Arch Pharm Res Vol 25, No 2, 158-164, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Immunomodulatory Activity of Protein-Bound Polysaccharide Extracted from *Chelidonium majus*

Jie-Young Song, Hyun-Ok Yang[1], Suhk-Neung Pyo[2], In-Sung Jung, Seh-Yoon Yi, and Yeon-Sook Yun

Laboratory of Immunology, Korea Cancer Center Hospital KAERI, 215-4, Gongneung-dong, Nowon-Ku, Seoul, 139-706, Korea, [1]Department of Traditional Medicines, Asan Institute for Life Sciences, Ulsan University, 388-1, Pungnap-dong, Songpa-Ku, Seoul, 138-736, Korea, and [2]Laboratory of Immunology, College of Pharmacy, Sungkyunkwan University, Chunchun-dong, Changahn-ku, Suwon, 440-746, Korea

(Received December 15, 2001)

In the course of searching immunomodulators from natural sources, the protein-bound polysaccharide, CM-Ala, has been isolated from the water extract of *Chelidonium majus* L. (Papaveraceae). The immunostimulatory characteristics have been investigated in several experiments such as generation of activated killer (AK) cells, proliferation of splenocytes, activation of macrophages and granulocyte macrophage-colony forming cell (GM-CFC) assay. Of the fractions obtained using Sephacryl S200 column chromatography, CM-Ala was the most effective fraction that augmented the cytotoxicity against Yac-1 tumor cells from 0.88% to 34.18% by culturing with splenocytes for 5 days. CM-Ala also enhanced nitric oxide production by two fold in peritoneal macrophages and exhibited antitumor activity. It showed mitogenic activity on both spleen cells and bone marrow cells. CM-Ala induced proliferation of splenocytes by 84 fold and increased GM-CFC numbers by 1.48 fold over than the non-treated. On the contrary, CM-Ala had cytotoxic activity to a diverse group of tumor cells. From the above results, we proposed that CM-Ala has a possibility of an effective antitumor immunostimulator.

Key words: *Chelidonium majus*, Protein-bound polysaccharide, Immunostimulator, Mitogenic activity

INTRODUCTION

The discovery and development of immune response modifying agents has become a promising and important goal in immunotherapy. The administration of various naturally occurring or synthetic biological response modifiers (BRMs) has been reported to enhance host immune reactivity (Terry and Rosenberg, 1982; Herbermann, 1985; Talmadge and Herbermann, 1986). However, the full therapeutic potential of the BRMs has not been realized due to their undesirable side effects when used at the concentration required for maximum biological activity (Lotze *et al.*, 1985; Lotze *et al.*, 1986; Urba and Longo, 1986). Thus, the identification and characterization of new compounds without deleterious side effects and activating all effector arms of antitumor immune system would enhance the prospects of immunotherapy as a practical and effective cancer treatment modality. To develop the new antineoplastic immunomodulator from natural sources, we have screened 68 kinds of folkloric and traditional medicines, which are previously reported about their immunostimulatory activities (Song *et al.*, 1998). In this study, protein-bound polysaccharide was isolated from the water-soluble fraction of *Cheidonium majus* L. (CM). It has already been reported that some alkaloids such as chelidonine, chelirubine, protopine, stylopine, sanguinarine, and chelerythrine from CM have the effective activities for the analgesic, antispasmodic and hypotensive (Jee and Lee, 1988; Lenfeld *et al.*, 1981; Wyczolkowska *et al.*, 1996). Antitumor and antiviral activities were also reported by Ukrain-a semisynthetic compound (Kery *et al.*, 1987; Voltcheck *et al.*, 1996; Liepins and Nowicky, 1992), however, water soluble fractions of this plant have not been extensively studied. In the present report, we show the immunomodulatory activities of polysaccharides from CM by the proliferation assay of splenocytes, generation of activated killer cells, activation of macrophages, and granulocyte-macrophage

Correspondence to: Yeon-Sook Yun, Laboratory of Immunology, Korea Cancer Center Hospital KAERI, 215-4, Gongneung-dong, Nowon-Ku, Seoul, 139-706, Korea
E-mail: immu@mail.kcch.re.kr

colony forming unit assay.

MATERIALS AND METHODS

Materials

The herbs of CM were purchased from Kyungdong traditional medicine market in Seoul, Korea. The recombinant IL-2 (rhIL-2) were purchased from BM (Mannheim, Germany), [^{3}H]-thymidine and $Na_2[^{51}Cr]O_4$ from NEN (Boston, MA, USA).

Animals

Six- to 8-weeks old female BALB/c and male C3H/HeN mice were purchased from Charles River Breeding Laboratory (Charles River Japan, Inc. Atsugi Breeding Center, Yokohama, Japan). The mice were kept for 7 days in our animal quarters to allow adaptation. Sterile standard mouse chow (NIH-7 open formula) and water *ad libitum* were given. They were housed randomly at 60% humidity and about 22 ± 2°C on a 12 h light-dark cycle.

Isolation of polysaccharide from CM

The activity-guided purification procedure of polysaccharide from CM is as shown in Fig. 1. Briefly, the crude drug of CM (400 g) was refluxed with methanol. The residue was dried absolutely at room temperature, and subsequently extracted with distilled water at 4°C for 24 h. The water extracts (CM-A, 28.6 g) was ultrafiltrated by 10 K membrane filter and lyophilized. The high molecular mass fraction (CM-AI, 2.8 g, M.W.>10 K) and the low molecular mass fraction (CM-AII, 24.6 g, M.W.<10 K) were obtained. The fraction, CM-AI (40 mg) was applied to a column (2.5 × 40 cm) of sephacryl S200, and eluted with 0.05 M sodium phosphate (pH 7.5). Fractions were collected with 4 ml/vial and monitored for protein and sugar contents using absorbance at 280 nm, Bradford assay (Bradford, 1976) and anthrone-sulphuric acid test (Dimler *et al.*, 1952). The vial numbers were plotted versus absorbance and sugar contents, affording a chromatogram that showed three parts of sugar contents. Three fractions were pooled, desalted with dialysis and lyophilized (CM-AIa, 3.24 mg, b, 2.28 mg, and c, 7.12 mg). This preparation was repeated giving reproducible profiles that permitted the pooling of like-fractions. Less than 0.05ng of endotoxin was detected in 1 mg of CM-AIa as measured by Limulus amebocytes lysate assay (Pyrotell, Associate of Cape Code. Inc., woods Hole, MA, USA) according to the manufacturer's instruction.

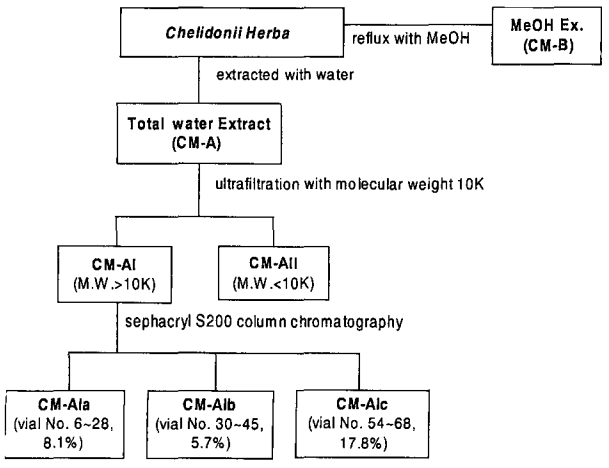

Fig. 1. Fractionation of Chelidonii Herba

General procedure

The total carbohydrate and protein contents were determined by the anthrone-H_2SO_4, and Lowry methods (Lowry *et al.*, 1951) using glucose and bovine serum albumin as the respective standards. The molecular weight was estimated by gel filtration using standard molecular weight markers (Bio-Rad. Hercules, CA); vitamine B-12 (MW 1,350), methylene blue (MW 7,000), ovalbumin (MW 44,000), γ-globulin (MW 158,000) and tyroglobulin (MW 670,000). CM-AIa was hydrolyzed by 1N-H_2SO_4, and then methylesterified. The product was analyzed by gas chromatography on Hewlett Packard model 5890 gas chromatograph equipped with an Ultra-1 capillary column.

Generation of activated killer cells

A 4 h [^{51}Cr]- release assay was used to determine the cytotoxic activity of AK cells (Mule and Rosenberg, 1991). Spleen cells were brought to a concentration of 3×10^6 cells/2 ml in RPMI1640 supplemented with 5% fetal bovine serum, 2×10^{-2} M HEPES buffer, 2×10^{-3} M glutamine, 1×10^{-3} M pyruvate, $5x10^{-5}$ M 2-mercaptoethanol, and 1% non-essential amino acid and antibiotics (GIBCO BRL, Gaithersburg, MD). The cells were cultured in 24 well plates at 37°C, 5% CO_2 incubator with rhIL-2, OK-432 (Picibanil, Chugai Pharmaceutical Co., Tokyo, Japan), and CM fractions for 5 days. After culture, cells were harvested and adjusted to the desired viable cell concentration. AK cells were incubated for 4 hours in 96 well U bottomed microplates containing ^{51}Cr-labeled target cells. The molony virus induced leukemia Yac-1 (H-2^a) was used as target cells. The plates were harvested and the radioactivity released in the supernatants was determined by using γ-counter (Beckmann Inst., Palo Alto. CA, USA). The percentage of cytotoxicity was calculated from the following formula; (ER-SR) / (MR-SR) × 100 where ER is the mean count from the experimental group, SR is the target cells incubated in medium alone. MR is the mean count from target cells treated with 5% triton X-100.

Tumoricidal activity of peritoneal macrophage

Macrophages were isolated from the peritoneal exudate cells as described by Klimetzek and Remold (Klimetzek and Remold, 1980). Peritoneal exudate cells were obtained from 6-7 weeks old C3H/HeN female mice by intraperitoneal injection of 1 ml Brewer thioglycollate broth (4.05 g /100 ml) (Difco Labs, Detroit, MI) and lavage of the peritoneal cavity with 5 ml of medium 3-4 days later. The cells were washed twice and resuspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The assay for macrophage cytotoxicity is based on an assay described previously (Ghaffar *et al.*, 1975). Cytotoxic activity was determined by measuring the radioactivity incorporated into tumor target cells after cocultivation with macrophages for 48 h. Macrophages (2×10^5 cells/well) were first incubated in either medium alone or in medium supplemented with CM-Ala for 24 h in 96-well plates. Macrophages were washed with RPMI-FBS to remove CM-Ala and co-incubated with 2 μCi/ml [^{3}H] thymidine labelled Yac-1 tumor cells (1×10^4 cells/well, effector: target cell ratio of 20:1) at 37°C in a 5% CO_2 incubator for 24 h. At the end of the incubation period, cells were harvested using an automatic multiwell harvester. The amount of radioactivity incorporated in the target cells was counted in a liquid scintillation counter. Under these conditions macrophages do not incorporate ^{3}H-TdR. Cytotoxicity was expressed as following formula.

$$\% \text{ cytotoxicity} = 1 - \frac{\text{Target counts/min with macrophages}}{\text{Target counts/min without macrophages}} \times 100$$

Proliferation of spleen cells

Single-cell suspension of spleen cells was prepared, treated with hypotonic solution to lyse erythrocytes. Spleen cells at 1.5×10^5 cells/ml were seeded in triplicate 0.2 ml cultures with various doses of CM fractions or concanavalin A (con A, Sigma) in 96-well tissue culture plates. Cultures were incubated for 72 h and pulsed with 2 μCi/well of [^{3}H]-thymidine during the last 4h. [^{3}H]-Thymidine uptake was determined in a liquid scintillation counter.

Granulocyte and macrophage colony-forming cell (GM-CFC) assay

Bone marrow cells were collected by flushing femurs and tibias, then single cell suspensions were obtained by repeated aspiration through needles. The bone marrow cells were plated in 35-mm tissue culture dishes (1×10^5/dish) containing Isacoves modified Dulbeccos medium (IMDM, Gibco, NY) supplemented with 0.3% agar, 20% horse serum and 10% of culture supernatant of NIH3T3 cells engineered to produce GM-CSF by transduction with MFG-GM-CSF retroviral vector. Various concentrations of CM-Ala or CM-Ala conditioned medium were tested in triplicate. CM-Ala conditioned medium was prepared by culturing spleen cells with CM-Ala for 24 h. The cultures were maintained at 37°C in 5% CO_2 in air for 7 days. Colonies of more than 50 cells were counted.

Colorimetric MTT assay

The mouse plasmacytoma MOPC315, mastocytoma P815, melanoma B16F10, human myelomonocytic U937 cell and human acute T lymphoma Jurkat cell lines were used for cytotoxicity assay. The cells were maintained in exponential growth in media and added 100 μl to 96 well flat-bottomed microplate. After addition of 50 μl samples, it was incubated for 2 days at 37°C, 5% CO_2 incubator. MTT solution was added 15 μl and incubated for 4 h. Culture was stopped by adding 150 μl 0.04N-HCl isopropanol into each well and the optical density at 540 nm was measured by Titertek Multiscan Plus (Flow Laboratories Inc.).

Statistical analysis

Statistical comparisons were performed by Students *t*-test.

RESULTS

Isolation of polysaccharide from CM

CM-AI was obtained from the water extract of CM by membrane filtration and fractionated on sephacryl S200 column chromatography, three kinds of fractions were obtained (Fig. 1). Of these fractions CM-Ala contained $28.2 \pm 3.03\%$ of protein, $69.8 \pm 6.04\%$ of carbohydrate. The carbohydrates of CM-Ala consisted mainly with galactose, mannose, glucose in the molar proportion of 5:4:1 as the neutral component sugars.

CM-Ala did not exhibit any lectin properties such as hemeagglutination, inhibition by sugar of the proliferation of lymphocytes and lectin-dependent cell mediated cytotoxicity (data not shown).

Effects of CM-Ala on the generation of activated killer (AK) cells

Spleen cells cultured for 5 days with CM-A fractions showed substantially increased cytotoxic antitumor activity (Fig. 2). In the presence of CM-AI or CM-Ala, the lytic activity of spleen lymphocytes to Yac-1 tumor cells increased from 0.88% to 29.39%, 34.18%, respectively. The optimal dose of CM-Ala for AK cells generation was 5 μg/ml.

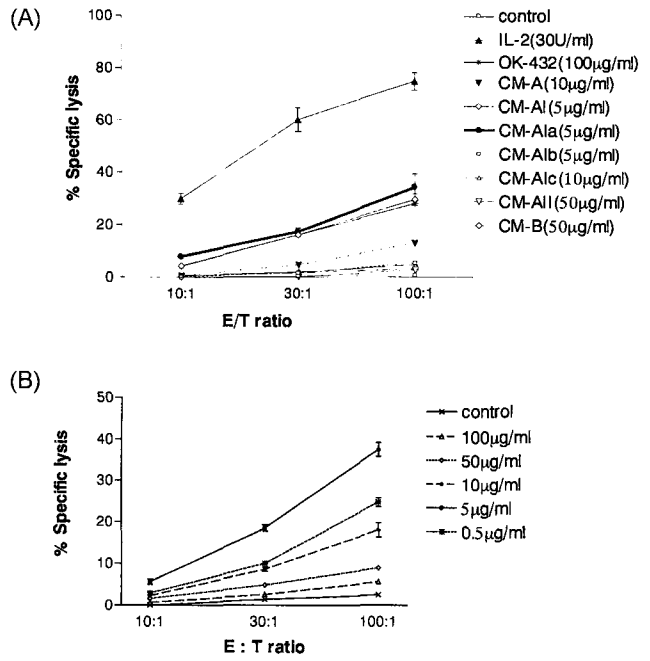

Fig. 2. Effects of CM fractions on mouse AK cell activity. A. Mouse splenocytes (3×10^6/ml) were cultured in the presence of rIL-2 (30U/ml) or each optimal concentration of CM fractions for 5 days. Cells were collected and assayed for AK cell activity. B. The dose-response curve of CM-Ala. The results represented the mean ± SEM of values obtained from five replicated experiments.

Tumoricidal activity of peritoneal macrophage

Macrophages showed the increased cytotoxicity from 24.5% to 57.0% by culturing with CM-Ala at 10 μg/ml (Table I). The peritoneal macrophages produced nitric oxide more than 2 fold by culturing with CM-Ala as compared to control (from 21.8 μM to 48.0 μM). This value was comparable with that of IFN-γ (50 U/ml) producing 55.6 μM of NO.

Mitogenic activity of the polysaccharide

Mitogenic activity of polysaccharide on murine splenocytes was measured (Table II, III). CM-B, CM-Alb, CM-Alc, and CM-AII showed no activity, whereas the CM-AI and CM-Ala at optimal concentration displayed stimulatory activity 75 and 84 times that of the control. Since CM-Ala was suggested to contain some protein molecules, CM-Ala was heated at 100°C for 1 h or treated with pronase at 37°C for 48 h. Neither of these treatments affected the potent mitogenic activity. These results suggested that carbohydrate region was involved in induction of mitogenic activity of CM-Ala, but that the protein moiety was not.

Table I. Antitumor activity by peritoneal macrophage cultured with CM-Ala

Treatment[a]	Dose	[^{3}H]-thymidine incorporation (cpm)	% of inhibition
None	-	55964.0 ± 992.1	24.5
IFN-γ	50 U/ml	34256.0 ± 988.5*	53.8
CM-Ala	10 μg/ml	31897.5 ± 977.9*	57.0
	50 μg/ml	34368.0 ± 1417.7*	53.7
	100 μg/ml	36766.0 ± 1279.9*	50.4
Target only	-	74156.5 ± 2204.1*	

[a]Peritoneal macrophages (PM) were cultured in the presence of IFN-γ or CM-Ala for 24 h and then further co-cultured for 24 h with targets at an initial effect/target cell ratio of 20 :1.
Effects on target cell viability are expressed as net percent [^{3}H] thymidine incorporated.
The results are mean ± S.D. of values obtained from three replicated experiments.
*Significantly different from control (no treatment): $p < 0.01$

Table II. Mitogenic effects of CM fractions on murine spleen cells

Treatment[a]	optimal concentration	^{3}H-TdR incorporation (cpm) ± S.D.
SC + Control	-	1488 ± 254
SC + Con A	2.5 μg/ml	138230 ± 7048*
SC + LPS	10 μg/ml	85347 ± 7948*
SC + CM-A	125 μg/ml	83883 ± 12015*
SC + CM-AI	62.5 μg/ml	111819 ± 8545*
SC + CM-Ala	31.3 μg/ml	124864 ± 13358*
SC + CM-Alb	250 μg/ml	10154 ± 722
SC + CM-Alc	250 μg/ml	4893 ± 677
SC + CM-AII	250 μg/ml	3653 ± 1001
SC + CM-B	250 μg/ml	3328 ± 210

[a]BALB/c spleen cells (SC, 1.5×10^5 cells/well) were cultured with various doses of CM fractions on microtiter plates. After 3 days, the growth of lymphocytes was determined by the incorporation of [^{3}H]-thymidine after 4 h pulsing with 2 μCi of [^{3}H]-thymidine. Results are presented the mean ± S.D. for quintuplicate from a representative experiments.
*Significantly increased versus control: $p<0.001$

CM-Ala induction of CSF secretion and GM-CFC *in vitro*

Spleen cells were cultured with 10, 50, 100 μg/ml of

Table III. The determination of optimal concentration of CM-Ala exhibiting mitogenic activity

CM-Ala (μg/ml)	-	500	250	125	62.5	31.3	15.7
^{3}H-TdR incorporation (cpm) ± S.D.	1479 ± 316	4226 ± 515	93250 ± 3048	125621 ± 14609	133494 ± 10690	143443 ± 9217	101136 ± 9339

The data represent the mean ± S.D. of values obtained by six replications. All the CM-Ala treated group showed significantly higher than that of control ($p< 0.001$).

CM-Ala. The supernatants were collected after 24 h and assayed for CSF activity by soft agar technique described by *Materials and Method*. As can be seen in Table IV, CSF secretion was increased by 14% that of control, but no statistically differences were noted. On the other hand, significantly higher numbers of the GM-CFU were observed when bone marrow cells were cultured with CM-Ala at 10 μg/ml directly in the agar plates (increase from 40.67 to 60.33 colonies/10^5 BM cells).

Cytotoxic activity of CM-Ala on tumor cells

In order to examine whether the CM-Ala also could inhibit proliferation of tumor cells, we tested the cytotoxicity by MTT assay (Fig. 3). In contrast with CM-Ala proliferated the lymphocytes as shown in Table II, CM-Ala inhibited the growth of a diverse group of tumor cells. The growth inhibition appeared dose-dependent manner. CM-Ala showed 51.0% cytotoxicity for P815 cell line and 52.7% for B16F10 cell line at 100 μg/ml.

DISCUSSION

Recently, biological response modifiers (BRMs) have attracted much attention for adjuvant immunotherapy of malignant diseases. The ideal BRM should initiate balanced immunostimulation with capability of nonspecifically activating all effector pathways (T cells, NK cells, and macrophages) without cytotoxicity to normal tissues. The model agent should be able to proliferate effector lymphocytes and macrophages. In addition, enhancement of hematopoiesis and endogenous generation of cytokines would exhibit the advantage of a sustained effect preferred in this BRM application (Bruce, 1989).

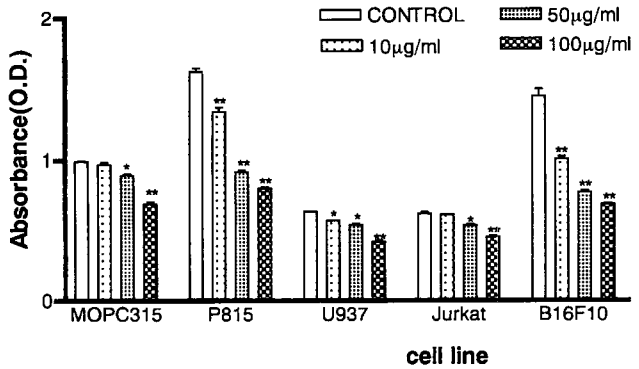

Fig. 3. Effect of CM-Ala on tumor cell survival measured by MTT test. Tumor cells (1×10^4 cells/well) were incubated with CM-Ala at 37°C, 5% CO_2 incubator. Three days later, 15 ml of MTT dye was added and after another 4 h incubation, 150 ml of 0.04N-HCl isopropanol was added. Each well was pipetted vigorously and O.D value was determined using microplate reader at 540nm. Significantly different from non-treated control of each cell line: $^*p<0.05$, $^{**}p<0.001$.

Some types of polysaccharide, such as PSK or Krestin (Chihara *et al.*, 1970), Lentinan (Tsukagoshi *et al.*, 1984) and OK-432 (Hoshina and Uchida, 1984) have been shown to have immunomodulating activity due to enhancement of NK activity and secretion of Th1 type cytokines. In this study we isolated protein-bound polysaccharide CM-Ala from water extracts of CM showing potent immunomodulatory activities. *Chelidonii Herba* has often contributed in the treatment of several diseases, such as gastrodynia, gastric ulcer, chronic bronchitis, inflammation, pertussis (Jee and Lee, 1988). However, its immunomodulatory activity was not reported.

The level of AK cell generation by CM-Ala was 38.7 times higher than that of control, and the activity was as strong as that of OK-432 in our system (Fig. 2). It has been known that AK cells are generated from both NK cells and T cells by culturing with IL-2, IFNγ or TNFα that is synergized with low dose of IL-2 (Lee *et al.*, 1997; Kim *et al.*, 1998). *In vitro* culture of lymphocytes with rIL-2 generates LAK cells that are cytotoxic to freshly isolated autologous and allogenic tumor cells and NK-resistant tumor cell lines (Lotze *et al.*, 1981; Grimm *et al.*, 1982; Rosenstein *et al.*, 1984), but the clinical trials of LAK cell and rIL-2 showed limited success (Rosenberg *et al.*, 1985; West *et al.*, 1987). Therefore, attempts have been made in various laboratories to find ways to generate LAK cells with higher cytotoxic activity and to minimize the toxicity.

In cytotoxicity assays, we have shown that CM-Ala elicited peritoneal macrophages demonstrated cytotoxic for Yac-1 tumor cells as IFNγ-elicited macrophages did (Table I). Hibbs *et al.* have demonstrated that activated macrophages produce NO, which inhibits mitochondrial respiration and results in cytotoxicity against target cells (Hibbs *et al.*, 1987; Hibbs *et al*, 1988). In the present studies suggested that reactive nitric oxide induced by CM-Ala is one of the effector molecules involved in macrophage mediated tumor cytotoxicity. We have recently found that CM-Ala induces mRNA expression of a variety of cytokines such as IL-2, IFN-γ, IL-4, IL-5, GM-CSF, IL-1β, TNFα (submitted). Thus it has been suggested that CM-Ala activates lymphocytes and macrophages to produce cytokines and thereby generates the effector cells to tumor cells.

CM-Ala induced proliferation of spleen cells by 84-fold compared to non-treated cells (Table II), and the heat inactivated or pronase treated CM-Ala did not change the mean value of cpm (data not shown). Therefore the carbohydrate region of CM-Ala was the main moiety of mitogenic activity. CM-Ala also slightly increased the level of CSF by spleen cells in dose-independent manner. When CM-Ala was inserted directly into the agar culture,

Table IV. Increase of the numbers of the GM-CFU in the bone marrow cells by culturing with CM-Ala

Treatment[a]	Dose	No. of colonies/1 × 10^5 BM cells
None	-	40.67 ± 4.67
CM-Ala	10 μg/ml	60.33 ± 4.37*
	50 μg/ml	42.67 ± 2.60
	100 μg/ml	29.00 ± 2.00
CM-Ala conditioned medium	10 μg/ml	44.50 ± 1.50
	50 μg/ml	45.50 ± 6.50
	100 μg/ml	42.50 ± 0.50

[a]BM cells from BALB/c mouse were cultured in agar plates in the presence of CM-Ala or CM-Ala conditioned medium as described in *Material and Method*. The data represent the mean ± SEM of values obtained from three replicated GM-CFC assays in which specimen was cultured in duplicate.
*Significantly different from control (no treatment) : $p<0.05$

the number of GM-CFC was increased 1.48 fold. This effect may be mediated by direct stimulation of GM-CFC proliferation and/or stimulation of production of the hematopoietic cytokines. The GM-CSFs probably play an important role in the hematopoietic generation of GM-progenitor cells and in the differentiation of myeloid leukemia cells.

On the other hand, CM-Ala at 100 μg/ml has been shown to have a direct growth inhibitory effect on tumor cells. This suggested that the effective dose to augment effector immune cells was different from the toxic dose to show killing activity against tumor cells, this was another advantage of a useful application of BRM.

According to many *in vitro* and *in vivo* studies of immunomodulators, it has not been unusual to observe a bell-type dose-response curve for therapeutic activity (Talmadge and Herbermann, 1986). Similarly, our results exhibited the bell shape dose-response curve as shown above. Therefore, the determination of the optimal immunostimulatory dose is one of the most important things to acquire predictive *in vivo* and clinical data.

Further studies on the purification and chemical characterization of CM-Ala are now in progress.

REFERENCES

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254 (1976).

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y., and Fukuoka, F., Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk) Sing. (an Edible mushroom). *Cancer Res.*, 30, 2776-2781 (1970).

Dimler, R. J., Schaefer, W. C., Wise, C. S., and Rist, C. E., Quantitative paper chromatography of D-glucose and its oligosaccharides. *Anal. Chem.*, 24, 1411-1415 (1952).

Ghaffar, A., Cullen, R. T., and Woodruff, M.F.A., Further analysis of the anti-tumor effect in vitro of peritoneal exudate cells from mice treated with *Corynebacterium parvum*. *Brit. J. Cancer*, 31, 15-24 (1975).

Grimm, E. A., Mazumder, A., Zhang, H. Z., and Rosenberg, S. A., Lymphokine-activated killer cell phenomenon, lysis of natural killer-resistant fresh solid tumor cells by interleikin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.*,155, 1823-1841 (1982).

Herbermann, R. B., Biological response modifiers for the treatment of cancer. In: E. Tsubura, I. Urashizaki, T. Aoki, S. Sone and Y. Kaneto. (Eds.). *Rationale of Biological response Modifiers in Cancer Treatment.* Excepta Medica, Princeton, pp. 240-255, (1985)

Hibbs, J. B., Taintor, R. R., and Vavrin, Z., Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogrn oxidation to nitrite. *Science*, 235, 473-476 (1987).

Hibbs, J. B., Taintor, R. R., Vavrin, Z., and Rachlin, E. M., Nitric oxide; a cytotoxic-activated macrophage effector molecule. *Biochem. Bioph. Res. Co.*, 157, 87-94 (1988).

Hoshina, T. and Uchida, A., OK-432 (picibanil): Property, action and chemical effectiveness. In: T. Hoshino. (Eds.), Excepta Medica, Amsterdam. pp. 3-21, (1984).

Jee, H. J. and Lee, S. I., Implication of Korean medicinal plants. Korea medical Index Inc., Seoul, pp. 153, (1988).

Kery, A., Horvath, J., Nasz, I., Verzar-petri, G., Kulcsar, G., and Dan, P., Antiviral alkaloids in Chelidonium majus. *Acta Pharm. Hungarica*, 57, 19-25 (1987).

Kim, K. H., Lee, Y. S., Jung, I. S., Park, S. Y., Chung, H. Y., Lee, I. R., and Yun, Y. S., Acidic polysaccharide from *Panax ginseng*, Ginsan, induces Th1 cell and macrophage cytokines and generate LAK cells in synergy with rIL-2. *Planta Med.*, 64, 110-115 (1998).

Klimetzek, V. and Remold, H.G., The murine bone marrow macrophage, a sensitive indicator cell for murine migration inhibitory factor and a new method for their harvest. *Cell. Immunol.* 53, 257-266 (1980).

Lee, Y. S., Jung, I. S., Lee, I. R., Kim, K. H., Hong, W. S., and Yun, Y. S., Activation of multiple effector pathway of immune system by the antineoplastic immunostimulator acidic polysaccharide Ginsan isolated from *Panax ginseng*. *Anticancer Res.*, 17, 323-332 (1997).

Lenfeld, J., Kroutil, M., Marsalek, E., Slavik, J., Preininger, V., and Simanek V., Antiinflammatory activity of quarternary benzophenanthridine alkaloids from *Chelidonium majus*. *Planta Med.*, 43, 161-165 (1981).

Liepins A. and Nowicky J. W., Activation of spleen cell lytic activity by the alkaloid thiophosphoric acid derivative: ukrain. *Int. J. Immunopharmaco.*, 14, 1437-1442 (1992).

Lotze, M. T., Frana, L. W., Sharrow, S. O., Robb, R. J., and Rosenberg, S. A., In vivo administration of purified human

interleukin 2. I. Half-life and immunologic effects of Jurkat cell line-derived interleukin 2. *J. Immunol.*, 134, 157-166 (1985).

Lotze, M. T., Grimm, E. A., Mazumder, A., Strausser, J. L., and Rosenberg, S. A., Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor. *Cancer Res.*, 41, 4420-4425 (1981).

Lotze, M. T., Matory, Y. L., Rayner, A. A., Ettinghausen, S. E., Vetto, J. T., Seipp, C.A., and Rosenberg, S.A., Clinical effects and toxicity of interleukin 2 in patients with cancer. *Cancer Res.*, 58, 2764-2772 (1986).

Lowry, O. H., Rosenberg, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Mule, J. J. and Rosenberg, S. A , Measurement of cytotoxic activity of LAK/NK cells. In: J.E Coliga, A.M. Kruisbeek, D.H. Margalies, E.M. Shevach and W. Strober.(Eds.). Current protocols in immunology. John Willey and Sons, New York, pp 7.18.1-7.18.7, (1991).

Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Maltory, Y. L., Skibber, J. M., Shiloni, E., and Vetto, J. T., Observations on the systemic administraion of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *New Engl. J. Med.* 313, 1485-1492 (1985).

Rosenstein, M., Yron, I., Kaufman, Y., and Rosenberg, S. A., Lymphokine-activated killer cell: Lysis of fresh syngeneic natural killerresistant murine tumor cells by lymphocytes cultured in interleukin 2. *Cancer Res.*, 44, 1946-1953 (1984).

Song, J. Y., Yang, H. O., Park, S. Y., Pyo, S. N., Kim, K. W. Son, E. W., and Yun, Y. S., Screening of antineo-plastic immunomodulator from herbal medicines. *Yakhak Hoeji*, 42, 132-139 (1998).

Talmadge, J. E. and Herbermann, R. B., The preclinical screening laboratory:Evaluation of immunomodulatory and therapeutic properties of biological response modifiers. *Cancer Treat. Reports*, 70, 171-182 (1986).

Terry, W. D. and Rosenberg, S. A., Immunotherapy of Human Cancer. Elsevier North-Holland. New York, (1982).

Tsukagoshi, S., Hashimoto, Y., Fujii, G., Kobayashi, H., Nomoto, K. and Orita, K., Krestin(PSK), *Cancer Treat. Rev.*, 11, 131-155 (1984).

Urba, W. J. and Longo, D. L., a-Interferon in the treatment of modular lymphoma. *Semin. Oncol.*, 13 (suppl.5), 40-47 (1986).

Voltchek, I., Kamyshentsev, M., Lavinsky, Y., Nowisky, J., Medvedev, Y., and Litvinchuk, L., Comparative study of the cytostatic effects of oliphen and ukrain. *J. Chemotherapy*, 8, 144-146 (1996).

West, W. H., Tauer, K. W., Yannelli, J. R., Marshall, G. D., Orr, D. W., Thurman, G. B., and Oldham, R. K., Constant infusion recombinant interleukin 2 in adaptive immunotherapy of advanced cancer. *New Engl. J. Med.*, 316, 898-905 (1987).

Wimer, B. M., The ideal biological response modifier. *Mol. Biotherapy*, 1, 311-317 (1989).

Wyczolkowska, J., Michon, T., and Nowicky, J. W., Inhibitory effect of thiophosphoric acid alkaloid derivatives from Chelidonium majus (ukrain) on ovalbumin antigenicity and anti-ovalbumin IgE antibody response in mice. *Drug. Exp. Clin. Res.*, 22, 225-228 (1996).