Original

Effect of GM and Non-GM Soybeans on the Immune System of BN Rats and B10A Mice

(Received January 13, 2000)

Reiko TESHIMA, Hiroshi AKIYAMA, Haruyo OKUNUKI, Jun-ichiro SAKUSHIMA,
Yukihiro GODA, Hiroshi ONODERA, Jun-ichi SAWADA and Masatake TOYODA

(National Institute of Health Sciences: 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan)

Subchronic animal feeding studies to examine the effect of glyphosate-tolerant soybeans, which contain the bacterial 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, on the immune system were conducted with BN rats and B10A mice. The studies were designed to compare the feeding value of a line of genetically modified glyphosate-tolerant soybeans (GM soybeans) to that of closely-related and one-parent same cultivar (non-GM soybeans). Heat-treated soybean meal was incorporated into the diets of the rats and mice at a concentration of 30%. The study duration was 15 weeks. Growth, food intake and weights of the liver and the spleen were compared between animals fed the non-GM and GM lines. The histopathology of the thymus, liver, spleen, mesenteric lymph node, Peyer's patches, and small intestine, and the production of soybean-specific IgE and IgG antibodies in the sera were also compared. Growth, feeding value, and the histopathology of immune-related organs showed no significant differences between animals fed GM and non-GM lines. The production of soybean-specific IgE was not detected in the sera of either group, and the increase in soybean-specific IgG was identical in the GM and non-GM groups. No immunotoxic activity was found in GM-soybean-fed rats or mice.

Key words: GM soybeans; CP4-EPSPS; BN rat; B10A mouse; immune system

Introduction

Genetically modified (GM) soybeans contain an introduced bacterial gene (5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 [CP4 EPSPS]) that confers tolerance to the herbicide glyphosate¹⁾⁻³⁾. This gene produces a glyphosate-insensitive homologue of the natural plant enzyme EPSPS, which is involved in aromatic amino-acid biosynthesis. An acute toxicity study in mice showed that the introduced EPSPS protein was non-toxic⁴⁾. The possibility that unintended changes had taken place was evaluated by comparing compositional data and nutritional studies for GM and conventional soybeans¹⁾. The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle was not altered by the genetic incorporation of glyphosate tolerance⁵⁾. The expression of unknown toxins, antinutrients or allergens causing unrecognized adverse effect in GM foods is unlikely. However, for a further proper risk estimation of new foods, it is important to characterize the transgenic progeny and to conduct a subchronic toxicity study in animals. There is no report about the equivalence of the composition of the transgenic progeny and non-GM cultivar, or on the effect of these cultivars on the immune system of animals, especially with regard to mucosal immunity and allergy. In our study, we used a commercially available

line of genetically modified, glyphosate-tolerant soybeans (GM soybeans), which are representative of transgenic progeny, and a closely-related and one-parent same cultivar (non-GM soybeans) as a control. The Brown Norway (BN) rat has been shown to have a high response to IgE and thus resembles atopic humans in terms of the propensity to develop allergic reactions⁶⁾. In addition, the BN rat has a similar profile of allergenicity and epitopes of ovalbumin to that of man and can be sensitised via the oral route^{7), 8)}. B10A mice have also been reported to be sensitive to the oral intake of ovalbumin and other antigens⁹⁾.

In the present study, the effects of GM and non-GM soybean feeding on mucosal immunity were examined in BN rats and B10A mice to determine the effects of genetic modifications on the allergenicity of food crops.

Materials and Methods

(1) Diet composition

A genetically modified, glyphosate-tolerant soybean (GM-soybean) line and a closely-related and one-parent same cultivar (non-GM soybean) line, produced in Ohio, U.S.A. and purchased from a Japanese trading company, were utilized in all animal feeding studies. The soybeans were heated at 100°C for 30 min in an autoclave, and feed containing 30% soybean meal was prepared by Oriental Yeast Corporation (Tokyo, Japan).

Table 1. Composition of Diets

Ingredients	g/100 g dry matter	
Soybean	30.0	
Corn oil	7.0	
Casein	8.0	
Cellulose	5.0	
Minerals*	3.5	
Vitamins**	1.0	
Choline	0.25	
TBHQ	0.0014	
Corn starch	33.2486	
Sucrose	10.0	
CaCO ₃	0.9	
CaHPO ₄	1.1	
Analyzed	Ca	0.67
	Mg	0.064
	P	0.42
	protein	17–18

* Tracer mineral premix provided the following (mg/kg diet): manganese, 10; zinc, 38; iron, 45; copper, 6; selenium, 0.18; iodine, 0.2.

** Vitamin premix (AIN-93VX) provided the following (mg/kg diet): thiamin mononitrate, 6.0; riboflavin, 6.0; calcium *D*-pantothenate, 16.0; pyridoxine, 7.0; biotin, 0.2; vitamin B₁₂, 0.025; nicotinic acid 30; folic acid, 2.0; all-*rac*- α -tocopherol, 15; retinol acetate, 8; vitamin D₃, 2.5.

The composition of the diets is shown in Table 1. The composition of the AIN-93M diet¹⁰⁾ was partially modified, and sucrose was used as the carbohydrate source in each diet. Calcium, magnesium, and phosphorus contents were adjusted¹¹⁾. Soybean compositional assays were performed at the Oriental Yeast Corporation and the Japanese Food Analytical Center (Tokyo, Japan). For the control diet group, CRF-1 diet (Oriental Yeast Corporation) was used.

(2) Experimental animals

Female BN rats and female B10A mice (purchased from Shizuoka Laboratory Animal Corporation, Shizuoka, Japan, at 6 weeks of age) were acclimated for one week on a CRF-1 diet after delivery from the breeding house and then subdivided and randomized into 3 groups with comparable body weights. These 3 groups (5 animals/group) consisted of the control diet group (CRF-1), the 30% GM-soybean diet group, and the 30% non-GM-soybean diet group. The 3 groups were then fed their respective diets for 15 weeks. Sera of each animal was obtained from blood at 10 and 15 weeks. At the end of the study, all test animals were anesthetized with ether and necropsied. Liver and spleen were weighed, and immune-related organs were collected from each animal and stored in formalin.

(3) Histopathological examination

Immune-related organs obtained from each animal and fixed in neutralized formalin as described above, were embedded in paraffin, processed, and stained with hematoxylin and eosin (HE). HE-stained thin sections

were examined under a light microscope. Histopathological analyses of thymus, liver, spleen, mesenteric lymph node, and Peyer's patches were done to evaluate the composition and arrangement of the cells. To assess immunotoxicity to the gut in terms of mucosal immune response, the structure of the crypt and the composition of cells (especially goblet cells and intraepithelial lymphocytes) of the mucosa of the small intestine were observed¹²⁾.

(4) ELISA

The mouse serum titer of soybean-specific IgE was determined using the method described by Teshima *et al.*¹³⁾, with some modifications. Fifty microliter of GM-soybean extract (50 μ g/mL) dissolved in a 50 mmol/L sodium carbonate buffer (pH 9.6) was added to each well of a 96-well microtiter plate, which was incubated overnight at 4°C. The solution was then discarded, and each well was washed 4 times with 200 μ L of PBS containing 0.05% Tween 20 (PBS/Tween). To minimize nonspecific binding of the serum proteins to unoccupied solid-phase sites, 200 μ L of 0.1% casein in PBS was added, and incubated for 1 hour at room temperature. After washing, 50 μ L of diluted serum was added to each well and the plates were incubated for 20 hours at 4°C. The solution was then removed, and the wells were washed. Fifty microliters of rabbit anti-mouse IgE, anti-mouse IgG1, anti-rat IgE or anti-rat IgG (10⁻³ dilution in PBS containing 0.1% casein [Nordic Immunology, Tilburg, the Netherlands]) was added to each well, and the plates were incubated for 1 hour at room temperature. The solution in each well was removed, and the wells were washed. Fifty microliter of β -galactosidase-linked goat anti-rabbit Ig conjugate (10⁻³ dilution in PBS containing 0.1% casein, [Amersham, UK]) was added to each well, and the plates were incubated for 1 hour at room temperature. After washing, the wells were then incubated for 1 hour at 37°C with 100 μ L of PBS containing 0.1 mmol/L 4-methylumbelliferone- β -galactoside (Sigma). Finally, 25 μ L of 1 mol/L anhydrous sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored at 317 and 374 nm for excitation and emission, respectively, using a Titertek Fluoroscan reader (Flow Laboratories Inc., Costa Mesa, CA, USA).

Results and Discussion

1) Compositional analysis of soybeans

The data on the fatty acid and amino acid contents of both soybean lines are shown in Table 2 and Table 3, respectively. No remarkable differences in the levels of any of the 5 fatty acids and 18 amino acids examined were found between the GM line and the closely-related non-GM line. We also found no marked difference in the contents of secondary metabolites such as isoflavone and soyasaponin in these two lines (unpublished data). Therefore, the composition of GM-soybean seeds seems to be equivalent to that of non-GM-soybeans. To avoid the deleterious effects of the trypsin inhibitor in un-

Table 2. Summary of Fatty Acids Contents (g/100 g) in Soybeans

Fatty acid	Seed line	
	Non-GM	GM
C16:0	1.77	2.09
C18:0	0.69	0.76
C18:1	3.74	3.92
C18:2	8.70	10.1
C18:3	1.53	1.54

Table 3. Summary of Amino Acid Contents (g/100 g) in Soybeans

Amino acid	Seed line	
	Non-GM	GM
Aspartic acid	4.31	4.14
Threonine	1.47	1.44
Serine	1.91	1.86
Glutamic acid	6.47	6.42
Proline	1.84	1.79
Glycine	1.56	1.55
Alanine	1.56	1.53
Valine	1.70	1.69
Isoleucine	1.64	1.60
Leucine	2.81	2.75
Tyrosine	1.21	1.21
Phenylalanine	1.86	1.81
Histidine	1.04	0.98
Lysine	2.30	2.29
Arginine	2.65	2.53
Cysteine	0.59	0.56
Methionine	0.53	0.55
Tryptophan	0.40	0.46

treated soybeans¹⁴), the soybeans were heated at 100°C for 30 minutes in an autoclave. The trypsin inhibitor unit value (TIU/mg) in both soybean lines decreased dramatically after heat treatment (before heating: 41.0 to 47.5 TIU/mg; after heating: 2.5 to 3.5 TIU/mg) (data not shown). Processed soybean meal from each line was incorporated into the rodent diets at 30% and the protein content was adjusted to be close to that of rodent diet used commercially⁵) (17–18 g/100 g).

2) Body weight and feed intake of BN rats and B10A mice

All the test animals appeared to be healthy throughout the 15-week study, and no meaningful differences in body weight (Table 4), cumulative body weight gain (Fig. 1), or food consumption (Table 4) were found between the animals fed the non-GM line and those that were fed the GM line. When the results for the control group (fed the commercial rat diet CRF-1) were compared with those of the other two groups, the body weights and cumulative body weight gain were slightly lower in the B10A mice that were fed a diet of processed soybeans. The lower values are probably attributable to differences in the processing conditions that may have affected the palatability of the diets.

Fig. 1. Body weight curves for B10A mice and BN rats fed ground soybeans

Values are means of 5 animals. B10A mice (a) or BN rats (b) were fed a diet containing 30% processed GM (—○—) or non-GM (—●—) soybeans. Control animals (—□—) were fed CRF-1.

3) Liver and spleen weights of BN rat and B10A mice

No significant differences in absolute liver and spleen weights were found between the control animals and the animals fed the processed soybean meals (Table 5).

4) Soybean-specific IgE and IgG production in BN rat and B10A mice

To examine the differences in the potency of antibody production in response to GM and non-GM soybean proteins, the titer (the reciprocal of a serum dilution whose fluorescence intensity was 50% of the maximum level) of GM-soybean-specific IgE and IgG antibodies in the sera was examined using an indirect ELISA. As shown in Table 6, no meaningful differences in the levels of soybean-specific IgE and IgG were found between animals fed a diet containing GM soybeans and those fed non-GM soybeans. When compared with

Table 4. Body Weight and Food Intake of BN Rats and B10A Mice

1) BN rats			
Group	Final body weight (g)	Diet intake (g/animal/day)	Soybean intake (g/kg/day)
GM-soybean	178.6±6.03	6.80	11.14
Non-GM-soybean	174.4±5.37	7.17	12.33
Control (CRF-1)	182.6±6.27	—	—

Values are means±S.D., (n=5)

2) B 10 A mice

Group	Final body weight (g)	Diet intake (g/animal/day)	Soybean intake (g/kg/day)
GM-soybean	24.2±0.91	2.77	33.91
Non-GM-soybean	24.1±1.90	2.82	35.49
Control (CRF-1)	26.5±2.02	—	—

Values are means±S.D., (n=5)

Table 5. Liver and Spleen Weights of BN Rats and B10A Mice

Group	Liver (g)		Spleen (g)	
	BN rats	B10A mice	BN rats	B10A mice
GM-soybean	4.639±0.151	0.974±0.051	0.390±0.067	0.083±0.007
Non-GM-soybean	4.549±0.142	0.914±0.092	0.495±0.094	0.077±0.005
Control (CRF-1)	4.669±0.111	1.103±0.127	0.417±0.033	0.092±0.014

Values are means±S.D., (n=5)

Table 6. Soybean-specific IgE and IgG Production in BN Rats and B10A Mice Fed with Ground GM Soybeans or Non-GM Soybeans

1) BN rats		
Group	ELISA-titer (n=5)	
	Soybean IgE	Soybean IgG
GM-soybean	143±100	1,576±1,207
Non-GM-soybean	152±149	1,950± 348*
Diet control	66± 37	643± 511

Values are means±S.D.

*: Different from control, $p < 0.05$.

2) B10A mice

Group	ELISA-titer (n=5)	
	Soybean IgE	Soybean IgG
GM-soybean	61±12	100±112
Non-GM-soybean	88±71	57± 16
Diet control	51± 0.9	173±226

Values are means±S.D.

the results for the control group, the level of soybean-specific IgG in BN rats was slightly higher in animals fed a diet containing processed soybean meal. The increase seems to be dependent on soybean intake and does not seem to reflect any difference between GM and non-GM soybeans. Thus, BN rats appear to respond to antigens that are common to both GM and non-GM soybeans. As a significant increase in soybean-specific

IgE was not detected in BN rats, the antigenicity of soybeans seems to be low. Thus, the antigenicity of GM and non-GM soybeans appears to be the same.

5) *Histopathology of immune organs*

No diagnostically significant abnormality was observed histopathologically in the HE-stained thin sections of liver derived from either the control animals or the soybean-fed animals. Therefore, no difference of the nutrient intake between soybean-fed animals and the control animals was found. No histopathologic differences of either the composition or the arrangement of the cells in immune-related organs, such as the spleen, thymus, mesenteric lymph node and Peyer's patches, were found between rats and mice raised on a diet of GM soybeans and those receiving non-GM soybeans. Furthermore, no diagnostically significant abnormality was observed histopathologically in the mucosa of the small intestine in animals fed either GM or non-GM soybeans, as shown in Fig. 2. Especially, no difference of the structure of the crypt or the frequency of the appearance of goblet cells was observed between GM- and non-GM-fed animals. Therefore, histopathologically, immunotoxicity of gut as represented by mucosal immune responses was not found.

Conclusion

We studied the equivalence of the composition of a line of transgenic progeny (GM soybeans) and a closely-related and one-parent same cultivar (non-GM soy-

Fig. 2. Histopathology of the mucus membrane of the small intestine
HE-stained thin section of small intestine in BN rats fed GM soybeans (a) and non-GM soybeans (b).

beans). Then animal feeding studies were conducted with BN rats and B10A mice to examine the effect of GM soybeans on the immune system. The results obtained were as follows: (1) No remarkable compositional differences in fatty acids or amino acids were found between GM and non-GM soybeans. The composition of GM soybeans seems to be equivalent to that of non-GM soybeans. (2) No significant difference of growth, food intake, or weights of the liver and the spleen was found between animals fed the non-GM and GM lines. (3) The histopathology of immune-related organs (the thymus, liver, spleen, mesenteric lymph node, Peyer's patches, and small intestine) was similar in animals fed GM and non-GM lines. (4) The production of soybean-specific IgE was not detected in the sera of either group, and the increase in soybean-specific IgG was the same in both GM and non-GM groups. All the results indicate that immuno-toxic activity and allergy-augmenting activity were not caused by GM soybeans in either rats or mice under the conditions used in our experiments.

Acknowledgement

We thank Dr. Esashi of National Institute of Health and Nutrition for his advice on preparing soybean meal feed.

References

- 1) Padgett, S. R., Kolacz, K. H., Delannary, X., Re, D. B., LaVallee, B. J., Tinius, C. N., Rhodes, W. K., Otero, Y. I., Barry, G. F., Eichholtz, D. A., Peschke, V. M., Nida, D. L., Taylor, N. B., Kishore, G. M., Development, identification and characterization of a glyphosate-tolerant soybean line. *Crop Sci.*, **35**, 1451-1461 (1995).
- 2) Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Goda, Y., Sebata, T., Isshiki, K., Toyoda, M., Hino, A., A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. *J. Food Hyg. Soc. Japan*, **40**, 149-157 (1999).
- 3) Toyoda, M., Current status of foods derived by biotechnology and its safety evaluation: refer to genetically modified plant products. *J. Food Hyg. Soc. Japan*, **37**, 247-259 (1996).
- 4) Harrison, L. A., Bailey, M. R., Naylor, M. W., Ream, J. E., Hammond, B. G., Nida, D. L., Burnette, B. L., Nickson, T. E., Mitsky, T. A., Taylor, M. L., Fuchs, R. L., Padgett, S. R., The expressed protein in glyphosate-tolerant soybeans, 5-enolpyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp strain CP4 is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. *J. Nutr.*, **126**, 728-740 (1996).
- 5) Hammond, B. G., Vicini, J. L., Hartnell, G. F., Naylor, M. W., Knight, C. D., Robinson, E. H., Fuchs, R. L., Padgett, S. R., The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *J. Nutr.*, **126**, 717-727 (1996).
- 6) Knippels, L. M., Penninks, A. M., van Meeteren, M., Houben, G. F., Humoral and cellular immune responses in different rat strains on oral exposure to ovalbumin. *Food Chem. Toxicol.*, **37**, 881-888 (1999).
- 7) Atkinson, H. A. C., Miller, K., Assessment of the Brown Norway rat as a suitable model for the investigation of food allergy. *Toxicology*, **91**, 281-288 (1994).
- 8) Atkinson, H. A. C., Johnson, I. T., Gee, J. M., Grigoriadou, F., Miller, K., A Brown Norway rat model of food allergy: Effect of plant components on the development of oral sensitisation. *Fd. Chem. Toxicol.*, **34**, 27-32 (1996).
- 9) Rizzo, L. V., Silver, P., Wiggert, B., Hakim, F., Gazzinelli, R. T., Chan, C. C., Caspi, R. R., Establishment and characterization of a murine CD4⁺ T cell line and clone that induce experimental autoimmune uveoretinitis in B10A

- mice. *J. Immunol.*, **156**, 1,654–1,660 (1996).
- 10) American Institute of Nutrition, Report of the AIN ad hoc committee on standards for nutritional studies. *J. Nutr.*, **107**, 1,340–1,348 (1997).
- 11) Hanai, M., Esashi, T., Bioavailability of magnesium contained in roasted and ground soybean (kinako) as evaluated by serum and bone magnesium contents, kidney calcification, and magnesium absorption. *J. Nutr. Sci. Vitaminol.*, **44**, 437–446 (1998).
- 12) Kawabata, T. K., Immunotoxicology of the gastrointestinal tract. "Experimental Immunotoxicology", Smialowicz, R. J., Holsapple, M. P. (eds), CRC Press, p. 143–155 (1996).
- 13) Teshima, R., Kawase, M., Tanaka, T., Hirai, K., Sato, M., Sawada, J., Ikebuchi, H., Ichinoe, M., Terao, T., Production and characterization of a specific monoclonal antibody against mycotoxin zearalenone. *J. Agric. Food Chem.*, **38**, 1,618–1,622 (1990).
- 14) Kakada, M. L., Hoffa, D. E., Leiner, I. E., Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. *J. Nutr.*, **103**, 1,772–1,778 (1973).