of 5-FU in sarcoma 180-bearing mice Solid-type sarcoma 180 was prepared by subcutaneous transplantation of 1.5×10^6 cells into the backs of mice on day 0. 5-FU (12.5 mg/kg body weight) or 5-FU (12.5 mg/kg body weight) plus chitosan (150, 375 and 750 mg/kg) dissolved and suspended, respectively, in distilled water was administered orally twice (7:00 a.m. and 7:00 p.m.) daily for 8 consecutive days, starting 12 h after implantation of tumor cells. Control mice were given distilled water alone on the same schedule. On day 9, blood was obtained by venous puncture under anesthetic with diethylether, and the tumor, small intestine, liver, adipose tissue and spleen were removed and weighed for evaluation of antitumor activity and side effects. The blood samples were chilled in test tubes containing heparin, and the leukocyte numbers were measured using a Coulter Counter (Japan Scientific Instruments Co., Ltd., Tokyo).

Measurement of sucrase activity in small intestinal mucosa membrane in sarcoma 180-bearing mice The small intestine was washed with cold 0.9% NaCl to remove the contents. Then the mucosa was scraped off with a glass slide and homogenized with 80 mM sodium phosphate buffer (pH 7.0) in a final volume of 2 ml using a Polytron homogenizer (Kinematica, Switzerland). Sucrase activity was determined by the methods of Kimura *et al.*^{11,12} Briefly, the assay was performed in a reaction mixture containing the homogenate (50 μl) in 5 mM sucrose, 80 mM sodium phosphate buffer (pH 7.0), in a total volume of 0.5 ml at 37°C for 30 min. The glucose liberated was determined using glucose oxidase reagents.

Measurement of incidence day of diarrhea induced by 5-FU in sarcoma 180-bearing mice Solid-type sarcoma 180 was prepared by subcutaneous transplantation of 1×10^6 cells into the backs of mice on day 0. 5-FU (25 mg/kg body weight) or 5-FU (12.5 mg/kg body weight) plus chitosan (150 and 750 mg/kg body weight) dissolved and suspended, respectively, in distilled water was administered orally twice (7:00 a.m. and 7:00 p.m.) daily until incidence of diarrhea, starting 12 h after implantation of tumor cells. Control mice were given distilled water alone on the same schedule.

Measurement of lymphocyte number and T cell population (CD8⁺ and NK1.1⁺ T cells) in C57BL/6 mice 5-FU (12.5 mg/kg body weight) or 5-FU (12.5 mg/kg body weight) plus chitosan (150 and 750 mg/kg) dissolved and suspended, respectively, in distilled water was administered orally twice (7:00 a.m. and 7:00 p.m.) daily for 7 days. Control mice were given distilled water alone on the same schedule. On day 8, the mice were killed by cervical dislocation and their spleens were quickly removed. The organs were gently teased to release cells by means of dissecting forceps in cold phosphate-buffered saline (PBS, pH 7.4). Five milliliters of the cell suspension was layered on 5 ml of Lympholyte-Mouse and centrifuged at $1,500g$ for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed three times with PBS (pH 7.4). The number of lymphocytes was measured using a Coulter Counter. The cell concentration was adjusted to 2×10^6 cells/100 μl , then 10 μl of FITC-labeled anti-mouse CD8 or PE-labeled anti-mouse NK1.1 was added to 100 μl of the cell suspension. After incubation for 30 min at 4°C , lymphocytes were rinsed three times with 1 ml of PBS and centrifuged at $700g$ for 5 min, and then CD8⁺ and NK1.1⁺ T cell populations were analyzed by flow cytometry using a FACS Calibur (Becton & Dickinson, Mountain View, CA).

Measurement of 5-FU in blood of mice 5-FU (12.5 mg/kg) or 5-FU (12.5 mg/kg) plus chitosan (750 mg/kg) was administered orally to mice. Blood taken by venous puncture under anesthesia 5, 15, 30, 60, 90 and 120 min after the administration of 5-FU or 5-FU plus chitosan, was centrifuged at $1,500g$ for 10 min at 4°C to separate the serum. The serum sample (1 ml) was shaken with 5 ml of chloroform for 10 min. The mixture was centrifuged at $1,500g$ for 10 min at 4°C , and the organic phase was removed. 5-FU in the remaining aqueous layer was extracted twice with 4 ml of ethyl acetate, and the ethyl acetate extract was concentrated at 40°C under a stream of nitrogen gas. Then the residues were dissolved in distilled water, and the 5-FU contents were determined by reverse-phase high-performance liquid chromatography (HPLC) under the following chromatographic conditions; monitoring wavelength, 280 nm; flow rate, 1 ml/min; mobile phase, 5 mM tetrabutylammonium solution containing 2% methanol adjusted to about pH 5 with dilute formic acid.

Measurement of 5-FU incorporation into perchloric acid (PCA)-soluble and RNA fractions of tumor, small intestine and spleen tissues in sarcoma 180-bearing mice [$6\text{-}^3\text{H}$]5-FU (12.5 mg/kg; 18.5 MBq/kg) or [$6\text{-}^3\text{H}$]5-FU (12.5 mg/kg; 18.5 MBq/kg) plus chitosan (150 and 750 mg/kg) was administered to sarcoma 180-bearing mice on day 9 after tumor implantation. One or 4 h later, the mice were killed and their tumor, small intestine and spleen tissues were removed and promptly frozen and stored at -80°C until use. Tumor tissue (500 mg), small

intestine (1.0 g) and spleen (100 mg) were homogenized with 5 volumes of cold 10% PCA and centrifuged at 1,500g for 10 min at 4°C. The 5-FU radioactivity incorporated into the PCA-soluble fraction of tumor, small intestine and spleen, which was due to a mixture of 5-FU and its nucleosides and nucleotides, was measured using a liquid scintillation counter. The radioactivity incorporated into the RNA fraction present in the PCA-precipitable material was extracted using a slight modification of the method of Schneider¹³ for determination of the amount of 5-FU incorporated into RNA.

Measurement of 5-FU phosphorylation Enzyme solutions to be assayed were prepared from sarcoma 180 cells. Tumor cell pellets were homogenized with 4 volumes of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 105,000g for 60 min at 4°C, and the supernatant fraction was used for the assay of 5-FU phosphorylation by the method of Ikenaka *et al.*¹⁴

Statistical analysis and pharmacokinetic parameters All values are expressed as mean±standard error (SE). Statistical analysis was performed with the Dunnett test to determine significance using Super ANOVA Software. Pharmacokinetic parameters were calculated using PK

for Mac ver 2.0 Software (Meiji Seika Pharm. Res. Center, Tokyo).

RESULTS

Combined antitumor activity of 5-FU and chitosan in sarcoma 180-bearing mice Fig. 1 shows the changes in body weights of the groups during the experiments. There was no significant difference between the sarcoma 180-bearing mice (control group), 5-FU-treated group and 5-FU plus chitosan-treated group (Fig. 1). As shown in Fig. 2, 5-FU reduced the tumor weight in sarcoma 180-bearing mice. 5-FU plus chitosan (150, 375 and 750 mg/kg body weight×2 times/day) also inhibited the tumor growth (Fig. 2). However, there was no significant difference between the 5-FU-treated group and 5-FU plus chitosan (150, 375 and 750 mg/kg×2 times/day) group. These results indicate that chitosan did not decrease the antitumor activity of 5-FU.

Effects of chitosan on myelotoxicity and gastrointestinal toxicity induced by 5-FU in sarcoma 180-bearing mice Next, we examined the effects of chitosan on some

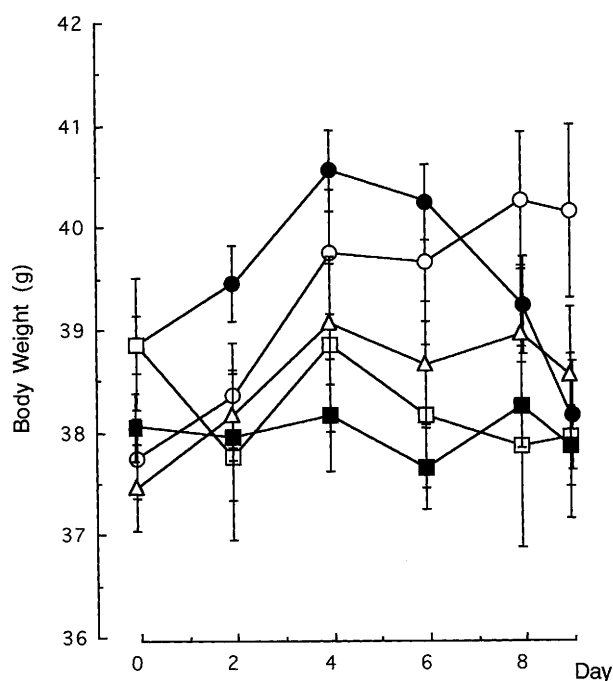


Fig. 1. The combined effects of 5-FU and chitosan on body weight in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. ○ sarcoma 180-bearing mice (control), ● 5-FU (12.5 mg/kg×2/day), □ 5-FU+chitosan (150 mg/kg×2/day), ■ 5-FU+chitosan (375 mg/kg×2/day), △ 5-FU+chitosan (750 mg/kg×2/day).

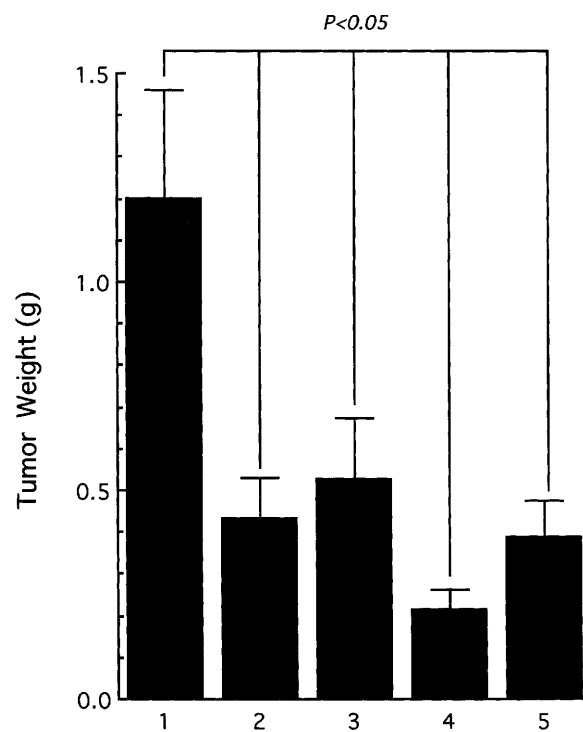


Fig. 2. The combined antitumor activity of 5-FU and chitosan on activity in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. 1, sarcoma 180-bearing mice (control); 2, 5-FU (12.5 mg/kg×2/day); 3, 5-FU+chitosan (150 mg/kg×2/day); 4, 5-FU+chitosan (375 mg/kg×2/day); 5, 5-FU+chitosan (750 mg/kg×2/day).

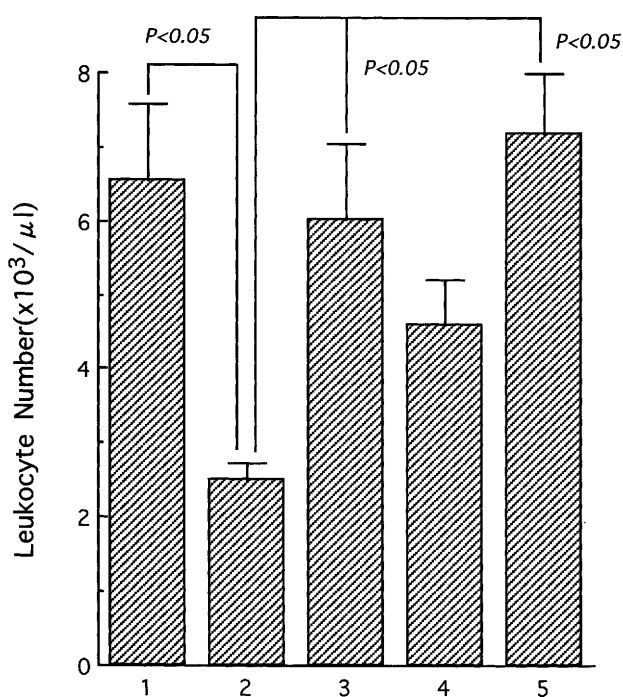


Fig. 3. Inhibitory effect of chitosan on myelotoxicity of 5-FU in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. 1, sarcoma 180-bearing mice (control); 2, 5-FU (12.5 mg/kg×2/day); 3, 5-FU+chitosan (150 mg/kg×2/day); 4, 5-FU+chitosan (375 mg/kg×2/day); 5, 5-FU+chitosan (750 mg/kg×2/day).

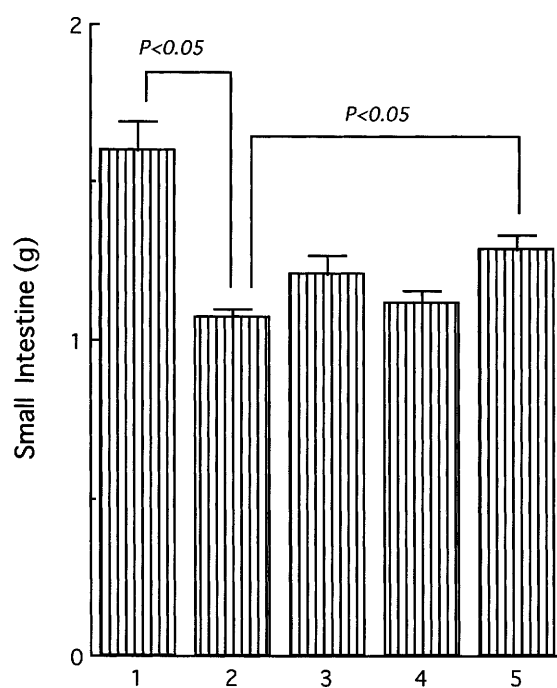


Fig. 4. Inhibitory effect of chitosan on gastrointestinal toxicity (reduction of small intestine weight) of 5-FU in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. 1, sarcoma 180-bearing mice (control); 2, 5-FU (12.5 mg/kg×2/day); 3, 5-FU+chitosan (150 mg/kg×2/day); 4, 5-FU+chitosan (375 mg/kg×2/day); 5, 5-FU+chitosan (750 mg/kg×2/day).

side effects induced by 5-FU: myelotoxicity, gastrointestinal toxicity and reduction of immunocompetence. Treatment with 5-FU reduced the leukocyte number and the weight of the small intestine (Figs. 3 and 4). These facts showed that 5-FU caused myelotoxicity and gastrointestinal toxicity. As shown in Fig. 3, the reduction of leukocyte number caused by 5-FU administration was significantly inhibited by the oral administration of chitosan (150 and 750 mg/kg×2 times/day). The reduction of small intestinal weight caused by 5-FU was also inhibited by chitosan (750 mg/kg×2 times/day) (Fig. 4). Sucrase activity in small intestinal mucosa was reduced by the oral administration of 5-FU in sarcoma 180-bearing mice (Fig. 5), indicating that 5-FU injured the small intestinal membrane mucosa. 5-FU plus chitosan (150, 375 and 750 mg/kg×2 times/day) showed significantly less reduction of sucrase activity in small intestinal mucosa. Furthermore, as shown in Table I, the administration of a 2-fold higher dose (25 mg/kg×2 times/day) of 5-FU caused diarrhea after 5 days. The onset of diarrhea induced by 5-FU (25 mg/kg×2 times/day) was delayed by the oral administration of chitosan (150 and 750 mg/kg×2 times/day) (Table I).

Effects of chitosan on the reduction of immunofunction of spleen induced by 5-FU It has been reported that 5-FU causes immunosuppression and immunotoxicity¹⁵⁻¹⁹ as well as a reduction in the weights of the spleen and thymus. As shown in Fig. 6, the reduction of spleen weight induced by 5-FU (12.5 mg/kg×2 times/day) was inhibited by the oral administration of chitosan (750 mg/kg×2 times/day) in sarcoma 180-bearing ICR mice. Therefore, to clarify the prevention by chitosan of immunofunctional toxicity induced by 5-FU, we investigated the effects of chitosan on the numbers of lymphocytes and CD8⁺ and NK.1.1⁺ T cells in spleen after 5-FU (12.5 mg/kg×2 times/day) administration for 7 days in C57BL/6 mice. As shown in Table II, chitosan (150 and 750 mg/kg×2/day) had no effect on the spleen weight or the numbers of lymphocyte, CD8⁺ and NK.1.1⁺ T cells in C57BL/6 mice. On the other hand, the numbers of lymphocytes and CD8⁺ T cells in spleen were significantly reduced by the administration of 5-FU. The spleen weight and NK.1.1⁺ T cell number also tended to be reduced by the administration of 5-FU, although the reductions were not significant. Chitosan inhibited the reductions of lymphocyte, CD8⁺ T cell

and NK.1.1⁺ T cell numbers induced by 5-FU (Table II). Moreover, the spleen weight and lymphocyte, CD8⁺ and NK.1.1⁺ T cell numbers tended to be increased by chitosan (750 mg/kg×2 times/day), although the increases were not significant (Table II). Further studies are needed to

clarify the effects of 5-FU and 5-FU plus chitosan on tumor growth in tumor-bearing C57BL/6 mice.

5-FU levels in the plasma of mice after oral co-administration of 5-FU and chitosan As shown in Fig. 7, the 5-FU levels in the blood of mice were about 160 and 200

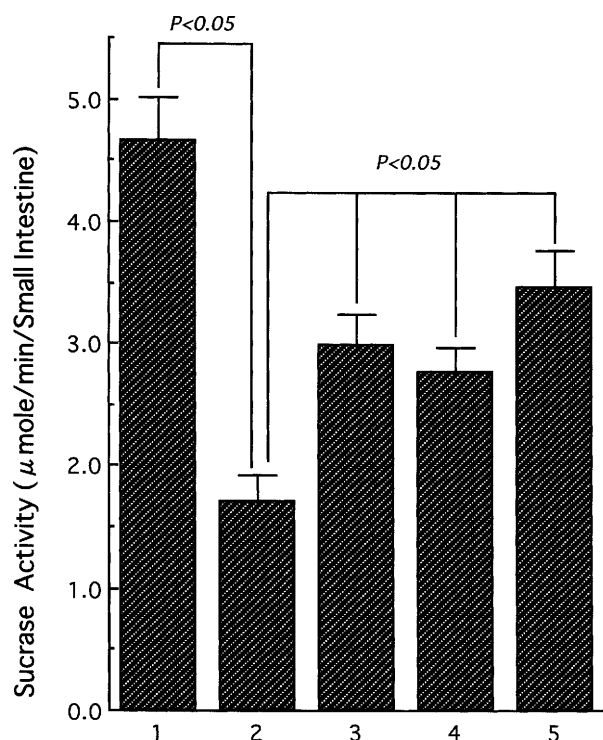


Fig. 5. Inhibitory effects of chitosan on gastrointestinal toxicity (reduction of sucrase activity in small intestinal mucosa) induced by 5-FU in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. 1, sarcoma 180-bearing mice (control); 2, 5-FU (12.5 mg/kg×2/day); 3, 5-FU+chitosan (150 mg/kg×2/day); 4, 5-FU+chitosan (375 mg/kg×2/day); 5, 5-FU + chitosan (750 mg/kg×2/day).

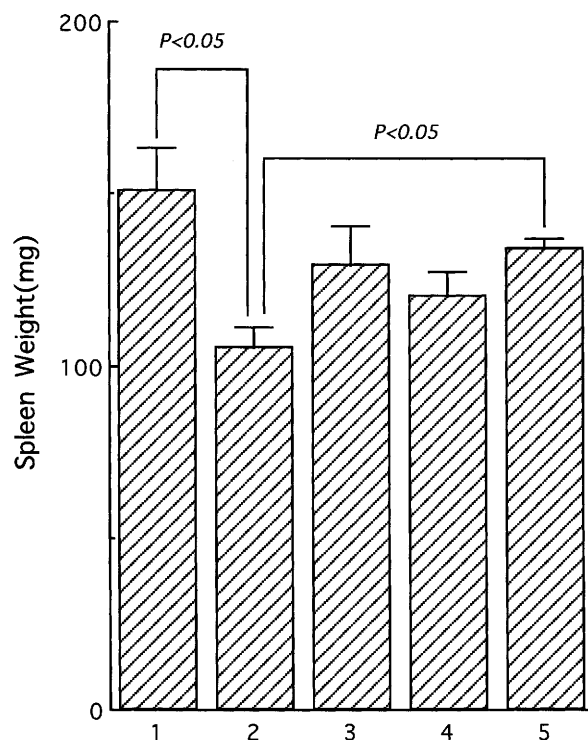


Fig. 6. Inhibitory effects of chitosan on immunocompetent organic toxicity (reduction of spleen weight) induced by 5-FU in sarcoma 180-bearing mice. Results are expressed as mean±SE of 9 mice. 1, sarcoma 180-bearing mice (control); 2, 5-FU (12.5 mg/kg×2/day); 3, 5-FU+chitosan (150 mg/kg×2/day); 4, 5-FU+chitosan (375 mg/kg×2/day); 5, 5-FU+chitosan (750 mg/kg×2/day).

Table I. Inhibitory Effects of Chitosan on Gastrointestinal Toxicity (Incidence of Diarrhea) of 5-FU in Sarcoma 180-bearing Mice

Day	Incidence of diarrhea (number)										
	0	1	2	3	4	5	6	7	8	9	
Sarcoma180-bearing mice (control)	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
5-FU (25.0 mg/kg×2/day)	0/8	0/8	0/8	0/8	0/8	1/8	1/8	5/8	5/8	7/8	
5-FU + chitosan (150 mg/kg×2/day)	0/8	0/8	0/8	0/8	0/8	0/8	0/8	3/8	3/8	7/8	
5-FU + chitosan (750 mg/kg×2/day)	0/8	0/8	0/8	0/8	0/8	0/8	0/8	2/8	2/8	5/8	

Results are expressed as the incidence of diarrhea of 8 mice.

Table II. Combined Effects of 5-FU and Chitosan on the Spleen Weight and the Numbers of Lymphocytes, CD8⁺ and NK1.1⁺ T Cells in Spleens of C57BL/6 Mice

	Spleen (mg) Mean±SE	Spleen cell number (× 10 ⁶ /spleen) Mean±SE		
		Lymphocyte	CD8 ⁺ T cell	NK1.1 ⁺ T cell
Control (1)	63.8±4.28	30.2±1.94*	3.38±0.278*	1.38±0.138
5-FU (12.5 mg/kg×2/day)	54.3±3.21	17.9±1.44	2.12±0.165	0.81±0.096
5-FU + chitosan (150 mg/kg×2/day)	55.4±3.54	23.2±3.81	3.23±0.576	0.97±0.177
5-FU + chitosan (750 mg/kg×2/day)	59.2±3.88	29.4±4.81*	3.75±0.521*	1.41±0.410
Control (2)	67.0±1.92	29.1±4.32	3.52±0.474	1.58±0.206
+ Chitosan (150 mg/kg×2/day)	72.4±1.72	26.9±3.29	2.99±0.346	1.44±0.125
+ Chitosan (750 mg/kg×2/day)	81.3±9.43	40.2±7.84	4.37±0.819	3.00±0.656

Results are expressed as mean±SE of 5 mice. * Significantly different from the administration of 5-FU alone; *P*<0.05.

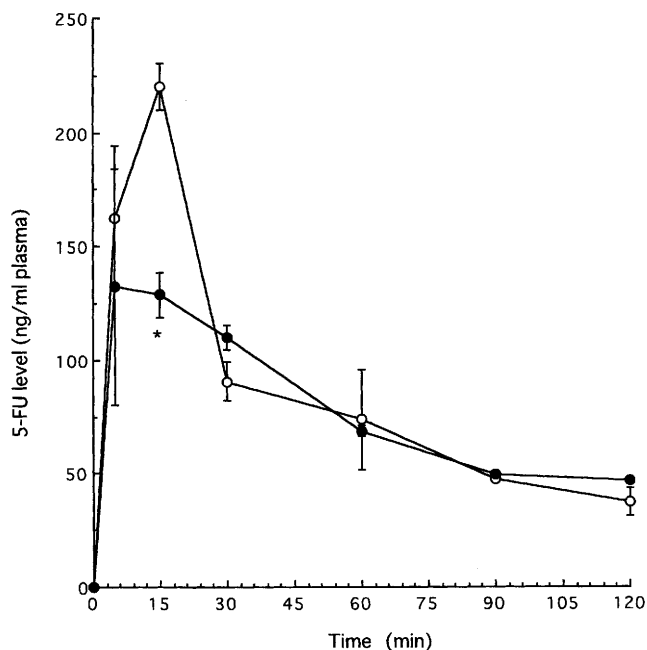


Fig. 7. 5-FU levels in the plasma of mice after oral co-administration of 5-FU and chitosan. Results are expressed as mean±SE of 5 mice. * Significantly different from the administration of 5-FU alone; *P*<0.05. ○ 5-FU (12.5 mg/kg), ● 5-FU+chitosan (750 mg/kg).

ng/ml, respectively, at 5 and 15 min after the oral administration of 5-FU (12.5 mg/kg) and then decreased rapidly. Table II showed that *T*_{1/2}, *T*_{max}, *C*_{max} and AUC (0–120 min) for the 5-FU administration were 30.9 min, 10.1 min,

Table III. Pharmacokinetic Parameters after Co-administration of 5-FU Plus Chitosan in Mice

	Pharmacokinetic parameter			
	<i>T</i> _{1/2} (min)	<i>T</i> _{max} (min)	<i>C</i> _{max} (ng/ml)	AUC (0–120 min) (ng·h/ml)
5-FU (12.5 mg/kg)	30.9	10.1	195.4	168.5
5-FU + chitosan (750 mg/kg)	59.6	7.5	136.4	159.2

Pharmacokinetic values were calculated using PK for Mac ver 2.0 Software (Meiji Seika Pharm. Res. Center).

195.4 ng/ml and 168.5 ng·h/ml, respectively. On the other hand, the 5-FU levels in the blood of mice were 132, 129, 110 and 69 ng/ml, respectively, at 5, 15, 30 and 60 min after the co-administration of 5-FU (12.5 mg/kg) plus chitosan (750 mg/kg) (Fig. 7). The pharmacokinetic parameters, *T*_{1/2}, *T*_{max}, *C*_{max} and AUC (0–120 min), for the 5-FU plus chitosan co-administration were 59.6 min, 7.5 min, 136.4 ng/ml and 159.2 ng·h/ml, respectively (Table III). Thus, *C*_{max} of blood 5-FU levels was reduced by the oral co-administration of 5-FU with chitosan, while the AUC (0–120 min) of blood 5-FU levels was not affected.

Effects of chitosan on 5-FU incorporation into PCA-soluble and RNA fractions of tumor, small intestine and spleen in sarcoma 180-bearing mice As shown in Table IV, chitosan (750 mg/kg) inhibited [³H]5-FU incorporation into PCA-soluble and RNA fractions of spleen and small intestine 1 and 4 h after the oral administration of [³H]5-FU (12.5 mg/kg; 18.5 MBq/kg). On the other hand, the [³H]5-FU incorporation into PCA-soluble and

Table IV. Effects of Chitosan on [$6\text{-}^3\text{H}$]5-FU Incorporation into PCA-soluble and RNA Fractions of Tumor, Small Intestine and Spleen Tissues in Sarcoma 180-bearing Mice

	[$6\text{-}^3\text{H}$]5-FU incorporation					
	Tumor (dpm $\times 10^{-3}$ /g) Mean \pm SE		Small intestine (dpm $\times 10^{-3}$ /g) Mean \pm SE		Spleen (dpm $\times 10^{-3}$ /100 mg) Mean \pm SE	
	PCA fraction	RNA fraction	PCA fraction	RNA fraction	PCA fraction	RNA fraction
1 h						
5-FU (12.5 mg/kg; 18.5 MBq/kg)	266.0 \pm 31.9	5.97 \pm 0.834	289.3 \pm 24.4	22.2 \pm 2.47	29.8 \pm 2.22	0.609 \pm 0.048
5-FU + chitosan (150 mg/kg)	248.6 \pm 30.8	5.74 \pm 1.37	201.6 \pm 9.76*	18.0 \pm 0.992	26.4 \pm 1.07	0.534 \pm 0.034
5-FU + chitosan (750 mg/kg)	212.9 \pm 23.3	6.18 \pm 1.21	166.0 \pm 13.1*	12.6 \pm 1.51*	21.8 \pm 1.44*	0.364 \pm 0.074*
4 h						
5-FU (12.5 mg/kg; 18.5 MBq/kg)	211.6 \pm 17.8	3.82 \pm 0.766	193.1 \pm 6.36	17.9 \pm 0.806	29.2 \pm 2.00	0.551 \pm 0.054
5-FU + chitosan (150 mg/kg)	229.2 \pm 87.1	3.95 \pm 2.41	169.2 \pm 16.7	13.8 \pm 1.95	26.8 \pm 2.24	0.549 \pm 0.033
5-FU + chitosan (750 mg/kg)	222.3 \pm 53.5	3.62 \pm 1.69	177.4 \pm 15.8	14.5 \pm 1.47	19.22 \pm 1.52*	0.378 \pm 0.047*

Results are expressed as mean \pm SE of 5 mice. * Significantly different from the administration of 5-FU alone; $P < 0.05$.

RNA fractions of the tumor was not inhibited by the administration of chitosan (150 and 750 mg/kg) (Table IV). These results suggest that chitosan might prevent the gastrointestinal toxicity and immunocompetent organic toxicity induced by 5-FU without reducing antitumor activity through inhibiting 5-FU incorporation into normal cells, but not into tumor cells.

DISCUSSION

5-FU and its derivatives, such as 5'-DFUR, tegafur and BOF-A₂, have been used extensively in the treatment of certain types of cancer.^{5-8, 20-23} However, there are a number of reports that gastrointestinal toxicity, myelotoxicity and immunotoxicity are induced by the administration of 5-FU through its phosphorylation in the small intestine, bone marrow and spleen.^{9, 10, 16-19} Recently, the clinical application of the combination of 5-FU and a modulator, such as leucovorin, methotrexate or cisplatin, has resulted in relatively high response rates in patients with colorectal cancer, lung cancer and breast cancer.²⁴⁻³⁰ However, in these treatments, severe gastrointestinal toxicity with diarrhea and mucositis and hematologic toxicity with leukopenia appeared to be dose-limiting factors.³¹⁻³⁴ Shirasaka *et al.*³⁵ reported that oxonic acid (5-azaorotic acid) inhibited gastrointestinal toxicity induced by 5-FU and its derivatives without loss of the antitumor activity of 5-FU in Yoshida sarcoma-bearing rats.

Regarding the effects on cancer chemotherapy, the combination of mitomycin C and chitosan derivatives (glycolchitosan and N-succinyl-chitosan) caused the greatest increase in life span at 10 mg of mitomycin C eq/kg in tumor-bearing mice, although the lethally toxic dose of mitomycin C was 20 mg eq/kg.³⁶ In this study, we examined the combined effects of 5-FU and chitosan on tumor growth and side effects in tumor-bearing mice. Chitosan had no direct antitumor activity (data not shown). Chitosan inhibited side effects such as gastrointestinal toxicity (injury to the small intestinal mucosa membrane and the incidence of diarrhea) and myelotoxicity (reduction of blood leukocyte number) induced by 5-FU administration without reducing the antitumor activity of 5-FU. Chitosan had no effect on the phosphorylation of 5-FU in sarcoma 180 cells, small intestine tissue, spleen cells or bone marrow cells *in vitro* (data not shown). Therefore, the prevention of 5-FU-induced gastrointestinal toxicity and myelotoxicity by chitosan could not be explained in terms of the inhibition of 5-FU phosphorylation in small intestine, spleen and bone marrow tissues. Fujii *et al.*³⁷ reported that about 58 ng/ml of 5-FU in the blood is sufficient for antitumor activity (T/C (mean tumor weight in drug-treated animals/mean tumor weight in control animals)=66%) without reducing leukocyte numbers, but levels of 176 ng/ml and over caused loss of body weight and myelotoxicity with a potent antitumor activity ($T/C=2\%$ below), when given by continuous venous infusion

of 5-FU and a 5-FU degradation inhibitor 3-cyano-2,6-dihydropyridine over 24 h for 6 consecutive days in Yoshida sarcoma-bearing rats. Fujii *et al.* suggested that the long-term infusion at a low dose of 5-FU with a 5-FU degradation inhibitor, which maintained a level of about 150 to 300 ng/ml of 5-FU in the blood, has a potent antitumor activity, greater than that of bolus injection or infusion at a high dose over a short period. However, myelotoxicity and gastrointestinal toxicity were caused by this system. Caballero *et al.*³⁸⁾ reported that nausea, vomiting, myelosuppression, and alopecia were not observed on long-term continuous intravenous infusion of 5-FU alone for 54–324 days. In this study, C_{\max} of 5-FU in the blood was reduced by the co-administration of 5-FU plus chitosan, but the AUC was not, as compared to that of 5-FU alone. The range of about 70–130 ng/ml of the 5-FU in the blood was maintained until 60 min after the oral co-administration of 5-FU plus chitosan. There are a number of reports that chitosan can be used as an absorption enhancer of drugs.^{39–42)} Sugimoto *et al.*⁴³⁾ reported that ampicillin absorption by poly(vinyl alcohol)-gel spheres was enhanced by the chitosan combination, and that poly(vinyl alcohol)-gel spheres prepared with chitosan prolonged the small intestinal transit time more than poly(vinyl alcohol)-gel spheres. Singh and Udupa⁴⁴⁾ reported that the antitumor activity in Ehrlich ascites tumor-bearing mice given methotrexate-loaded chitosan microspheres was better when compared with plain methotrexate on oral administration, and the plasma methotrexate levels were more sustained. Furthermore, it has been reported that the release rate of the drug from microspheres prepared with high-molecular-weight chitosan was slow in comparison with that prepared from medium- and low-molecular-weight chitosan.⁴⁵⁾ It seems likely that 5-FU may be adsorbed on the cationic polymer chitosan and consequently, the release rate of 5-FU from 5-FU-chitosan complex is slow so that the small intestinal transit time is prolonged, compared with 5-FU administration alone. Therefore, the blood level of 5-FU after co-administration of 5-FU plus chitosan is lower than that after the 5-FU administration alone in the first 30 min. On the basis of these facts, this result suggests that the maintenance of a lower concentration of 5-FU after the co-administration of 5-FU plus chitosan prevented side effects such as myelotoxicity and gastrointestinal toxicity induced by 5-FU alone. Furthermore, chitosan reduced the 5-FU incorporation into PCA-soluble and RNA fractions of small intestine and spleen tissues without affecting the 5-FU incorporation into tumor tissues. These results suggest that the effects of chitosan might be due to selective inhibition

of 5-FU uptake into small intestine and spleen tissues. It has been reported that 5-FU causes immunosuppression and immunotoxicity^{15–19)} together with a reduction of the spleen and thymus weights. We reported that chitosan enhances the natural killer (NK) activity of mouse lymphocytes *in vitro*.¹⁾ Lim *et al.*⁴⁶⁾ reported that the administration of chitosan reduced serum immunoglobulin (Ig) A and IgE concentrations, and increased interferon (IFN)- γ and tumor necrosis factor α production induced by concanavalin A in isolated mesenteric lymph node lymphocytes. Shibata *et al.*⁴⁷⁾ reported that C57BL/6 mice pretreated with monoclonal antibodies against mouse IFN- γ or NK1.1 showed a markedly decreased level of alveolar macrophage priming following injection of chitin particles. They suggested that the alveolar macrophage priming mechanisms were due to direct activation of macrophages by IFN- γ , which is produced by NK1.1⁺ and CD4⁻ T cells in the spleen. Thus, it is suggested that chitosan may act as an immunomodulator in the spleen and intestinal immune systems of animals. In the present study, it was found that 5-FU reduced the numbers of lymphocytes, CD8⁺ and NK1.1⁺ T cells of the spleen in C57BL/6 mice. The co-administration of 5-FU and chitosan prevented the reductions of lymphocyte and CD8⁺ T cell numbers in spleen, and consequently chitosan prevented the reduction of immune function induced by 5-FU. Furthermore, spleen weight and lymphocyte, CD8⁺ and NK1.1⁺ T cell numbers tended to be increased by the oral administration of chitosan (750 mg/kg \times 2 times/day), although the increases were not significant. These effects of chitosan might be partly due to the selective inhibition of 5-FU uptake into spleen tissue and/or the prevention of 5-FU-induced immunotoxicity by immunomodulator action of chitosan. We conclude that the combination of chitosan with 5-FU or its derivatives might be useful for the prevention of gastrointestinal toxicity, immunotoxicity and myelotoxicity caused by 5-FU and its derivatives. Experiments are now in progress to examine the clinical usefulness in cancer chemotherapy of the administration of chitosan together with 5-FU and its derivatives.

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