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Effect of Subchronic Feeding of Genetically Modified Corn (CBH351) on Immune System in BN Rats and B10A Mice

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Subchronic animal feeding studies to examine the effect on the immune system of genetically modified corn CBH351, which contains the Cry9C protein derived from *Bacillus thuringiensis* subspecies *tolworthi*, were conducted in female BN rats and B10A mice.

The studies were designed to compare the effect of a line of genetically modified corn CBH351 (GM corn) with that of isoline corn (non-GM corn). Heat-treated corn meal was incorporated into the diets of the rats and mice at a concentration of 50%. The study duration was 13 weeks. Growth, food intake, and organ weights of the thymus, spleen, and liver were compared between animals fed the non-GM and GM lines. The histological findings in thymus, spleen, mesenteric lymph nodes, Peyer's patches, small intestines, liver, kidney, and bone marrow, and the presence of Cry9C-specific IgE, IgG, IgG1 and IgA antibodies in serum were also compared. The results showed no significant differences in growth, feeding value, or the histological findings in immunity-related organs between the animals fed the GM and non-GM lines. Production of Cry9 C-specific IgE and IgA was not detected in the serum of either group. Production of Cry9C-specific IgG and IgG1 was slightly increased in the 50% GM groups of BN rats. No Cry9C-specific IgG or IgG1 was detected in the serum of BN rats fed the diet containing 5% GM-corn In conclusion, no immunotoxic activity was detected in the GM-corn-fed rats and mice in this subchronic dietary study.

Key words: GM corns, Cry9C, BN rats, B10A mice, immune system

Introduction

As of April 2002, the commercialization of 40 varieties of genetically modified (GM) crops, including soybean, corn, canola, potatoes, cotton, and sugar beets, had been authorized in Japan by the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF). Some GM crops contain genes for insecticidal crystal protein (ICP); for example, several strains of GM-corn (MON810, Bt11, Event176, DBT418, *etc.*) contain the cry1Ab gene derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1. Cry1Ab is an ICP. It exhibit insecticidal activity against corn earworm[1)-3)], and its digestibility by SGF (simulated gastric fluid) has been reported to be relatively quick[4)]. By contrast, another strain of GM-corn, CBH351, which has not yet been authorized as safe for use in foods and feeds in Japan, contains the *cry9C* gene derived from *Bacillus thuringiensis* serovar *tolworthi*[5)]. Cry9C is also an ICP, and it exhibits toxic activity against lepidopteran larvae. One of the characteristics of Cry9C is that its digestibility by SGF has been reported to be relatively slow*[1]. To predict the safety of GM foods, compositional data and nutritional studies of GM and conventional lines are compared to evaluate the possibility of unintended changes occurring[6)]. Animal feeding studies of GM and conventional lines are not obligatory in Japan; however, they are sometimes useful for assessing the safety of transgenic progeny and of crops expressing poorly digestible proteins as a result of the introduction of new genes. In a previous paper, we reported a subchronic toxicity study of the transgenic progeny of GM-soybeans and a non-GM cultivar[7)]. We mainly assessed their effect on the immune system of animals, especially on mucosal immunity and allergy. In the present study, we used a line of genetically modified Cry9C-containing corn (GM (CBH351) corn), which has poor digestibility in gastric fluid, and an isoline cultivar (non-GM corn) as a control. Our purpose was to compare the subchronic toxicity of GM-corn and non-GM corn, and to assess the antigenicity of the newly expressed proteins. We used two strains of animals, Brown Norway (BN) rats and B10A mice, that have been reported to be high responders to allergic reactions. The BN rat has been shown to be a high IgE responder and thus resembles atopic humans in terms of the propensity to develop allergic

*1 http://www.epa.gov/oscpmont/sap/2001/july/agencypositionpaper.pdf

reactions[8]. In addition, the BN rat has a similar profile of allergenicity and epitopes of ovalbumin to that of man and can be sensitized *via* the oral route[9, 10]. B10A mice have also been reported to be sensitive to orally administered ovalbumin and other antigens[11].

In this report, we first examined the effect of GM and non-GM corn feeding on the immunotoxicity of BN rats and B10A mice to assess the effect of genetic modifications on the potential adverse effects, including allergenicity, of these crops.

Materials and Methods

1. Diet composition

The genetically modified CBH351 corn line (GM-corn) and an isogenic non-GM corn line produced in Texas, U. S.A. and provided by Aventis Cropscience were utilized in all animal feeding studies. The corns were heated at 100℃ for 10 minutes in an autoclave, and feed containing 50% corn meal was prepared by Oriental Yeast Corporation (Tokyo, Japan). The composition of the diets is shown in Table 1. The composition of the AIN-93M diet[12] was partially modified, and sucrose was used as the carbohydrate source in each diet. Casein was used as a protein source to adjust the protein content to 14–15%. Tryptophan was added to compensate for the deficiency of tryptophan in corn[13]. The corn composition assays were performed at the Oriental Yeast Corporation and the Japanese Food Analytical Center (Tokyo, Japan). The AIN-93M diet (Oriental Yeast Corporation) was used in the control diet group. Feed containing 5% corn meal was prepared by mixing 50% corn meal and AIN-93M diet at the ratio of 1 : 10.

2. Experimental animals and design

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 AIN-93M diet after delivery from the breeding house and then subdivided and randomized into 2 groups with comparable body weights. These 2 groups (10 animals/group) were fed either the 50% GM-corn diet group or the 50% non-GM-corn diet group. The 2 groups were then fed their respective diets for 13 weeks. An additional control diet (AIN-93M) group of ten animals was used for histopathological examination to evaluate spontaneous histopathological changes in the group and to allow comparison with those observed in the corn-fed group. During the experimental period, general condition was checked daily, and body weight and feed consumption were recorded every week. Blood for serum samples of each animal was obtained from the ocular vein at 4, 8, and 13 weeks. At the end of the study, all test animals were anesthetized with ether and blood samples for hematological examination and serum biochemistry were collected from the abdominal aorta. The animals were then necropsied. Thymus, spleen and liver were weighed, and immune-related organs including thymus, spleen, mesenteric lymph nodes, small intestines, bone marrow, liver and kidneys were excised from each animal and fixed with 10% neutral buffered formalin.

Table 1. Composition of Diets

Ingredients	g/100 g dry matter
Corn	50
L-Cystine	0.18
Casein	10.5202
Corn starch	15.5
Sucrose	10
Soybean oil	4
Cellulose	5
AIN-93 minerals	3.5
AIN-93 vitamins	1
Choline	0.25
TBHQ	0.0008
Tryptophan	0.049
Analyzed protein	14–15

To assess antibody production in BN rats, we added two animal groups (8 animals/group), a group fed the 5% GM-corn diet, and a group fed the 5% non-GM-corn diet.

3. Hematological examination and serum biochemistry

Red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC) count, were determined with an auto hematology analyzer (M-2000, Sysmex Corp., Japan). The percentages of neutrophils (Neut), eosinophils (Eosino), basophils (Baso), lymphocytes (Lympho), monocytes (Mono) and erythroblasts (Ebl) among white blood cells were determined with the auto blood cell analyzer (Microx Heg-120A, Tateishi Electronic Corp., Japan). For the examination of serum biochemistry, total protein and albumin/globulin ratio (A/G) in the sera were measured at the SRL Corporation (Tokyo, Japan). The concentration of serum histamine was examined by the post-column HPLC method, as described previously[14].

4. Histopathological examination

Immune-related organs, liver, kidney and bone marrow 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 sections were observed under a light microscope and evaluated histopathologically. To assess immunotoxicity to the gut, the structure of the crypt and the composition of cells (especially goblet cells and intraepithelial lymphocytes) of the mucosa of the small intestine (duodenum, jejunum, ileum) were observed[15].

5. ELISA for antibody production

The Cry9C-specific antibody in the sera of B10A mice and BN rats fed 50% or 5% corn diet was examined by ELISA as follows. The mouse and rat serum titers of Cry9C-specific IgE, IgG, IgG1, and IgA were determined

by the method described by Teshima *et al.*,[16] with some modifications. Fifty microliters of purified Cry9C protein (20 μg/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℃. The solution was then discarded, and each well was washed 4 times with 200 μL of PBS containing 0.05% Tween 20 (PBS/Tween). To minimize non-specific binding of the serum proteins to unoccupied solid-phase sites, 200 μL of 0.1% casein in PBS was added, and the mixture was incubated for 1 hour at room temperature. After washing, 50 μL of diluted serum was added to each well and the plates were incubated for 20 hours at 4℃. The solution was then removed, and the wells were washed. Fifty microliters of rabbit anti-mouse IgE, anti-mouse IgG, anti-mouse IgG1, anti-mouse IgA, anti-rat IgE, anti-rat IgG, anti-rat IgG1, or anti-rat IgA (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 microliters 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 plates were incubated for 1 hour at 37℃ with 100 μL of PBS containing 0.1 mmol/L 4-methylumbelliferone-β-galactoside (Sigma). Finally, 25 μL of 1 mol/L anhydrous sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored at 317 nm and 374 nm for excitation and emission, respectively, with a Titertek Fluoroscan reader (Flow Laboratories Inc., Costa Mesa, CA, USA). As the positive control serum for Cry9C protein, we used serum collected from immunized BN rats and B10A mice intraperitoneally injected 4 times with a mixture of 5 μg of Cry9C and 1 mg of alum in 0.2 mL of PBS.

6. *ELISA for Cry9C*

The Cry9C content of CBH351 GM corn was determined with a GMO-Bt9 corn ELISA kit (Azmax Co., Ltd., Tokyo, Japan). The content of Cry9C in GM-corn was 12.3±0.9 μg/g (n=4).

7. *Statistical analysis*

Differences of body weight, feed intake, organ weight, hematological changes, serum biochemistry, and serum histamine concentration between GM and non-GM fed animals were evaluated by means of Student's t test, and a p value less than 0.05 was considered to be statistically significant.

Results and Discussion

1. *Compositional analysis of corns*

The nutritional components, the fatty acid contents and the amino acid and phytate[17] content of the corn lines are shown in Tables 2, 3 and 4, respectively. Since no remarkable differences in the levels of any of the 9 fatty acids or 18 amino acids measured were found between the GM line and non-GM isoline, the composition of the GM-corn was considered to be equivalent to

Table 2. Nutritional Components of the Corns

Nutrient	Seed line		
	Non-GM	GM	(Unit)
Moisture	13.2±0.1	13.3±0	(g/100 g)
Crude protein	8.6±0	8.9±0.4	(g/100 g)
Crude fat	3.7±0	3.6±0.1	(g/100 g)
Carbohydrates	71.8±0.1	71.4±0.1	(g/100 g)
Crude fiber	1.5±0.1	1.6±0	(g/100 g)
Ash	1.3±0.1	1.2±0	(g/100 g)
Energy	350 ±0.7	349 ±0.7	(kcal/100 g)
Calcium	8.2±0.2	12.3±3.9	(mg/100 g)
Phosphorus	288 ±8.5	274 ±3.5	(mg/100 g)
Potassium	353 ±0.7	357 ±9.9	(mg/100 g)
Magnesium	103 ±0	109 ±1.4	(mg/100 g)

Values are means ±S.D. of duplicate determinations.

Table 3. Summary of the Fatty Acid Content of the Corns

Fatty acid	Seed line	
	Non-GM	GM
C16 : 0	12.45±0.07	12.85±0.07
C18 : 0	1.65±0.07	1.6 ±0
C18 : 1	20.0 ±0.28	20.0 ±0
C18 : 2	63.35±0.49	62.8 ±0.14
C18 : 3	1.6 ±0	1.75±0.07
C20 : 0	0.4 ±0	0.4 ±0
C20 : 1	0.2 ±0	0.2 ±0
C22 : 0	0.15±0.07	0.2 ±0
C24 : 0	0.2 ±0	0.2 ±0

Values are means ±S.D. of duplicate determinations.

Table 4. Summary of the Amino Acid and Phytate Contents of the Corns (g/100 g)

Amino acid	Seed line	
	Non-GM	GM
Aspartic acid	0.60±0.01	0.63±0.04
Threonine	0.30±0	0.32±0.01
Serine	0.40±0	0.42±0.01
Glutamic acid	1.53±0.01	1.60±0.01
Proline	0.71±0.01	0.74±0.01
Glycine	0.29±0	0.30±0
Alanine	0.63±0	0.66±0
Valine	0.37±0	0.39±0.01
Isoleucine	0.28±0.01	0.29±0
Leucine	1.07±0.01	1.12±0.01
Tyrosine	0.27±0.01	0.30±0.02
Phenylalanine	0.40±0	0.42±0.01
Histidine	0.22±0.01	0.23±0.01
Lysine	0.24±0	0.25±0.01
Arginine	0.32±0.01	0.33±0.01
Cystine	0.18±0	0.18±0
Methionine	0.17±0	0.17±0
Tryptophan	0.06±0	0.06±0
Phytate	0.46±0.06	0.44±0.01

Values are means ±S.D. of duplicate determinations.

that of non-GM-corn. To mimic the usual process of preparing frozen corn, the corn samples were heated at 100℃ for 10 minutes in an autoclave. Processed corn meal from each line was incorporated into the rodent diets at 50%, and the protein content was adjusted so that it approximated that of the commercial rodent diet used[13] (14–15 g/100 g).

2. General condition, body weight and feed intake of BN rats and B10A mice

All the test animals appeared to be healthy throughout the 13-week study, and no meaningful differences in cumulative body weight (Fig. 1), or feed consumption (Fig. 2) were found between the animals fed the non-GM line and those that were fed the GM line.

3. Thymus, spleen, and liver weights of BN rats and B10A mice

No significant differences in absolute thymus, spleen or liver weight were found between the animals fed the non-GM line and those fed the GM line (Table 5).

4. Hematological examination and serum biochemistry

As shown in Table 6, no significant differences in RBC, MCV, MCH, MCHC, WBC, or differential cell counts were found between the animals fed the non-GM line and those fed the GM line. Nor were there any significant differences in serum biochemistry values (total protein and the ratio of A/G) or serum histamine

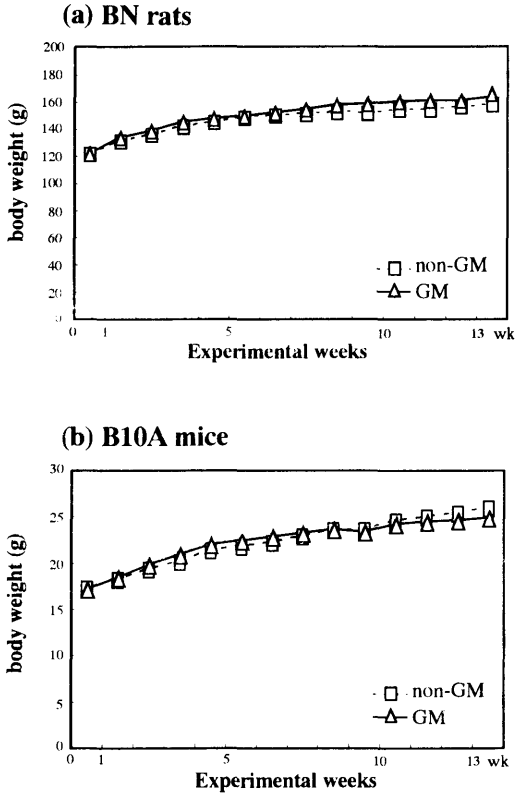

Fig. 1. Body weight curves for BN rats and B10A mice fed ground corn
Values are means of data for 10 animals. BN rats (a) and B10A mice (b) were fed a diet containing 50% processed GM corn (—△—) or non-GM (···□···) corn.

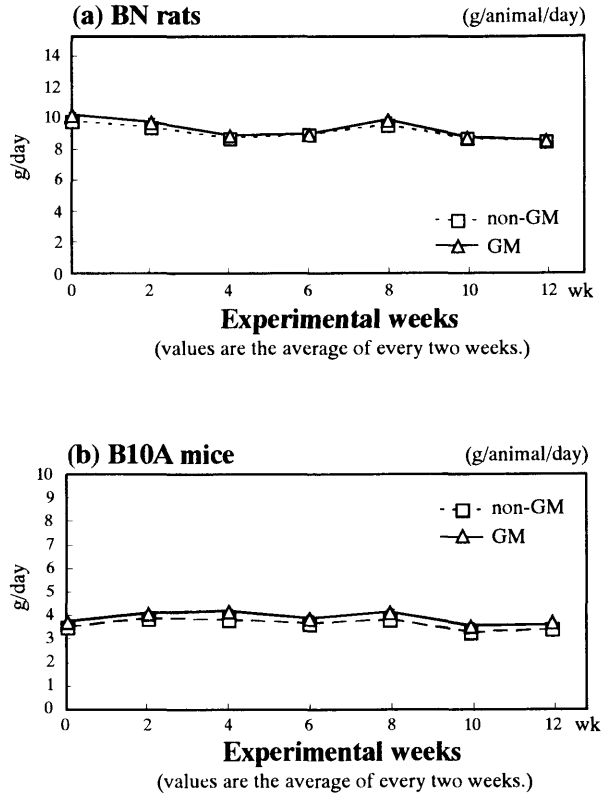

Fig. 2. Changes in diet intake of BN rats and B10A mice fed ground corn
Values are means of data for 10 animals. BN rats (a) or B10A mice (b) were fed a diet containing 50% processed GM corn (—△—) or non-GM (···□···) corn.

Table 5. Organ Weights of BN Rats and B10A Mice

1) BN rats

	Groups	
	Non-GM	GM
Body weight (g)	153.7 ±6.970[a]	157.3 ±6.302
Absolute (g)		
Thymus	0.165±0.027	0.177±0.029
Spleen	0.266±0.023	0.273±0.022
Liver	3.833±0.169	3.749±0.204

[a] Mean ±S.D. ($n=10$)

2) B10A mice

	Groups	
	Non-GM	GM
Body weight (g)	25.83± 1.789[a]	23.91±1.544
Absolute (mg)		
Thymus	43.75± 10.09	42.70±7.409
Spleen	78.34± 20.62	73.42±7.516
Liver	996.4 ±147.6	930.9 ±75.91

[a] Mean ±S.D. ($n=10$)
No significant difference was observed between non-GM and GM groups.

Table 6. Hematological Changes in BN Rats and B10A Mice

1) BN rats

		Groups	
		Non-GM	GM
RBC	($\times 10^4/\mu L$)	883.6 ±45.91[a)]	911.8 ±28.04
MCV	(fL)	52.81 ± 0.331	52.98 ± 0.329
MCH	(pg)	16.47 ± 0.652	16.83 ± 0.275
MCHC	(g/dL)	31.19 ± 1.232	31.78 ± 0.507
WBC	($\times 10^2/\mu L$)	23.00 ± 6.912	21.63 ±14.84
Differential cell counts (%)			
	Neut	17.96 ±14.12	20.61 ±11.82
	Eosino	3.122± 1.977	1.900± 2.558
	Baso	0	0
	Lymph	77.26 ±14.25	76.19 ±12.55
	Mono	1.667± 1.000	1.300± 0.949
	Ebl	0	0

[a)] Mean ±S.D. ($n=10$)

2) B10A mice

		Groups	
		Non-GM	GM
RBC	($\times 10^4/\mu L$)	983.3 ±165.8[a)]	958.0 ±117.7
MCV	(fL)	52.59± 0.446	51.13± 0.701
MCH	(pg)	14.13± 0.437	14.41± 0.621
MCHC	(g/dL)	27.38± 0.824	28.15± 1.133
WBC	($\times 10^2/\mu L$)	24.60± 5.177	26.00± 4.243

[a)] Mean ±S.D. ($n=10$)

No significant difference was observed between non-GM and GM groups.

Table 7. Serum Biochemistry Data of BN Rats According to Diet

	Groups	
	Non-GM	GM
Total protein (g/dL)	5.410±0.534[a)]	5.150±0.366
Albumin/globulin (A/G)	2.530±0.231	2.520±0.220

[a)] Mean ±S.D. ($n=10$)

No significant difference was observed between non-GM and GM groups.

Table 8. Determination of Serum Histamine Levels

1) BN rats

	Groups	
	Non-GM	GM
Serum histamine (nmol/L)	58.69±17.80	64.16±20.80

($n=8$)

2) B10A mice

	Groups	
	Non-GM	GM
Serum histamine (nmol/L)	85.26±59.8	62.28±42.0

($n=8$)

No significant difference was observed between non-GM and GM groups.

level between the animals fed the non-GM line and those fed the GM line (Tables 7 and 8). Serum histamine was measured as a marker for hypersensitivity.

5. *Cry9C-specific IgE, IgG and IgA production in BN rats and B10A mice*

To determine whether there was any difference in antibody production between animals fed the GM or non-GM corn diet, the titers (reciprocal of the serum dilution whose fluorescence intensity was 50% of the maximum level) of Cry9C-specific IgE, IgG, and IgA antibodies in serum were determined by indirect ELISA. As shown in Table 9, the production of Cry9C-specific IgE or IgA was not found in the serum of BN rats or B10A mice fed the diet containing 50% GM corn or non-GM corn. On the other hand, marginally higher values of the production of Cry9C-specific IgG and IgG1 were found in the serum of BN rats fed a diet containing 50% GM corn. As shown in Table 10, the production of Cry9C-specific IgG or IgG1 was not found in the serum of BN rats fed a diet containing 5% GM corn. The production of Cry9C-specific IgG or IgG1 was not found in the serum of B10A mice fed a diet containing 50% corn (Table 9). Since no evidence of production of Cry 9C-specific IgE was detected in the BN rats or B10A mice fed 50% CBH corn, the possibility of induction of immediate-type hypersensitivity in the animals seems

Table 9. Cry9C-specific-IgE, IgG1, IgG and IgA Production in BN Rats and B10A Mice Fed with Ground 50% GM-corn or Non-GM-corn

1) BN rats

Group	ELISA-titer ($n=10$)			
	Cry9C-IgE	Cry9C-IgG1	Cry9C-IgG	Cry9C-IgA
GM-corn	<50	560±74*	710±177*	<500
Non-GM-corn	<50	<500	<500	<500

* Values are means ±S.D.

2) B10A mice

Group	ELISA-titer ($n=10$)			
	Cry9C-IgE	Cry9C-IgG1	Cry9C-IgG	Cry9C-IgA
GM-corn	<50	<500	<500	<500
Non-GM-corn	<50	<500	<500	<500

Table 10. Cry9C-specific IgE, IgG1, IgG Production in BN Rats Fed with Ground 5% GM-corn or Non-GM-corn

1) BN rats

Group	ELISA-titer ($n=8$)		
	Cry9C-IgE	Cry9C-IgG1	Cry9C-IgG
GM-corn	<50	<500	<500
Non-GM-corn	<50	<500	<500

to be extremely low. Slight increases in the titer of Cry9C-specific IgG and IgG1 were observed in the serum of BN rats fed 50% CBH corn (IgG: 710 and IgG1: 560), but the titers were much lower than the titers in serum obtained from BN rats intraperitoneally injected with Cry9C, whose titers of Cry9C-specific IgG and IgG1 were approximately 500,000, and 300,000, respectively. It has been reported that when serum concentrations of antigen-specific IgG are high, the antigen–antibody complexes may induce an Arthus reaction (type III hypersensitivity)[18]. However, since the increase in Cry9C-specific IgG titer in the BN rats fed 50% GM corn was extremely small, the possibility of formation of antigen–antibody complexes and induction of type III hypersensitivity in the animals is considered to be extremely low.

6. Histopathology of immune system organs

No significant finding was detected in immune-related organs, such as the spleen, thymus, mesenteric lymph node and Peyer's patches, of rats and mice fed the GM corn diet, those fed the non-GM corn diet, and those fed the control AIN93M diet. Moreover, no significant abnormal findings were detected histopathologically in the mucosa of the small intestine in animals fed either the GM-corn or non-GM corn diet. In particular, no differences in the structure of the crypts or the frequency of the appearance of goblet cells were observed between the GM-corn and non-GM-corn-fed animals. Thus, no histopathological evidence of an immunological response of gut-associated lymphoid tissue was found.

Conclusion

We compared the composition of a line of GM-corn and an isoline of non-GM corn and then conducted animal feeding studies on BN rats and B10A mice to assess the potential effect of the GM corn on the rat and mouse immune systems. The results obtained were as follows. (1) No remarkable compositional differences in fatty acids, amino acids or phytate were found between the GM and non-GM corns. The composition of the GM corn was equivalent to that of non-GM corn. (2) No significant differences in growth, food intake, or weight of the liver, spleen, or thymus were found between the animals fed the non-GM and GM lines. (3) The histopathology of immune-related organs (thymus, spleen, mesenteric lymph nodes, Peyer's patches, small intestine, and liver) was similar in animals fed GM and non-GM lines. (4) No evidence of production of Cry9C-specific IgE (specific marker of allergenicity) or IgA was detected in the serum of either group, and an minor increase in Cry9C-specific IgG (marker of exposure to the new protein) was found in the serum of BN rats fed 50% GM corn, but not in those fed 5% GM corn. All of the results indicate that the subchronic (13 weeks) dietary intake of the CBH351 GM corn does not have any effect on immune-related organs or allergenic potential in rats or mice under the conditions used in our experiments.

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