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Immunomodulatory activities of edible beans and related constituents from soybean

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Abstract

To explore the health-modulating constituents of common edible beans, their immunomodulatory activity on human peripheral blood mononuclear cells (PBMC) was evaluated. Studies were conducted on lymphocyte transformation by BrdU immunoassay, secretion of interferon-gamma (IFN-γ) and interleukin 10 (IL-10) and elucidation of the responding cells by flow cytometry. The results at 20 μg/mL showed that genistein, phytic acid and syringic acid induced a Th1-predominant immune response because they significantly suppressed the secretion of IL-10 and augmented the IFN-γ production. The present study concludes that several non-nutritional ingredients of soybean such as flavonoids, plant acids and plant hormones most likely to be important in modulation of human immunity.
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Keywords: Immunomodulation; Interferon-gamma (IFN-γ); Interleukin 10 (IL-10); Leguminosae; Flavonoids

1. Introduction

Mungbean (*Phaseolus radiatus* L. var. *typicus* Prain), rice bean (*Phaseolus calcaratus* Roxb.), soybean (*Glycine max* Merr.), small bean (*Phaseolus radiatus* L. var. *aurea* Prain) and wild soybean (*Glycine soja* sieb & zucc.) are common edible beans of Leguminosae. They contribute many phytonutrients including immunomodulatory nutrients such as vitamins (A, B2, C, and E) and minerals (copper, zinc, iron, selenium) (Bhaskaram, 2002; Chandra, 1991). Soybean also contains many bioactive phytochemicals including flavonoids (genistein, quercitrin) and organic acids (indole-3-acetic acid, phytic acid, syringic acid) (Duke, 1992). Despite their food and traditional nutritive uses, these beans have also been valued as folkloric herbal remedies for the treatment of edema, beri-beri, jaundice, pyodermas, enteritis, diarrheas, sore eyes, night blindness, night sweating, erysipelas, dizziness, headache, constipation, lumbago, blurred vision, rheumatic pains, food or drug poisioning and infantile malabsorption and malnutrition (Hsiao, 1990; Huang, 1994).

Soybean is a widely eaten high nutritive food in many cultures of the world. It possesses many traditional phytonutrients and several bioactive phytochemicals including flavonoids and saponins, which have a variety of potential health benefits, such as anti-inflammatory (Yamaki et al., 2002), anti-oxidative (Cos et al., 1998), anti-mutagenic (Sangwan, Shanker, Sangwan, & Kumar, 1998), anti-carcinogenic (Dixon & Ferreira, 2002; Ren & Lien, 1997; Wattenberg, 1983; Zhou et al., 1998), anti-arterial contraction (Ho, Chen, Huang, & Chen, 2002), anti-diarrhea (Galvez et al., 1995), antiviral (Hayashi, Hayashi, Hiraoka, & Ikeshiro, 1997) and hepatoprotective effects (Kinjo et al., 1999). Soybean constituents with estrogenic activity, including isoflavones (daidzein, biochanin A, genistein, fomononetin), flavonoids (quercetin, kaempferol), and phytosterols (campesterol, coumesterol), may additionally play a role

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in cancer prevention, moderation of menopausal symptoms and other health effects (Duke, 1992; Su, Chow, Kung, Hung, & Chang, 2003; Tham, Gardner, & Haskell, 1998; Zhou et al., 1998). Several phytochemicals were recently reported to possess immunomodulatory properties, including phenolic compounds such as caffeic, chlorogenic, ferulic and *p*-coumaric acids, which have been reported as common organic acids in the soybean (Chiang, Ng, Chiang, Chang, & Lin, 2003a; Duke, 1992). To further explore the potentially health-modulating role of the most popular foods of Leguminosae, the immunomodulatory activities of the crude extracts and several common constituents of pure compounds from the soybean on human PBMC were evaluated.

2. Materials and methods

2.1. Crude extracts and chemicals

Dry seeds were obtained from Known-You Seed Co., Ltd. (Kaohsiung, Taiwan) and were authenticated by Dr. Shing-Ginn Lee, Chief of the Taitung agricultural improvement station, Taitung, Taiwan. The dry seeds (500 g) were boiled in 1000 mL of distilled water for 1 h and filtered by gauze. The aqueous extract was concentrated in vacco, and then lyophilized. The aqueous extract was collected and stored at 4 °C until use. Heparin, Ficoll–Hypaque, dimethyl sulfoxide (DMSO), phytohemagglutinin (PHA-L), concanavalin A (Con A), genistein, indole-3-acetic acid, phytic acid, quercitrin and syringic acid were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Each was more than 98% pure, excluding the crude extracts. 5-Bromo-2′-deoxyuridine (BrdU) labeling and detection kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). The human IFN-γ and IL-10 immunoassay kits were purchased from R&D System Inc. (Minneapolis, MN, USA).

2.2. Lymphocyte transformation

Peripheral blood from four healthy volunteers (20–35 years old) was collected in a sterile syringe containing sufficient heparin to give a final concentration of 100 units/mL. Mononuclear cells were obtained by centrifuging the whole blood and a normal saline (1/1, v/v) mixture on Ficoll–Hypaque (2.4:1, v/v) gradients as described by the manufacture's protocol (Sigma–Aldrich). After centrifugation, 0.05 mL (5.0×1.0^5) unfractionated PBMC was placed in a 5 mL test tube containing 0.15 mL fetal calf serum and 0.75 mL of RPMI 1640, with 0.05 mL of test samples, mitogen (positive control), medium (negative control) or additional medium with dimethyl sulfoxide (DMSO: solvent control). After gentle mixing, 200 μL/well was added into the wells of a 96-well microculture plate in triplicate. The culture plate was then allowed to incubate for three days at 37 °C in a 5% CO_2 incubator. BrdU immunoassay was completed as described by Chiang et al. (2003a). The optical densities were determined with a ELISA reader at a test wavelength of 450 nm. The stimulation index (SI) was determined by the ratio of optical density of test substance to the optical density of negative control.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The solid-phase sandwich ELISA procedure was performed according to the standard protocol of IFN-γ or IL-10 ELISA kit. The cultivation and treatment of peripheral blood mononuclear cells was completed as previously described (Chiang et al., 2003a). After three days, particulates were removed from the supernatants by centrifugation and the samples were stored at −70 °C until use. The sample diluent (100 μL) was added to each well and then 100 μL/well of IFN-γ or IL-10 standard or supernatant sample was added, covered with a new adhesive strip, and incubated for 2 h at room temperature. The washing process was repeated four times. The IFN-γ or IL-10 conjugate (200 μL/well) was added, covered with a new adhesive strip, and incubated for 2 h at room temperature. The washing process was repeated four times. The substrate solution (200 μL/well) was added and incubated for 30 min at room temperature. Finally, the stop solution (50 μL/well) was added and the optical density of each well determined within 30 min, using a ELISA reader at a wavelength of 450 nm and a reference wavelength of 540 nm.

2.4. Flow cytometry analysis

All fluoroscein isothiocyanate (FITC-) and phycoerythrin (PE-) conjugated monoclonal antibodies (mAbs) were purchased from Becton Dickison (San Jose, CA, USA). Optimal concentrations of mAbs were determined for each mAb by titration. The isolated PBMC were cultured in triplicate with test drugs (20 μg/mL), PHA (5 μg/mL, positive control), DMSO (0.1%, solvent control) or medium (negative control) for three days in 37 °C of a 5% CO_2 incubator. Then the numbers of NK cells ($CD3^-$, $CD16^+$, $CD56^+$), activated PBMC ($CD25^+$), T cell subsets ($CD3^+$, $CD4^+$, $CD8^+$), total T cells ($CD3^+$), total B cells ($CD3^-$, HLA-DR^+) and active T cells ($CD3^+$, HLA-DR^+) at zero or three days were determined by standard FACScan procedures with mAbs according to the manufacture's protocol. The stained PBMC were analyzed by a flow cytometric analyzer (FACSCalibur, Becton Dickinson, Cookeysville, MD) with Cell Quest software (Becton Dickinson).

2.5. Statistical analysis

Results were expressed as mean ± standard error. The 2-tails of Student's *t* test (two groups) or one-way ANOVA and multiple comparison of Dunnett's *t* test (>2 groups) were used to evaluate the difference between the control and test samples by the SPSS Base 8.0 software for

Windows. The P value of less than 0.05 was considered to be significant difference in all experiments.

3. Results and discussion

3.1. Immunomodulatory activity evaluated by lymphocyte activation and IFN-γ secretion

The results from the aqueous extracts of the five edible beans showed that the soybean's extracts significantly stimulated the proliferation of human PBMC and the secretion of IFN-γ, whereas extracts from the mungbean, rice bean and small bean only significantly augmented the proliferation of human PBMC (Table 1). To further explore the immunostimulating phytochemicals, we examined several known bioactive compounds such as flavonoids (genistein, quercitrin), plant acids (phytic acid, syringic acid) and plant hormone (indole-3-acetic acid), which have been reported to be present in the soybean (Duke, 1992). IFN-γ secretion, well-known to be associated with lymphocyte activation after stimulation by antigens or mitogens, polyclonal mitogen (PHA, Con A), genistein (2 μg/mL) and phytic acid (2 μg/mL), showed this classical response. However, genistein at 20 μg/mL exhibited a different pattern of immunomodulation, significantly augmenting lymphocyte activation but suppressing IFN-γ secretion. Quercitrin, syringic acid and indole-3-acetic acid were also found to be potent activators for the proliferation of PBMC, but not for elevation of IFN-γ secretion. In addition, phytic acid at 20 μg/mL expressed another response, significantly promoting the secretion of IFN-γ but not modulating the proliferation of PBMC (Table 2).

3.2. Immunomodulatory activity elucidated by flow cytometry analysis

After culturing for three days, some cell fractions were significantly changed, a decrease of total B cells at medium control but an increase of active T cells at solvent control (Table 3). There were four types of immunomodulation after treatment with phytochemicals including type 1 (enhanced lymphocyte activation and secretion of IFN-γ), type 2 (elevated lymphocyte activation and suppressed IFN-γ secretion), type 3 (augmented lymphocyte activation) and type 4 (increased secretion of IFN-γ). The immunomodulatory effects of the type 1 response by adding positive control (PHA) exhibited significant elevation of $CD8^+$ T cells and activated PBMC (Table 4). The type 2 immunomodulating response of genistein (20 μg/mL) showed that it significantly increased $CD4^+$ T cells and decreased active T cells (Table 5). The type 3 immunomodulatory effect of syringic acid exhibited similar changes of immune cells as genistein at 20 μg/mL and also a significant decline of the active T cells (Table 6). Finally, the type 4 immunomodulating response by phytic acid showed that it significantly decreased the total B and active T cells, whereas it did not significantly elevate the $CD4^+$ T cells (Table 7).

3.3. Responding subtype of $CD4^+$ T cells

To further define the responding subtype of $CD4^+$ T cells after stimulation by soybean's constituents, we determined the unique cytokine of different subtypes, such as IFN-γ for Th1 cells and IL-10 for Th2 cells. Results showed that at 20 μg/mL, genistein, phytic acid and syringic acid significantly decreased the secretion of IL-10, whereas IFN-γ production was moderately augmented by phytic acid but slightly suppressed by genistein (Table 8).

Since ancient times several species of Leguminosae such as the mungbean, rice bean, small bean, soybean and wild soybean have been used as common foods in many different cultures of the world. They provide many dietary phytonutrients including immunomodulatory nutrients of vitamins and minerals and also contain a variety of phytochemicals of unknown function (Bhaskaram, 2002; Chandra, 1991; Duke, 1992). Recently, our studies have demonstrated that wild vegetables of Plantaginaceae possess antiviral, antitumor and immunomodulatory properties and many ingredients such as chlorogenic acid,

Table 1
The immunomodulatory activity of aqueous extracts from common edible beans of Leguminosae

Drug		Stimulation index at dose (μg/mL)[b]			γ-Interferon (pg/mL)[c]
		50	100	200	
Medium[a]	1.00 ± 0.02				11.7 ± 1.12
Con A[a]	5.98 ± 0.58*				523 ± 47*
PHA[a]	5.56 ± 0.38*				541 ± 36*
Glycine max		1.65 ± 0.17*	1.42 ± 0.20*	1.13 ± 0.08	43.5 ± 7.20*
Glycine soja		1.18 ± 0.16	1.12 ± 0.08	1.24 ± 0.28	11.5 ± 1.12
Phaseolus calcaratus		1.24 ± 0.13	1.25 ± 0.19	1.38 ± 0.22*	11.3 ± 1.42
Phaseolus radiatus L. *var. aurea*		1.24 ± 0.38	1.40 ± 0.21*	1.41 ± 0.23*	10.5 ± 1.74
Phaseolus radiatus L. *var. typicus*		1.29 ± 0.17	1.46 ± 0.25*	1.54 ± 0.27*	12.0 ± 1.76

[a] Negative control (medium), positive control (Con A : 10 μg/mL, PHA : 5 μg/mL).
[b] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. One-way ANOVA and multiple comparisons of Dunnett's t test were used to evaluate the difference between test drug and medium control (*$P < 0.05$).
[c] The data were the response of the maxium stimulation index group of each drug.

Table 2
The immunomodulatory activity of flavonoids and plant acids from soybean

Drug	Dose (μg/mL)	Stimulation index[a]	γ-Interferon (pg/mL)
DMSO	0.10%	1.00 ± 0.02	11.8 ± 1.14
Con A	10	5.98 ± 0.58*	523 ± 47*
PHA	5	5.56 ± 0.38*	541 ± 36*
Genistein	2	1.38 ± 0.25*	13.6 ± 1.53*
	20	1.32 ± 0.22*	6.00 ± 0.54*
Quercitrin	2	1.21 ± 0.12	12.0 ± 1.28
	20	1.29 ± 0.08*	11.4 ± 1.21
Phytic acid	2	1.26 ± 0.12*	14.2 ± 1.56*
	20	0.97 ± 0.08	14.7 ± 1.37*
Syringic acid	2	1.32 ± 0.08*	10.0 ± 1.03
	20	1.32 ± 0.05*	10.0 ± 1.12
Indole-3-acetic acid	2	1.32 ± 0.21*	11.7 ± 1.21
	20	1.15 ± 0.12	13.0 ± 1.26

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. One-way ANOVA and multiple comparisons of Dunnett's t test were used to evaluate the difference between test drug and DMSO control (*$P < 0.05$).

ferulic acid, caffeic acid and *p*-coumaric acid elevated the activity of human PBMC proliferation and secretion of IFN-γ (Chiang et al., 2003a, Chiang, Chiang, Chang, & Lin, 2003b). The above mentioned bioactive phytochemicals have also been reported to be present in the soybean (Duke, 1992). Additionally, the soybean contains several bioactive constituents including flavonoids, saponins and phenolic compounds, which are reported to have anti-inflammation (Yamaki et al., 2002), anti-oxidation (Cos et al., 1998), anti-carcinogenesis (Dixon & Ferreira, 2002; Ren & Lien, 1997; Wattenberg, 1983; Zhou et al., 1998; Su et al., 2003), estrogenic (Tham et al., 1998) and anti-infection effects (Hayashi et al., 1997; Nakashima et al., 1989).

An unsuitable immunity has been well-known to have a central role in a variety of ailments including infections,

Table 3
The cell fractions of controls

Cells	Day 0	Day 3[a]	
		Medium	DMSO (0.1%)
Total T	0.597 ± 0.066	0.595 ± 0.053	0.609 ± 0.065
Total B	0.229 ± 0.021	0.175 ± 0.012*	0.216 ± 0.042
Active T	0.128 ± 0.029	0.139 ± 0.015	0.271 ± 0.029*
$CD4^+$ T	0.341 ± 0.036	0.391 ± 0.026	0.307 ± 0.066
$CD8^+$ T	0.404 ± 0.042	0.379 ± 0.021	0.421 ± 0.041
NK cell	0.296 ± 0.067	0.318 ± 0.055	0.336 ± 0.063
Activate PBMC	0.060 ± 0.008	0.092 ± 0.010	0.075 ± 0.013

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The controls were determined the difference between day 0 and day 3 by ANOVA and multiple comparisons (*$P < 0.05$).

Table 4
The cell fractions in type 1 immuno modulatory activity of control drugs

Cells	DMSO (0.1%)	PHA (5 μg/mL)[a]
Total T	0.609 ± 0.065	0.632 ± 0.056
Total B	0.216 ± 0.042	0.173 ± 0.032
Active T	0.271 ± 0.029	0.262 ± 0.016
$CD4^+$ T	0.307 ± 0.066	0.273 ± 0.029
$CD8^+$ T	0.421 ± 0.041	0.513 ± 0.053*
NK cell	0.336 ± 0.063	0.323 ± 0.045
Activated PBMC	0.075 ± 0.013	0.344 ± 0.003*

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The difference between the positive control (PHA) and DMSO was determined by Student's t test (*$P < 0.05$).

Table 5
The cell fractions in type 2 immunomodulatory activity of genistein

Cells	DMSO (0.1%)	Genistein (20 μg/mL)[a]
Total T	0.609 ± 0.065	0.618 ± 0.087
Total B	0.216 ± 0.042	0.175 ± 0.039
Active T	0.271 ± 0.029	0.176 ± 0.023*
$CD4^+$ T	0.307 ± 0.066	0.375 ± 0.036*
$CD8^+$ T	0.421 ± 0.041	0.415 ± 0.031
NK cell	0.336 ± 0.063	0.319 ± 0.074
Activated PBMC	0.075 ± 0.013	0.056 ± 0.008*

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The difference between genistein and DMSO was determined by Student's t test (*$P < 0.05$).

Table 6
The cell fraction in type 3 immunomodulatory activity of syringic acid

Cells	DMSO (0.1%)	Syringic acid (20 μg/mL)[a]
Total T	0.609 ± 0.065	0.623 ± 0.064
Total B	0.216 ± 0.042	0.152 ± 0.019*
Active T	0.271 ± 0.029	0.196 ± 0.005*
$CD4^+$ T	0.307 ± 0.066	0.389 ± 0.012*
$CD8^+$ T	0.421 ± 0.041	0.394 ± 0.032
NK cell	0.336 ± 0.063	0.317 ± 0.052
Activated PBMC	0.075 ± 0.013	0.058 ± 0.004

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The difference between syringic acid and DMSO was determined by Student's t test (*$P < 0.05$).

Table 7
The cell fractions in type 4 immunomodulatory activity of phytic acid

Cells	DMSO (0.1%)	Phytic acid (20 μg/mL)[a]
Total T	0.609 ± 0.065	0.622 ± 0.061
Total B	0.216 ± 0.042	0.156 ± 0.015*
Active T	0.271 ± 0.029	0.202 ± 0.008*
$CD4^+$ T	0.307 ± 0.066	0.365 ± 0.034
$CD8^+$ T	0.421 ± 0.041	0.407 ± 0.027
NK cell	0.336 ± 0.063	0.314 ± 0.054
Activated PBMC	0.075 ± 0.013	0.050 ± 0.003

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The difference between phytic acid and DMSO was determined by Student's t test (*$P < 0.05$).

Table 8
Phytoconstituents modulated the secretion of IL-10 and IFN-γ

	Dose (μg/mL)	IL-10 (pg/mL)	IFN-γ (pg/mL)
Medium		1.00 ± 0.12[a]	1.00 ± 0.09[b]
DMSO	0.1%	1.15 ± 0.03[c]	1.37 ± 0.13
PHA	5	1.15 ± 0.13	62.9 ± 4.19*
Genistein	20	0.08 ± 0.03*	0.69 ± 0.06
Phytic acid	20	0.46 ± 0.13*	1.71 ± 0.15
Syringic acid	20	0.43 ± 0.06*	1.16 ± 0.13

[a] 141 ± 16.9 pg/mL.
[b] 8.6 ± 0.12 pg/mL.
[c] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate of four volunteers. The difference between medium control and drugs was determined by ANOVA and multiple comparisons (*$P < 0.05$).

cancer, allergy, aging and many disorders of various organs (Peakman & Vergani, 1997). Although the above edible beans provide phytonutrients, the exact roles in promoting human health from non-nutritional ingredients by immunomodulation have yet to be fully studied. We therefore evaluated the immunomodulatory activities of the crude extracts and five constituents from soybean on human PBMC by using two common immunoassay methods including lymphocyte transformation and secretion of IFN-γ (Chiang et al., 2003a, 2003b). The results from the crude extracts of the five edible beans from Leguminosae showed that four of the extracts significantly stimulated the proliferation of human PBMC and/or the secretion of IFN-γ. The immunostimulating activity may probably be due to the phenolic compounds such as caffeic, chlorogenic, ferulic and *p*-coumaric acids, which have been shown to possess this effect and to be present in the soybean (Chiang et al., 2003a; Duke, 1992). However, this study showed that flavonoids (genistein, quercitrin), plant acids (phytic acid, syringic acid) and plant hormone (indole-3-acetic acid) may also contribute to the immuno-enhancing activity of lymphocyte activation and/or IFN-γ secretion. Therefore, dietary phytonutrient of some vitamins and minerals are presumed to be important in modulation of human immunity, and other food constituents, such as flavonoids, plant acids and plant hormones of non-nutrients, most likely provide immunomodulation as well.

To determine which types of lymphocytes directed the patterns of immunomodulation by flow cytometry, the results showed that polyclonal mitogen PHA increased $CD8^+$ T cells, whereas constituents of the soybean elevated $CD4^+$ T cells. Thus enhanced secretion of IFN-γ might be due to the $CD8^+$ T cells because they were significantly elevated in the PHA but not in the soybean's ingredients. Despite the different species tested, the results that $CD8^+$ T cells were the main cell type to secrete IFN-γ were in accordance with the observation made by Sad, Marcotte, and Mosmann (1995). To elucidate which type of $CD4^+$ T cells regulated the patterns of immunomodulation, the results indicated that immunostimulation by soybean's constituents might be due to Th1 cells, owing to the fact they slightly elevated IFN-γ but significantly inhibited IL-10 secretion. In this study, we proposed another way the edible beans and soybean's ingredients acted to affect health as immunomodulating agents, that is, directly enhancing of lymphocyte activation and/or the secretion of multipotent cytokine IFN-γ.

4. Conclusions

The aim of the present study was to evaluate the immunostimulating activity of five common edible beans and their related ingredients. Studies were conducted on lymphocyte transformation by BrdU immunoassay, secretion of IFN-γ and IL-10 using an ELISA assay and elucidation of the responding cells by flow cytometry. Among the five common edible beans tested, the soybean's extracts significantly stimulated the proliferation of human PBMC and the secretion of IFN-γ. At non-cytotoxic concentrations, soybean constituents including flavonoids, plant acids and plant hormone exhibited four types of immunostimulation, which induced the proliferation of $CD8^+$ T cells in a type 1 response, but elicited the proliferation of $CD4^+$ T cells in another type of immunostimulation. The results at 20 μg/mL showed that genistein, phytic acid and syrigic acid enhanced a Th1-predominant immune response because they augmented the IFN-γ (Th1 cytokine) production and significantly suppressed the secretion of immunosuppressive IL-10 (Th2 cytokine). The present study concludes that dietary phytonutrient of some vitamins and minerals of common edible beans are presumed to be important in stimulation of human immunity and that other edible bean's constituents such as flavonoids, plant acids and plant hormones of non-nutrients most likely provide immunostimulation as well.

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