Immunostimulatory activities of yam tuber mucilages

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ABSTRACT. The purified mucilages from three Taiwanese yam cultivars, including *Dioscorea alata* L. cv. Tainong 1 (TN1), *D. alata* L. cv. Tainong 2 (TN2), and *D. alata* L. var. purpurea (Roxb.) cv. Ming-Jen (MJ), and the commercial lentinan (Len) were used to evaluate the immunostimulatory effects on the murine innate and adaptive immunity. BALB/c mice were grouped and administrated orally with 0.5 ml of TN1, TN2, MJ daily for 5 weeks. The positive and negative controls were fed with lentinan and distilled water, respectively. Blood samples were drawn from the retroorbital sinus on day 7 and 21, and the lymphocyte subpopulation, phagocytosis of granulocyte and monocyte were analyzed by flow cytometry. The mice were sacrificed on day 36, and the splenocytes were harvested for determinations of natural killer (NK) cell cytotoxicity activity. The stimulation index on the phagocytosis of peritoneal macrophages and the RAW264.7 cell line by yam mucilage were also determined *in vitro*. The results showed that all three mucilages, especially MJ yam, could elevate the number of T helper cells in the peripheral blood and enhance the phagocytic activity of granulocyte, monocytes and macrophages both *ex vivo* and *in vitro* tests. Increased splenic cytotoxic activity following the administration of mucilages from MJ yam was observed. Furthermore, the production of specific anti-ovalbumin (OVA) antibody and OVA-stimulated splenic cell proliferation were also enhanced by all mucilage groups. It is suggested that the tuber mucilage may function as an immunomodulatory substance.

Keywords: *Dioscorea*; Immunostimulatory; Lentinan; mucilage; Nk cell; Phagocytic activity; Yam.

Introduction

Yam (*Dioscorea* species) is a member of the monocotyledonous family Dioscoreaceae and is a staple food in West Africa, Southeast Asia, and the Caribbean (Akoruda, 1984). The fresh tuber slices are widely used as functional foods in Taiwan, and the dried slices are used as traditional Chinese medicines (Liu et al., 1995). Yam tuber contains mucilages, mannan-protein macromolecules (Misaki et al., 1972; Tsai and Tsai, 1984). Recently, yam tuber mucilage was reported to exhibit antioxidant (Hou et al., 2002; Lin et al., 2005), angiotensin converting enzyme inhibitory activities (Lee et al., 2003) and hypoglycemic activities (Hikino et al., 1986; Bailey and Day, 1989). Furthermore, Chinese yam (*D. alata* cv. Tainong No. 2) feeding resulted in antioxidant effects in hyperhomocysteinemia rats (Chang et al., 2004).

Many isolated polysaccharides are reported to have immunomodulatory activities (Brown and Gordon, 2003;

Feizi, 2000), and medicinal mushrooms (Wasser, 2002) have been intensively investigated for their beneficial effects as immunomodulatory and antitumor agents. Lentinan (Len), the (1→3)-β-glucan isolated from *Lentinus edodes*, has been demonstrated to have an anti-tumor activity against Sarcoma 180 *in vivo* and *in vitro* (Zhang et al., 2005). Reishi (*Ganoderma lucidum*) polysaccharides were reported as immune potentiators (Chang and Lu, 2004; Zhu and Lin, 2005; Hsu, et al., 2004). The cold-water extracts of dietary mushrooms, including *Hypsizigus mamoreus*, *Agrocybe aegerita*, and *Flammulina velutipes*, were showed to have antiproliferative activity against human leukemic U937 cells (Ou et al., 2005). The immunomodulatory activity by an isolated α-glucan-protein complex from mycelium of *Tricholoma matsutake* has also been documented (Hoshi et al., 2005). Several food-grade microalgae, including *Spirulina platensis*, *Aphanizomenon flos-aquae*, and *Chlorella pyrenoidosa*, are also known to contain polysaccharides, potent immunostimulators of human monocytes and macrophages (Pugh et al., 2001). In this study, orally administered mucilages from three different Taiwanese yam cultivars, including *Dioscorea alata* L. cv. Tainong 1 (TN1), *Dioscorea alata* L. cv. Tainong 2

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(TN2), and *D. alata* L. var. *purpurea* (Roxb.) cv. Ming-Jen (MJ), were evaluated for their immunostimulatory effects on the innate and adaptive immunity of BALB/c mice.

MATERIAL AND METHODS

Plant materials

Local fresh tubers of Taiwanese yam, including *D. alata* L. cv. Tainong 1 (TN1), *D. alata* L. cv. Tainong 2 (TN2), and *D. alata* L. var. *purpurea* (Roxb.) cv. Ming-Jen (MJ), were kindly provided by Dr. Liu, Sin-Yie (Taiwan Agricultural Research Institute, Wu-Feng, Taichung, Taiwan). The TN1 tuber is a round or elliptic shape with white fleshes in the brown peel. The TN2 tuber is a cylindraceous shape with white fleshes in the brown peel. The MJ tuber is a cylindraceous shape with purple fleshes in the purplish-red peel.

Extraction and purification of yam tuber mucilage

After washing and peeling, the yam tubers were cut into strips for mucilage extraction and purification as described elsewhere (Hou et al., 2002; Lee et al., 2003). Briefly, yam tuber was homogenized with four volumes (W/V) of 50 mM Tris-HCl buffer (pH 8.3) containing 1% vitamin C. After centrifugation at 14,000 ×g for 30 min, the supernatants were mixed with isopropanol to a final concentration of 70%, and stirred quickly at 4ºC overnight. The precipitates were filtrated and dehydrated with 100% isopropanol, then, rinsed with acetone. After drying at 40ºC in an oven, the crude mucilage (CM) was ground and collected for further purification by both SDS and heating procedures. One gram of CM powder was dissolved in 200 ml distilled water and kept in a 50ºC water bath. Forty mililiters of 5% SDS solution (dissolved in 45% ethanol) were added to the CM solution. The mixture was stirred gently at 50ºC for 30 min, then, at room temperature for another 2 h. The mucilage solution was then placed in an ice bath to quickly reduce the temperature in order to precipitate the SDS-protein complex. After centrifugation as above, the supernatants were precipitated with isopropanol and dried in a 40ºC oven as described earlier. The mucilage was again ground, dissolved, and then heated in boiling water for 20 min. After centrifugation, the supernatants were mixed with isopropanol to a final concentration of 70%. The partially purified mucilage was filtrated, dehydrated, rinsed with acetone, dried, and then collected for further use.

Experimental animals

Five weeks old male BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and divided randomly into five groups (n=8). Each group was housed individually in wire-bottomed stainless steel cages in a temperature- and humidity-controlled room (at 22ºC) with a 12-h light/dark cycle and free access to AIN-76 feeds and water. All animal experimental procedures followed the published guidelines (COA, 2004). For assessment of innate immunity, each 0.5 ml of TN1, TN2, MJ (10 mg/m) and commercial Len (0.05 mg/ml) were administrated orally once a day for 5 weeks. Distilled water was used for the control group. Blood samples were drawn from the retroorbital sinus on days 7 and 21, and the lymphocyte subpopulation and both phagocytosis of granulocyte, and monocyte were analyzed by flow cytometry as described below.

Lymphocyte subpopulation assay

The labeled primary antibodies used for lymphocyte subpopulation, including T cell, B cell, T helper cell, and cytotoxic T cell, by flow cytometry (Viau et al., 2005) were showed in Table 1. All labeled monoclonal antibodies were purchased from Serotec Company (Oxford, UK). Fifty microliters of blood isolated from the retroorbital sinus on days 7 and 21 were put in the Falcon tube (Falcon 2052). Labeled monoclonal antibodies (5 μl) were added and incubated at room temperature under light protection for 20 min. After lysis and washing, the lymphocyte subpopulation was determined by the flow cytometry and analyzed by CellQuest software (Becton Dickinson FACS Calibur^{TM}, CA).

Phagocytosis of granulocyte and monocyte

FITC-labeled *Escherichia coli* (Molecular Probes, USA) powder (5 mg) was suspended in 0.5 ml of Hank's balanced salt solution (HBSS) and used for phagocytosis analysis by flow cytometry (Butcher et al., 2001; Gaforio et al., 2002). One hundred microliters of blood from the retroorbital sinus on days 7 and 21 were mixed with 20 μl of FITC-labeled *E. coli* solution at 37ºC for 10 min. The Falcon tube was immersed in an ice bath to stop the phagocytosis. One hundred microliters of of trypan blue (1.25 mg/ml) were added to quench the residual FITC-labeled *E. coli*. After lysis and washing, 5 μl of propidium iodide (PI, 2 mg/ml) were added for 10 min, and the phagocytosis of granulocyte and monocyte was determined by flow cytometry (Becton Dickinson FACS Calibur^{TM}, CA).

Table 1. The labeled primary antibody used for lymphocyte subpopulation assay by flow cytometry.

Lymphocyte subpopulation	Surface marker	Monoclonal antibody used
T cell	CD3$^+$	Phycoerythrin (PE)-anti-mouse CD3
B cell	CD19$^+$	Fluorescein isocyanate (FITC)-anti-mouse CD19
T helper cell	CD4$^+$	FITC-anti-mouse CD4
T cytotic cell	CD8$^+$	PE-anti-mouse CD8

Phagocytosis of peritoneal macrophage and the RAW264.7 cell line *in vitro*

The peritoneal macrophages were obtained at 3 day after intraperitoneal injectionior with 2 ml of thioglycollate broth (Becton Dickinson, CA) (Choi and Hwang, 2002; Choi et al., 2004). RAW264.7 cell line (Sosroseno et al., 2003) was cultured in Dulbecco's modified eagle medium (DMEM, GibcoBRL, USA) containing 5% fetal bovine serum (FCS, GibcoBRL, USA), adjusted to 2×10^6 cell/ml, and seeded in a 96-well plate (100 μl/well). Ten microliters of TN1, TN2, MJ (1 mg/ml), and commercial Len (0.1 μg/ml) were added to the 96 wells and cultured in a 5% CO_2 humidified incubator at 37ºC for 60 min. After removing the supernatants, 20 μl of FITC-labeled *E. coli* was added for 2 h. Two hundred microliters of trypan blue (1.25 mg/ml) were added in each wells one min for quenching. The stimulation index (%) for phagocytosis of peritoneal macrophage and RAW264.7 cells by yam mucilages was determined by fluorescent analyzer.

Splenocyte-mediated cytotoxicity assay

Treated mice were sacrificed at day 36 and splenocytes were harvested for determinations of NK cell activity (Choi et al., 2004; Zhu and Lin, 2005). Briefly, isolated splenocytes were washed with PBS for 3 times and adjusted to 4×10^6 cell/ml with RPMI-1640 medium (GibcoBRL, USA) as effector cells. The YAC-1 cells, as target cells, were washed with HBSS and adjusted to 2×10^6 cell/ml. Target cells at 200 μl were mixed with 2 μl of $DiOC_{18}$ (3 mM) (Molecular Probes, USA) at 37ºC for 20 min. Target cells were resuspended in 200 μl of RPMI-1640 medium (GibcoBRL, USA) for further uses. The effector cells were mixed with target cells (40:1, 20:1, 10:1, and 5:1) in serial dilutions and were co-cultured in 5% CO_2 humidified incubator at 37ºC for 2 h. The supernatants were removed, and the same volume of PI solution (0.2 mg/ml) was added. The splenocyte-mediated cytotoxicity was determined by flow cytometry (Becton Dickinson FACS Calibur[TM], CA).

Effects of yam mucilage on the production of the specific anti-Ova antibody (adaptive immunity assessments)

Mice were divided in 5 groups, each consisting of 8 animals and administered orally with 0.5 ml of TN1, TN2, MJ (10 mg/ml) and commercial Len (0.05 mg/ml) once a day for 5 weeks. The ovalbumin (OVA, 2 mg/ml was used on day 0, and 6 mg/ml was used on day 21) was pre-mixed with an equal volume of aluminum adjuvant (2.5 mg/ml) for mice immunizations. Each mouse was injected intraperitoneally on day 0 and day 21 with 0.2 ml of OVA/aluminum adjuvant. Blood samples were drawn from the retroorbital sinus before and on days 7, 32, and 43 after immunization. The production of specific anti-OVA antibody was analyzed by ELISA method (Mlčková et al., 2001; Okamoto et al., 2003). The 96-well plate was coated with OVA (1 to 5 μg/ml) and then blocked with bovine

serum albumin (10 mg/ml). Peroxidase-conjugated goat anti-mouse IgG (diluted in $1{:}10^4$) or -conjugated goat anti-mouse IgM (diluted in $1{:}10^4$) was added and incubayed at room temperature for 2 h. The amounts of anti-OVA-IgG and anti-OVA-IgM were measured by adding hydrogen peroxide and TMB for 15 min and then recording at 450 nm by ELISA reader (TECAN Sunrise microplate reader, Männedorf, Switzerland).

Effects of yam mucilage on the splenocyte proliferations after specific Ag stimulation (adaptive immunity assessments)

Twenty-four days after the second booster, the mice were sacrificed, and the splenocytes were harvested for proliferation determinations by MTT assay. Briefly, isolated splenocytes were washed with PBS for 3 times, adjusted to 1×10^6 cell/ml with RPMI-1640 medium (GibcoBRL, USA), and seeded in a 96-well plate (100 μl/well). Fifty microliters of OVA（1 μg/ml）were added to each test well and then cultured in a 5% CO_2 humidified incubator at 37ºC for 48 h. Five microliters of MTT (5 mg/ml) were then added under light protection for 4 hrs and 100 μl of 10% SDS in 0.01 N HCl were added for 18 hrs. Absorbance at 595 nm was determined by ELISA reader (TECAN Sunrise microplate reader, Männedorf, Switzerland). The stimulation index (%) for proliferation of splenocytes by OVA was calculated following the equation: (A_{595} with yam mucilage treatment)÷(A_{595} without yam mucilage treatment)×100%.

Statistical analysis

Means of triplicates were measured. Student's t-test was used for comparison between two treatments. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**).

RESULTS AND DISCUSSION

Yam tuber contains mucilages that were reported to be a mannan-protein macromolecule (Misaki et al., 1972; Tsai and Tai, 1984). Our previous report revealed that the total recovery of purified yam tuber mucilages was about 48% that of the crude mucilages (Hou et al., 2002). In this report, mucilages from three cultivars of native Taiwanese yam were reported to have immunomodulatory activities in innate immunity and adaptive immunity.

Lymphocyte subpopulation assay

The results showed that the number of T cells in TN2 or MJ yam mucilages-treated animals is increased as compared with the control group in the first week (Figure 1A) and MJ yam mucilages-treated ones in the third week (Figure 1B) ($P<0.01$). This cell increment was due to elevated number of T helper cells and T cytotoxic cells (Figures 1C and 1D) ($P<0.05$). The increased T helper cells were also found after glutamine supplements in rats with gut-derived sepsis (Yeh et al., 2004). Sinclair (1998) found that the
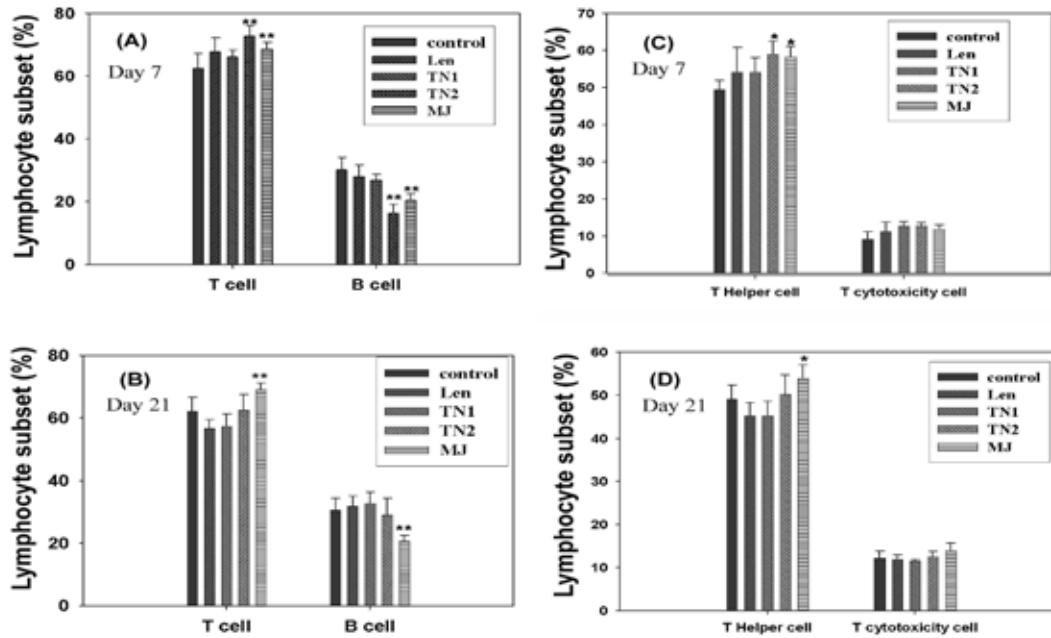
Figure 1. The effects of yam mucilages on the lymphocyte subpopulations. The blood from the retroorbital sinus on day 7 and day 21 was analyzed for both T and B cells (A and B) and T cell subsets (C and D) using flow cytometry. Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**). Bar represents standard deviation.

isolated polysaccharides from *Astragalus membranaceus* (Huang-gi, Chinese herb) elevated the T cell and T cytotoxic cell numbers. The increased T helper cell subpopulations in Th1 or Th2 subsets (Mosmann and Sad, 1996) and the secreted cytokines will be further investigated.

Phagocytosis of granulocyte and monocyte isolated from blood of BALB/c mice

In the first week (Figure 2A), the phagocytic activity of the granulocyte population showed no apparent differences from the control group. However, phagocytic activity by monocyte population significantly increased in TN1, TN2, and the MJ groups as compared with that in the control ($P<0.05$ for TN1 And TN2 and $P<0.01$ for MJ). The phagocytic activity in Len-treated animals was elevated in

the third, but not the first week as compared with the control ($P<0.01$). In the third week (Figure 2B), the phagocytic activities of granulocyte and monocyte populations significantly increased in the Len ($P<0.01$), TN1 ($P<0.05$), TN2 ($P<0.05$), and MJ ($P<0.01$) groups. Our present data reveal that the oral administration of native Taiwanese yam mucilages can elevate the phagocytic cell populations of BALB/c mice *ex vivo*. In mammals, phagocytosis is a very important defense against pathogen invasions and apoptotic cell scavenging, which is performed by phagocytes like macrophages, dendritic cells, and granulocytes (Stuart et al., 2005). Monocytes and other leukocytes are recruited to the inflammatory site and differentiate in advance to inflammatory macrophages (Van den Berg et al., 2001).
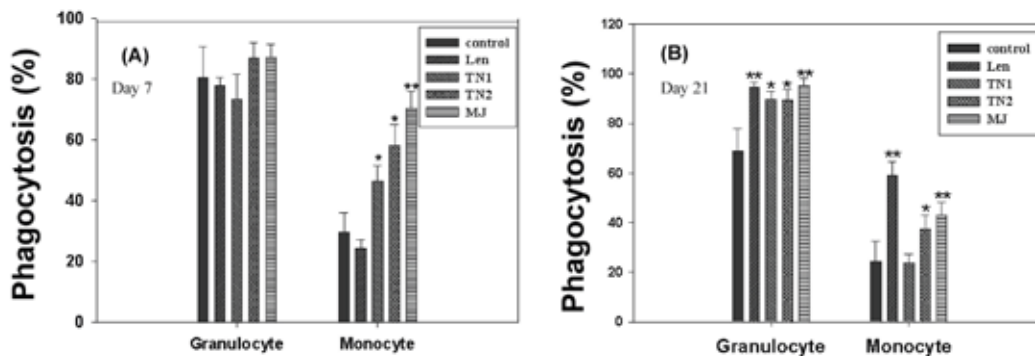

Figure 2. The effects of yam mucilages on the phagocytosis of granulocyte and monocyte on day 7 (A) and day 21 (B). Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**). Bar represents standard deviation.
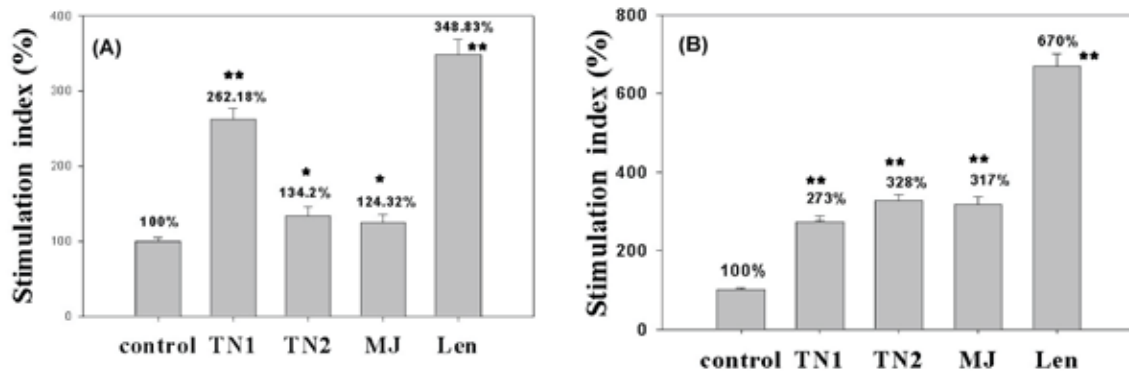
Figure 3. The effects of yam mucilages on the phagocytic activity of peritoneal macrophage (A) and the RAW264.7 cell line (B). Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**). Bar represents standard deviation.

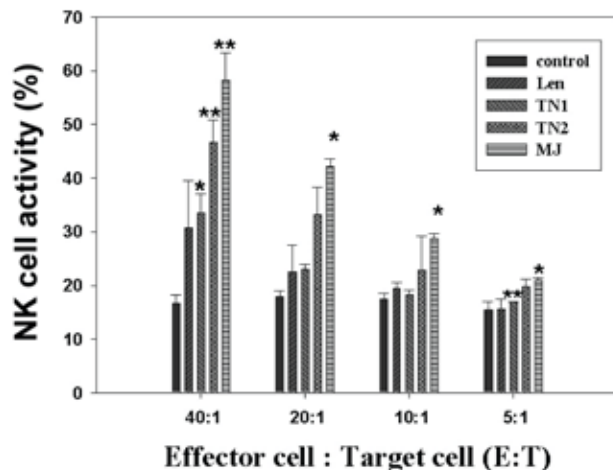## Phagocytosis of peritoneal macrophage and the RAW264.7 cell line

Figure 4. The effects of yam mucilages on the splenocyte-mediated cytotoxicity. Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**). Bar represents standard deviation.

The peritoneal macrophages and the RAW264.7 cells were frequently used for phagocytic analysis *in vitro* (Choi et al., 2004; Sosroseno et al., 2003). All the yam mucilages enhanced phagocytosis by murine peritoneal macrophages as compared with the control ($P<0.01$ for TN1 and $P<0.05$ for TN2 and MJ, Figure 3). Similarly, all the yam mucilages augmented phagocytosis by RAW264.7 cells as compared with the control ($P<0.01$, Figure 3). Choi et al. (2004) reported on the yam mucilages with stimulated activity of phagocytosis. Our present result showed that the stimulation index of peritoneal macrophage treated with TN1, TN2, MJ, and commercial Len were 2.62, 1.34, 1.24, and 3.48-folds, respectively (Figure 3A), and the stimulation index of RAW264.7 cell line treated with TN1, TN2, MJ, and commercial Len were 2.73, 3.28, 3.17, and 6.7-folds, respectively (Figure 3B). High mannuronic acid-containing alginate was reported to stimulate the murine peritoneal macrophage phagocytosis (Son et al., 2001). From the results of Figures 2 and 3, the mucilages from three Taiwanese yams may exhibit a stimulatory effect on phagocytic activity by granulocyte and monocyte (*ex vivo*), on peritoneal macrophages, and on the RAW264.7 cells (*in vitro*).
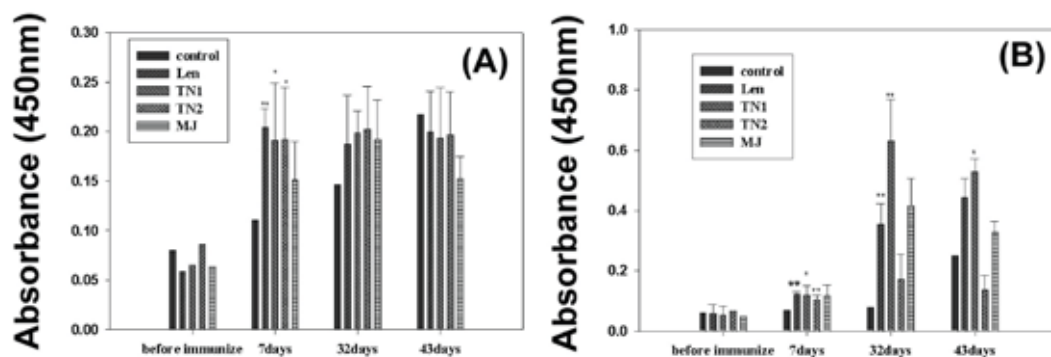

Figure 5. Effects of yam mucilages on the production of the serum specific anti-ovalbumin antibody of IgM (A) or IgG (B). Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when $P<0.05$ (*) or $P<0.01$ (**). Bar represents standard deviation.

Splenocyte-mediated cytotoxicity assay

The results of the present study showed that oral administration of mucilages promoted NK cell activity. The MJ mucilages showed significant differences (40:1, *P*<0.01; 20:1, 10:1, 5:1, *P*<0.05) from the control group. Several reports have shown that polysaccharides can activate NK cell activity (Wasser, 2002; Choi et al., 2004; Vetvicka and Yvin, 2004; Hoshi et al., 2005; Zhu and Lin, 2005). NK cells are large granular lymphocytes that lyse a variety of transformed and infected cells and are sufficiently developed to control infection or tumor (Diefenbach and Raulet, 2001). The potential use of MJ mucilages for anti-cancer treatment will be further investigated.

Effects of yam mucilage on the production of the specific anti-Ova antibody

For evaluating the effects of mucilages on the adaptive immunity, the production of specific IgM (Figure 5A) or IgG (Figure 5B) against OVA was determined. It was found that one week after immunization the oral administrations of mucilage could elevate the amounts of specific IgM (Figure 5A) and specific IgG (Figure 5B) against OVA compared to the control group (P<0.01, for TN1 and TN2). After the second booster of OVA (day 21), the specific IgM production (day 32 and day 43) remained constant, and no significant differences among groups appeared. However, the IgG production apparently increased at day 32, and the Len and TN1 groups significantly outperfomed the control group (*P*<0.01). It was found that the specific antibodies rapidly responses against foreign proteins (or antigens) in the presence of yam mucilages. It might be possible to use the yam mucilages as immune adjuvants for immunomodulations.

Effects of yam mucilage on the splenocyte proliferations

Following immunization with OVA, mice were sacrificed on day 45, and the splenocytes were harvested for proliferation determinations. As seen in Figure 6, out of yam mucilages investigated, TN1 and MJ significantly increased cell proliferation as compared with the control (*P*<0.05). It meant that the yam mucilages might be acted as mitogens. The increased lymphocyte subpopulation needed investigations further.

In conclusion, the effects on the innate immunity (Figures 1 to 4) and adaptive immunity (Figures 5 and 6) of oral administrations of three mucilages from Taiwanese yams on BALB/c mice seem significant. It is suggested that the tuber mucilage might function as an immunostimulatory substance. The production of cytokines and anti-cancer treatments will be investigated in the future.
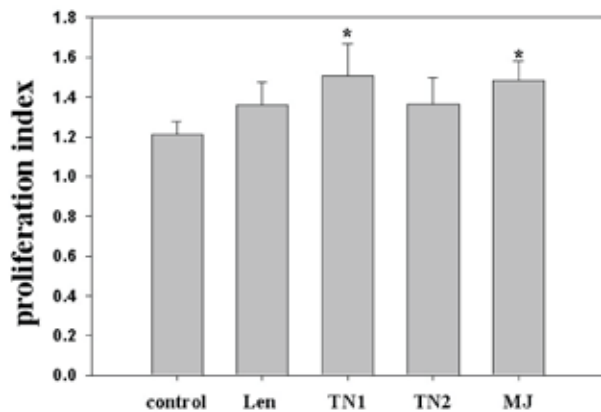
Figure 6. Effects of yam mucilages on the splenocyte proliferations. Means of triplicates were measured. A difference between the control and each treatment was considered statistically significant when *P*<0.05 (*) or *P*<0.01 (**). Bar represents standard deviation.

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山藥黏多醣免疫調節活性的研究

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　　從三種山藥「台農一號（TN1）、台農二號（TN2）及名間長紅（MJ）」抽取的黏多醣（10 mg/ml），以及香菇多醣（Len，0.05 mg/ml）進行餵食 BALB/c 小鼠五週，觀察對於非特異性免疫活性與特異性免疫活性之免疫調節活性評估。非特異性免疫活性包含淋巴細胞亞群的分佈、顆粒性白血球和單核球細胞的吞噬作用、自然殺手細胞活性的分析。同時亦測試山藥黏質多醣對老鼠腹腔巨噬細胞與巨噬細胞株（RAW264.7）體外吞噬作用之影響。而特異性免疫活性之評估則以 OVA 抗原（ovalbumin）免疫老鼠後，利用 ELISA 分析專一性抗體產生濃度的高低；並以 MTT 定量法測試老鼠脾臟細胞受 OVA 刺激後的分裂能力。本研究結果顯示，TN2 及 MJ 組老鼠周邊血液中 T cell（CD3$^+$）總數顯著增加（$p < 0.01$），而其中增加的又以輔助 T 細胞（T helper cell, CD4$^+$）（$p < 0.05$）為最顯著。而 BALB/c 小白鼠周邊血液中單核球細胞的吞噬能力在第一週時 MJ 組（$p < 0.05$）、TN1 和 TN2 組（$p < 0.01$）有顯著性增加，而顆粒性白血球的吞噬能力則皆無明顯變化。但至第三週時，四種樣品皆顯著促進顆粒性白血球之吞噬能力；而單核球細胞之吞噬能力除 TN2 組外其餘三種樣品皆呈顯著增加。而四種樣品在試管中亦均能促進老鼠腹腔巨噬細胞與巨噬細胞株（RAW264.7）之吞噬能力。自然殺手細胞毒殺能力的測定結果發現：Effector cell 和 Target cell 比例從 5：1 至 40：1 時，MJ 組自然殺手細胞毒殺能力均比對照組有顯著增加（$p < 0.01$）。以 OVA 抗原免疫 BALB/c 小白鼠後，結果發現香菇多醣和三種山藥黏質多醣皆可促進專一性抗 OVA 抗體 IgG 和 IgM 之生成，而其中以 TN1 和香菇多醣效果較顯著。比較 OVA 免疫後老鼠的脾臟細胞在 OVA 抗原刺激後之分裂能力，亦發現香菇多醣和三種山藥黏質多醣組老鼠脾臟細胞增生的能力均較對照組強。

關鍵詞：免疫促進；香菇多醣；山藥黏多醣；自然殺手細胞；吞噬活性；山藥。