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Original Article

Modifying Effects of Maharishi Amrit Kalash 4 and 5 on Phagocytic and Digestive Functions of Macrophages in Male ICR Mice

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Abstract

A study was carried out to examine modifying effects of Maharishi Amrit Kalash 4 (MAK 4) and Maharishi Amrit Kalash 5 (MAK 5) on phagocytic and digestive functions of macrophages in male ICR mice. Mice at 4 week of age were divided into 3 groups: no treatment group (control), MAK 4 treated group (MAK 4 group) and MAK 5 treated group (MAK 5 group). MAK 4 and MAK 5 were given p.o. at 50 mg/kg per day (5 days/week) for 7 weeks. Phagocytic function of reticuloendothelial system evaluated by the carbon clearance was enhanced by the treatment of MAK 4 and MAK 5. Superoxide anion (O_2-) production of peritoneal macrophages increased significantly in both MAK 4 and MAK 5 groups. The acid phosphatase activity of peritoneal macrophages increased significantly in MAK 4 group compared to the control group, but not in MAK 5 group. The activities of β-glucuronidase and lactate dehydrogenase in both MAK 4 and MAK 5 groups increased significantly when compared to the control group. These results suggest that MAK 4 and MAK 5 promote the phagocytic and digestive functions of macrophages and have a stimulatory effect on macrophages.

Key words: Ayurvedic food supplement, Macrophage, Lysosomal enzyme, Reticuloendothelial system, Mouse.

Introduction

Ayurveda is the oldest medical system that originated in India at about 6,000 B.C.[1]. The concept of this medical system consisted of physical exercise and special herbal food supplements called Rasayana. Rasayana is believed to enhance resistance to infection and diseases, and give longevity[2]. Zaman[3] described that the traditional medicines including Ayurveda are useful for the maintenance of healthy life in South-East Asia. However, there are few reports[4-9] for their pharmacological actions examined from the standpoint of modern medicine. Recently in Japan, many people direct their attention to health. Therefore, it might be important to investigate the effects of Rasayana scientifically with respect to preventive medicine.

Maharishi Amrit Kalash 4 (MAK 4) and 5 (MAK 5) are two versions of Rasayana prepared according to the ancient Ayurvedic recipe[2]. MAK 4 and MAK 5 are also expected to potentiate immune system to prevent infectious diseases, but the studies on its immunological action[4, 5, 9] are limited. Recently, our research group[10, 11] reported the dose-dependent activation of immune function by short term administration (10 or 20 consecutive days) of MAK 4 and MAK 5 in mice. We found that 50 mg/kg is the appropriate dose to enhance not only macrophage function but also lymphocyte responsiveness for the gastric intubation both of MAK 4 and MAK 5. However, the effects of the long-term administration of MAK 4 and MAK 5 on macrophage functions are not elucidated.

The purpose of the present study was to investigate the effects of the long-term administration of MAK 4 and MAK 5 on phagocytic and digestive functions of macrophage as primary stage of the host defense system in mice.

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Materials and Methods

1. Animals

Sixty male ICR mice, 3 weeks old, weighing 10 to 12 g were obtained from Japan SLC. Inc. (Hamamatsu, Japan). They were housed, five per cage, with pellet food (CE-2: Nihon Clea, Japan) and water *ad libitum*, in an animal room under a 12 h light-dark cycle at a temperature of 22 ± 1℃ and a humidity of 60 ± 5%. After a week acclimation, they were used for the experiment.

2. Ingredients of MAK 4 and MAK 5, and treatment

MAK 4 and MAK 5 were obtained from Maharishi Ayurveda Products International (Lancaster, USA). The ingredients of MAK 4 and MAK 5 have been described elsewhere[7, 8]. The ingredients in MAK 4 are: Indian gallnut (terminalia chebula), 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 ingredients in MAK 5 are: gymnema aurentiacum (meda milkweed), black musale, heart-leaved moonseed, sphaeranthus indicus, butterfly pea, licorice, vanda spatulatum, elephant creeper and indian wild pepper. The exact composition of various ingredients in MAK 4 and MAK 5 are not disclosed by the supplier, but the quality control (e. g., minimal variation from batch to batch) was assured. MAK 4 and MAK 5 suspended in distilled water were given to mice p.o. at 50 mg/kg per day (5 days/week) for 7 weeks. Control mice were given water as the vehicle (0.1 ml/10 g of body weight). The dose of MAK 4 and MAK 5 in this study was decided based on our previous study[10, 11]. In order to remove the acute effects of the treatment of MAK 4 and MAK 5, the animals were sacrificed by bleeding 72 hours after the last administration under ether anesthesia for the following experiments.

3. Assay of carbon clearance activity

The phagocytic activity of the reticuloendothelial system was determined by measuring clearance of colloidal carbon from the peripheral blood as described by Weir et al.[2] The carbon suspension (a mixture of 3 ml of Perikan Drawing ink 17 Black, 4 ml of physiological saline solution and 4 ml of 3% gelatin) was injected intravenously at 0.1 ml/10 g of body weight under ether anesthesia. Blood (25 μl) samples were collected into heparinized capillary tubes from the retro-orbital plexus of mice anesthetized with ether at 5 and 10 min after the injection. Immediately after the collection, the blood was mixed with 2 ml of 0.1% sodium carbonate solution. Optical density (OD) of the mixture was measured at 675 nm with a spectrophotometer (Hitachi U-2000A: Tokyo, Japan). Each group consisted of 10 mice.

The phagocytic index (K) and the corrected phagocytic index (α) were calculated according to the following equation[12, 13]:

$$K = (\log OD_{t5} - \log OD_{t10})/(t_{10} - t_5)$$

$$\alpha = \sqrt[3]{K} \cdot Pc/Po$$

where t_5 = 5 min, t_{10} = 10 min, OD_{t5} = optical density at 5 min after the injection of carbon solution, OD_{t10} = optical density at 10 min after the injection of carbon solution, Pc = whole body weight (g), Po = total weight (g) of liver and spleen.

The mice were killed by bleeding under ether anesthesia at 60 min after the injection of carbon solution and their livers and spleens were removed. Carbon taken up by the liver and spleen were measured according to the method of Fisher et al.[14] Livers and spleens were individually immersed into a mixture of 1 ml 10% potassium hydroxide in 70% ethanol solution and 2 ml of 2% aqueous gum acacia and digested at 37℃ overnight. Each of the digested material were then diluted to 10 ml with distilled water and the OD was measured at 800 nm.

4. Isolation of peritoneal macrophages and determination of superoxide anion (O_2-) production

All procedures were conducted under aseptic conditions. Each group consisted of 10 mice.

Mice were sacrificed by bleeding under ether anesthesia and peritoneal cells were obtained by intraperitoneal injection of Hanks' solution (Nissui Seiyaku Co., Ltd., Tokyo, Japan). The peritoneal exudated cells were suspended in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS: Gibco Laboratories Life Technologies, Inc., New York, USA) (10% FCS-RPMI 1640 medium) and incubated in culture plate (Corning Laboratory Sciences Co., New York, USA) for 2 h at 37℃ in a 5% CO_2 incubator. After removing non-adherent cells by washing the plate with Hanks' solution, the adherent cells were harvested from the bottom using rubber policeman and resuspended in 10% FCS-RPMI 1640 medium. The cells were used for experiments as resident peritoneal macrophages. Cell viabilities checked by the trypan blue dye exclusion test were more than 95%.

O_2- production of peritoneal macrophages was measured by the nitro blue tetrazolium (NBT) reduction method[15]. A 0.1 ml of macrophage cell suspension (2.0×10^6 cells/ml) in 10% FCS-RPMI 1640 medium was placed in a flat-bottomed 96-well tissue culture plate (Corning Laboratory Sciences Co., New York, USA) and was incubated for 2 hours. After aspiration of the solution, 0.1 ml of nitro blue tetrazolium (NBT: 4 mg/ml, Nacalai Tesque, Inc., Kyoto, Japan), 10% FCS-RPMI 1640 solution and 0.1 ml of phorbol 12-myristate 13-acetate (PMA: 0.3 μg/ml, Sigma Chemical Co.) were added to each well. After 30 min incubation in 5% CO_2 at 37℃, medium was removed from the well. The remaining cells were washed twice with RPMI 1640 medium, and dissolved with 0.1 ml of 2N potassium hydroxide solution and 0.1 ml of dimethyl sulfoxide. Then the OD at 630 nm was measured by a microplate reader (Corona Electric, Co., Ltd., MTP-120, Tokyo, Japan).

5. Assay of APH, GLU and LDH activities in peritoneal macrophages

A 3.0 ml of suspension containing 2.0×10^6 cells/ml macrophages were centrifuged at 1,000 rpm at 4℃ for 5 min. The resulting cell pellet was dissolved with 3.0 ml of 0.1% Triton X-100 and the intracellular activities of acid phosphatase (APH), β-glucuronidase (GLU) and lactate dehydrogenase (LDH) of the solution were measured by the APH kit (Wako Pure Chemical Industries, LTD.), GLU kit (Sigma Chemical Co.) and LDH kit (Wako Pure Chemical Industries, LTD.), respectively. The APH activity was expressed as International Unit (IU) per 2.0×10^5 cells, GLU activity was expressed as IU per 4.0×10^5 cells and LDH activity was expressed as IU per 1.0×10^5 cells.

Statistics

Differences among the three groups were evaluated by using

one way analysis of variance (ANOVA). A P value of 0.05 was accepted as statistically significant. All data were presented as the mean ± SE.

Results

The body weights of the control mice, MAK 4 and MAK 5 treated mice at 4 weeks of age were 18.9 ± 0.3g (n = 20), 18.8 ± 0.4g (n = 20) and 18.4 ± 0.4g (n = 20), respectively. After 7 weeks, the body weights of the control mice, MAK 4 and MAK 5 treated mice were 43.6 ± 0.6g (n = 20), 44.3 ± 1.1g (n = 20) and 43.4 ± 0.7g (n = 20), respectively. The food intake of the control mice, MAK 4 and MAK 5 treated mice were 38.0 ± 0.3g/week/mouse, 37.4 ± 0.8g /week/mouse and 37.7 ± 0.5g/week/mouse, respectively. The MAK 4 and MAK 5- long-term administration scarcely affected the body weight gain and the food intake. Figure 1 shows the effects of MAK 4 and MAK 5 on the phagocytic index (K) and the corrected phagocytic index (α) of the reticuloendothelial system in mice. The MAK 4 and MAK 5 treated groups had a significantly higher phagocytic index (K) and the corrected phagocytic index (α) compared to the control group ($P < 0.05$ or $P < 0.01$). The uptake of carbon in liver and spleen of carbon-injected mice is shown Figure 2. The uptakes of carbon in liver of the groups treated with MAK 4 and MAK 5 were significantly higher than that in the control group ($P < 0.05$ or $P < 0.01$), but there is no significant difference in the spleen in all groups.

As shown in Figure 3, O_2- production of peritoneal macrophages in the absence of PMA was significantly enhanced in the MAK 4 treated group compared to the control group ($P < 0.01$), and the MAK 5 treated group tended to increase. O_2-production of peritoneal macrophages in the presence of PMA was significantly high in the MAK 4 and MAK 5 treated groups compared to the control group ($P < 0.01$).

Figure 4 illustrates the effects of MAK 4 and MAK 5 on intracellular activities of APH and GLU of peritoneal macrophages in mice. The APH activity of peritoneal macrophages in MAK 4 group was significantly higher than the control group ($P < 0.01$). No significant difference in APH activity was observed between the MAK 5 group and control. The GLU activity of peritoneal macrophages in the MAK 4 and MAK 5 groups increased

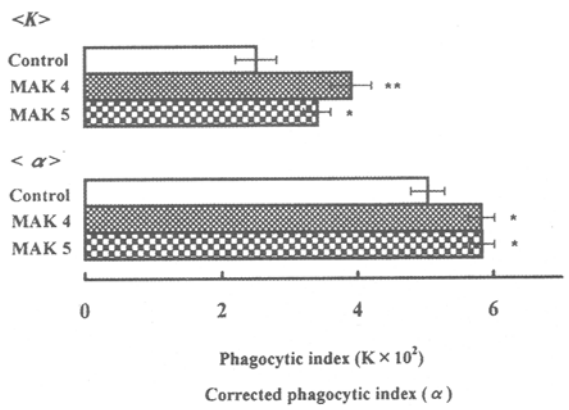

Fig. 1 Effects of MAK 4 and MAK 5 on the phagocytic index (K) and the corrected phagocytic index (α) of the reticuloendothelial system in mice. Each horizontal and error bar represents the mean ± SE. Statistically significant difference from the control at $^*P < 0.05$ and $^{**}P < 0.01$, respectively. MAK 4: Maharishi Amrit Kalash 4, MAK 5: Maharishi Amrit Kalash 5.

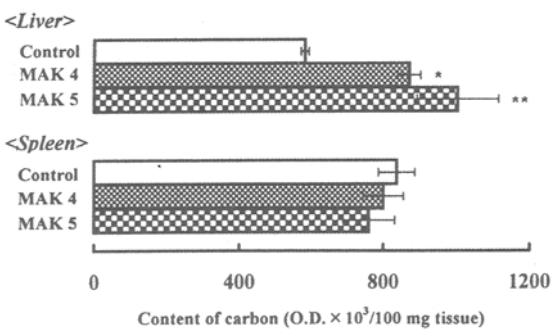

Fig. 2 Effects of MAK 4 and MAK 5 on the hepatic and splenic uptakes of carbon in mice given an injection of carbon. Each horizontal and error bar represents the mean ± SE. Statistically significant difference from the control at $^*P < 0.05$ and $^{**}P < 0.01$, respectively. MAK 4: Maharishi Amrit Kalash 4, MAK 5: Maharishi Amrit Kalash 5.

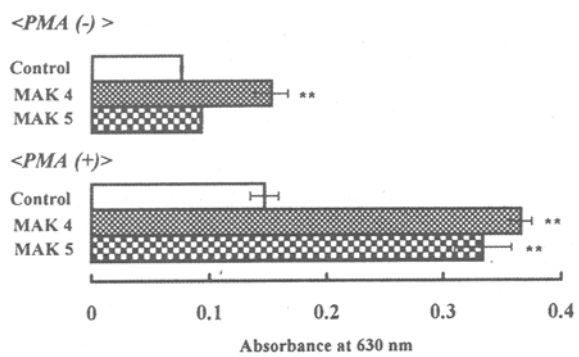

Fig. 3 Effects of MAK 4 and MAK 5 on superoxide anion (O_2-) production of peritoneal macrophages in the absence and presence of phorbol 12-myristate 13-acetate (PMA) in mice. Each horizontal and error bar represents the mean ± SE. Statistically significant difference from the control at $^{**}P < 0.01$. MAK 4: Maharishi Amrit Kalash 4, MAK 5: Maharishi Amrit Kalash 5.

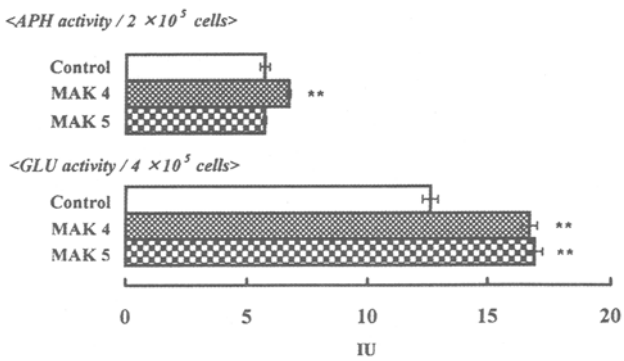

Fig. 4 Effects of MAK 4 and MAK 5 on intracellular activities of acid phosphatase (APH) and β-glucuronidase (GLU) of peritoneal macrophages in mice. Each horizontal and error bar represents the mean ± SE. Statistically significant difference from the control at $^{**}P < 0.01$. MAK 4: Maharishi Amrit Kalash 4, MAK 5: Maharishi Amrit Kalash 5.

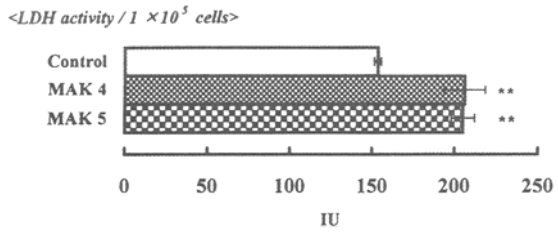

Fig. 5 Effect of MAK 4 and MAK 5 on intracellular activity of lactate dehydrogenase (LDH) of peritoneal macrophages in mice. Each horizontal and error bar represents the mean ± SE. Statistically significant difference from the control at **$P < 0.01$. MAK 4: Maharishi Amrit Kalash 4, MAK 5: Maharishi Amrit Kalash 5.

significantly compared to the control group ($P < 0.01$).

Figure 5 shows the effects of MAK 4 and MAK 5 on LDH activity of peritoneal macrophages in mice. LDH activities of peritoneal macrophages in the MAK 4 and MAK 5 treated mice were significantly higher than those in the control group ($P < 0.01$).

Discussion

In this study male ICR strain mice aged 4 weeks were used to examine the effects of MAK 4 and MAK 5 on phagocytic and digestive functions of macrophages as primary stage of the host defense system. At the end of the experiment, there were no differences in the body weight gain and food intake of mice among all groups.

In the present study, the effects of MAK 4 and MAK 5 on phagocytic activity of reticuloendothelial system were estimated by the carbon clearance method in mice.

It has generally been found that phagocytosis and elimination of the foreign substances are typical functions in the reticuloendothelial system. The phagocytosis of reticuloendothelial system is performed by the Kupffer cells[16]. Biozzi et al.[17] clearly demonstrated that almost all the carbon injected is found in the liver and spleen. The carbon clearance was evaluated by the phagocytic index (K), the corrected phagocytic index (α) and the uptake of carbon in the liver and spleen. We found that the MAK 4 and MAK 5 treated mice had significantly higher values of K and α compared to the control group. Moreover, MAK 4 and MAK 5 treated mice enhanced the hepatic uptake of carbon but not the splenic uptake of carbon. The results described here suggest that MAK 4 and MAK 5 enhance the phagocytosis of carbon *in vivo* through improving function of the Kupffer cells in liver.

In the current study, we evaluated the function of the elimination stage of the phagocytic process by measuring the O_2- production, lysosomal enzymes such as APH and GLU, and cytoplasmic enzyme such as LDH of peritoneal macrophages in mice. We observed that the O_2- production capacity of peritoneal macrophages in the absence of PMA was significantly enhanced in the MAK 4 treated group compared to the control group, and it tended to increase in the MAK 5 treated group. On the other hand, O_2- production stimulated by PMA was significantly higher in the MAK 4 and MAK 5 treated groups compared to that in the control group. We[9] reported earlier that the O_2- production capacity of peritoneal macrophages in the absence of PMA was not affected by oral treatment with MAK 4 for 10 consecutive days at the dose of 50 or 100 mg/kg. The discrepancy between these results might be due to the difference of the experimental duration. It has been shown that O_2- production by macrophages are related to the primary stage of changes in the macrophage function[18] and the pentose phosphate pathway in glycolysis[9]. Therefore, it can be considered that MAK 4 and MAK 5 act on the primary stage of changes in the macrophage function, and has the potency to activate the pentose phosphate pathway in peritoneal macrophages.

It is known that after the activation of macrophage, activities of lysosomal enzymes and cytoplasmic enzymes in the macrophages were increased[20, 21]. In this study, except for the APH activity of peritoneal macrophages in the MAK 5 treated mice, the intracellular activities of APH, GLU and LDH in the peritoneal macrophages were enhanced significantly by the treatment of MAK 4 and MAK 5. These results suggest that MAK 4 and MAK 5 affect the ability of the peritoneal macrophage to respond appropriately to foreign substances.

In this study, APH activity was enhanced in the MAK 4 treated group, but not in the MAK 5 treated group. APH, GLU and LDH were synthesized under the different mechanisms *in vivo*, as reported by Allison et al.[21]. The findings that MAK 5 did not affect the APH activity of the peritoneal macrophages suggest the difference in susceptibility of each enzyme to the MAK 4 and MAK 5 stimulation. Moreover, MAK 4 contains indian gallnut (terminalia chebula)[7], while MAK 5 has gymnema aurentiacum (meda milkweed)[8] as the main ingredient. Thus, it is suspected that the different main ingredient may affect the APH activity of peritoneal macrophages. Further study will be necessary to elucidate the action of main ingredient in MAK 4 and MAK 5 on macrophage functions.

Although it is not easy to apply the results in this study to human, it is likely that macrophage function in human may be improved by the intake of MAK 4 and MAK 5. High level of the host's defense function may be maintained by the intake of food supplemented with MAK 4 and MAK 5 that may improve immune function as shown in the present study. Even if foreign substances, antigens, and/or bacteria intrude into human body, they will be processed by macrophages, namely primary nonspecific immune responses.

In conclusion, it is suggested that the long-term administration of MAK 4 and MAK 5 enhances the phagocytic and digestive functions of macrophage as primary stage of the host defense system and exerts a stimulatory effect on macrophage, based on the findings of the increase in the O_2- production capacity, lysosomal and cytoplasmic enzyme activity of peritoneal macrophages in mice.

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