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Original Article

Effects of *Cordyceps Militaris* Extract on Tumor Immunity

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Background and Aims : Even though various strategies for cancer treatment have advanced with the remarkable development of genomic information and technology, it is far from giving relief to cancer patients. Recently there is accumulating evidence that the immune system is closely connected to anti-tumor defense mechanisms in a multistage process. This includes tumorigenesis, invasion, growth and metastasis. *Cordyceps Militaris*, a well-known oriental herbal medicine, is a parasitic fungus that has been used as an immune enhancing agent for a long period of time. However, little is known about the cancer-related immunomodulatory effects and anti-tumor activities. In the present study, we aimed to investigate the effects of *Cordyceps Militaris* extract (CME) on immune modulating and anti-tumor activity.

Materials and Methods : To elucidate the effects of CME on macrophage and natural killer (NK) cell activity, we analyzed nitric oxide (NO) production, NK cytotoxicity and gene expression of cytokines related with macrophages and NK cell activity.

Results and Conclusions : CME activated and promoted macrophage production of NO. It also enhanced gene expression of IL-1 and iNOS in RAW 264.7 cells. CME promoted cytotoxicity of NK cells against YAC-1 cells and enhanced NK cell related gene expression such as IL-1, IL-2, IL-12, iNOS, IFN-γ and TNF-α in mice splenocytes. It also promoted protein expression of IL-10, IL-12, IFN-γ and TNF-α in mice splenocytes and inhibited lung tumor metastasis induced by CT-26 cell line compared with the control group. From these results, it could be concluded that CME is an effective herbal drug for modulating the immune system and anti-cancer treatment by promoting macrophage and NK cell activity.

Key Words : *Cordyceps Militaris*, immune modulating activity, anti-tumor activity

Introduction

Even though various strategies for cancer treatment have advanced with the remarkable development of genomic information and technologies, it is far from giving relief to patients with cancer. The main conventional cancer therapies, radiation and chemotherapy, generally show low efficacy and a high rate of undesirable side effects.

Recently evidence has been accumulating closely connecting the immune system with anti-tumor defense mechanisms in a multistage process including tumorigenesis, invasion, growth and metastasis [1].

The immune modulating and anti-tumor activities of various oriental herbal plants have been experimented extensively and reported over the world. Administration of these herbs are known to inhibit tumor growth and incidence, and prolong the tumor-bearing rodent survival in transplanted experimental models and also restore lowered host immune defenses[2,4].

Cordyceps Militaris, a well-known traditional

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oriental medicine, is a parasitic fungus that has been used as an immune potentiating agent for a long time. Over the past few decades, a considerable number of studies have been conducted on the effects of *Cordyceps* fungus on various diseases[5,10].

In these studies, various effects of anti-oxidation and stimulating the immune system have been shown. However, little is known about cancer-related immunomodulatory effects and anti-tumor activities.

Our previous research has demonstrated the anti-tumor effects of *Cordyceps Militaris* presented by anti-angiogenesis[11]. In the present study, to investigate effect of *Cordyceps Militaris* extract (CME) on immune modulating and anti-tumor activity, we especially analyzed the effect on macrophage and NK cell activity through the measurement of NO production of macrophages, NK cell cytotoxicity and several gene expressions of related cytokines.

Materials and Methods

1. Materials

Cordyceps Militar was obtained from Daejeon Oriental Medical Hospital. Fifty grams of *Cordyceps Militaris* was mixed with 2L of distilled water and left for 1 h at room temperature, and the whole mixture was then boiled for 2 h. The CME was filtered and then lyophilized. The yield of CME was 10.5% (w/w) in terms of the dried medicinal herbs. M-MLV RT, Taq polymerase, dNTP and 5X TBE buffer were obtained from Promega (Madison, WI). Other chemicals were purchased from Sigma (St Louis, MO).

2. Experimental animals

Specific pathogen-free BALB/c mice were obtained from a commercial animal breeder (Daehan BioLink, Korea). The animals were housed under normal laboratory conditions (23 ± 2℃ and 40-60% relative humidity) with 12 h light/dark cycle with free access to standard rodent food and water.

3. Cell culture

RAW 264.7 and HT1080 cells were obtained from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Hep G2, CT-26 cells from Korean Cell Line Bank (Seoul, Korea) and human fibroblast, 7250 cells from National Cancer Institute (USA). The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 units streptomycin and 100 units penicillin.

4. Measure of cytotoxicity

7250, HT1080, Hep G2 and CT-26 cells (1×103) were seeded into 96-well plates and cultured overnight. The cells were treated with CME (0, 50, 100, 200 ㎍/㎖). CCK-8 (20 ul) was added to each well. Three hours later, 150 ul medium was harvested to determine optical density. Cell proliferation was determined on days 0, 2, 4, and 6.

5. NO assay

RAW 264.7 cells were cultured with DMEM containing 10% FBS. RAW 264.7 (5×105 cells) were plated in 24-well plates (BD, NJ, USA) and treated with CME (0, 2, 20, 200 ㎍/㎖) and LPS (1 ㎍/㎖) and incubated at 37℃ with 5% CO2. NO formation was measured as the stable end product nitrite (NO2-) in the culture supernatant with Griess reagent. Briefly, an aliquot of culture supernatant (100 ㎕) was added to each well of 96-well plates and mixed with the same volume

Table 1. Oligonucleotide sequences of primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense Antisense	5'-AAG CTC TCA CCT CAA TGG A-3' 5'-TGC TTG AGA GGT GCT GAT GT-3'	302
IL-10	Sense Antisense	5'-TCC TTG GAA AAC CTC GTT TG-3' 5'-TCT CTT CCC AAG ACC CAT GA-3'	389
iNOS	Sense Antisense	5'-TGG TGG TGA CAA GCA CAT TT-3' 5'-CTG AGT TCG TCC CCT TCT CTC C-3'	229
TNF-α	Sense Antisense	5'-CTC CCA GGT TCT CTT CAA GG-3' 5'-TGG AAG ACT CCT CCC AGG TA-3'	195
β-actin	Sense Antisense	5'-ACC GTG AAA AGA TGA CCC AG-3' 5'-TCT CAG CTG TGG TGG TGA AG-3'	285

of Griess reagent (1:1[v/v]; 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride in H2O, 1% sulfanilamide in 5% H2PO4), and then the A540 was read with a microplate reader (Molecular Devices, USA). Nitrite concentration was determined by using dilutions of sodium nitrite in culture medium as standards. By adding CME to standard solutions of sodium nitrite, it was confirmed that CME did not interfere with the nitrite assay.

7. NK cell 51Cr release assay

51Cr release assay was performed as described previously with modifications[15]. Spleen cell suspensions were prepared in ice-cold DMEM from BALB/c mice. After adjusting to final concentration (1×107 cells/㎖), 100 ㎕ of suspension (4×106, 2×106 and 1×106 cells/well) were plated onto round bottom 96-well plates (4 wells per group) with various concentrations of CME (0.2, 2, 20, 200 ㎍/㎖) and IL-2 (300 U/㎖). These cells were incubated for 14 h at 37℃ with 5% CO2 and prepared as effector cells.

YAC-1 cells (5×106) were cultured for using as target cells of NK cell. After labeling the target cells by incubating for 2 h (37C, 5% CO2) with 51Cr (200 μ Ci), washing and lysis of unhealthy cells, the labeled target cells were centrifuged for 5 min at 400 ×g, and adjusted to 2×105 cells/㎖. Fifty microliter cell suspension (1×104 cells) was added to effector cells and incubated for 4 h. Maximum release groups were added with 150 ㎕ of 2% NP-40, and spontaneous release groups with 150 ㎕ of complete medium. After 4 h, the cells were concentrated by centrifugation at 500×g for 10 min, and cell-free supernatant was harvested from each well for assessment of radioactivity. Then gamma irradiation from each well was assessed in a scintillation counter (Packard Instruments). The percentage of specific lysis was calculated by the following equation:

$$\text{Specific killing activity (\%)} = \frac{\text{CME release - spont. release}}{\text{max. release - spont. release}} \times 100$$

8. Gene expressions of IL-1, IL-2, IL-4, IL-10, IL-12, TNF-α, IFN-γ, TGF-β, iNOS in splenocytes

BALB/c mice were sacrificed and spleens removed to PBS. After RBC lysis, the cells were washed twice with PBS. The splenocytes (2×107 cells) were treated with various concentrations of CME (0, 0.2, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) in 6-well plates and incubated for 6 and 12 h at 37℃ with 5% CO2. Total RNA was isolated by the easy-BLUE reagent (iNtRON, Korea) and all processes of first strand cDNA and polymerase chain reaction were done according to the manufacturer's instructions.

Briefly, PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95 ℃ for 10 min; then 27 cycles for β-actin and 35 cycles for other genes at 95 ℃ for 1 min, 60 ℃ for 40 seconds and 72 ℃ for 40 seconds. The PCR products were run on a 1 % agarose gel in 0.5 × TBE buffer. The primers used are described in Table 2.

9. Cytokine expressions of IL-2, IL-10, TNF-α, IFN-γ in splenocytes

BALB/c mice were sacrificed and spleens removed to PBS. After RBC lysis with lysing buffer, the cells were washed twice with PBS. The splenocytes (5×106 cells) were treated with CME (0, 2, 20, 200 ㎍/㎖), Con A (0.5 ㎍/㎖), or LPS (0.5 ㎍/㎖) in 24-well plates and incubated for 24 and 48 h at 37℃ with 5% CO2. Supernatant was harvested and cytokines were determined by ELISA kit (BD, USA).

10. Splenocyte proliferation

BALB/c mice were sacrificed and spleens removed to PBS. After RBC lysis with lysing buffer, the cells were washed twice with PBS. The splenocytes (1×106 cells) were seeded into a 96-well plate and treated with CME (0, 2, 20, 200 ㎍/㎖) with 3 ㎍/㎖ Con A (T cell mitogen), or 3 ㎍/㎖ LPS (B cell mitogen). The plate was incubated for 72 h at 37℃ with 5% CO2. Cellular proliferation was determined using CCK-8.

11. Pulmonary colony assay

Mice were divided into 2 groups (10 mice per group). The mice were treated with 200 mg of CME per kg body weight and the mice in the

Table 2. Oligonucleotide sequences of primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense Antisense	5'-AAG CTC TCA CCT CAA TGG A-3' 5'-TGC TTG AGA GGT GCT GAT GT-3'	302
IL-2	Sense Antisense	5'-TGC TCC TTG TCA ACA GCG-3' 5'-TCA TCA TCG AAT TGG CAC TC-3'	391
IL-4	Sense Antisense	5'-TCA ACC CCC AGC TAG TTG TC-3' 5'-TGT TCT TCA AGC ACG GAG GT-3'	254
IL-10	Sense Antisense	5'-TCC TTG GAA AAC CTC GTT TG-3' 5'-TCT CTT CCC AAG ACC CAT GA-3'	389
TNF-α	Sense Antisense	5'-CTC CCA GGT TCT CTT CAA GG-3' 5'-TGG AAG ACT CCT CCC AGG TA-3'	195
IFN-γ	Sense Antisense	5'-GGA TAT CTG GAG GAA CTG GC-3' 5'-GAG CTC ATT GAA TGC TTG GC-3'	250
TGF-β	Sense Antisense	5'-TGA GTG GCT GTC TTT TGA CG-3' 5'-TTC TCT GTG GAG CTG AAG CA-3'	310
iNOS	Sense Antisense	5'-TGG TGG TGA CAA GCA CAT TT-3' 5'-CTG AGT TCG TCC CCT TCT CTC C-3'	229
β-actin	Sense Antisense	5'-ACC GTG AAA AGA TGA CCC AG-3' 5'-TCT CAG CTG TGG TGG TGA AG-3'	285

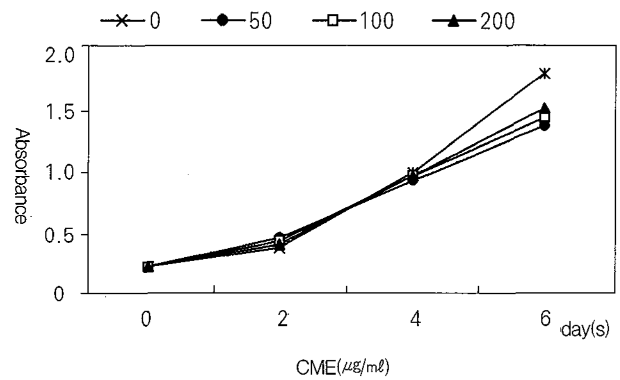

Fig. 1. Effect of CME on cytotoxicity of 7250 cell. Human fibroblast cell line, 7250 cells (1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CME (0, 50, 100, 200 ㎍/㎖), and OD450-560 was determined at 3 h after CCK-8 addition on experiments day 0, 2, 4, 6

control group were administrated distilled water. CME was treated from 7 days before CT-26 injection to 7 days after CT-26 injection. CT-26 cells (2×104) were inoculated to tail vein. The mice were sacrificed 14 days after tumor inoculation and lungs were removed. Lung tumor colonies were counted.

12. Histopathological observations

For the histomorpathological evaluation, a portion of lung tissue was removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded lung section (4 ㎛ in thickness) was stained with hematoxylin & eosin (HE) for histopathological examination.

13. Statistical analysis

Results are expressed as the mean ± SD. Statistical analysis of the data was carried out by Student's t-test. A difference from the respective control data at the levels of $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ was regarded as statis-

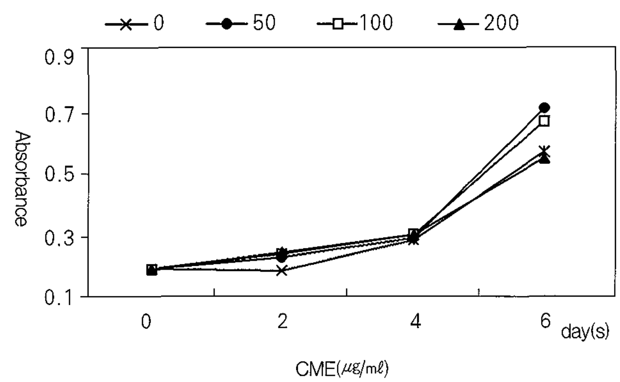

Fig. 2. Effect of CME on cytotoxicity of HT 1080. HT1080 cells(1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CME (0, 50, 100, 200 ㎍/㎖), and OD450-560 was determined at 3 h after CCK-8 addition on experiments day 0, 2, 4, 6

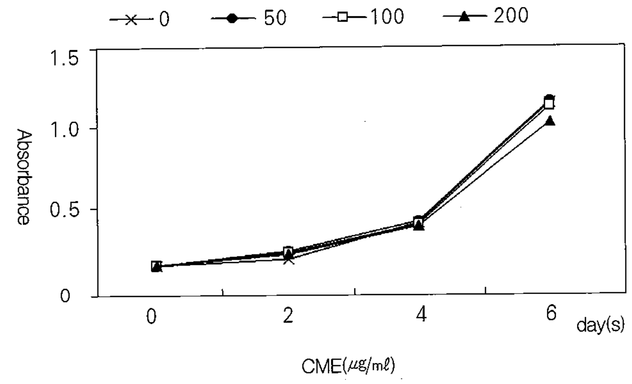

Fig. 3. Effect of CME on cytotoxicity of Hep G2. Hep G2 cells(1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CME (0, 50, 100, 200 ㎍/㎖), and OD450-560 was determined at 3 h after CCK-8 addition on experiments day 0, 2, 4, 6.

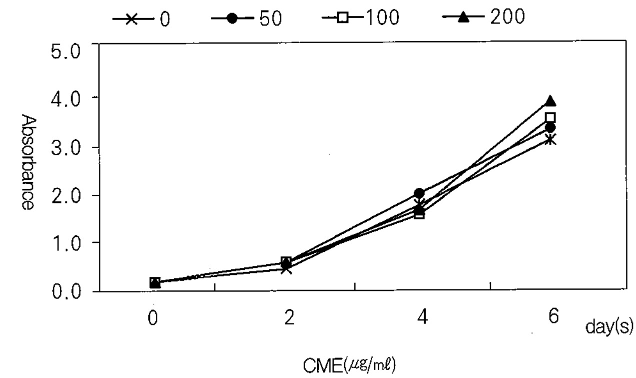

Fig. 4. Effect of CME on cytotoxicity of CT-26. CT-26 cells(1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CME (0, 50, 100, 200 ㎍/㎖), and OD450-560 was determined at 3 h after CCK-8 addition on experiments day 0, 2, 4, 6.

tically significant.

Results

1. Cytotoxicity

Human fibroblast (7250), HT1080, Hep G2, CT-26 cells (1×103) were seeded into 96-well plates and cultured overnight. The cells were treated with various concentrations of CME (0, 50, 100, 200 ㎍/㎖). As shown in Fig. 1-4, no effect of CME on cell growth was observed.

2. NO production

NO production was measured after treatment with various concentrations of CME (0, 2, 20, 200 ㎍/㎖). NO production was significantly increased at 200 ㎍/㎖ of CME. However, CME did not affect NO production in low concentrations, below 20 ㎍/㎖ (Fig. 5).

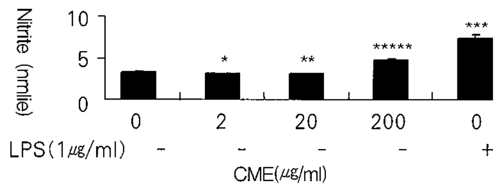

Fig. 5. Effect of CME on NO production of RAW 264.7 cells. RAW264.7 cells were treated with CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖). Each data represents the mean ± SD. Statistically significant value compared with control by T-test. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$)

3. Changes in gene expression in RAW 264.7 cells

To investigate gene expression of cytokines in RAW 264.7, macrophage cell line, RAW 264.7 cells were treated with various concentrations of CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) for 12 and 24 h. IL-1β mRNA expression was up-regulated by CME treatment in a dose-de-

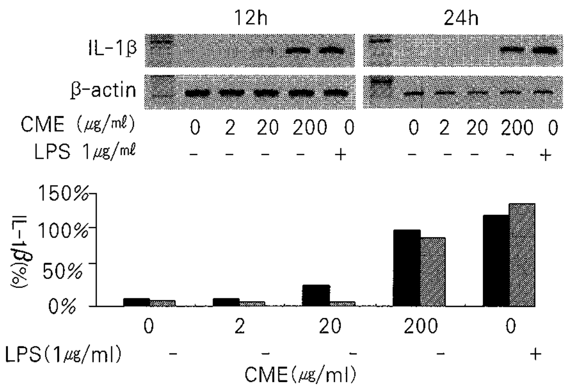

Fig. 6. IL-1β gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of IL-1 to β-actin.

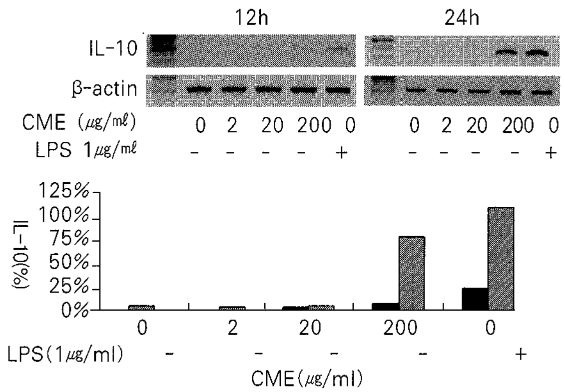

Fig. 7. IL-10 gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CME (0, 2, 20, 200 ㎍/㎖)) or LPS (1 ㎍/㎖) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of IL-10 to β-actin.

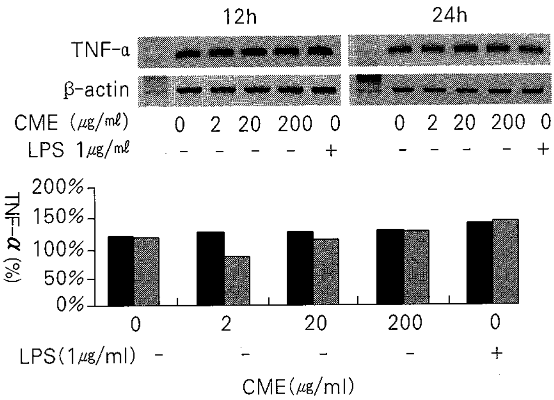

Fig. 8. TNF-α gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of TNF-α to β-actin.

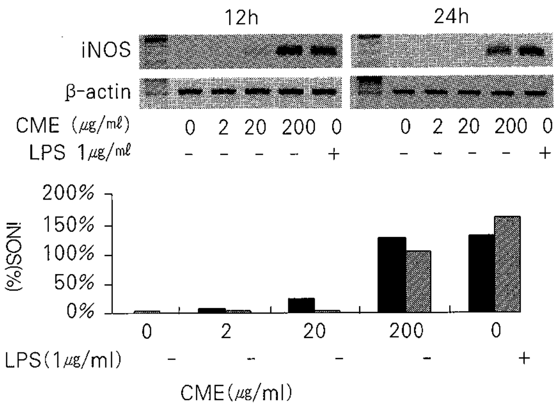

Fig. 9. iNOS gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of iNOS to β-actin.

pendent manner. Especially, IL-1β mRNA expression increased 10-fold compared with the control (Fig. 6). IL-10 gene expression also up-regulated at 200 μg/mℓ CME. However, CME did not affect IL-10 gene expression at low concentrations, below 20 μg/mℓ CME (Fig. 7). TNF-α gene expression was not affected by CME treatment (Fig. 8). iNOS gene expression was increased by CME treatment in a dose-dependant manner.

4. NK cell activity

CME showed a significant effect on NK cell cytotoxic activity compared with the control at 0.2, 2, and 20 μg/mℓ CME at all ratios of effector cell to target cell. Especially, at ratio of 50 (effector cell) : 1 (target cell), CME (0.2, 2, 20 μg/mℓ) showed significantly different efficacy of

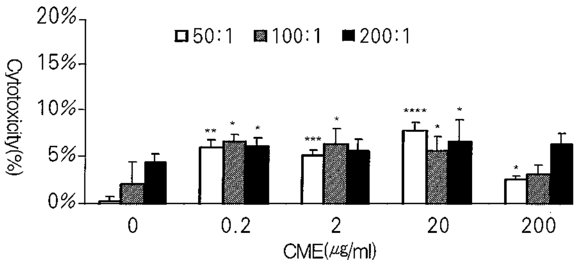

Fig. 9. Effect of CME on NK cell activity. Spleen cells (effector cell) were treated with CME (0, 2, 20, 200 μg/mℓ) and IL-2 (300 U/ml) for 14 h. Yac-1 cells (taget cell) labeled with ^{51}Cr were mixed to effector cells for 4 h. Cell-free supernatant containing released ^{51}Cr was counted by using gamma scintillating counter. Each data represent the mean±SD. Statistically significant value compared with control by T-test.(*: p<0.05, **: p<0.01, ***: p<0.001).

the NK cell cytotoxic activity.

5. Changes in gene expression in splenocytes

To investigate gene expression of cytokines in murine splenocytes, splenocytes were isolated from BABL/c and treated with various concentrations of CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6 and 12 h. IL-1β mRNA expression were up- regulated in a dose-dependent manner at both 6 and 12 h after treatment (Fig. 10). IL-2 gene expression also up-regulated in the concentration of CME over 2μg/mℓ at 6 h after CME treatment. But at 12 h after CME treatment, IL-2 gene expression decreased in a dose-dependant manner. IL-4, IL-10 and TGF-β gene expression increased slightly at 6 h after CME treatment. However, there was not any change at 12 h after CME treatment (Fig 11, 13, 18). CME up-regulated IFN-γ gene expression remarkably and dose-dependently and at 200 ug/ml CME, the gene expression increased to over 10 times of control.

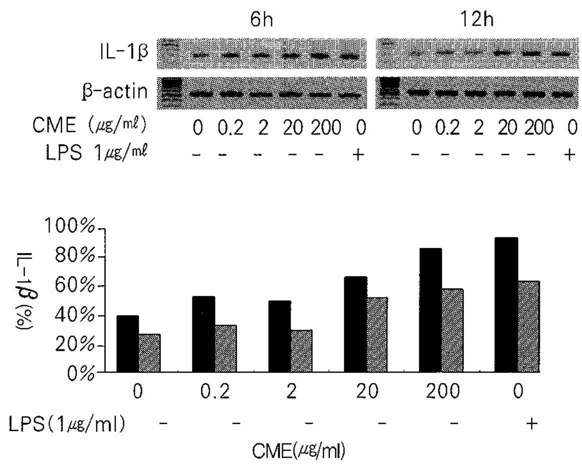

Fig. 10. IL-1β gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.

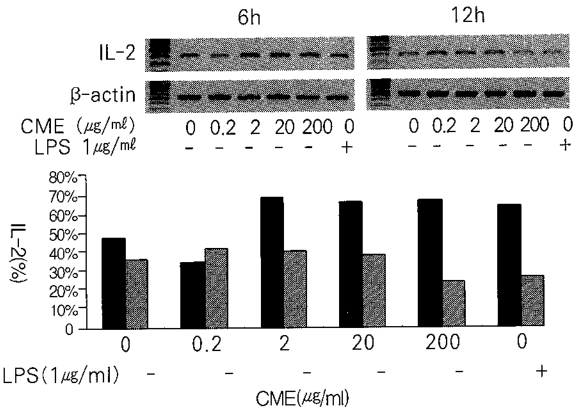

Fig. 11. IL-2 gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

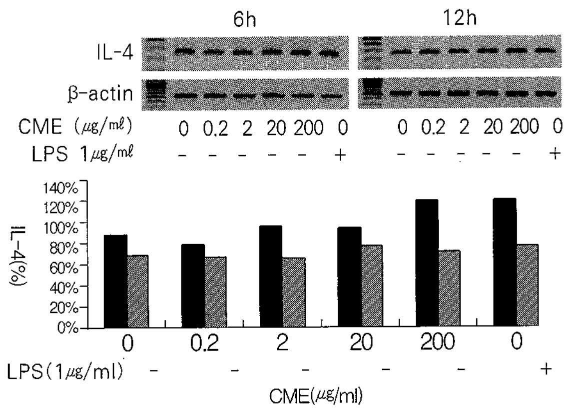

Fig. 12. IL-4 gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

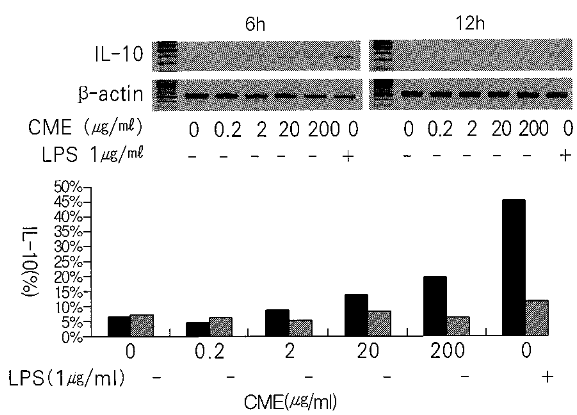

Fig. 13. IL-10 gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

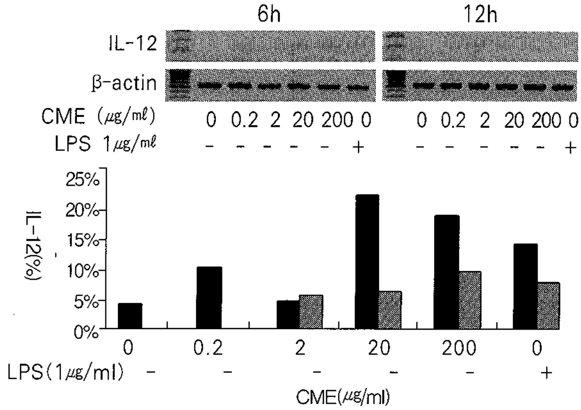

Fig. 14. IL-12 gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

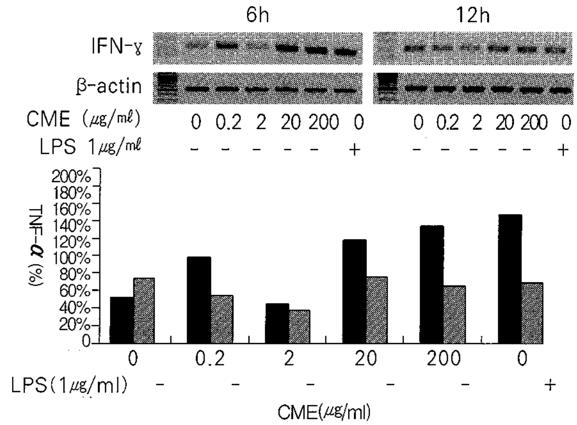

Fig. 15. TNF-α gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

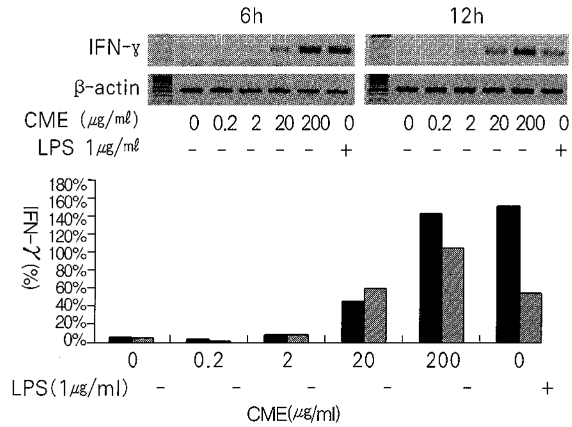

Fig 16. IFN-γ gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

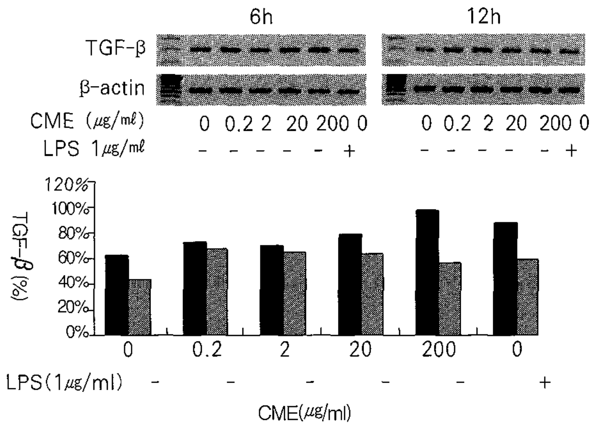

Fig. 17. TGF-β gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

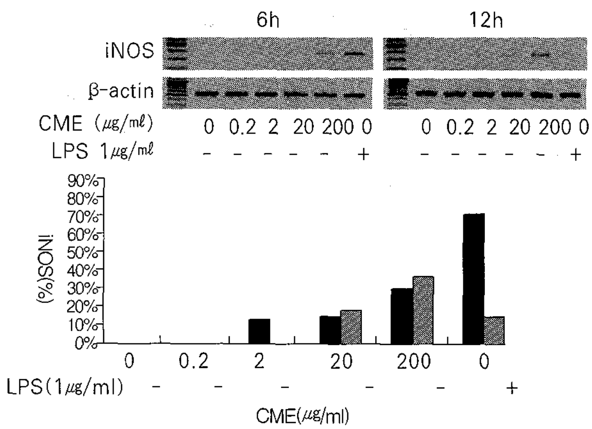

Fig. 18. iNOS gene expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▒). The gene expression was shown as the percentage to β-actin.

CME up-regulated IL-12 and TNF-α gene expression slightly and increased iNOS gene expression on dose-dependant manner.

6. Change in protein levels of cytokines with splenocytes

To investigate protein expression in mouse splenocytes, splenocytes were treated with various concentrations of CME (0, 2, 20, 200 μg/mℓ) and LPS (1 μg/mℓ) for 24 h and 48 h. Protein expression was determined by ELISA kit. CME increased IL-2 expression only at high con- centrations. TNF-α expression also increased under CME treatment in a dose-dependant manner within 24 h. CME increased IL-10 expression continuously within 48 h and dose- dependently. At 24 h after treatment of 0 and 200 μg/mℓ CME, the concentrations of IL-10 were 41 and 331 pg/ml respectively. IFN-γ expression also increased remarkably by 15-fold at 200 μg/mℓ CME. The ratio of IFN-γ to IL-10 increased by 9.6 and 4.5 (24 and 48 h respectively) at 200 μg/mℓ CME.

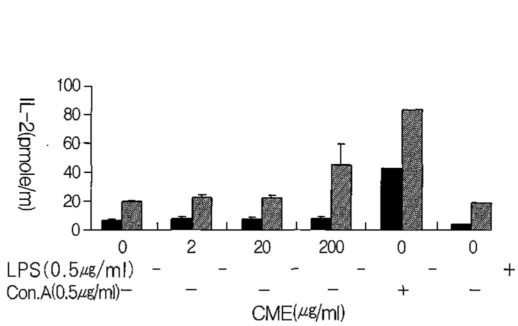

Fig. 19. IL-2 protein expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖), Con A (0.5 ㎍/㎖) or LPS (0.5 ㎍/㎖) for 24(■) and 48 h(▒). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

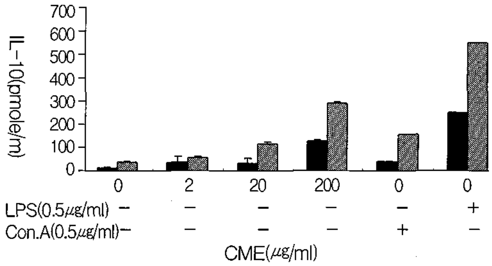

Fig. 20. IL-10 protein expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖), Con A (0.5 ㎍/㎖) or LPS (0.5 ㎍/㎖) for 24(■) and 48 h(▒). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

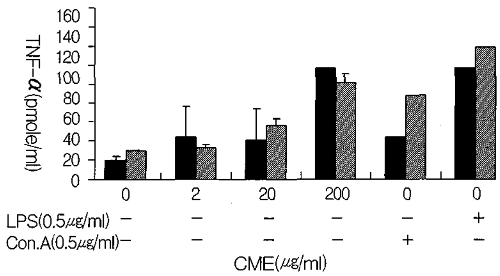

Fig. 21. TNF-α protein expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖), Con A (0.5 ㎍/㎖) or LPS (0.5 ㎍/㎖) for 24(■) and 48 h(▒). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

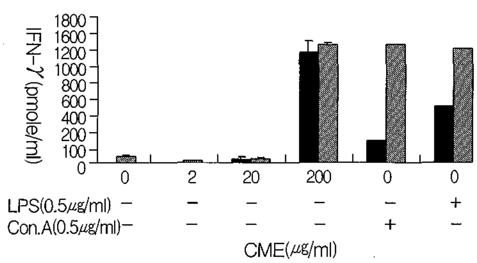

Fig. 22. IFN-γ protein expression in splenocytes. Splenocytes were treated with CME (0, 2, 20, 200 ㎍/㎖), Con A (0.5 ㎍/㎖) or LPS (0.5 ㎍/㎖) for 24(■) and 48 h(▒). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

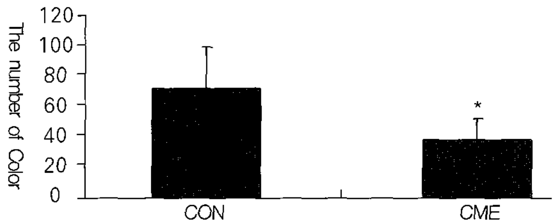

Fig. 23. Pulmonary colony assay. Each group were administered water (CON), or CME (200 mg/5ml/kg) from 7 days before CT-26 (2×10^4 cells) injection to 7 days after CT-26 injection. The mice were sacrificed 14 days after tumor inoculation, and lungs were removed and tumor colonies were counted. The results were expressed as mean ± SD

7. Inhibition of pulmonary colony

The mice were treated with 200 mg of CME per kg body weight; the mice in the control group were administrated distilled water. Pulmonary colonization was observed on day 14 after CT-26 inoculation. In the CME treatment group, tumor implantation was inhibited by 46% compared with the control group.

8. Histopathological finding

Mice were divided into 2 groups (10 mice per each group). The mice were treated with 200 mg of CME per kg body weight and the mice in control group were administrated with distilled water. CME were treated from 7 days before CT-26 injection to 7 days after CT-26 injection. CT-26 cells (2×10^4) were inoculated to tail vein. The mice were sacrificed 14 days after tumor inoculation and lungs were removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded lung section (4 ㎛ in thickness) was stained with hematoxylin & eosin (H&E).

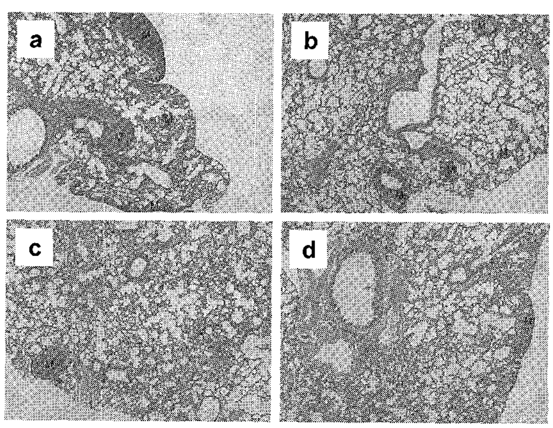

Fig. 24. Histopathological examination of lung of mice inoculated CT-26. Each group were administered with water (upper), or CME (under) from 7 days before CT-26 (2×10^4 cells) injection to 7 days after CT-26 injection. The mice were sacrificed 14 days after tumor inoculation, and lungs were removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded lung section (4 ㎛ in thickness) was stained with hematoxylin & eosin (H&E). M is the metastasis region of CT-26.

Discussion

Recently, cancer immunosurveillance has been thought one of the most important factors for controlling both cancer itself and patients suffering from tumors. During host-tumor interaction, tumor immunity considerably affects the whole process including tumor incidence or development, invasion, growth and metastasis.

Even when tumor cells can be recognized and attacked by various cellular and humoral components in early stages, most cancer cells are less immunogenic and develop various strategies to evade the immune system. According to the characteristic of innate immunity that it can work on with less tumor specific antigen dependent manners, it is strongly assumed that non-specific effector cells like macrophages and NK cells play more critical roles than others[12,14].

Macrophages and NK cells are distributed throughout the body and extravasate and migrate to various tissue sites. They possess or produce mediators for killing tumor cells, such as NO or perforin, and several cytokines including TNF, INF-γ and IL-12. They should be ready to employ for elimination of cancer cells without previous notice[15,18]. On the other hand, it is virtually impossible to pinpoint a physiological role of these cytokines in the natural mechanism of defense against tumors because of their complex and pleiotropic influence[12,19].

There are many therapies and thousands of plant candidates of cancer agents in the oriental medical field, especially herbal plants showing the effects of immune modulation and anti-tumor activity[2,4]. *Cordyceps Militaris* has been used as a plant medicine for a long time for patients suffering from cancer. It has also been used for cancer patients in the East-West Cancer Center of Daejeon University Oriental Hospital and shown to have some clinical efficacy. Cordycepin is a very important compound which is present in *Cordyceps Militaris*. The nucleoside analogue cordycepin (3'-deoxyadenosine) has been shown to inhibit the growth of various tumor cells[20,21].

In our previous study, *Cordyceps Militaris* has demonstrated anti-tumor effects related with anti-angiogenesis[11]. In the historical background, *Cordyceps Militaris* has been prescribed for invigorating the lungs and kidneys, and stopping bleeding and dissipating phlegm effects. Thus, in the present study, we investigated CME on immune modulating and anti-tumor activity through measuring cytotoxicity, immune modulating and anti-tumor activity, NO production, NK cytotoxicity and gene expressions of cytokines related with macrophages and NK cells.

To know the direct drug effect of CME on inhibition of cancer cell growth including normal cells, we measured cytotoxicity on 7250, HT1080, Hep G2, and CT-26 cells. CME didn't affect the cell growth of any, implying that CME has no direct anti-tumor effect through cell cytotoxicity.

Activated macrophages express inducible nitric oxide synthase (iNOS), whose product (NO) has a major role in bactericidal and anti-tumor function[22,24]. We evaluated the effects of CME on activation of macrophages by detection of NO release from RAW 264.7 cells treated with CME. NO production significantly increased at 200 ㎍/㎖ of CME. Although CME did not affect NO production in low concentrations, below 20 ㎍/㎖, CME also enhanced iNOS gene expression in a dose-dependant manner. In gene expression of cytokines, TNF-α gene expression was not affected by CME treatment. However, IL-1β mRNA expression was significantly increased in a dose-dependent manner. CME did not affect IL-10 gene expression at low concentrations, below 20 ㎍/㎖.

IL-1 usually is secreted by activated macrophages or other antigen presenting cells. TNF also acts directly on many other types of immune and inflammatory cells. IL-10 is secreted by tumor cells (and also by cells of the immune system) of generally immunosuppressive mediators[22,25]. Partial up-regulation of IL-10 is still a matter of debate.

Next, to investigate the effects of CME on activation of NK cells, we measured cytotoxicity on Yac-1 cells which are loss of class I MHC molecules and gene expression of cytokines related with NK cell activity. CME showed a significant effect on NK cytotoxic activity compared with control in all ratios of effector cell : target cell. Moreover, it showed a significant increase IL-1 and iNOS in splenocytes, the same as in RAW 264.7 cells. Also, CME up-regulated IL-2 gene expression in the concentrations of CME over 2 ㎍/㎖ at 6 h after CME treatment in contrast to no significant difference in immunosuppressive mediators such as IL-4, IL-6, IL-10 and TGF-β . TGF-β is known to acts as an effective tumor suppressor at early stages of carcinogenesis but later during tumor development it might exert oncogenic activity by promoting invasiveness and metastasis[26,28].

CME up-regulated gene expression of iNOS, IL-12, TNF-α and increased protein expression in mouse splenocytes, too. On the other hand, CME also promoted gene expression of IL-10 protein expression in mouse splenocytes. However, the ratio of IFN-γ to IL-10 was increased by 9.6 and 4.5 (24 and 48 h respectively) at 200 ㎍/㎖ CME. From this result, we suppose that CME activates cellular immunity rather than acts as an immunosuppressive mediator even if CME increased IL-10 expression dose-dependently.

Especially, Il-12 and IFN-γ expression also increased remarkably at 200 ㎍/㎖ CME. IL-12, produced mainly by monocytes/macrophages and dendritic cells, is a cytokine that regulates the transition from innate to adaptive immunity. It induces secretion of IFN-γ and other cytokines from T and NK cells, and is a central regulator of Th1 cell development and exerts strong anti-tumor effects when administered to mice[29]. In NK cell activity related with cytokines, IFN-γ is one of the most important immune mediators in tumor immunity. IFN-γ can be secreted by NK cells or activated Th1 cells, and strongly activates macro-phages and NK cells themselves, which mainly play roles in destroying tumor cells[30]. These results indicate that CME may have an anti-tumor property by promotion of NK cell activity as lysis of Yac-1 cells and activation of IL-1, IL-2, iNOS, TNF-α , IL-12 and IFN-γ .

We also observed that CME significantly inhibited pulmonary colonization of CT-26 cells compared with the control group in vivo.

From the above results, it could be summarized that CME has significant properties to activate macrophages and NK cells relating with up-regulation of cytokines like IL-1, IL-2, IL-12, IFN-γ and iNOS. Also, we may conclude that CME presents anticancer effects by modulating immune response currently connected with macrophages and NK cells. Therefore, it could be a potential anti-cancer agent after learning more about their underling mechanisms of action and clinical evaluation.

Conclusion

This experimental study was carried out to evaluate immune modulating and anti-tumor activity of CME. To elucidate the effects of

CME on the macrophage and NK cell activity, we analyzed NO production, NK cytotoxicity and gene expressions of cytokines related with macrophage and NK cell activity.

The results were as follows.

1. CME had no cytotoxic effects on 7250, HT1080, Hep G2, and CT-26 cells.

2. CME activated and promoted macrophages to product NO.

3. CME up-regulated gene expression of IL-1 and iNOS in RAW 264.7 cells.

4. CME promoted cytotoxicity of NK cells against YAC-1 cells.

5. CME up-regulated NK cell related gene expression such as IL-1, IL-2, IL-12, iNOS, IFN-γ and TNF-α in mice splenocytes.

6. CME promoted protein expression of IL-10, IL-12, IFN-γ and TNF-α in mice splenocytes.

7. CME inhibited lung tumor metastasis induced by CT-26 cell line compared with the control group.

From these results, it could be concluded that CME is an effective herbal drug for immune modulation and anti-cancer therapy by promoting activity of macrophages and NK cells.

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