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Original Article

Dose-Dependent Activation of Immune Function in Mice by Ingestion of Maharishi Amrit Kalash 5

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Abstract

This study was carried out to evaluate the dose-effects of ingestion of Maharishi Amrit Kalash 5 (MAK 5), an Ayurvedic food supplement, on the immune function in 10 week 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 5 by gastric intubation of an aqueous emulsion at the doses of 10, 50, 100 and 200 mg/kg once a day for 20 days. Glucose consumption of peritoneal macrophages in the MAK 5-treated mice at all doses after 24 hours of incubation, and only at the dose of 200 mg/kg after 48 hours of incubation were significantly higher than those in the control group. O_2^- production of peritoneal macrophages in the presence of stimulator was significantly higher in the MAK 5-treated group at the dose of 200 mg/kg than in the control group. Activities of β-glucuronidase and lactate dehydrogenase in the peritoneal macrophages were significantly increased in the MAK 5-treated mice at all doses. MAK 5 did not enhance spontaneous splenic lymphocyte proliferation at any dose in mice. Stimulation indices in the MAK 5-treated groups at the doses of 50, 100 and 200 mg/kg were significantly higher than that of the control group. These results indicate that gastric intubation of MAK 5 once a day at the dose of 50 mg/kg enhances not only macrophage function but also lymphocyte responsiveness 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. The ancient Ayurvedic system provides an approach to the prevention and cure of disease, and emphasizes the philosophy of maintaining excellent health by normalizing the cellular functions and improving the inherent vitality of tissues.

Maharishi Amrit Kalash 5 (MAK 5), one of the Ayurvedic food supplements, belongs to a group of substances known as Rasayana [1]. MAK 5 is a commercially available Rasayana that is composed of a variety of herbs, minerals and dairy products [2]. MAK 5 and other Rasayanas are believed to enhance the body's resistance to infections and disease, and enhance longevity [1]. Recently, Dileepan [2, 3] and our research group [4] reported that MAK 5 has immunomodulatory potency. However, dose-dependent activation of immune function by MAK 5 has not been reported yet. The purpose of this study was to investigate the dose-dependent effects of MAK 5 on immune function and its mechanism in inbred BALB/c mice.

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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 indoors 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 5 was obtained from Maharishi Ayurveda Products International (Lancaster, USA). The ingredients in MAK 5[5] are: gymnema aurentiacum, black musale, heart-leaved moonseed, sphaeranthus indicus, butterfly pea, licorice, vanda spatulatum, elephant creeper and Indian wild pepper. The exact amounts of the various ingredients in MAK 5 were not disclosed by the supplier, but quality control (e. g., minimal variation from batch to batch) was assured.

MAK 5 was suspended in 0.2% carboxymethyl cellulose sodium salt (CMC) (Nacalai Tesque, Kyoto, Japan) solution and given to mice p. o. at doses of 10 mg/kg, 50 mg/kg, 100 mg/kg and 200 mg/kg per day for 20 consecutive days. The control mice were given 0.2% CMC solution (0.1 ml/10 g of body weight) p. o. The animals were killed by bleeding 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 celles (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 (Nissui Seiyaku Co. Ltd., Tokyo, Japan) 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 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] × 100.

O_2^- production of macrophages was assayed by the nitro blue tetrazolium (NBT) (Nakarai Tesque Inc., Kyoto, Japan) reduction method[9]. First, 100 μl (2×10^5 cells) of macrophage suspension was incubated for 2 hours. After aspiration of the solution, 100 μl of NBT (4 mg/ml) and 100 μl of phorbol 12-myristate 13-acetate (0.3 μg/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.

A 2.0 ml 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)[8]. 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%.

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 (Corning Laboratory Sciences Co., New York, USA) 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 μl 0.04N 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 following equation.

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

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 $p<0.05$.

Results

The effects of MAK 5 on the body weight and the food consumption for 20 days in mice are shown in Table 1. There were no significant differences among the MAK 5 treated groups and the control group in body weight and food consumption.

Figure 1 shows the effect of MAK 5 on the glucose consumption of peritoneal macrophages in mice. Glucose consump-

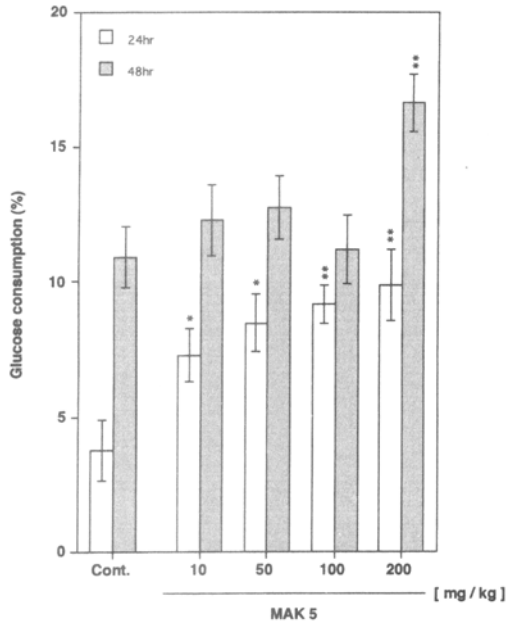

Fig. 1 Effects of Maharishi Amrit Kalash 5 (MAK 5) on glucose consumption of peritoneal macrophages in mice. Each value represents the mean±SE of triplicate sets. *p<0.05, **p<0.01, compared with the controls. Cont., Control.

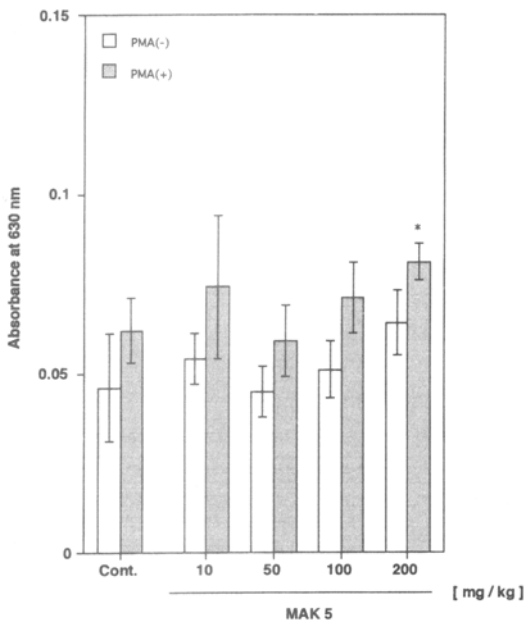

Fig. 2 Effects of Maharishi Amrit Kalash 5 (MAK 5) on superoxide anion (O_2^-) production of peritoneal macrophages in mice. Each value represents the mean±SE of triplicate determinations. *p<0.05, compared with the controls. Cont., Control.

tion of peritoneal macrophages in the MAK 5-treated mice at the doses of 10 mg/kg ($p<0.05$), 50 mg/kg ($p<0.05$), 100 mg/kg ($p<0.01$) and 200 mg/kg ($p<0.01$) after 24-hour incubation, and only at the dose of 200 mg/kg ($p<0.01$) after 48 hours of incubation were significantly high compared with the control group.

As shown in Fig. 2, O_2^- production in the absence of PMA was not enhanced in MAK 5-treated groups at any dose. On the other hand, O_2^- production stimulated by PMA was significantly

Table 1 Effects of MAK 5 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 ± 0.4	21.5 ± 0.3	3.11 ± 0.09
MAK 5				
10mg/kg	12	19.6 ± 0.3	21.1 ± 0.2	3.01 ± 0.07
50mg/kg	12	19.7 ± 0.4	21.2 ± 0.3	3.16 ± 0.10
100mg/kg	12	19.9 ± 0.3	20.9 ± 0.3	3.10 ± 0.07
200mg/kg	12	19.5 ± 0.5	21.3 ± 0.4	3.04 ± 0.08

Each value represents the mean ± SE.
MAK 5, Maharishi Amrit Kalash 5; N, Number of mice used.

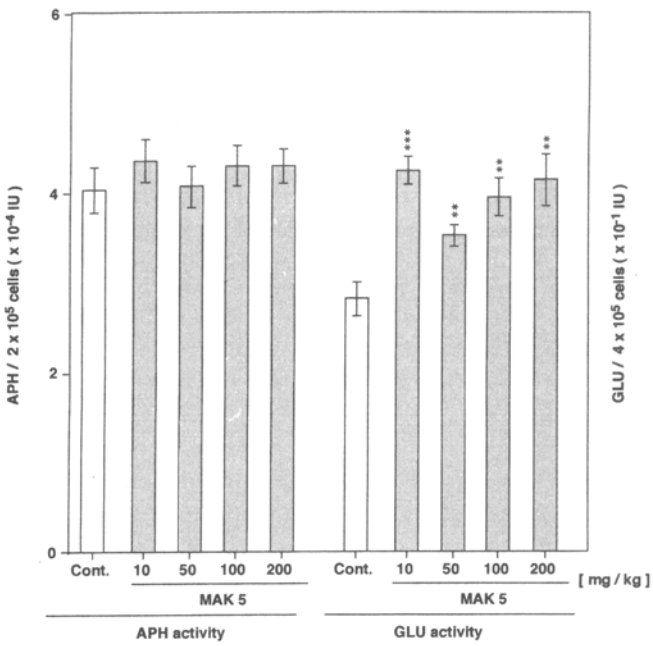

Fig. 3 Effects of Maharishi Amrit Kalash 5 (MAK 5) on acid phosphatase (APH) and β-glucuronidase (GLU) activities of peritoneal macrophages in mice. Each value represents the mean±SE of triplicate determinations. **p<0.01, ***p<0.001, compared with the controls. Cont., Control.

high only in the MAK 5-treated group at the dose of 200 mg/kg compared with that in the control group ($p<0.05$).

Figures 3 and 4 show the effects of MAK 5 on APH, GLU and LDH activities of peritoneal macrophages in mice. There were no significant differences among MAK 5-treated groups and the control group in the APH activities of peritoneal macrophages. GLU and LDH activities of peritoneal macrophages in the MAK 5-treated mice at all doses were significantly higher than those in the control group ($p<0.01$).

MAK 5 did not enhance spontaneous splenic lymphocyte proliferation in mice at any dose. Figure 5 shows the effect of MAK 5 on the proliferation of splenocytes induced by Con A in mice. Stimulation indices in the MAK 5-treated groups at the doses of 50 mg/kg ($p<0.05$), 100 mg/kg ($p<0.05$) and 200 mg/kg ($p<0.01$) were significantly higher than that of the control group.

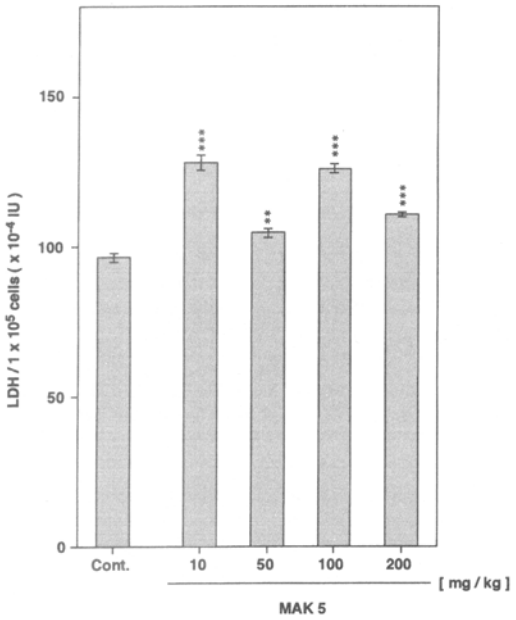

Fig. 4 Effects of Maharishi Amrit Kalash 5 (MAK 5) on lactate dehydrogenase (LDH) activities of peritoneal macrophages in mice. Each value represents the mean±SE of triplicate determinations. ** p<0.01, ***p<0.001, compared with the controls. Cont., Control.

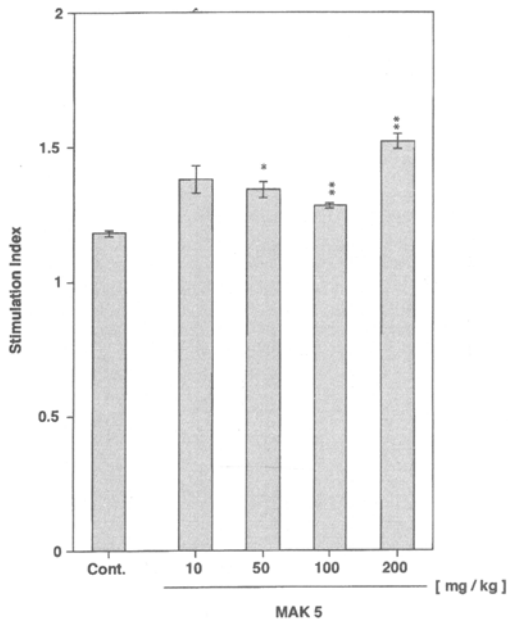

Fig. 5 Effects of Maharishi Amrit Kalash 5 (MAK 5) on proliferation of splenocytes induced by Con A in mice. Each value represents the mean±SE of 4 mice. *p<0.05, **p<0.01, compared with the controls. Cont., Control.

Discussion

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 5-treated mice at the doses of over 10 mg/kg after 24-hour incubation, and at the dose of 200 mg/kg after 48-hour incubation were significantly high compared with the control group. These results suggested that MAK 5 had the potency to activate peritoneal macrophages in the priming stage and that it was necessary to treat mice with MAK 5 at doses of over 10 mg/kg at least once a day to activate the peritoneal macrophages by 24 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 5 for 20 days at the dose of 200 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 affected significantly by daily oral treatment with MAK 5 for 10 days at the dose of 50 mg/kg. On the other hand, Dileepan et al.[2] reported that macrophage O_2^- production of male Sprague Dawley rats (weighing 210-225g) was not affected by treatment with 50 mg/rat/day for 10 or 20 days. The discrepancy between these results may be attributable to the difference of animal strain or animal species. At any rate, our results suggested that macrophage O_2^- generation, at least in the presence of activators, was altered by MAK 5 ingestion. However, further studies are needed to determine the optical doses of MAK 5 needed to enhance macrophage O_2^- production.

Ryan et al.[11] and Pantalone et al.[12] 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 GOT in the macrophages were increased. In the present study, we observed that activities of GLU and LDH in the peritoneal macrophages were increased significantly in the MAK 5-treated mice at all doses. These results suggested that MAK 5 can induce peritoneal macrophages to phagocytose 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 our previous[4] and present studies, we concluded that gastric intubation of MAK 5 at the dose of 50 mg/kg was sufficient to enhance macrophage function.

The results showing that MAK 5 did not enhance spontaneous splenic lymphocyte proliferation at any dose in female BALB/c mice in the present experiment were in accordance with those reported by Dileepan et al.[2,3]

We[4] previously reported that the index for stimulation of spleen cells by Con A in mice is significantly higher in groups treated with MAK at the doses of 50 and 100 mg/kg than in controls. In the current study, we observed that the stimulation index of spleen cells by Con A was increased significantly by the treatment with MAK 5 at the doses of 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 5 at doses of over 50 mg/kg. Lymphocyte proliferation is a complex event involving, among other factors, the participation of interleukins 1 and 2 and interleukin-2 receptor expression[13]. It is possible that MAK 5 ingestion enhances the production of various cytokines and/or T-cell-receptor expression. Further studies are needed to clarify the mechanism of the enhancement of lymphocyte proliferation by MAK 5.

It has been shown that lectins from a variety of beans, including that from kidney beans (phaseous vulgaris)[14], can trans-

form lymphocytes in vitro and enhance their proliferation. Several other lectins and alkaloids with mitogenic properties have also been identified[15, 16]. 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 study and reported by others[3], and lack of macrophage-mediated tumor cell killing[3] in MAK 5-treated mice suggest that direct activation by these compounds is unlikely.

In conclusion, oral administration of MAK 5 once a day (over 50 mg/kg) enhances not only macrophage function but also lymphocyte responsiveness.

References

1) Glaser JL. Maharishi Ayurveda: An introduction to recent research. Mod Sci Ved Sci 1988; **2**: 89-108.
2) Dileepan KN, Patel V, Sharma HM, Stechschulte DJ. Priming of splenic lymphocytes after ingestion of an Ayurvedic herbal food supplement: Evidence for an immunomodulatory effect. Biochem Archiv 1990; **6**: 267-74.
3) Dileepan KN, Varghese ST, Page JC, Stechschulte DJ. Enhanced lymphoproliferative response, macrophage mediated tumor cell killing and nitric oxide production after ingestion of an Ayurvedic drug. Biochem Archiv 1993; **9**: 365-74.
4) Inaba R, Sugiura H, Iwata H. Immunomodulatory effects of Maharishi Amrit Kalash 4 and 5 in mice. Jpn J Hyg 1995; **50**: 901-5.
5) Sharma H, Dwivedi C, Statter BC, Abou-Issa H. Antineoplastic properties of Maharishi Amrit Kalash, an Ayurvedic food supplement, against 7, 12-dimethylbenz (*a*) anthracen-induced mammary tumors in rats. J Ed Ind Med 1991; **3**: 1-8.
6) Adachi Y, Ohno N, Ohsawa M, Oikawa S, Yadomae T. Changes of biological activities of (1-3)- β -D-glucan from Glifola frondosa upon molecular weight reduction by heat treatment. Chem Pharm Bull 1990; **38**: 477-81.
7) Barbara BH, Stanley MS. Selected methods in cellular immunology. San Francisco: Freeman WH and Company, 1980 (Japanese translation by Imai K, Kawaguchi S, Harada T. Tokyo: Rikogakusya, 1982: 15-6) .
8) Sugiura H, Maeno H, Ueya E, Inaba R, Iwata H, Nishida H. Effects of ingestion of "BON-NARINE" on immune functions in mice. Jpn J Hyg 1994; **49**: 914-23.
9) DiGregorio KA, Cilento EV, Lantz RC. Measurement of superoxide release from single pulmonary alveolar macrophages. Am J Physiol 1987; **252**: 677-83.
10) Sugawara I, Ishizaka S, Tsuji T, Nishiyama T. MTT assay-rapid colorimetric assay applicable to cellular proliferation and cytotoxicity assays. J Clin Exp Med 1984; **128**: 733-5.
11) Ryan JL, Glode LM, Rosenstreich DL. Lack of responsiveness of C3H/HeJ macrophages to lipopolysaccharide: The cellular basis of LPS-stimulated metabolism. J Immunol 1979; **122**: 932-5.
12) Pantalone R, Page RC. Enzyme production and secretion by lymphokine-activated macrophages. J Reticuloendothel Soc 1979; **21**: 343-57.
13) Smith KA. Interleukin-2: Inception, impact, and implications. Science 1988; **240**: 1169-76.
14) Nowell P. Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytosis. Cancer Res 1960; **20**: 462-6.
15) Kaneko Y, Yatsuzaka M, Endo Y, Oda T. Activation of human lymphocytes by tumor promotor teleocidin. Biochem Biophys Res Comm 1981; **100**: 888-93.
16) Collins M, Rosengurt E. Stimulation of DNA synthesis in murine fibroblasts by the tumor promotor teleocidin: Relationship to phorbor esters and vasopressin. Biochem Biophys Res Comm 1982; **104**: 1159-66.

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