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Original Article

Dose-Dependent Activation of Immune Function in Mice by Ingestion of Maharishi Amrit Kalash 4

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Abstract

This study was carried out to evaluate the dose-effects of ingestion of Maharishi Amrit Kalash 4 (MAK 4), an ayurvedic food supplement, on the immune function in female inbred BALB/c mice. Superoxide anion (O_2^-) production of peritoneal macrophages and the response of spleen cells to concanavalin A (Con A) were examined in mice given MAK 4 by gastric intubation of an aqueous emulsion at the dose of 10, 50, 100 or 200 mg/kg once a day for 20 days. Glucose consumption of peritoneal macrophage in the MAK 4-treated mice at the doses of 10 and 50 mg/kg after not only 24-hour but also 48-hour incubations were significantly high compared with the control group. Glucose consumption of peritoneal macrophages in the MAK 4-treated mice at the doses of 100 and 200 mg/kg after 48 hours of incubation were significantly lower than that of the control group. O_2^- production in the absence of a stimulator was significantly enhanced in the MAK 4-treated groups at the doses of 10 and 50 mg/kg. On the other hand, O_2^- production in the presence of a stimulator was significantly high in the MAK 4-treated groups at the doses of 10 and 50 mg/kg, and was significantly low in the MAK 4-treated groups at the doses of 100 and 200 mg/kg compared with that in the control group. Activities of acid phosphatase in the peritoneal macrophages were significantly low in the MAK 4-treated groups at the doses of 100 and 200 mg/kg compared with those in the control group. Activities of β -glucuronidase (GLU) and lactate dehydrogenase (LDH) in the peritoneal macrophages were significantly increased in the MAK 4-treated mice at the doses of 10 and 50 mg/kg. GLU and LDH activities of peritoneal macrophages in the MAK 4-treated mice at the doses of 100 and 200 mg /kg were significantly low compared with those in the control group. MAK 4 did not enhance spontaneous splenic lymphocyte proliferation at any dose in mice. Stimulation indices in the MAK 4-treated groups at all doses were significantly higher than those of the control group. These results indicate that 10 and 50 mg/kg per day might be appropriate doses to enhance not only macrophage function but also lymphocyte responsiveness for the gastric intubation of MAK 4 in mice.

Key words: Ayurvedic food supplement, Immune function, Dose-effect, Macrophage, Splenocyte

Introduction

Ayurveda (ayu=life, veda=knowledge, meaning science of life) is an ancient science that originated from Vedic scripture and is widely practiced in India[1]. The ancient Ayurvedic system provides an approach to the prevention and cure of disease, and emphasizes the philosophy of maintaining excellent

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health by normalizing the cellular functions and improving the inherent vitality of tissues. The World Health Organization has recognized its potency[1].

Maharishi Amrit Kalash 4 (MAK 4) and 5 (MAK 5), which are the Ayurvedic food supplements, belong to a group of substances known as Rasayana[2]. MAK 4 and MAK 5 are commercially available Rasayanas that are composed of variety of herbs, minerals and dairy products[3,4]. They are believed to enhance the body's resistance to infection and disease, and enhance longevity[2]. Recently, we found the immunomodulatory potency of MAK 4 as well as MAK 5[4]. In addition, our recent work revealed dose-dependent activation of MAK 5 in immune function using BALB/c female mice[5]. Such effect of MAK 4 has not been examined. The purpose of this study was to investigate the dose-dependent effects of MAK 4 on immune function and its mechanism in inbred BALB/c female mice and to compare these effects to those of MAK 5.

Materials and Methods

Animals and treatment

Sixty BALB/c female mice (10 weeks old, initial weights of 17-18g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were randomly divided into 5 groups (12 mice/group), and kept in a holding room controlled at a temperature of 21-23℃ and a humidity of 60 ± 5% under light-dark cycle (Light : 0800-2000). They were acclimated for 1 week prior to the experiment. Food (CE-2, Nihon Clea, Tokyo, Japan) and water were supplied ad libitum.

MAK 4 was obtained from Maharishi Ayurveda Products International (Lancaster,USA). The ingredients in MAK 4[3] are : Indian gallnut, Indian gooseberry, dried catkins, Indian pennywort, nutgrass, white sandalwood, evalyulus alsinoides, embella, aloewood, licorice, cardamom, cinnamon, cyperus, turmeric, honey, raw sugar and ghee (clarified butter).The exact amounts of the various ingredients in MAK 4 were not disclosed by the supplier, but quality control (e.g.,minimal variation from batch to batch) was assured.

MAK 4 was suspended in a 0.2% carboxymethyl cellulose sodium salt (CMC) (Nacalai Tesque, Kyoto, Japan) solution and given to mice p.o. at a dose of 10 mg/kg, 50 mg/kg, 100 mg/kg or 200 mg/kg per day for 20 consecutive days. The control mice were given a 0.2% CMC solution (0.1 ml/10g of body weight) p.o. The animals were killed by decapitation 48 hours after the final administration under ether anesthesia for the following experiments.

Isolation of macrophages and determination of glucose consumption and superoxide anion (O_2^-) production

Peritoneal exudate cells (PECs) were collected from the sacrificed mice using 5 ml of Hanks' solution (Nissui Seiyaku Co. Ltd., Tokyo, Japan). The PECs were pooled in each group which consisted of 8 mice. Macrophages in the PEC suspension were isolated by the cell-adhesion method[6]. The recovered cells were washed twice with Hanks solution by centrifugation at 1,000 rpm for 5 min, and suspended in 4 ml of RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS-RPMI 1640) (Gibco Laboratories Life Technologies, Inc.,USA) at a concentration of 2.0×10^6 cells/ml. The viability of peritoneal macrophages determined by the trypan blue dye exclusion test[7] was more than 98%.

Glucose remaining in the peritoneal macrophage culture supernatant was measured by the glucose B-test Wako (Wako Co., Osaka, Japan)[6]. The supernatants (10 μl) obtained from the macrophage culture sampled (2×10^5 cells/well) at 24 h and 48 h at 37℃ were incubated with 3.0 ml of color reagent for 20 min at 37℃. The optical density of the solution was measured at 505 nm and remaining glucose was determined from a calibration curve with a standard glucose solution. The results were expressed as percent glucose consumption, calculated from the following equation[8] : [1-(glucose content in culture medium cultured with macrophages/glucose content in culture medium without macrophages)] $\times100$.

O_2^- production of macrophages was assayed by the nitro blue tetrazolium (NBT) (Nacalai Tesque Inc., Kyoto, Japan)reduction method[9]. First, 100μl (2×10^5 cells)of macrophage suspension was incubated for 2 hours. After aspiration of the supernatant, 100μ 1 of NBT (4 mg/lm) and 100μ 1 of phorbol 12-myristate 13-acetate (0.3μ g/ml) (PMA) (Sigma Chemical Co., St. Louis, USA) solutions in FCS-RPMI 1640 were added to wells of the 96-well flat-bottomed microplate (Corning Laboratory Sciences Co., New York, USA). After 30-min incubation in 5% CO_2., at 37℃, the medium was removed and the cells in the wells were fixed by 2.0 ml of 70% ethyl alcohol. After removal of the alcohol, the fixed cells were dissolved by 0.1 ml 2N KOH and 0.1 ml dimethyl sulfoxide. Optical density of the solution at 630 nm was measured using a microplate reader (Corona Electric Co. Ltd., MTP 120, Tokyo, Japan) for use as the index of the amount of O_2^- produced by the activated macrophages. The results are presented as the mean ± SE of triplicate determinations.

Determination of activities of acid phosphatase, β-glucuronidase and lactate dehydrogenase.

2.0ml of suspension containing 2.0×10^6 cells/ml of macrophages was centrifuged at 3,000 rpm for 10 min. After aspiration of the supernatant, the resulting cell pellet was dissolved with 2.0ml of 0.1% Triton X-100 and the activities of acid phosphatase (APH), β-glucuronidase (GLU) and lactate dehydrogenase (LDH) of the solution were measured. Activity of APH of macrophages was assayed by the *p*-nitrophenylphosphate method (acid phospha B-test Wako, Wako Co., Osaka,Japan)[8]. Activity of GLU of macrophages was assayed using a kit for determination of GLU (Sigma Chemical Co., St. Louis, USA)[9]. The LDH activity of macrophages was assayed by the tetrazolium salt method (Lactate Dehydrogenase C II-test Wako, Wako Co., Osaka, Japan)[8]. Activities of APH, GLU and LDH were expressed as International Units (IU) per 2×10^5 cells, IU per 4×10^5 cells and IU per 1×10^5 cells, respectively.

Mitogen-induced proliferation of splenic lymphocytes.

A single-cell suspension was prepared by pressing the spleen between two glass slides. After washing in Hanks' solution, the cells were suspended in FCS-RPMI 1640 (4×10^6 cells /ml). The viability of splenocytes determined by trypan blue dye exclusion test was more than 98%. Each group consisted of 4 mice.

The cell suspension (50 μl) and concanavalin A (Sigma

Chemical Co., St. Louis, USA) solution (25 μl, Con A 10 μg/ml) in FCS-RPMI 1640 were added to wells of a 96-well flat-bottomed microplate and incubated for 72 hr in 5% CO_2 at 37 ℃. The proliferation of spleen cells was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)[10]. Six hours before the end of the incubation, 10 μl of 0.5% MTT (Sigma Chemical Co., St.Louis,USA) dissolved in PBS(-) was added to the wells. After incubation, 150 μl0.04N HCl-isopropanol was added to each well. Then optical density at 570 nm was measured using a microtiter plate reader (Corona Electric Co. Ltd., MTP 120, Tokyo, Japan). The experiments were done in triplicate. The stimulation index (S. I.) was calculated by means of the following equation.

$$S.\ I. = \frac{\text{mean optical density of the cells stimulated with Con A}}{\text{mean optical density of the cells not stimulated with Con A}}$$

Statistics

The statistical significance of the results was tested with student's t-test or Welch's t-test. The level of significance was set at $p<0.05$.

Table 1 Effects of MAK 4 on body weight and food consumption in mice.

Group	N	Body weight (g)		Food consumption (g/day/mouse)
		Initial	After 20 days	
Control	12	19.5 ± 0.3	21.5 ± 0.3	3.13 ± 0.07
MAK 4				
10mg/kg	12	19.3 ± 0.3	21.3 ± 0.4	3.11 ± 0.07
50mg/kg	12	19.3 ± 0.3	21.4 ± 0.3	3.26 ± 0.05
100mg/kg	12	19.7 ± 0.4	21.2 ± 0.3	3.10 ± 0.07
200mg/kg	12	19.4 ± 0.4	21.7 ± 0.4	3.34 ± 0.08

Each value represents the mean ± SE.
MAK 4, Maharishi Amrit Kalash 4; N, Number of mice used.

Results

The effects of MAK 4 on the mean body weight and food consumption during the study (20 days) in mice are shown in Table 1. There were no significant differences among the MAK 4-treated groups and the control group in body weight and food consumption.

Figure 1 shows the effects of MAK 4 on the glucose consumption of peritoneal macrophages in mice. Glucose consumption of peritoneal macrophages in the MAK 4-treated mice at the doses of 10 mg/kg ($p<0.001$) and 50 mg/kg ($p<0.001$) after not only 24-hour but also 48-hour incubations were significantly high compared with the control group. Glucose consumption of peritoneal macrophages in the MAK 4-treated mice at the doses of 100 mg/kg ($p<0.001$) and 200 mg/kg ($p<0.001$) after 48 hours of incubation were significantly lower than that of the control group.

As shown in Fig. 2, O_2^- production in the absence of PMA was significantly enhanced by the administration of MAK 4 at the doses of 10 mg/kg ($p<0.05$) and 50 mg/kg ($p<0.01$). O_2^- production stimulated by PMA was significantly high in the MAK 4-treated groups at the doses of 10 mg/kg ($p<0.05$) and 50 mg/kg, and was significantly low in the MAK 4-treated groups at the doses of 100 mg/kg ($p<0.01$) and 200 mg/kg ($p<0.05$) compared with that in the control group.

Figures 3 and 4 illustrate the effects of MAK 4 on the activities of APH, GLU and LDH in peritoneal macrophages in mice. APH activities of peritoneal macrophages were significantly low in the MAK 4-treated groups at the doses of 100 mg/kg and 200 mg/kg compared with the control group ($p<0.001$). GLU and LDH activities of peritoneal macrophages in

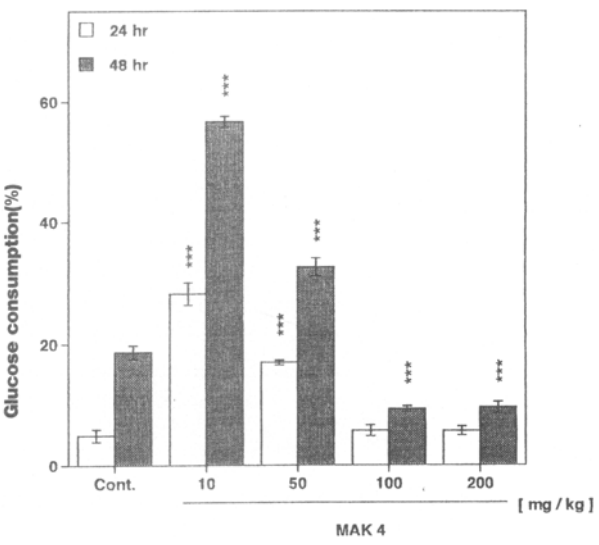

Fig. 1 Effects of Maharishi Amrit Kalash 4 (MAK 4) on glucose consumption of peritoneal macrophages in mice.
Each value represents the mean±SE of triplicate sets. ★★★ $p<0.001$, compared with the controls. Cont., Control.

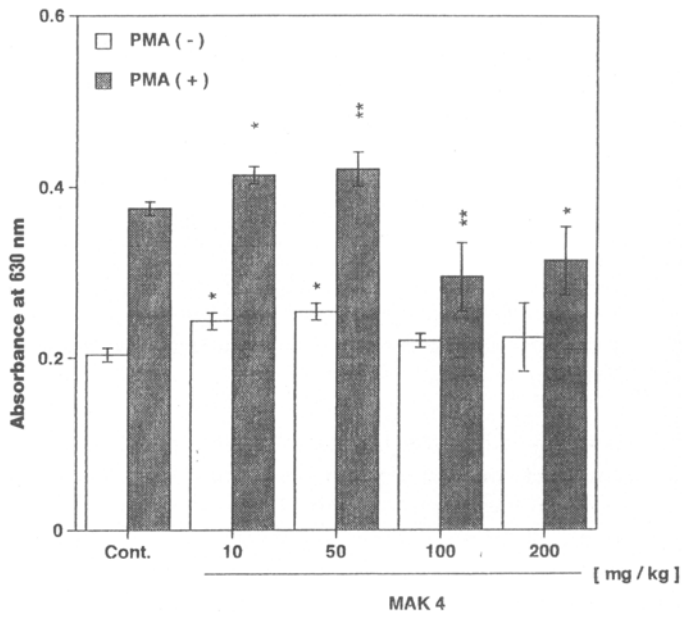

Fig. 2 Effects of Maharishi Amrit Kalash 4 (MAK 4) on superoxide anion (O_2^-) production of peritoneal macrophages in mice.
Each value represents the mean±SE of triplicate determinations. ★ $p<0.05$, ★★ $p<0.01$, compared with the controls. Cont., Control.

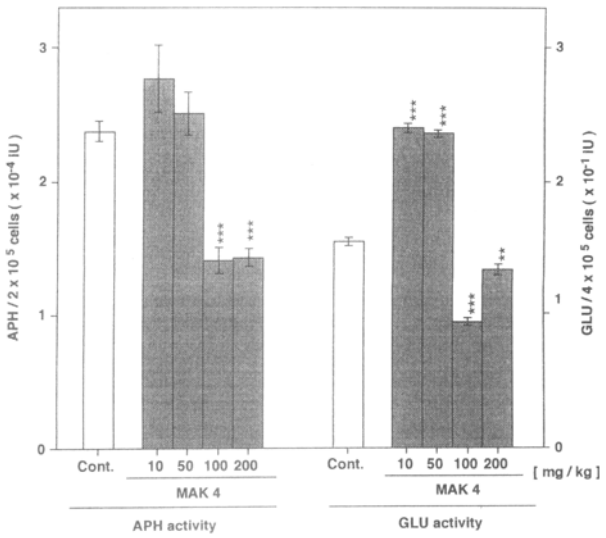

Fig. 3 Effects of Maharishi Amrit Kalash 4 (MAK 4) on acid phosphatase (APH) and β-glucuronidase (GLU) activities of peritoneal macrophages in mice.
Each value represents the mean±SE of triplicate determinations. ★★ $p<0.01$, ★★★ $p<0.001$, compared with the controls. Cont., Control.

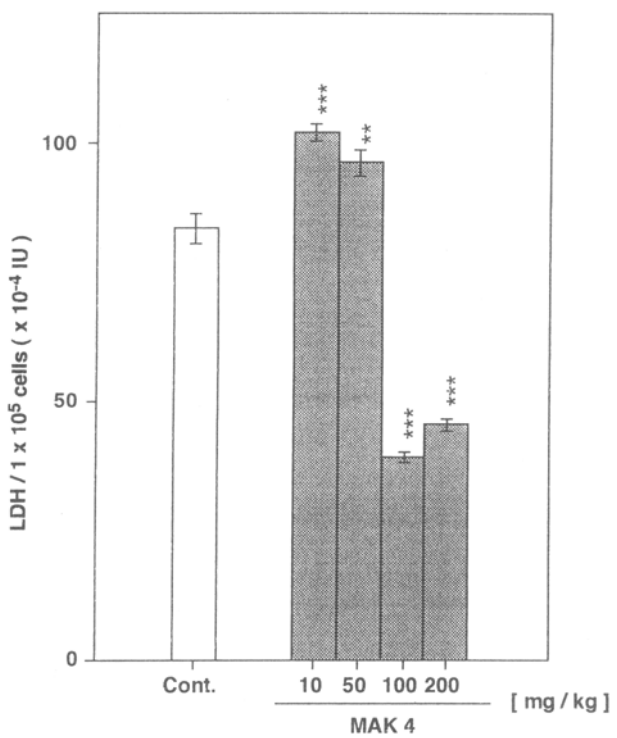

Fig. 4 Effects of Maharishi Amrit Kalash 4 (MAK 4) on lactate dehydrogenase (LDH) activities of peritoneal macrophages in mice.
Each value represents the mean±SE of triplicate determinations. ★★ $p<0.01$, ★★★ $p<0.001$, compared with the controls. Cont., Control.

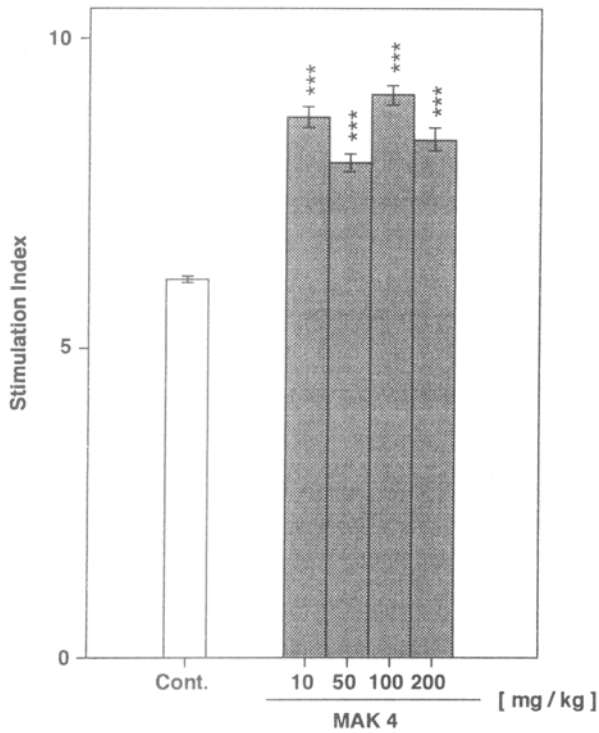

Fig. 5 Effects of Maharishi Amrit Kalash 4 (MAK 4) on proliferation of splenocytes induced by Con A in mice.
Each value represents the mean±SE of 4 mice. ★★★ $p<0.001$, compared with the controls. Cont., Control.

the MAK 4-treated mice at the doses of 10 mg/kg and 50 mg/kg were significantly higher than those in the control group ($p<0.01$ or $p<0.001$). GLU and LDH activities of peritoneal macrophages in the MAK 4-treated mice at the doses of 100 mg/kg and 200 mg/kg were significantly low compared with those in the control group ($p<0.01$ or $p<0.001$).

MAK 4 did not enhance spontaneous splenic lymphocyte proliferation in mice at any dose. Figure 5 shows the effect of MAK 4 on the proliferation splenocytes induced by Con A in mice. Stimulation indices in the MAK 4-treated groups at all doses were significantly higher than those of the control group ($p<0.001$).

Discussion

We reported earlier that two kinds of Maharishi Amrit Kalash (MAK 4 and MAK 5) have immunomodulatory potency[4]. The ingredients in MAK 4 are completely different from those in MAK 5[4]. Recently, we reported from the experiments of dose-effects of ingestion of MAK 5 that oral administration of MAK 5 once a day (over 50 mg/kg) enhances not only macrophage function but also lymphocyte responsiveness in BALB/c female mice[5].

It is known that glucose consumption in culture by macrophages increases as a result of the activation of macrophages at the priming stage[11]. In the present study, we observed that glucose consumption of peritoneal macrophages in the MAK 4-treated mice at the doses of 10 and 50 mg/kg after not only 24-hour but also 48-hour incubation were significantly high compared with the control group. These results

suggested that MAK 4 had the potency to activate peritoneal macrophages in the priming stage and that it was necessary to treat mice with MAK 4 at doses of 10 or 50 mg/kg at least once a day to activate the peritoneal macrophages by 24 hours and 48 hours.

In the present study, we observed that the ability of macrophages to generate O_2^- in the presence of PMA, but not in its absence, in inbred female BALB/c mice was increased significantly by daily oral treatment with MAK 4 for 20 days at the doses of 10 and 50 mg/kg. We[4] reported earlier that the ability of macrophages to produce O_2^- in the presence of PMA in outbred male ddy mice was not affected by daily oral treatment with MAK 4 for 10 days at the dose of 50 or 100 mg/kg. The discrepancy between these results may be attributable to the difference of animal strain, sex or the experimental duration. At any rate, our results suggested that macrophage O_2^- generation, at least in the presence of activators, was altered by MAK 4 ingestion. However, further studies are needed to determine the optimal doses of MAK 4 needed to enhance macrophage O_2^- production. It is known that glucose consumption and O_2^- production by macrophages are related to the pentose phosphate pathway in the glycolysis[11]. Therefore, it can be considered that MAK 4 has the potency to activate the pentose phosphate pathway in peritoneal macrophages.

Ryan et al.[12] and Allison et al.[13] reported that after the activation of macrophages in the priming stage, activities of lysosomal enzymes such as APH and GLU, and cytoplasmic enzymes such as LDH and leucine-2-naphthylamidase in the macrophages were increased. In the current study, activities of GLU and LDH in the peritoneal macrophages were increased significantly by the treatment of MAK 4 at the doses of 10 and 50 mg/kg. These results suggested that MAK 4 affects the ability of peritoneal macrophages to respond appropriately to foreign substances.

In this study, we evaluated the macrophage function by measuring the O_2^- production and the activity of phagocytosis of foreign substances of macrophages. Based on the results obtained in the present study, we concluded that gastric intubation MAK 4 at the dose 10 mg/kg was sufficient to enhance macrophage function.

In contrast with the case of MAK 5[5], glucose consumption of peritoneal macrophage in the MAK 4-treated mice at the doses of 100 and 200 mg/kg after 48 hours of incubation was significantly lower than that of the control group. In addition, O_2^- production of peritoneal macrophages stimulated by PMA was significantly decreased by the treatment with MAK 4 at the doses of 100 and 200 mg/kg. Also, APH, GLU and LDH activities of peritoneal macrophages were significantly low in the MAK 4-treated groups at the doses of 100 and 200 mg/kg compared with those in the control group. The differences in these parameters measured in mice gavaged with MAK 4 and MAK 5 may be explained by the different composition in MAK 4 and MAK 5. However, it can be said that MAK 4 could enhance the macrophage function when applied less than 50 mg/kg/day but not over 100 mg/kg/day. This may suggest the existence of the appropriate dose of MAK 4 in contrast with MAK 5.

The results that MAK 4 did not enhance spontaneous splenic lymphocyte proliferation at any dose in female BALB/c mice in the present experiment were in accordance with our previous study.[4]

We[4] previously reported that the index for stimulation of spleen cells by Con A in male ddy mice was significantly higher in groups treated with MAK 4 at the doses of 50 and 100 mg/kg than in the controls. In the current study, the stimulation index of spleen cells by Con A was increased significantly by the treatment with MAK 4 at the doses of 10, 50, 100 and 200 mg/kg. These results suggested that for enhancing splenic lymphocyte proliferation in mice it might be necessary to treat mice with MAK 4 at doses of over 10 mg/kg.

Lymphocyte proliferation is a complex event involving, among other factors, the participation of interleukins 1 and 2 and interleukin-2 receptor expression[14]. It is possible that MAK 4 ingestion enhances the production of various cytokines and/or T-cell-receptor expression. Further studies to clarify the mechanism of the enhancement of lymphocyte proliferation by MAK 4 are underway in our laboratory.

It has been shown that lectins from a variety of beans, including those from kidney beans (phaseous vulgaris)[15], can transform lymphocytes in vitro and enhance their proliferation. Several other lectins and alkaloids with mitogenic properties have also been identified[16,17]. Since Ayurvedic drugs and food supplements contain a variety of herbs, minerals and dairy products, the presence of such compounds is certainly possible. However, the lack of enhanced spontaneous (basal) lymphocyte proliferation found in the current and previous experiments[4] in MAK 4-treated mice suggests that direct activation by these compounds is unlikely.

In conclusion, 10 and 50 mg/kg per day might be appropriate doses to enhance not only macrophage function but also lymphocyte responsiveness for the gastric intubation of MAK 4 in mice.

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