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IMMUNOMODULATION BY A UNICELLULAR GREEN ALGAE (CHLORELLA PYRENOIDOSA) IN TUMOR BEARING MICE

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SUMMARY
A unicellular algae, *Chlorella pyrenoidosa,* was used as a biological response modifier. In C57BL/6/(B6), C3H/He and DDD/1 mice, both intraperitoneal or oral administrations of autoclaved *Chlorella* cells or heat-extracted substance were carried out every other day for 10 days before mouse mammary carcinoma (MM-2) or Ehrlich ascites cells were transplanted into the peritoneal cavity. In case of mouse leukemia cells 9EL-4), subcutaneous transplantation was carried out. All control mice died within 20 days after each tumor cell transplantation while 73.3-80% of the treated groups survived over 60 days in the combination of MM-2 vs. C3H/He and EL-4 vs B6 respectively. The cytotoxic activities against tumor cells, that were abolished by treatment with anti-Thyl. 2 monoclonal antibody plus complement, were evidenced in the experimental host. Since *Chlorella* cells and derivatives showed not indication of direct in vitro cytotoxicity to either tumor or mouse spleen cells, the antitumor effects documented may be mediated by host immune response.

INTRODUCTION

Several types of immunopotentiators have been developed recently and are being studied for possible use in the treatment of patients suffering from malignant disease. Immunopotentiating activities have been found in several bacterial genera. E.g. *Mycobacterium* (Old et. al., 1959. 1961;Mathe et al,1969, Azuma etc al, 1974 Morton and Goodnight, 1978) *Nocardia* (Azuma et al., 1976) *Streptococcus* (Suzuki et al., 1975; Yoshida et al. 1987) and *Propionibacterium* (Scott. 1974; Woodruff and Warner, 1977). These have been tested in tumor bearing animals and patients in the form of viable.cells of avirulent or attenuated strains, such as *Basillus Calmette-Guerin* or of individual cell components such as the cell wall skeleton (Azuma et al., 1974) *Salmonella* species (Kurashige et al., 1981) are also expected to have immunopotentiating activity since they induce productive inflammation and a proliferative reaction of the reticuloendothelial system similar to that induced by mycobacteria (Okonogi et al., 1959). However, treatment with bacterial agents is usually accompanied by significant side effects such as local and systemic fevers and sepsis. The use of biologically modified or degraded materials generally has reduced their biological activities. (Takamura and Yoshida, 1986) The present paper deals with the effect of an unicellular algae *Chlorella pyrenoidosa,* on tumor activity in mice.

EXPERIMENTAL PROCEDURES

Animals and drugs : C57BL/6(B6), C3H/He and DDD/1 mice were delivered by Kurea Japan Ltd. And kept in an animal room at Kanazawa Medical Unversity under the SPF (specific pathogen free) system. Streptococcal agent (OK-432) was kindly supplied by Chugai Pharmaceutical Co. Ltd., Tokyo.
Tumor and tumor transplantation : Mouse mammary carcinoma cells (MM-2, Ca-755, Ehrlich) and leukemia cells (EL04) were maintained in vivo in C3H/He, C57BL/6 (B6) and DDD/1 mice in the ascites form. These four tumor cells were collected from ascites fluid 7 days after the intraperitoneal (i.p.) inoculation with 1 x 10 6 of each of the tumor cells, washed twice with RPMI-1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) and resuspended in the same medium at a concentration of 5 x 106 or 5 x 10 7/ml. C3H/He mice were transplanted i.p. with 0.1ml of MM-2 tumor cell suspension (5 x

105/mouse). For B6 mice, 0.1ml of EL-4 AND Ca-755 (1 x 106/ml) was administrated subcuraneously (s.c.) Mice of DDD/1 strain were transplanted s.c. with 0.1 ml of Ehrlich cell suspension (5 x 106/mouse).
Determination of tumor growth : Mice given the s.c. transplantation of Ehrlich cells were sacrificed 30 and 60 days after tumor cell transplantation. Their tumors were removed and weighed and the ratio of tumor growth was calculated according to the following formula.

Inhibition index +	Tumor weight in experimental group (g)
	Tumor weight in control group (g)

When EL-4, MM-2 and CA-755 tumor cells were transplanted i.p. into mice, survival time was monitored up to 60 days after the tumor transplantation.
Spleen cells for cytotoxicity test : Spleen cells (SPC) of B6 mice were prepared using conventional methods to get a single cell suspension (Hashimoto and Sudo, 1971). Briefly, SPC suspended in RPMI-1640 medium containing 10% fetal calf serum were incubated in a 90-mm plastic dish at 37° C for 60 min in a CO2 incubator to separate adherent cells. The remaining non-adherent cells were used with or without further purification by antiserum. Non-adherent SPC were fractionated by anti-Thy1.2 monoclonal antibody and guinea pig complement in order to clarify the activity of T-cell population. Macrophage-rich (93%) adherent cells of SPC were isolated by the method of Kumagai (Kumagai et al., 1979).
Target cell for cytotoxicity assay procedures : The tumor cells employed as target cells, namely mouse leukemia (EL-4), mouse mammary tumor (MM-2) and Ehrlrich ascites cells, were prepared by in vitro culture.
Cytotoxicity assays : The cytolytic activity of effector cells in SPC of B6 mice were tested by the 3H-uridine method with some modification (Hashimoto and Sudo, 1971). In brief, the target cells were treated with 1 m Ci of 3H-uridine for 5h at 37° C at a concentration of 5 x 105 cells/ml. Aliquots (0.5ml) of the target cell suspension were introduced into test tubes and incubated for 3h at 37° C. For each tube, 0.5ml of effector cell suspension was mixed into each tube and the mixture was incubated for 20h at 37° C. The cell suspensions were then centrifuged and washed twice with cold 5% trichloroacetic acid solution. The sedimented cells were dissolved in 0.3ml of Suluene (Packard Instrument Co Ltd., USA). The radioactivity of the digested cell solution was counted in a toluene-based scintillation medium by a Packard liquid scintillation counter (Model 4450). The percentage of net lysis was calculated as 1- (cpm in culture with effector cell/cpm in target cell alone) 100.
Preparation of Chlorella cells : Five types of samples were prepared from *C. pyrenoidosa* cells; (i) intact chlorella cells (ITC), (ii) autoclaved cells (ACC) prepared by heating *Chlorella* cells at 120° C for 3 min, (iii) a hot water protein rich extract (CCF-prf), (iv) a cell wall fraction (CWF) where ACC were further heated at 100° C for 30 min. broken up by ultrasonication and homogenization and centrifuged at 10,000 x g for 1 h to yield a precipitate and (v) a protein free hot water extract (CGF-pff) prepared by removing protein from CGF-prf by heating at 100° C for 9 min. Each sample was dissolved at appropriate concentrations in physiological saline. The original *Chlorella* cells were produced and supplied by YSK International Co Ltd. (Kyoto, Japan),
Treatment of mice : Mice were treated with samples before or after tumor cell transplantation. Pretreatment was carried out by giving the sample orally or intraperitoneally every other day from 7 to 2 days before the tumor transplantation. Post-treatment was done in the same manner except 2 to 7 days after transplantation.

RESULTS
Effect of treatment upon the survival time of EL-4 bearing mice : In order to test the effect of oral pretreatment with *Chlorella* on the survival time of tumor-bearing mice, three groups of B6 mice were pretreated with (i) 1 x 106 of ACC, (ii) 1 mg of CFG-prf and (iii) 1mg of CFG, respectively. All mice were

transplanted i.p. with 5 x 104 of mouse leukemia cells 9EL-4). Mice stimulated with none of these agents were used as the tumor –bearing control. In both groups receiving ACC or CGF-prf, antitumor activities were successfully established by CFG-pff failed in this regard. In case of oral administration, 11 mice (73%) in the former two groups survived more than 60 days after tumor transplantation and all the mice in the CGF-pff group and control group died far earlier (approximately 20 days after the tumor transplantation) as shown in Fig. 1 When ACC and CFG-prf were introduced i.p., 10 of the 15 mice (66.7%) survived more than 60 days. The control and CGF-pff again died within 20 days.

Effect of treatment upon the life-span of MM-2 bearing mice : To test the effect of *Chlorella* upon a different tumor celltype, mouse mammary carcinoma cells (MM-2) were transplanted before and after the treatment with *chlorella* derivatives. Experimental schedules were the same as above except that the tumor cell line (MM-2) dosage of cells (5 x 105mouse) and mice strain (C3H/He) were different. As shown in Fig. 2 eight mice (80%) receiving CGF-prf orally were alive more than 60 days and none of the mice receiving CGF-pff survived. In case of i.p. administration, approximately the same percentage of mice receiving ACC and CGF-prf survived. Again, CGF-pff was not protective.

Effect of time between sample administration and MM-2 tumor cell transplanation on survival :In the first experiment, four agents (ACC, CWF, CFG-prf and OK-432, the Streptococcal antitumor agent) were administrated i.p. pre or post MM-2 tumor cell transplantation. A dosage of each sample per mouse per day was 1 x 108 cells, 2mg 1mg and 1 KE(Kliniche Einheit, corresponding to 0.1mg of dried bacterial cells), respectively, and administrated ever other day for five times. As seen in Table 1, ACC and CGF-prf were effective in elongation of survival only when given prior to MM-2 cell transplantation, while the cell wall fraction was not effective regardless of the administration schedule.

The second experiment use Ehrlich tumor cells and DDD/1 mice. Three *Chlorella* agents (ITC, ACC and CWF) were used. As shown in Table 2, ACC and CWF were effective in inhibiting tumor growth irrespective of the time schedule, while ITC was effective for inhibiting tumor growth only when given before the tumor inoculation

TABLE 1
Effect of i.p. administration of time of samples on survival of mice after mm-2 cell transplantation

Group	Chlorella samples	Time of treatment	death time (days)	Mean death time(days)	60-day survivors
1	-	-	16, 18, 18, 19, 19, 21, 21, 21, 21, 22.	19,6	0/10
2	ACC	Pre	16, 9, --, --, -- , --, --, --, --, --.	$\geq$ 51,5*	8/10
3	ACC	Post	19, 10, 22, 22, 22, 22, 23, 26, 27, 30.	23,3	0/10
4	CWF	Pre	18, 18, 19, 19, 19, 20, 21, 22, 22, --.	$\geq$ 23,8	1/10
5	CWF	Post	19, 21, 21, 22, 22, 22, 23, 23, 24, 27.	22,4	0/10

6	CGF-prf	Pre	17, 17, 21, --, -- , --, --, --, --, --.	≥47,5**	7/10
7	CGF-prf	Post	16, 16, 18, 19, 19, 20, 20, 25, 26, --.	≥ 23,9	1/10
8	OK-432	Pre	12, 18, 20, 21, 25, 27, 27, --, --, --.	≥ 33*	3/10
9	OK-432	Post	17, 18, 19, 20, 20, 22, 22, --, --, --.	≥ 31,8*	3/10

Significance relative to Group1 (Control): *P < 0.001,, **P < 0.0005 by Student's *t*-test

TABLE 2
Effect of i.p. Administration time of sample on Tumor growth in mine after Ehrilich Ascites Tumor Cell Transplantation

Group	Chlorella sample	Time of treatment	Tumor growth (inhibition index)
1	--	--	1.00
2	ITC	Pre	0.54**
3	ITC	Post	1.12
4	ACC	Pre	0.49*
5	ACC	Post	0.57***
6	CWF	Pre	0.61
7	CWF	Post	0.73

Significance relative to Group 1 (Control): *P < 0.05, ** P < 0.01, *** P< 0.001 by Student's *t*-test

Specificity of antitumor activity : C57BL/6 mice were pretreated with ACC and then transplanted with EL-4 tumor cells as mentioned above. The mice surviving over 60 days were divided into two groups. One was rechallenged by the same tumor cells 9EL-4) and the other challenged by mouse mammary carcinoma 9CA-755), then both tested for median survival days. Normal mice were used as controls and treated in the manner previously described. As indicated in Table 3, surviving mice were resistant to rechallenge by the same tumor cell line, but not to the other line, Ca-755. Thus the antitumor activities in mice augmented by ACC and CGF-prf were regarded as a specific immune reaction.

Table 3
Effect of Rechallenge on El-4 cured mice by ACC

Group	mice	tumor cell	death time	Mean death time (days)	30-day survivors (quantal)

1	Cured	EL-4	--, --, --, --	> 30*	4/4
2	Normal	EL-4	19,19,21,21,21,22	20,5	0/6
3	Cured	Ca-755	15,16,16,16	15,8	0/4
4	Normal	Ca-755	13,13,13,14,14,15	13,7	0/6

Significance relative to Group 2 (Normal control)) * P <0.01 by Student's *t*-test

Induction of cytotoxic cells in the spleen of experimental mice : In an attempt to define the mechanism of antitumor effect augmented by the *Chlorella* derivatives, adherent and non-adherent cells were prepared 14 days after tumor cell transplantation. Target EL-4 cells were labelled by 3H-uridine and the cytolytic activity of the effector cells were judged by the method reported by Hashimoto and Sudo (1971). As shown in Table 4, a cytolytic cell was generated in non-adherent spleen cells of experimental mice and the activity was augmented by in vitro secondary culture with the same strain of tumor cells. Cytotoxic activity was reduced after treatment with anti-Thyl.1 monoclonal antibody and complement. Adherent cells did not shown significant toxicity for the target cell

TABLE 4
Induction of cytolytic cells in the spleen of experimental mice

Treatment of host[a]	Tumor cell transplant to the host	Secondary culture with[b]	Specific lysis of tumor cell (%)		
			By non-adherent cells treated with:		By adeherent cells
			Only C	Anti-Thy 1.2 + C	
Normal Control	-	Medium MMC-EL4 MMC-L1210	9.5 ± 2.6 8.6 ± 2.1 6.5 ± 2.0	8.6 ± 1.6 10.3 ± 2.3 12.6 ± 2.8	10.5 ± 4.5 11.5 ± 3.5 9.8 ± 4.5
ACC	+	Medium MMC-EL4 MMC-L1210	25.6 ± 4.6 53.6. ± 8.2** 20.2 ± 3.6	10.3 ± 1.8 16.5 ± 1.9 13.3 ± 3.1	13.8. ± 3.6 11.6 ± 4.5 12.2 ± 2.9
CGF-prf	+	Medium MMC-EL4 MMC-L1210	28.2 ± 5.6 64.3 ± 10.2*** 16.5 ± 3.3	8.6 ± 3.6 15.8 ± 4.2 10.3 ± 2.3	15.6 ± 3.8 13.8 ± 2.9 12.2 ± 2.8
CGF-pff	+	Medium MMC 2.0-EL4 MMC-L1210	10.3 ± 2.8 15.3 ± 3.3 13.3 ± 2.6	6.5 ± 2.0 10.3 ± 1.8 11.2 ± 3.2	9.8 ± 6.5 10.2 ± 3.8 10.5 ± 4.2
OK0-432[c]	+	Medium MMC-EL4 MMC-L1210	25.6 ± 7.8 30.1 ± 8.6* 18.3 ± 5.5	18.6 ± 4.2 24.6 ± 4.1 13.3 ± 3.3	16.3 ± 8.5 13.5 ± 6.3 12.3 ± 3.3

a Treatment of tumor-bearing host was stated 7 days before tumor transplantation

b Adherent or non-adherent spleen cells that were further separated and treated with anti-Thy 1.2 plus complement were cultured in vitro with mitomycin-C treated cells for days.

c Antitumor agent associated with *Streptococcal* species. Significance relative to the MMC EL4 group of control (*P <0.01, ***P: 0.001) by Student's test.

TABLE 5
Direct Cytotoxicity of the samples in Vitro

Target Cell[a] cultured with		Dead cell (%)			
		Normal Spleen Cell	EL-4	MM-2	Ehrlich Ascites
None (control)		2.3	4.2	4.8	3.8
ACC	1.x107/ml 1 x 108/ml	3.8 4.3	2.1 2.5	3.6 3.8	4.2 2.3
CGF-prf	1mg/ml 10mg/ml	5.2 8.3	4.2 3.3	3.2 2.5	2.9 3.6
CFG-pff	1mg/ml 10mg/m	4.3 3.6	2.5 2.2	3.8 3.6	3.1 1.8
OK-432[b]	0.5KE/ml 5 KE/m	13.3* 28.6***	12.2* 20.3**	20.6** 25.3***	22.2** 32.3***

[a] Each of the target cells were incubated in vitro for 12 h and dead cells counted by the method of trypan-blue dye exclusion.
[b] Antitumor agent associated with *Streptococcal* species (1 Kriniche Einheit corresponds to 0.1mg of driedbacteria).
Significance (*P <0.05, ** P<0.01, *** P ,0.001) vs. control using the Student's *t*-test

In vitro toxicity of Chlorella derivatives against mouse tumor cells and normal spleen cells: The direct cytotoxicity of each *Chlorella* derivative was tested in vitro again EL-4,, MM-2 and Ehrlich tumor cells as well as normal spleen cells. As shown in Table 5, none of derivatives from *Chlorella* evidenced cytotoxicity for the tumor cells and spleen cells but significant activity was observed for the streptococcal agent, OK-432.

DISCUSSION

It has been reported that both specific and non-specific immunities, are depressed in tumor-bearing animals and humans (Kishida et al., 1978; Karashige and Mitsuhashi, 1982). The former surely allows tumor cells to proliferate within the host and the latter results in serious infectious diseases even by low or normally non-pathogenic microorganisms. An agent which augments a non-specific immunity in these hosts is also expected to activate specific anti-tumor immunity. Many kinds of immunomodulators have been developed recently and are being examined for use in the treatment of patients suffering from malignant diseases

Bacterial cells of the *Mycobacterium* species (Mitsuhashi et al., 1959; Mathe et al., 1969; Hellstrom et al., 1973) and *Corynebacterium* species (Scott, 1974; Morton and Goodnight, 1978) enhance the immune responses in the hosts and can be clinically used for immunotherapy of malignant tumor. Previously in our laboratory, tumor-bearing hosts were tested for their immune responsiveness such as antibody-forming cell production, blastogenesis of lymphocytes by phytohemagglutinin-P and the chemotactice, phagocytic and bactericidal activities of macrophages. It was revealed that among these responses, the chemotactic activity of macrophages was dramatically reduced as early as a week after tumor transplantation. All of the other responses were essentially unchanged for 3 or 4 weeks but were finally depressed in the last stage of life.
In a preceding attempt designed to recover the reduced chemotactic activity of macrophages, Chinese herbs were employed. The chemotactic activity in mice pretreated with the Chinese herbs was sustained at normal levels after tumor transplantation (Hamada et al., 1988)
In the present paper, the effect of a unicellular algae, *Chlorella pyrenoidosa,* on tumor-bearing animals were tested. ACC and CGF-prf were effective in prolonging survival days when given prior to i.p. MM-2 tumor transplantation; moreover, ACC and CWF were observed to be effective in inhibiting the Ehrlich solid tumor transplanted s.c. into the hind back. In another study, a hot-water extract of *Chlorella vulgalis*has been shown to be effective for another tumor/mouse system (Konishi et al., 1985).
Mice surviving the treatment with the agent prior to the tumor transplantation were resistance to rechallenge by the same tumor cell but not to a different tumor cell line. Further, ACC and CGF-prf proved to enhance macrophage activity and cytotoxic activity of non-adherent lymphocytes; thus the antitumor effect of the samples may be due to a synergistic effect of activated macrophages and cytotoxic T-cells (Azuma et al., 1976). *Chlorella* showed no toxicity to cultured spleen cells or tumor cells in vitro but showed significant indirect effects in mice. In preliminary studies, *Chlorella* derivatives did not enhance humoral antibody production (unpublished observations). Therefore, we support the view that the antitumor effect of these samples on tumor-bearing mice is mostly dependent upon the enhancement (or restoration) of macrophage chemotactic activity which is inhibited more rapidly than the other various activities of immuno-competent cells. Thus the antitumor effect by *Chlorella* agents on tumor-bearing mice may represent host-mediated cellular immunity

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