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Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): Resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes

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Summary. *When a hot water extract of Chlorella vulgaris (CE) was injected into the peritoneal cavity of BALB/c mice inoculated with syngeneic Meth-A tumor cells, the survival times were strikingly prolonged. Furthermore, peritoneal exudate cells (PEC) rich in polymorphonuclear cells (PMN) obtained from normal mice 24 h after CE injection exhibited an antitumor effect in a Winn-type assay using normal recipients. Such an activity of PEC remained almost intact after T cell or macrophage depletion. However, such PEC did not express an antitumor effect in a Winn-type assay using irradiated recipients. It was suggested that CE-induced PEC, presumably PMN, expressed an antitumor effect in cooperation with a host- or recipient-derived element(s) sensitive to irradiation. The antitumor mechanism of CE may be different from that of OK-432, one of the biological response modifiers.*

Introduction

Many kinds of substances have been reported as factors in the induction of antitumor effects, each of which depends upon a different mechanism [4, 7, 19]. Macrophages [11], NK cells [26], T cells [18], antibodies [1], complement [12, 19], and interferon [9, 26] have been generally accepted as important elements in such resistance. Also, it has been reported that polymorphonuclear cells (PMN) participate in such an antitumor resistance in vivo [5, 8, 13, 21] and in vitro [5, 6, 8, 16], although details of the mechanisms have remained unclear. *Chlorella,* a unicellular green alga, has been found to contain highly nutritious substances and to exert various biological effects [15, 20, 22]. We have reported elsewhere that a hot water extract from *Chlorella vulgaris* (CE) exhibited an antitumor effect against Meth A tumor following intratumor injection [23].

In the present study, CE exerted an antitumor effect after injection into the peritoneal cavity of animals inoculated with tumor cells. PMN appear to be involved in the development of such an antitumor effect, as described below.

Materials and methods

Animals. Female mice of an inbred BALB/c strain were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and were used at 8 weeks of age.

Tumors. Meth A, methylcholanthrene-induced fibrosarcoma of BALB/c origin, was maintained by weekly peritoneal passage in BALB/c mice.

Preparation of a hot water extract of Chlorella vulgaris. Dried *Chlorella vulgaris* cells (produced by Chlorella Ind. Co. Ltd) were suspended in distilled water at a concentration of 10% (w/v), boiled at 100° C for 20 min, and centrifuged at 10.000 rpm for 20 min. The supernatant was lyophilized. The dried *Chlorella vulgaris* extract (CE) was dissolved in phosphate-buffered saline (PBS) before injection.

OK-432. OK-432 (Chugai Pharmaceutical Co., Ltd, Tokyo) containing 0.1 mg dried group A *Streptococci* (Su strain) per clinical unit (KE) was donated by the company.

Preparation of peritoneal exudate cells. Mice were injected IP with saline, 200 mg/kg CE, 1 ml 0.2%–12% casein, or 2–50 KE/kg OK-432 to induce PEC. PEC were harvested from these mice with PBS containing 3 U heparin/ml. The cells were collected by centrifugation at 110 *g* for 10 min, washed twice with PBS, and counted with a hemocytometer. Smear specimens were stained with Giemsa solution to determine the numbers of PMN, lymphocytes, and macrophages.

Treatments of PEC in vitro. Anti-Thy 1.2 treatment. PEC were incubated at 10° C for 30 min at 1×10^7 cells/ml in a 1 : 1,600 dilution of anti-Thy 1.2 antibody supplied by Becton Dickinson. The cells were washed in PBS and incubated at the same concentration in a 1 : 3 dilution of guinea pig serum for 30 min at 37° C. After a further wash with PBS the cells were resuspended for injection.

Removal of adherent cells. PEC were suspended in PBS at a concentration 2–3 million cells/ml and 5–6 ml of this suspension was incubated at 37° C for 1 h in plastic dishes. After incubation, the nonadherent cells were collected, centrifuged at 150 *g* for 8 min, and adjusted to the required concentration with PBS.

Carrageenan treatment [14]. PEC were incubated with 400 μg/ml carrageenan (type IV) as final concentration at 37° C for 4 h, and then repeatedly washed. These conditions have previously been shown to selectively inactivate macrophages without obvious impairment of lymphocyte function [17].

X-Irradiation of recipient mice. Mice were exposed to 5 Gy of whole-body X-irradiation 1 day before inoculation of the

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mixture of PEC and tumor cells. The irradiation was delivered from a Shimazu 250 kV machine operating at 200 kV with 0.3 mm Cu and 1 mm Al filtration at 100 cm from the target focus.

Tumor growth inhibition tests in vivo. BALB/c mice were inoculated IP with 3×10^4 Meth-A tumor cells. CE was administered to the mice IP every other day, on three occasions starting 6 days before, and/or on five occasions after the tumor inoculation. The survival times of the tumor-inoculated mice were measured. In a Winn-type assay, the mixtures of 5×10^4 Meth-A tumor cells and 5×10^5 PEC, unless noted otherwise, were injected SC to the normal or irradiated mice [25]. The tumor growth inhibition was evaluated by measuring the longest diameter and the shortest diameter of developing tumors.

Results

Antitumor effect of CE against IP-inoculated Meth-A tumor

Effects of CE injected IP several times on the life span of mice bearing IP-inoculated Meth-A tumors were observed. Mice were inoculated IP with 3×10^4 Meth-A cells on day 0, and were injected with 50 mg/kg CE three times before tumor inoculation, on days −6, −4, and −2, and/or five times after tumor inoculation, on days 2, 4, 6, 8, and 10. As shown in Fig. 1, the survival periods of tumor-bearing mice were obviously prolonged after CE injections administered on five occasions all after the tumor inoculation, and 14 of 27 mice survived for 60 days after the tumor inoculation. Such an effect was reinforced by CE injections given on eight occasions distributed over a period both before and after the tumor inoculation, and 26 of 28 mice survived at 60 days after inoculation. Survival times were not, however, extended by CE injections given exclusively before the tumor inoculation compared with a control group. There was a dose-dependent effect on the survival periods when CE injections were started before and continued after tumor inoculation (Fig. 2). This was the most effective injection schedule.

Effects of IP injection of CE on cellular accumulation in the peritoneal cavity

Thus, an obvious antitumor effect of CE was observed following IP injections to BALB/c mice bearing IP-inoculated tumor (Figs. 1 and 2) and following intratumor injection to CDF1 mice bearing SC-inoculated tumor [23], while systemic injections of CE were not so effective (data were not shown). Several possible explanations might be considered for these results, viz. the induction of factors (cells) suppressive to tumor growth at the CE-injected site, the induction of a microenvironment capable of resisting a tumor establishment at the CE-injected site, and the direct killing effect of CE on tumor cells. First, we examined the populations of PEC after an IP injection of 200 mg/kg CE to normal mice, to check on the possibility that a host-mediated mechanism was involved. PMN increased significantly 24 h after CE injection (Table 1), while the total number pf PEC was decreased. This high PMN number was maintained up to day 3. The total number of macrophages was decreased at 1 day and substantially increased at 3 and 5 days. The total number of lymphocytes was decreased at 1 and 2 days and had recovered approximately to the normal level at 3 days.

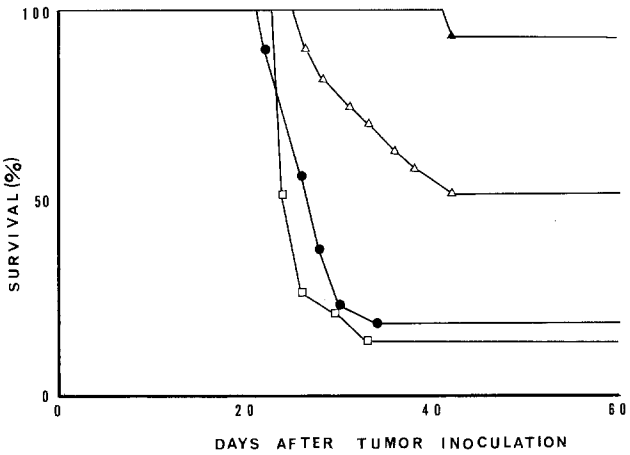

Fig. 1. Effects of various administration patterns of CE on the life span of mice inoculated IP with 3×10^4 Meth-A cells on day 0. Mice were injected IP with 50 mg/kg of CE on three occasions before tumor inoculation, on days −6, −4, and −2 (□———□), on five occasions after tumor inoculation, on days 2, 4, 6, 8, and 10 (△———△), or on eight occasions, some before and some after tumor inoculation, on days −6, −4, −2, 2, 4, 6, 8, and 10 (▲———▲), or with saline on eight occasions, some before and some after tumor inoculation on days −6, −4, −2, 2, 4, 6, 8, and 10 (●———●). The experiment was carried out with 27 or 28 mice per group. The CE-injected group treated exclusively after the tumor inoculation and the one treated both before and after tumor inoculation were different from saline group at the $\alpha = 0.05$ level according to the Mann-Whitney U-test

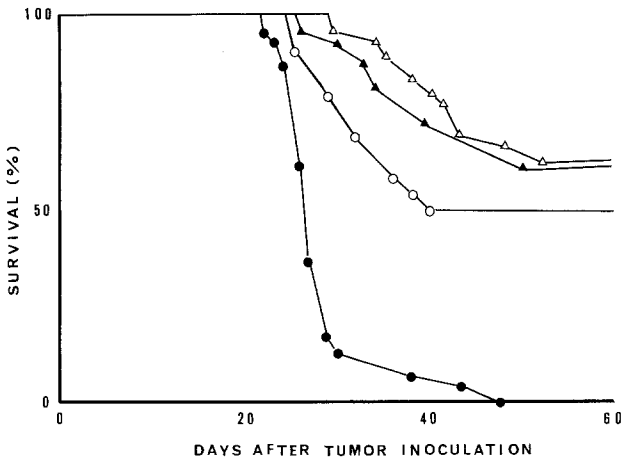

Fig. 2. Effects of various doses of CE on the survival of BALB/c mice inoculated IP with 3×10^4 Meth-A cells. Mice received eight IP injections of CE 10 mg/kg (○———○), 50 mg/kg (▲———▲), or 150 mg/kg (△———△) or saline as control (●———●), timed every other day starting from 6 days before tumor inoculation. The experiment was carried out with 30 animals per group. All CE-injected groups were different from saline group at the $\alpha = 0.05$ level according to the Mann-Whitney U-test

Antitumor activity of CE-induced PEC in an in vivo Winn-type assay

An antitumor activity of CE-induced PEC obtained from normal mice was examined. In a Winn-type assay, 5×10^4 cells of Meth A were mixed with 5×10^5 PEC, which were collected at various times after CE injection, and the mixtures were inoculated SC to normal recipients. A suppressive effect on tumor growth was observed in PEC obtained as early as 6 h and 24 h after CE injection (Table 2). Such an activity was not detected in PEC obtained 2, 3, 5, or 9 days after a single

Table 1. Effects of an IP injection of CE on peritoneal exudate cell accumulation and its components

Days after injection of CE	Number of PEC[a] per mouse ($\times 10^6$)	Number and percentage of each cell type					
		PMN		Macrophages		Lymphocytes	
		$\times 10^6$	%	$\times 10^6$	%	$\times 10^6$	%
–	3.84	0.13	(3.3)	2.83	(73.8)	0.88	(22.9)
1	1.44	0.74	(51.5)	0.64	(44.7)	0.06	(3.8)
2	2.04	0.87	(42.6)	1.09	(53.4)	0.08	(3.8)
3	10.24	0.59	(5.8)	8.78	(85.7)	0.87	(8.5)
5	7.83	0.32	(4.1)	6.67	(85.2)	0.84	(10.7)

[a] PEC were collected on the days indicated after IP injection of 200 mg/kg CE to the normal mice. Collected PEC from five mice were pooled and the total number and types of accumulating cells determined; cell numbers are expressed per mouse

Table 2. Antitumor effect of PEC collected from mice at various times after IP injection of CE in a Winn-type assay[a]

PEC	Number of mice	Tumor size (mm^2)		
		11 days[b]	16 days	20 days
–	11	29.2 ± 6.3[c]	63.2 ± 19.1	133.3 ± 32.4
Normal	8	26.3 ± 8.8	53.1 ± 24.0	106.3 ± 44.2
Saline	8	26.8 ± 7.0	40.1 ± 19.6	88.8 ± 44.9
CE 6 h	8	5.6 ± 10.5[d]	9.4 ± 17.8[d]	12.7 ± 23.5[d]
CE 1 d	8	2.0 ± 4.3[d]	6.5 ± 8.3[d]	10.9 ± 16.2[d]
CE 2 d	8	26.8 ± 12.3	53.4 ± 39.6	93.0 ± 68.2
CE 3 d	8	23.7 ± 8.9	49.2 ± 17.6	98.8 ± 53.8
CE 5 d	5	30.0 ± 13.6	56.5 ± 26.9	105.0 ± 51.2
CE 9 d	5	29.9 ± 17.7	46.0 ± 27.1	123.9 ± 99.6

[a] PEC were collected from mice 24 h after IP injection of saline or at the times indicated after an injection of 200 mg/kg CE. Normal PEC were collected from nontreated normal mice. The mixture of 5×10^4 cells of Meth A and 5×10^5 of PEC was injected SC into the right flank of normal recipient mice
[b] Days after tumor inoculation
[c] Values are means ± SD
[d] Significant at $P < 0.01$ (Student's *t*-test)

Table 3. Effects of various ratios of CE-induced PEC to tumor cells on tumor growth in Winn-type assay[a]

PEC	E/T[b]	Tumor size (mm^2)	
		10 days[c]	22 days
–	0 : 1	26.0 ± 6.1[d]	209.9 ± 130.2[e]
Saline	10 : 1	15.7 ± 18.7	205.8 ± 67.9[e]
CE	20 : 1	0.0 ± 0.0[e]	0.0 ± 0.0[e]
CE	10 : 1	0.0 ± 0.0[e]	0.0 ± 0.0[e]
CE	5 : 1	12.3 ± 15.8	59.4 ± 56.9[e]
CE	2.5 : 1	6.2 ± 10.4	48.2 ± 60.7[e]

[a] PEC were obtained 24 h after IP injection of saline or 200 mg/kg CE. The mixtures of tumor cells and PEC were injected SC into the right flank of normal recipient mice
[b] 5×10^4 cells of Meth-A tumor was used as target cells
[c] Days after the tumor inoculation
[d] Values are means ± SD from eight mice
[e] Significant at $P < 0.01$ (Student's *t*-test) compared with the control group

injection of CE. PEC obtained from normal or saline-injected mice did not exhibit any such activity. The antitumor effect of PEC obtained 24 h after CE injection was positive even at an E/T ratio as low as 2.5 : 1, compared with the tumor growth with target tumor cells alone (Table 3).

Characterization of suppressive factors (cells) for tumor cells

Various treatments were tried in an attempt to identify the cells responsible for an antitumor activity in the Winn-type assay. PEC were obtained 24 h after CE injection to normal mice. The antitumor activity of CE-induced PEC remained intact after their treatment with anti-Thy 1.2 antibody plus complement (Table 4). The following two treatments aiming at depletion of macrophages were applied. One was the removal of adherent cells. After this treatment, tumors were suppressed completely in four of six mice 36 days after, while tumors grew in the remaining two mice as well as in the group in which saline-induced PEC were used. The other was carrageenan treatment in vitro. Following this treatment, PMN accounted for over 90% of the remaining cells, and the antitumor activity was hardly reduced. The antitumor activity of CE-induced PEC may depend chiefly upon PMN and paritally upon macrophages.

Inability of casein- or OK-432-induced PEC to exhibit antitumor effects

It is well known that PMN are induced by various stimulators. An antitumor effect was examined using CE-induced PEC and other stimulator-induced PEC. PEC were obtained from mice injected with casein, a well-known irritant, IP at 6 h or 24 h before. Such PEC contained predominantly PMN, as many as CE-induced PEC did. Nevertheless, casein-induced PEC did not exhibit an antitumor effect in the Winn-type assay (Table 5). OK-432, which is known as one of the biological response modifiers and the activator of macrophages, induced abundant PMN in the early stage after injection. However, OK-432-induced PEC exhibited scarcely any inhibitory activity on tumor growth at 24 h after injection (Table 6). These results suggest that the characteristics of CE-induced PEC are different from those of PEC induced by other stimulators.

Inability of CE-induced PEC to express an antitumor effect in irradiated recipients

Meth A cells were mixed with PEC, which were obtained 24 h after CE injection to normal mice, and the mixture was

Table 4. Influence of various treatments of CE-induced PEC on antitumor activity in the Winn-type assay[a]

Inducer of PEC	Treatment of PEC	*n*	Tumor size (mm^2)		
			10 days[d]	22 days	27 days
Saline	Untreat	6	17.6 ± 3.3[e]	76.3 ± 29.6	160.3 ± 38.6
CE	Untreat	6	0.0 ± 0.0[f]	0.0 ± 0.0[f]	0.0 ± 0.0[f]
CE	α-Thy 1.2 + C'	6	0.0 ± 0.0[f]	0.0 ± 0.0[f]	0.0 ± 0.0[f]
CE	Removal of adherent cell[b]	6	5.7 ± 7.4	32.3 ± 50.1	59.7 ± 94.2[f]
CE	Carrageenan[c]	5	3.1 ± 4.5[f]	6.4 ± 8.8[f]	6.8 ± 6.8[f]

[a] PEC were obtained 24 h after IP injection of saline or 200 mg/kg CE, and 5×10^4 Meth-A cells were mixed with 5×10^5 whole or treated PEC and inoculated SC into the right flank of normal recipient mice
[b] Adherent cells were removed from CE-induced PEC by incubation at 37° C for 1 h in plastic dishes
[c] Cells were obtained from CE-induced PEC after incubation at 37° C for 4 h with 400 μg/ml carrageenan as final concentration
[d] Days after the tumor inoculation
[e] Values are means ± SD
[f] Significant at $P < 0.01$ (Student's *t*-test) compared with the saline group

Table 5. Inability of casein-induced PEC to exhibit an antitumor effect in the Winn-type assay[a]

PEC		Tumor size (mm^2)	
		10 days	22 days
Expt. 1			
–		26.0 ± 6.1[b]	209.9 ± 130.2
CE	24 h	1.1 ± 3.2[c]	18.0 ± 50.9[c]
0.2% casein	6 h	19.4 ± 3.7	193.5 ± 35.2
0.2% casein	24 h	15.7 ± 18.7	205.8 ± 67.9
Expt. 2			
–		38.9 ± 14.6	221.7 ± 116.9
CE	24 h	2.0 ± 5.7[c]	32.4 ± 47.7[c]
0.2% casein	24 h	44.4 ± 7.4	304.3 ± 54.3
2.0% casein	24 h	42.4 ± 7.5	311.9 ± 72.2
12.0% casein	24 h	40.3 ± 10.1	301.0 ± 68.0

[a] Casein-induced PEC were obtained by injection of 1.0 ml casein, and mixtures of 5×10^4 cells of Meth A and 5×10^5 PEC were inoculated SC into the flank of normal recipient mice
[b] Values are means ± SD from eight mice
[c] Significant at $P < 0.01$ (Student's *t*-test)

inoculated SC recipients that had been exposed to 5 Gy 24 h before. The antitumor effect of CE-induced PEC was not expressed in such irradiated recipients, in contrast to the positive effect in normal recipients (Table 7). The antitumor effect of CE-induced PEC in our Winn-type assay may require the involvement of some recipient-derived element(s) sensitive to irradiation.

Discussion

We can summarize the results in the present study as follows: (1) CE injected into the peritoneal cavity exerted an antitumor effect on the syngeneic ascites tumor cells; this effect was dose-dependent. (2) PEC obtained 6 h and 24 h after IP injection of CE to normal mice exhibited an antitumor activity in the Winn-type assay with normal recipients. The cells chiefly participating in the expression of such an activity were resistant to the treatment of anti-Thy 1.2 and complement, or were partially resistant to the treatments aiming at the removal of macrophages. (3) An antitumor activity of such CE-induced PEC was not exhibited in the Winn-type assay with irradiated recipients. CE-induced PEC may express an antitumor effect in cooperation with some host- or recipient-derived element(s) sensitive to irradiation. (4) CE might exert an antitumor effect by a mechanism(s) distinct from those of OK-432, one of the biological response modifiers.

IP injections of CE exert the effect on prolongation of the life span of IP-inoculated Meth A tumor-bearing mice. As shown in Fig. 1, a more pronounced effect was obtained with CE injections given on eight occasions distributed over both the pre- and postinoculation periods than with CE injections given on five occasions all after the tumor inoculation, and extension of life span was obtained with three CE injections all given before the inoculation. It is possible to explain this phenomenon with reference to the difference in frequency of CE injections, because the life span was not extended by one or two CE injection(s) given after tumor inoculation. In addition, it is possible that the antitumor action of CE injections given exclusively before the tumor inoculation was not exhibited as prolongation of life span. Moreover, a single injection of 50 mg/kg CE induced a reduction in body weight by a few percentage points; the body weight recovered almost to the normal level when several further CE injections were given on consecutive days (data not shown). This result suggests the possibility that some other influence counteracts the antitumor effect with a single injection of CE. It has not, however, been explained why a more pronounced antitumor effect was induced when CE injections were given both before and after tumor inoculation than when they were given after inoculation only, although preinoculation injections only did not have any effect. In contrast, CE exhibited scarcely any antitumor effect following systemic injection (data not shown). We came to the following conclusions: (1) CE induced antitumor elements at the site of CE injection; (2) tumor cells were killed directly by CE; or (3) CE induced an alteration of the microenvironment, making it unsuitable for tumor establishment. We have shown in a previous paper [23] that the antitumor effect of intratumorally injected CE on SC-inoculated tumor is mediated by host defense.

Primarily, the peritoneal cavity of normal mice was used as the site of the CE injection, cellular elements in CE-induced PEC were examined. As shown in Table 1, PMN accumulated in the peritoneal cavity in large numbers within 24 h, remaining

Table 6. Inability of OK-432-induced PEC to exhibit an antitumor effect in the Winn-type assay[a]

PEC	n	Tumor size (mm^2)		
		10 days	15 days	22 days
	8	44.6 ± 23.7[b]	73.8 ± 28.8	181.6 ± 71.9
CE (100 mg/kg)	5	8.0 ± 9.2[c]	8.1 ± 16.2[c]	55.0 ± 42.0[c]
CE (500 mg/kg)	5	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
OK-432 (2KE/kg)	8	45.9 ± 16.5	82.6 ± 32.7	190.4 ± 65.3
OK-432 (10KE/kg)	8	40.9 ± 13.8	62.7 ± 20.8	147.8 ± 72.2
OK-432 (50KE/kg)	8	45.7 ± 30.1	79.4 ± 33.3	157.0 ± 55.5

[a] CE- and OK-432-induced PEC were obtained 24 h after IP injection, and the mixtures of 1×10^5 cells of Meth A and 1×10^6 cells of PEC were inoculated SC into the flank of normal recipient mice
[b] Values are means ± SD
[c] Significant at $P < 0.05$ (Student's t-test)

Table 7. Inability of CE-induced PEC to express antitumor effect in Winn-type assay with the X-ray-irradiated recipient mice[a]

PEC	Recipient	Tumor size (mm^2)		
		11 days	16 days	20 days
–	Irradiated	44.8 ± 8.9[b]	91.4 ± 39.3	179.0 ± 27.7
Saline	Irradiated	44.9 ± 5.0	125.5 ± 12.6	224.7 ± 19.4
CE	Irradiated	57.6 ± 8.8	129.8 ± 19.6	227.0 ± 26.6
–	Normal	29.2 ± 6.3	63.2 ± 19.1	133.3 ± 32.4
Saline	Normal	26.8 ± 7.0	40.1 ± 19.6	88.8 ± 44.9
CE	Normal	2.0 ± 4.3[c]	6.5 ± 8.3[c]	12.7 ± 23.5[c]

[a] Irradiated recipient mice were exposed to 5 Gy of whole-body X-ray irradiation 24 h before inoculation of the mixtures of tumor cells and PEC. PEC were obtained from normal mice 24 h after IP injection of saline or 200 mg/kg CE. 5×10^4 cells of Meth-A tumor were mixed with 5×10^5 PEC and inoculated SC into the right flank of irradiated recipient mice
[b] Values are means ± SD from eight mice
[c] Significant at $P < 0.01$ (Student's t-test)

at higher levels than normal for 3–5 days. The total number of macrophages in the peritoneal cavity decreased in 1 day and increased twofold or threefold in 3–5 days compared with that in untreated controls. No increase in the number of lymphocytes was detected during the observation period of 5 days. Cellular accumulation similar to this pattern was also observed after IP injection of tumor cells and CE in the preliminary experiments. It seems possible that the local accumulation of cellular elements capable of participating in the host defense, especially of PMN and macrophages, contributes to the antitumor effect of CE. A direct tumor-suppressing effect of CE could not be expected, since CE exerted only a weak direct cytotoxic effect in an in vitro assay. The results reported in this and in previous papers may not support the concept of an altered microenvironment as the principal mechanism of the antitumor effect by CE, although a partial contribution of this mechanism and direct killing cannot be ruled out completely.

The antitumor activity of accumulated PEC was examined in a Winn-type assay in normal recipients (Tables 2–6). PMN-rich PEC obtained early (6 h and 24 h) after CE injection exhibited antitumor activity. However, macrophage-rich PEC obtained at 3 and 5 days after did not exhibit any such activity. The number of macrophages in CE-induced PEC decreased strikingly in comparison with resident or saline-induced PEC after its injection.

The antitumor activity of CE-induced PEC was slightly reduced by the removal of adherent cells. The purpose of this treatment was macrophage depletion, but many PMN were removed together with macrophages. The reduction of activity may be due to partial participation of macrophages or removal of easily adherent PMN. But the reduction is not statistically significant ($P > 0.2$). The antitumor activity was hardly reduced by carrageenan treatment of PEC in vitro to block the macrophages. These results support the conjecture that PMN may be an effective element in the growth-inhibitory activity of CE-induced PEC, although the involvement of macrophages cannot be ruled out completely.

On the other hand, the difference in the antitumor activity on day 1 and on day 2 was striking (Table 2), while the number and composition of PEC were very similar (Table 1). The following possible explanations are suggested for this discrepancy: (1) PMN activated on day 1 may already be inactivated or have died on day 2, since PMN is short-lived and easily destroyed. (2) Qualitatively different PMN may be induced on day 2 than on day 1. (3) The antitumor effect may be counteracted by the appearance of another suppressive factor on day 2 (e.g., suppressor macrophages). Even though the actual mechanisms are obscure, the first possibility may be acceptable, in view of the general quality of PMN, if PMN are an effective element in CE-induced PEC. CE must be retained in the peritoneum early after IP injection of CE. We can rule out the possibility that the antitumor activity of CE-induced PEC is due to a direct action of CE contained in PEC, since CE exerted only a weak direct cytotoxic activity in an in vitro assay, as noted above, and PMN-rich PEC were incapable of

expressing an antitumor effect alone, as shown in Winn-type assay using irradiated recipients (Table 7). Therefore, one of the most probable mechanisms for such an effect may be that PMN release some factor(s) capable of activating the final effector cells, which are radiosensitive, at least as to the activation.

Culture supernatant of casein-induced PMN has been reported to enhance mitogen responses (Con A, PHA) of thymocytes [10, 24, 27]. The antitumor effect of CE-induced PMN-rich PEC may be mediated by a mechanism similar to the one cited above, although casein-induced PEC did not express an antitumor effect in our experiment (Table 5). The antitumor activity of CE-induced PEC was compared with that of other biological response modifiers. It was reported that tumor destruction induced by OK-432 against ascites tumor in human may be mediated by PMN [13]. In our Winn-type assay, OK-432-induced PMN-rich PEC did not exhibit antitumor activity (Table 6), though it has been reported that macrophages obtained 4–7 days after injection of *C. parvum* exhibited a strong cytolytic activity [7] and that this activity was mediated by PMN [2, 3]. In the present study, macrophage-rich PEC induced by CE did not exhibit an antitumor effect on tumor growth, while PMN-rich PEC exhibited an antitumor effect. These results suggested that the characteristics of CE-induced PEC might be different from those of PEC induced by casein, OK-432, or other immunomodulators. Unfortunately, the final effector responsible for the elimination of tumor cells and the mechanism mediating the effect from CE-induced PMN to the final effector were not discussed during the present study.

The antitumor activity of CE given by IP injection was shown in mice bearing ascites tumor. This antitumor activity was also exhibited by PMN-rich PEC obtained early after CE injection in the present study. These PMN-rich PEC induced by CE, which have antitumor activity, did not contact with any tumor antigen. This antitumor activity of PMN-rich PEC was considered to be nonspecific in this study. On the other hand, it has already been reported that concomitant immunity is enhanced by intratumor injection of CE [23]. Taken together, these results suggest the possibility that PMN-rich PEC induced by CE, or factors released from such a cell population, stimulate the radiosensitive elements and enhance a specific immune response against the tumor. Further scrupulous study is necessary to determine the actual mechanism of the antitumor effect of CE.

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