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Antitumor and Immunopotentiating Effects of Manda Enzyme

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Abstract – This study was to evaluate the antitumor and immunopotentiation effects of Manda Enzyme (ME). Oral administration of ME (0.2 ml/mouse) to tumor bearing mice significantly prolonged survival rate compared to the control group with the prolongation ratio of 40%. The inhibition ratios for the first and the second experiments were 51.8% and 26.4%, respectively. Only the spleen index was significantly increased in the MEF-treated group, but not in the control group. Gamma globulin level of the MEF-treated group was elevated when mice were injected with sarcoma-180 cells on the left groin. Activities of natural killer (NK) and lymphokine-activated killer (LAK) cells were observed by ^{51}Cr-release method. Activities of NK cell against YAC-1 cells were significantly increased in the MEF treated group. And LAK cell activities against P815 cells were also significantly increased in the experimental group. These observations, therefore, suggest that ME may have an anticancer effect and immunopotentiating effect *in vivo*.

Key words – Natural products, antitumor activity, immunopotentiation effect

Introduction

Chemical compounds which have been commonly used as anticancer agents in the clinical fields often exhibit serious side effects. Therefore, several attempts (Lee *et al.*, 1988; Xu *et al.*, 1989; Chichara *et al.*, 1970; Miyazaki *et al.*, 1981) have been made to develop a new anticancer and cancer prophylactic agents from naturally occurring compounds, which lacks side effects.

It had been studied and reported that the extracts of several herbs, including Korean Ginseng, had a potent cytotoxic activities against some cancer cells both *in vitro* and *in vivo* (Hwang *et al.*, 1988a, 1980b; Shim *et al.*, 1988; Hwang *et al.*, 1986; Hwang *et al.*, 1988).

Manda Enzyme (ME) is one of the health food products which was prepared by fermentation of various natural products such as wild fruit and sea-weeds for the past 3 years (Matsuura *et al.*, 1994).

Okuda reported that an administration of ME inhibited the lipolytic activity of toxohormone-L which was purified from ascites of patients with hepatoma, and this treatment prevented liver injury induced by lipid peroxides (Okuda *et al.*, 1994).

It was also reported that ME inhibit alcohol toxicity by increasing the reduced level of ALDH activity, and also have some mod-

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ifying effect to normalize streptozotocin-induced diabetic rats (Joo, 1994).

ME was extracted with water and fractionated by ultrafiltration membrane (Hwang, 1994). Using these fractions, the cytotoxicity, size distribution curve, and the morphological changes on the several cancer cells were investigated. In the present study, low molecular weight compounds (M.W.<500) of ME extracts also showed the growth inhibition effect of cancer cells *in vitro* as similar to crude ME.

As a part of on-going investigations, this study was performed to confirm the anticancer activity *in vivo* and to investigate the immunopotentiating effect of ME as well.

Effects of ME on the survival time of tumor bearing mice, the growth inhibition of solid tumor, weight of the immune organs, γ-globulin level, and the activity of NK and LAK cells were observed.

Experimental

Animals – ICR mice were obtained from the Laborary Animal Center of Seoul National University. The weight was about 20-25 g.

ME – ME was supplied from ME Manufacturing Co., Ltd., in Japan. Original ME was used as a crude one. MEF was prepared as follows ; Manda 50 g was extracted with 50 ml of distilled water at room temperature to a total volume of 100 ml and filtered using YC 05 ultrafiltration membrane. And the filtrate was freeze-dried. Then freeze-dried MEF (M.W.<500) was dissolved in distilled water to the concentration of 2 g/ml.

Reagents – RPMI 1640, fetal bovine serum (FBS), trypsin-EDTA were products of Grand Island Biological Co. (GIBCO). IL-2 (interleukin-2) was purchased from Genzyme Co. ^{51}Chromium was a product of Amersham Co. Ficoll-paque was purchased from Pharmacia Biotech.

Apparatus – Petri dish was a product of Falcon. Round bottom 96-well microtiter plates were purchased from Nunc. Rohrentubes from Sarstedt and γ-counter from Berthold Co., were used. Electrophoresis kit of Helena was used.

Effect of ME on the survival time of tumor-bearing mice – (Lee *et al.*, 1992) All of ICR mice (body weight, 20~25g) were i.p. injected with 0.1 ml of sarcoma-180 (1×10^6 cells/ml) on day 0, and survived mice were estimated daily. Experimental group received 0.2ml of ME orally for 20 days starting from day -10 through day +9. For the first 10 days, crude ME (0.2 ml/mouse) was used and for the next 10 days MEF (MW <500, 0.2 ml/mouse) was used. Mice of control group were administered with saline (0.2 ml/mouse) during the same period.

* Prolongation ratio was calculated as follows;

$$\frac{\text{mean survival day of experimental group} - \text{mean survival day of control group}}{\text{mean survival day of control group}} \times 100$$

Growth inhibition test of solid tumor – (Ha *et al.*, 1991) At the beginning of the experiment, all mice (body weight, 20~25 g) were injected subcutaneously with 0.1 ml of sarcoma-180 cells on the left groin of mice on day 0.

Experimental group were then received 0.2 ml/mouse of ME orally for 20 times consecutively starting from day -10 ~ +9. For the first 10 days, the crude ME (0.2 ml/mouse) and for the next 10 days the MEF (M.W.<500) were used. Mice of control group were administered with saline (0.2 ml/mouse) during the same period. On the 7th day after the last treatment, all mice were sacrificed and their tumor mass were determined.

For the 2nd experiment, experimental group were received orally 0.2 ml/mouse of the MEF (M.W.<500) for 20 days. On the

15th day after the last treatment, all of the mice were sacrificed and their tumor mass were evaluated.
The rest of the method was identical with the first experiment.

* Inhibition ratio was obtained by calculating as follows;

$$\frac{\text{tumor weight of experimental group} - \text{tumor weight of experimental group}}{\text{tumor weight of control group}} \times 100$$

Effect of ME on the immune organ weight - (Kim *et al.*, 1988) ICR mice of experimental group were orally administered with MEF (M.W.<500, 0.2 ml/mouse) for 20 days. Mice of control group were orally administered with saline (0.2 ml/mouse). On the 15th day after the last treatment, all of the mice were sacrificed and the liver, spleen and thymus were dissected in order to determine their weights.

* Organ index was expressed as follows;

$$\frac{\text{organ weight}}{\text{body weight}} \times 100$$

Effect of ME on γ-globulin level - (Seo, 1982) All mice were injected with sarcoma-180 cells (1×10^6 cells/ml) on their left groin on day 0. ICR mice of the experimental group were then orally administered with MEF (0.2 ml/mouse) for 20 times starting from day -10 ~ +9. Control group were administered with saline (0.2 ml/mouse) during the same period. On the 15th day after the last treatment, serums from all mice were obtained. Protein concentrations were quantified. All serum samples were subjected to cellulose acetate electrophoresis and γ-globulin levels were analyzed.

* γ-globulin level;

$$\text{protein (g/dl)} \times \gamma\text{-globulin(\%)}$$

Effect of ME on NK cells activity - All of the mice were i.p. challenged sarcoma-180 cells (1×10^6 cells/ml) on day 0. Mice of experimental group were orally administered with MEF (M.W<500, 0.2 ml/mouse) for 11 times on day -5 ~ +5 and the control group were administered with saline (0.2 ml/mouse) during the same period. On day +8, spleen cells of mice were obtained and NK cell activities were evaluated by ^{51}Cr-release assay (Haward *et al.*, 1986; Ladazuri *et al.*, 1972; Reynolds *et al.*, 1984).

Effector cells:

The spleen of ICR mice was aseptically removed, tested apart, and raked through the needle of syringes to produce a single cell suspension in RPMI 1640 medium containing 5% FBS. Cells were washed twice in RPMI medium (contained 5% FBS) by centrifugation at 1500 rpm for 10 min.

And then, mononuclear cells were isolated with a Ficoll-paque by centrifugation (swing type, break off) at 1350 rpm for 30 min and washed twice with RPMI medium (contained 5% FBS). Effector cells (1x107cells/ml) were dispensed into a round-bottom 96-well microtiter plates by 0.1 ml.

Target cells:

Mouse lymphoma YAC-1 cell used in NK cells activity experiments was maintained as suspension cultures in RPMI 1640 medium supplemented with 5% FBS. YAC-1 target cells (5×10^6 cells) were labeled with 200 μl of ^{51}Cr solution for 1 hr in the 37 °C incubator. To minimize the spontaneous release of target cells during the assay, cells were washed with the media in 3 times. After washing, target cells were diluted to 1×10^5 cells per ml in RPMI (with 5% FBS) medium.

Cytotoxicity assay:

Cytotoxicity assay was done with ^{51}Cr labeled target cells by 4-hr ^{51}Cr release assay. Labeled target cells (1×10^4 cells/0.1 ml) were dispensed into 96-well round-bottomed plates containing effector cells (1×10^6 cells/0.

1 ml). Effector-to-target cell ratio used was 100:1. These plates were incubated at 37 °C for 4 hr, and the supernatant from each well was carefully transferred to Rohren-tubes, and counted by Brethold gamma counter.

* Percent cytotoxicity was calculated as follows ;

$$\frac{\text{experimental release - spontaneous releases}}{\text{maximal release-spontaneous release}} \times 100$$

where the spontaneous release was obtained from target cells alone in RPMI medium (with 5% FBS), and maximal release was from target cells resolved by triton X-100.

All samples were assayed in quintuplet. The values were presented as the mean× standard deviation of quintuplet determinations.

Effect of ME on LAK cells activity – (Gdimm *et al.*, 1983) IL-2 was reconstituted with 10 mM acetic acid to a final concentration of 100 g/ml. All of the mice were i.p. challenged with sarcoma-180 cells (1×10^6 cells/ml) on day 0. Mice of experimental group were orally administered with the MEF (M.W<500, 0.2 ml/mouse) for 11 times on day -5 ~ +5 and the control group, with saline (0.2 ml/mouse) during the same period. On day +8, the NK cell were obtained from the spleen of mice. IL-2 (1000unit/ well) was added to a round-bottom 96-well microtiter plates containing the NK cells. And the mixture was incubated for 72 hr at 37 °C. The LAK cells are derived from precursor cells that are distinguisable from NK cells. To measure LAK cell activity, mastocytoma cell line P815 were used as target cells. It was labeled with 200 μl of ^{51}Cr solution for 1 hr in the 37 °C incubator. Effector(E) to target (T) cell ratio used were E:T=100:1

The measurement of LAK cell activity was performed by the same method as that for NK cell activity.

$$\frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

where the spontaneous release was obtained from target cells alone in RPMI medium (with 5% FBS), and maximal release was from target cells resolved by Triton X-100.

Results and Discussion

Effect of Me on the survival time of tumor-bearing mice – As shown in Table 1, ME had a prolongation effect of 40.4% on the life-span of ICR mice inoculated with sarcoma-180 cells. In this experiment, there was no survived mice more than 30% of the

Table 1. Antitumor effect of ME on the life-span of ICR-mice inoculated with sarcoma-180 cells.

Group	Mean survival days	Prolongation ratio[1)]
Control	11.75	-
Treatment[2)]	16.5	40.5

Sarcoma-180 cells were peritoneally inoculated to both control and treatment group.

[1)]Prolongation ratio was obtained by calculating

$$\frac{\text{mean survival days of experimental group - mean survival days of control group}}{\text{mean survival days a of control group}} \times 100$$

[2)]ME was orally administered to treatment group.

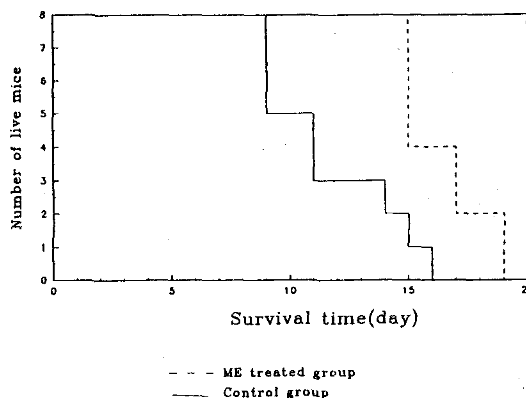

Fig. 1. Effect of ME on the life-span of ICR mice inoculated with sarcoma-180 cells.

total. The average life-span of experimental group was 16.5 days. And that of control group was 11.75 days in mean survival days. This indicates that administration of ME prolonged life-span of experimental group by 40% compared to the control. Fig. 1 shows survival time of experimental group and control group.

These results clearly suggest that the life-span of ME treated group has been prolonged against sarcoma-180 cells indicating anticancer effect of ME.

Growth inhibition test of solid tumor – The initial result of antitumor effects by oral injection of ME in ICR mice which were subcutaneously administered with sarcoma-180 cells was shown Table 2. Tumor weight obtained in experimental group was 0.94 ± 0.68 g and in control group 1.95 ± 0.49 g. ME inhibited the growth rate of solid tumor in experimental group by 51.8% compared to control group.

The second set of experimental results indicates that the average tumor weight of the treated group was 1.48±0.30 g while that of control group was 2.01±0.42 g, showing the inhibition ratio of 26.37% (Table 3).

Table 2. Antitumor effect of ME in ICR mice inoculated with sarcoma-180 cells.

Group	Tumor Weight (g)	Inhibition ratio (%)[1]
Control	1.95 ± 0.49	-
Treatment[2]	0.94 ± 0.68*	51.8

Sarcoma-180 cells were subcutaneously inoculated to both control and treatment group.
All values are expressed as mean ± standard deviation.
* $p<0.05$
[1]Inhibition ratio was obtained by calculating

$$\frac{\text{tumor weight of control group - tumor weight of experimental group}}{\text{tumor weight of control group}} \times 100$$

[2]ME was orally administered to treatment group.

Table 3. Antitumor effect of ME in ICR mice inoculated with sarcoma-180 cells (2nd experiment).

Group	Tumor Weight (g)	Inhibition ratio (%)[1]
Control	2.01 ± 0.42	-
Treatment[2]	1.48 ± 0.30*	51.8

All experimental conditions are same as in Table 2.

The results oftained from the two experiments by oral injection of ME clearly indicated that an anticarcinogenic effect of ME, evidenced by decreasing the size as well as weight of tumor mass.

It is noted that the inhibition ratio of the 2nd data was lower than the first result. It was considered that lower value was due to individual variation and poor condition of mice.

Effect of ME on the immune organ weight – Table 4 showed effect by oral injection of MEF (0.2 ml/mouse) on the immune organ weight of ICR mice. Liver and thymus index did not show any difference between control and experimental group. The spleen index of MEF treated group has been increased significantly (0.83±0.15) compared to that of the control group (0.63±0.17). Spleen is a peripheral lymphoid organ which produce antibodies and effector cell against foreign materials (Roitt *et al.*, 1985). It was therefore thought that MEF might influence the activation of immune system.

Effect of ME on γ-globulin level – Antibodies or immunoglobulins are found in the gamma globulin fractions of plasma proteins which can be identified by electrophoresis. We conducted electrophoresis of serum samples to evaluate γ-globulin level.

Table 4. Effect of ME on the weight of immune organs.

Group	Liver index	Spleen index	Thymus index[1]
Control	4.94 ± 1.30	0.63 ± 0.17	0.28 ± 0.11
Treatment[2]	4.80 ± 0.90	0.83 ± 0.15*	0.31 ± 0.05

[1]

$$\text{organ index} = \frac{\text{organ weight}}{\text{body weight}} \times 100$$

[2] ME was orally administered to treatment group.
[3] All values are expressed as mean ± standard deviation.
*$p<0.05$

Table 5. Effect of ME on the amount of serum protein.

Group	Total Protein	Albumin	α1	α2	β1	γ (g/dl)[1]
Control	5.78 ± 0.51	2.94 ± 0.31	0.57 ± 0.10	0.46 ± 0.12	1.43 ± 0.15	0.37 ± 0.12
Treatment[2]	5.74 ± 0.50	2.75 ± 0.23	0.53 ± 0.11	0.41 ± 0.14	1.47 ± 0.09	0.58 ± 0.24

[1]γ globulin level = protein(g/dl) × γ globulin(%)
[2]ME was orally administered to treatment group.
[3]All values are expressed are mean ± standard deviation.
*p<0.05

Table 6. Effect of ME on Natural Killer (NK) cells activity (% specific Cr^{51} release)

Group	Cytotoxicity (%)
Control	11.81 ± 4.01
Treatment[2]	16.61 ± 8.14

[1]% cytotoxicity =

$$\frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}}$$

[2]ME was orally administered to treatment group.
[3]All values are expressed as mean ± standard deviation.
*p<0.05

Table 7. Effect of ME on Lymphokine-activated killer (LAK) cells activity (% specific Cr^{51} release)

Group	Cytotoxicity (%)
Control	18.37 ± 1.79
Treatment[2]	

[1]% cytotoxicity =

$$\frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}}$$

[2]ME was orally administered to treatment group.
[3]All values are expressed as mean ± standard deviation.
*p<0.05

Table 5 showed effect of MEF on the serum proteins of tumor bearing mice. The amounts of total protein, albumin and α1-, α2-, β-globulin were not much different from control group except that of γ-globulin. The γ-globulin level of MEF treated group was shown to be 0.58±0.24 g/dl and that of the control was group was 0.37±0.12 g/dl.

It is likely that MEF influenced the immune system by increasing γ-globulin level. Therefore, it was estimated that ME caused immune reinforcement against sarcoma-180 cells.

Effect of ME on NK cells activity in mice – Morphologically NK cells belong to a class of large granular lymphocyte and have characteristics of non-adherent and non-phagocytosis. Unlike T lymphocytes or B lymphocytes, NK cells destroy tumors in a nonspecific fashion and do not require prior exposure for sensitization to the tumor antigens (Kim *et al.*, 1989). Effect of ME on NK cell activities was shown in Table 6. The cytotoxicity of control group was 11.81± 4.01%, while that of treatment group was 16.61±8.14%. The NK cell activities of experimental group were significantly enhanced compared to the control group.

In regard to the possible role of immune surveillance and in resistance to growth and metastasis of tumor (Herberman *et al.*, 1986), it was thought that increase of NK cell activities meant the activation of natural immunity. Based on the results indicating the prolongation of survival days and the inhibition of solid tumor growth, it is likely that ME might have anticancer effects caused by activation of the immune system.

Effect of ME on LAK cells activity – When NK cells were treated with IL-2, they produced LAK cells. IL-2 is a class of lymphokine which activates macrophage and NK cells (Roitt *et al.*, 1985). Recently it has been reported that LAK cells have an anticancer effect *in vitro* (Gills *et al.*, 1981) and *in vivo* (Mule *et al.*, 1985). Therefore, in recent years the use of LAK cells has been regarded as a new immuno-cancer therapy. We measured the LAK cell activities. Effect

of ME on LAK cell activities were shown in Table 7.

Cytotoxicity of the control group was 18.38± 1.79%, while that of the treatment group was 33.75±0.35%. It was shown that the LAK cell activities of the latter group were significantly enhanced than the former. The increase of LAK cell activities may have represented the immune reinforcement concomittant with the enhancement of NK cell activity. Because the effect of LAK cell activity was not reproducible in some experiment, further studies are needed.

In conclusion, we have observed that by administration of ME the survival days of tumor bearing mice were prolonged and the solid tumor growth was inhibited. The spleen weight, γ-globulin level as well as NK and LAK cell activities were also increased. Therefore, ME appears to have an anticancer effects *in vivo* by immune reinforcement. Further studies to develop ME as a new anticancer and cancer prophylactic agent are warranted.

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