NOTE *Immunology*

Induction of Interferon-Gamma (IFN-γ) and T Helper 1 (Th1) Immune Response by Bitter Gourd Extract

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ABSTRACT. Mice were inoculated intraperitoneally wih 34 different types of vegetable juices, and interferon-gamma (IFN-γ) and interleukin-4 (IL-4) were measured as markers for the induction of Th1 and Th2 cells, respectively. Serum IFN-γ level was markedly increased in mice inoculated with bitter gourd (*Momordica charantia*) juice, but IL-4 levels were not increased with any of the 34 vegetable juices. Testing of the various components of bitter gourd, including peel, pulp, and seed, showed that the pulp induced the highest levels of IFN-γ. Trial immunogen including the heat extract of the pulp induced specific IgG_{2a} antibody of the mice serum inoculated with this immunogen. These results demonstrate that bitter gourd pulp induced IFN-γ production and show its promise as a means of effective immunostimulatory therapy specific for Th1 cells and IFN-γ production.

KEY WORDS: bitter gourd, interferon-gamma, Th1.

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Immune function in mammals is a complex and important homeostasis mechanism involving a variety of white blood cells, such as B cells, T cells, natural killer (NK) cells, and phagocytes (macrophages and neutrophils). Recent studies have shown the importance of the balance of T helper 1 (Th1) and T helper 2 (Th2) cells in the host immune response to infection [11, 14, 15]. Normal mice usually have low susceptibility to *Neospora caninum*, but the interferon-gamma (IFN-γ) knockout mouse and the anti-IFN-γ antibody treated mouse are highly susceptible to infection with *N. caninum* [4, 7]. This protozoa is an intracellular parasite, so in addition to humoral immunity mediated by antibodies and complement, activation of Th1 cellular immunity, primarily involving macrophages and neutrophils, is also important [1]. To prevent these types of intracellular parasitic infections, vaccines using parasite antigens or recombinant parasite antigens have been developed. However, these vaccines with only antigens are often insufficiently effective, so supplemental immune therapy with substances capable of inducing Th1 cellular immunity is necessary [5].

Plant derived substances are often recognized as highly "foreign" by the host immune system. In other words, plant derived substances, by virtue of local accumulation and increase in immune cells, can have immunostimulatory effects [10]. In addition to parasite derived antigens in vaccines to control intracellular parasites, the use of immunostimulatory substances to control Th1 or Th2 cellular responses can dramatically increase the effectiveness of these vaccines.

Therefore, we have focused on finding plant substances, particularly those of vegetables, capable of non-specifically inducing Th1 and Th2 cells.

Experimental animals, 5-week-old female ddY mice, were purchased from Saitama Experimental Animal Supply Co. (Saitama, Japan).

For the preparation of *Propionibacterium acnes* suspension, *P. acnes* was incubated in brain heart infusion broth (containing 0.03% L-cysteine and 0.03% Tween 80) (Difco, U.S.A.) by stationary culture at 37°C for 24 hr. After incubation, the bacterial suspension was centrifuged (5,900 g, 20 min) and resuspended in 0.01 M phosphate buffered saline, pH 7.2, (PBS). The *P. acnes* suspension was inactivated by heating for 30 min at 60°C and then stored at 4°C until the use.

For inoculation of the mice with *P. acnes* suspension and blood sample collection, each mouse was inoculated intraperitoneally with 0.5 m*l* of *P. acnes* suspension (1 mg/m*l*). At 0, 2, 4, 6, 8, and 10 days after inoculation, blood was collected from the hearts of 3 animals.

In this study, 34 vegetables were used: eggplant, green pepper, tomato, pumpkin, watermelon, ginger, carrot, Japanese radish, turnip, lotus root, burdock, Irish potato, garlic, onion, green onion, leek, cabbage, komatsuna, Napa cabbage, lettuce, broccoli, cauliflower, Malabar spinach, Angelica, Jew's mallow, parsley, Japanese ginger, water shield, celery, bitter gourd, gumbo, spinach, yam, and taro. Each vegetable was pulped with a mixer, and the stock solutions were prepared by adding 2 m*l* of sterile distilled water to 1 g (final weight) of each vegetable. For some vegetables, higher dilution solutions were also prepared.

The bitter gourd was separated into peel, pulp, and seeds. Each was dried by incubation at 25°C for 24 hr, followed by resuspension in sterile distilled water for injection (1,000 mg/m*l* as × 1).

Three mice were inoculated intraperitoneally with 0.5 m*l* of one type of vegetable juice prepared as above. A control group of mice was similarly inoculated with 0.5 m*l* of PBS. Seven days after inoculation, whole blood was collected from the heart of each animal. Blood counts were measured and the remaining serum was separated and stored at –80°C until use.

Blood samples from mice inoculated with vegetable juice were immediately anticoagulated with heparin. Leukocytes were counted with a fully automated blood cell counter (Celltac alpha; Nihon Kohden Co., Tokyo, Japan).

Cytokines measured in the blood samples from the mice inoculated with *P. acnes* and each vegetable juice included IFN-γ, as a marker of Th1 cells, and interleukin-4 (IL-4), as a marker of Th2 cells. The assays were performed using mouse IFN-γ and mouse IL-4 ELISA kits (Endogen, U.S.A.) according to the manuals provided with the kits.

For the effect of extract of bitter gourd pulp on the immunization in mice, dried bitter gourd pulp was added to distilled water at a concentration of 20 mg/m*l*, and homogenized using an ART-MICCRA D-8 homogenizer at setting C for 5 min. The homogenate was heated at 100°C for 1 hr and centrifuged at 12,000 g for 30 min. The supernatant was filtrated using a 0.45-μm pore size Millex-HV filter (Millipore, U.S.A.) and used as heat extract of bitter gourd pulp (HEBGP).

For the preparation of two immunogens including *N. caninum* tachyzoite surface Nc-p43 recombinant antigen (rNc-p43), the method of Son *et al.* [17] was used as follows: 10 g squalane (Wako Pure Chemical Industries, Ltd., Osaka), 4 g rheodol (HLB 7.1; Kao Co., Ltd., Tokyo), 2 g Glycerol (Wako), 1 m*l* rNc-p43 (4.7 mg/m*l*), and 13 m*l* HEBGP or distilled water for control immunogen. Trial and control immunogens were inoculated 0.2 m*l*/head intramuscularly in 33 mice, respectively. After that blood was collected from 3 mice were measured every week from 0 to 10 weeks post inoculation and IgG_1 and IgG_{2a} antibodies were measured using the enzyme linked immunosorbent assay (ELISA) of Hohdatsu *et al.* [6] using rNc-p43 as antigen and anti-mouse IgG_1 and IgG_{2a} rat monoclonal antibodies (Zymed Laboratories Inc., U.S.A.) as conjugates.

To determine the optimum time for measurement of IFN-γ and IL-4 in the mice inoculated with vegetable juice, a preliminary study was at first performed using *P. acnes*, which is known to induce macrophages [8]. Figure 1 depicts the serum IFN-γ and IL-4 levels 0, 2, 4, 6, 8, and 10 days after inoculation of the mice with *P. acnes*. The IFN-γ levels peaked on the 6th and 8th day and decreased by day 10, whereas the IL-4 levels remained similar on post inoculation day 4 and thereafter. Based on the results of the preliminary test, we measured IFN-γ and IL-4 on day 7 after inoculation of mice with vegetable juice.

Table 1 shows the serum IFN-γ and IL-4 levels and leukocyte counts on day 7 after inoculation of mice with vegetable juice. By the time of scheduled blood sample collection, all the mice that were inoculated with Jew's mallow, bitter gourd, gumbo, spinach, yam, and taro juices at lower dilution ratios than those shown had died. Thus, for these vegetable juices, the mice were inoculated with higher dilution ratios. The mice survived and assays could be performed, at dilution ratios of 4 for Jew's mallow, spinach, yam, and taro; at a dilution ratio of 8 for gumbo; and at a dilution ratio of 16 for bitter gourd.

IFN-γ was below the limits of detection (< 10 *pg*/μ*l*) for

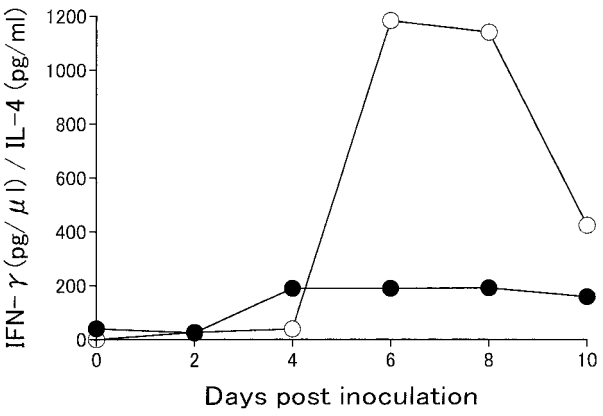

Fig. 1. Serum IFN-γ and IL-4 levels of mice inoculated with *Propionibacterium acnes*. Each mouse was inoculated intraperitoneally with 0.5 m*l* of *P. acnes* suspension (1 mg/m*l*). At 0, 2, 4, 6, 8, and 10 days after inoculation, blood was collected from the hearts of 3 animals each. ○: IFN-γ and ●: IL-4.

many vegetable juices, but in the mice inoculated with bitter gourd (*Momordica charantia*), there was marked induction of IFN-γ (mean, 1,101 *pg*/μ*l*). A slight IFN-γ induction, but not markedly high values, was also seen with carrots (167 *pg*/μ*l*), cauliflower (102 *pg*/μ*l*), and water shield (137 *pg*/μ*l*). However, IL-4 in the serum, as compared to the control group (185 *pg*/m*l*), was not increased or decreased with any of the vegetable juices. Leukocyte counts were higher with several vegetable juices, including green pepper (14,700/μ*l*), water shield (16,100/μ*l*), bitter gourd (20,600/μ*l*), and yam (15,000/μ*l*). No correlation was observed between leukocyte counts, IFN-γ or IL-4.

Bitter gourd induced high titer of serum IFN-γ, so further testing was performed by inoculating mice separately with peel, pulp, and seed juices of bitter gourd. The IFN-γ levels are shown in Table 2. All the mice inoculated with pulp juice at dilution ratios up to 1,280 died within 7 days after inoculation. However, pulp juice at dilution ratios of 2,560 and 5,120 induced higher levels of IFN-γ than peel juice and seed juice at corresponding dilution ratios of 2,560 and 5,120.

Immune responses in mice inoculated with trial and control immunogens are shown in Fig. 2. Serum IgG_1 antibody titers against Nc-p43 of the mice inoculated with both immunogens rose at 2 weeks and peaked at 6–7 weeks post inoculation. The ELISA values of both groups were not significantly different. On the other hand, the serum IgG_{2a} antibody titer did not increase in the mice inoculated with control immunogen. IgG_{2a} antibody in the mice inoculated with trial immunogen began to increase on the 2nd week and maintained a high level of antibodies until 9 weeks post inoculation. The antibodies in the mice with trial immunogen were higher than those with control immunogen.

Pharmacological research has shown that ingestion of a variety of food products can have antitumor effects by virtue of an increase in leukocytes and tumor necrosis factor-alpha

Table 1. IFN-γ and IL-4 levels and WBC counts of mice inoculated with various vegetable juices

Vegetable	Dilution	IFN-γ (*pg*/*μl*)	IL-4 (*p*g/m*l*)	WBC (/*μl*)
Eggplant	× 1	<10	164	3,700
Green pepper	× 1	<10	164	14,700
Tomato	× 1	<10	114	3,800
Pumpkin	× 1	<10	135	2,200
Watermelon	× 1	15	256	10,300
Ginger	× 1	15	214	11,300
Carrot	× 1	167	101	11,300
Japanese radish	× 1	<10	142	7,800
Turnip	× 1	27	126	7,000
Lotus root	× 1	51	173	5,600
Burdock	× 1	<10	74	7,100
Irish potato	× 1	<10	81	8,000
Garlic	× 1	<10	82	10,300
Onion	× 1	<10	60	5,000
Green onion	× 1	<10	125	4,700
Leek	× 1	15	82	3,500
Cabbage	× 1	51	223	7,600
Komatsuna[a)]	× 1	<10	90	6,600
Napa cabbage	× 1	<10	72	8,600
Lettuce	× 1	<10	112	5,600
Broccoli	× 1	15	98	9,400
Cauliflower	× 1	102	95	10,800
Malabar spinach	× 1	15	177	10,200
Angelica	× 1	27	83	12,700
Jew's mallow nalta jute	× 4[b)]	<10	174	9,600
Parsley	× 1	75	159	7,600
Japanese ginger	× 1	75	108	13,400
Water shield	× 1	137	131	16,100
Celery	× 1	<10	95	9,500
Bitter gourd	× 16[b)]	1,101	290	20,600
Gumbo	× 8[b)]	<10	87	10,800
Spinach	× 4[b)]	44	128	5,600
Yam	× 4[b)]	87	124	15,000
Taro	× 4[b)]	87	189	9,800
Control		<10	185	3,500

a) Komatsuna (*Brassica campestris*).
b) Mice did not die at dilution ratios of 4 for Jew's mallow, spinach, yam; at a dilution ratio of 8 for gumbo; and at a dilution ratio of 16 for bitter gourd.

Table 2. Induction of mouse IFN-γ by peel, pulp, and seed juices of bitter gourd

Part of Bitter gourd	Dilution[a)]	IFN-γ (pg/*μl*)
Peel	× 1,280	780
	× 2,560	590
	× 5,120	400
Pulp	× 1,280	d[b)]
	× 2,560	1,520
	× 5,120	1,640
Seed	× 1,280	500
	× 2,560	560
	× 5,120	340

a) Dilution 1: 1,000 mg/m*l*.
b) Mice died within 7 days post inoculation.

(TNF-α) [19, 21]. Vegetables that can markedly enhance macrophage production of TNF are cabbage, eggplant, Japanese radish, spinach, cucumbers, carrots, and onions [21]. This means that some of these vegetable extracts can be used to stimulate host immune responses [20]. In the present study, mice were inoculated intraperitoneally with 34 different types of vegetable juice, and IFN-γ and IL-4 were measured as markers for induction of Th1 cells and Th2 cells, respectively. Serum IL-4 levels, as compared with the control group, were not markedly increased by any of the 34 vegetable juices. However, serum IFN-γ levels were higher than the control group after inoculation with carrot, cauliflower, water shield, and bitter gourd juices. In particular, with bitter gourd (*M. charantia*), even at a dilution ratio of 16, IFN-γ was high at 1,101 *pg*/*μl*, showing very high IFN-γ induction. Testing of the separate components of bitter gourd, peel, pulp, and seeds, showed that the pulp induced the highest levels of IFN-γ. Bitter gourd contains momordicin, a glucoside-like substance that can lower blood glucose [9]. Steroids extracted from charantin, a stigmasterol, are protein alkaloid complexes with insulin-like effects [12, 13]. In addition, bitter gourd seeds have been shown to be effective in treatment of cancer [16]. Our results demonstrate that bitter gourd, especially its pulp, induced a high titer of IFN-γ in mice, and shows promise as an immunostimulatory therapy specific to Th1 cells and IFN-γ production [10].

Studies using the intracellular parasite *Toxoplasma gondii* have shown that administration of *T. gondii* immune serum alone is insufficient to prevent infection [18]. In addition, in *Leishmania* parasitic infections, the immune balance is skewed toward a Th2 response, with overproduction of IL-4 and inhibition of IFN-γ induced macrophage activation. The *Leishmania* parasites phagocytized by macrophages are not digested, thus worsening infection [2, 3]. This highlights the importance of Th1 cellular immunity in preventing intracellular parasitic infections. As demonstrated in our study, bitter gourd, especially its pulp juice, induced high levels of serum IFN-γ which activates Th1 cellular immunity, which is essential for preventing intracellular parasitic infection.

The trial immunogen using HEBGP as immunopotentiator induced a high IgG_{2a} antibody in mice inoculated with it, and bitter gourd induced Th1 cellular immunity in mice inoculated with the trial immunogen. As heat extract of bitter gourd pulp produced these results, we consider the constituents of the heat extract are mainly carbohydrates.

The vegetables tested in our study are often eaten in a daily meals, and safety is generally not a concern with oral ingestion. However, mice died that were inoculated with Jew's mallow, bitter gourd, gumbo, spinach, yam, and taro juices. This requires further investigation of the active ingredients and characteristics of these vegetable juices. Deaths in mice inoculated with whole or pulp juices of bitter gourd occurred within 3 to 5 days after inoculation, thus raising the possibility of infection due to the immunocompromised condition induced by IFN-γ overproduction, but it

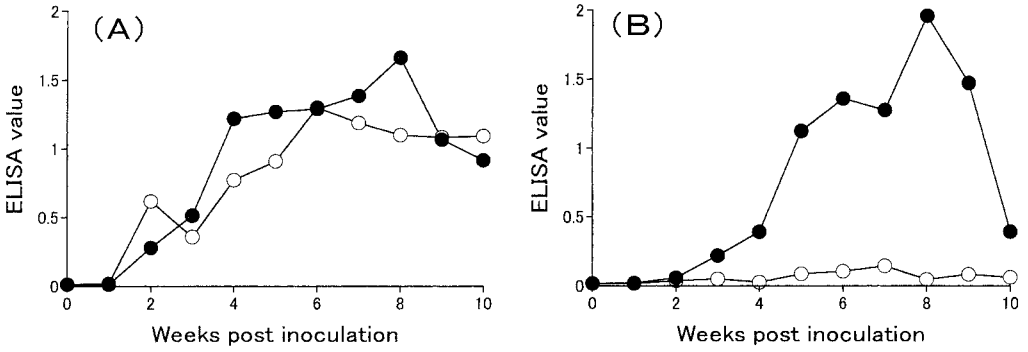

Fig. 2. Immune response against rNc-p43 in mice inoculated with trial and control immunogens, with and without the heat extract of bitter gourd pulp, respectively. Antibody titers were measured by ELISA using anti-mouse IgG_1 and IgG_{2a} rat monoclonal antibodies. Each titer was the average value from 3 mice. (A) Mouse IgG_1 antibody response. (B) Mouse IgG_{2a} antibody response. ● : Trial and ○: Control immunogen groups.

is not clear. Further studies should be conducted to characterize the active ingredients of bitter gourd pulp that induce high IFN-γ levels and to evaluate the ability of IFN-γ induction as adjuvant therapy.

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