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 (O2·-) 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 longevity2). Zaman3) described that the traditional medicines including Ayurveda are useful for the maintenance of healthy life in South-East Asia. However, there are few reports4-6) 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 recipe7). MAK 4 and MAK 5 are also expected to potentiate immune system to prevent infectious diseases, but the studies on its immunological actions8-10) are limited. Recently, our research group11-13) 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.
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°C 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. The ingredients in MAK 4 are: Indian gallnut (terminalia chebula), Indian gooseberry, dried catkins, Indian pennywort, nutgrass, white sandalwood, evalylus alstonoides, embella, aloewood, licorice, cardamom, cinnamon, cyperus, turmeric, honey, raw sugar and ghee (clarified butter). The ingredients in MAK 5 are: gymnema aurentiacum (meda millweed), black musale, heart-leafed 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. The dose of MAK 4 and MAK 5 in this study was decided based on our previous study. 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. 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 equations:

\[ K = \frac{\log \text{OD}_{t_0} - \log \text{OD}_{t_5}}{t_0 - t_5} \]
\[ \alpha = \frac{K \cdot P_c}{P_o} \]

where \( t_0 = 5 \text{ min}, t_5 = 10 \text{ min}, \text{OD}_{t_0} = \text{optical density at 5 min after the injection of carbon solution}, \text{OD}_{t_5} = \text{optical density at 10 min after the injection of carbon solution}, P_c = \text{whole body weight (g)}, P_o = \text{total weight (g) of liver and spleen} \).

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. 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°C 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°C in a 5% CO₂ 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%.

O₂⁻ production of peritoneal macrophages was measured by the nitro blue tetrazolium (NBT) reduction method. A 0.1 ml of macrophage cell suspension (2.0 × 10⁶ 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% CO₂ at 37°C, 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 × 10⁶ cells/ml macrophages were centrifuged at 1,000 rpm at 4°C 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 × 10⁶ cells, GLU activity was expressed as IU per 4.0 × 10⁶ cells and LDH activity was expressed as IU per 1.0 × 10⁶ cells.

Statistics

Differences among the three groups were evaluated by using