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26-Week Oral Safety Study in Macaques for Transgenic Rice Containing Major Human T-Cell Epitope Peptides from Japanese Cedar Pollen Allergens

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A study of repeated oral administration of transgenic rice containing a hybrid peptide of major human T-cell epitopes (7Crp) from Japanese cedar pollen allergens was carried out in cynomolgus macaques over 26 weeks. The monkeys were divided into three groups, each comprising three males and three females, administered a high dose of transgenic rice, a low dose of transgenic rice, or a high dose of the parental rice strain. The transgenic rice 7crp#10 and the parental nontransgenic control were polished, steamed, mashed, and prepared in water at 40% (w/v). Monkeys were orally administered a high or low dose of transgenic rice or the nontransgenic control by gavage every day. No adverse effects on general behavior or body weight of animals were observed during the study. Analysis of blood from monkeys administered for 26 weeks showed that, with few exceptions, there were no significant differences in hematological or biochemical values between them. Additionally, neither pathological symptoms nor histopathological abnormalities were observed. Thus, it was concluded that oral administration of transgenic rice containing T-cell epitopes from Japanese cedar pollen allergens has no adverse effects.

KEYWORDS: Transgenic rice; cynomolgus macaque; Japanese cedar pollen allergens; subchronic

INTRODUCTION

Genetically modified (GM) crops are now being commercially cultivated on about 140 million hectares worldwide (*1*). To date, genes encoding the 5-enol-pyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. CP4 (*2*) and phosphinothricin acetyltransferase from *Streptomyces hygroscopicus* (*3*) for herbicide tolerance, and/or Bt toxins from *Bacillus* spp. (*4*) for insect resistance are constitutively expressed in target crops under the control of CaMV35S or ubiquitin promoters. These GM crops are primarily of benefit to crop growers and seed companies and are called "first-generation" GM crops. These approaches result in relatively low levels of introduced gene expression that were sufficient for improvement of agronomic traits (*5*).

In contrast, the so-called second-generation GM crops with traits beneficial to consumers, such as high nutritional values (food quality), have been developed by fortification with essential amino acids (*6*), essential minerals (*7*), desired vitamins (*8*, *9*), or beneficial fatty acids (*10*) contained in their edible parts. Furthermore, third-generation GM crops functioning as protein factories for the production of vaccines or therapeutic agents with health benefits for the consumer (added-value products) have recently been developed by introducing genes encoding bioactive peptides, antigens, antibodies, cytokines, etc., into the crops (*11*−*15*). Higher concentrations of recombinant products have been produced in target tissues of selected crops by optimizing expression systems (promoter strength, specificity) and post-transcription events (mRNA stability, codon optimization, intracellular localization) to maximize the yields of the target proteins (*16*−*18*).

Before becoming acceptable as a food, the safety of GM crops must be ascertained. Any toxicity or allergenicity associated with the transfer of foreign genes must be compatible with the concept of substantial equivalence and familiarity according to Codex guidelines (*19*). Moreover, for plant-made vaccines, in addition to toxicity, several other risk assessments such as oral tolerance, detrimental effects on the environment, or gene transfer to nontransgenic crops by out-crossing should be considered (*20*). To this end, specific regulations and guidelines for pharmaceutical plants and their products have been established in several countries and continue to evolve (*21*).

Japanese cedar pollen allergy is an important public health problem in Japan. About 20% of the Japanese people are afflicted with this pollinosis from February to April each year, with the number of patients steadily increasing. To control this allergic disease, our approach has been to develop simple, safe, and convenient allergen-specific immunotherapy using GM crops. As a model experiment, mouse major T-cell epitopes from Japanese cedar pollen allergens, Cry j 1 and Cry j 2, were expressed as a fusion protein with soybean seed protein glycinin in transgenic

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rice seed in place of the intact native allergens associated with systemic anaphylaxis (*22*). The feasibility of this rice seed-based peptide vaccine has been demonstrated by feeding BALB/c mice transgenic rice containing mouse T-cell epitopes from Japanese cedar pollen allergens (*22*). In that study, not only immunological signs, such as levels of allergen-specific IgE, T-cell proliferative responses, and cytokines secreted by T-cells, but also allergy symptoms such as sneezing were observed (*22*). As the next step, transgenic rice containing a hybrid peptide composed of seven major human T-cell epitopes (7Crp) derived from Japanese cedar pollen allergens may be exploited for the control of pollen allergy in humans. The 7Crp was designed without IgE-mediated adverse effects by deleting B-cell epitopes and by alternating tertiary structure possesses required for allergen-specific IgE binding. It is known that 7Crp possesses the same level of immunogenicity (ability to induce immune modification in T-cell) as the native allergens, but without binding to allergen-specific IgE (allergenicity), thus representing an ideal safe tolerogen (*23*). As the next step, transgenic rice containing 7Crp was exploited for the control of pollen allergy in humans. The 7Crp accumulates predominantly in endoplasmic reticulum (ER)-derived prolamin protein bodies (PBs) in the transgenic rice seed (*24*). Epitope peptides in rice seeds are protected from digestion by gastrointestinal enzymes relative to the naked peptide by packaging (bioencapsulation) within their cell walls and ER-derived PBs (*25*). This provides an effective means of delivering bioencapsulated tolerogens to the mucosal immune system. Here, subchronic toxicity of transgenic rice seed containing 7Crp was investigated by oral administration to cynomolgus macaques for 26 weeks (182 days) to establish safety in a nonhuman primate model. No adverse effects related to 7Crp ingestion were observed. This assessment of the safety of subchronic administration of transgenic rice seed containing parts of the allergens used for allergen-specific immunotherapy against pollen allergy disease is the first demonstration of the potential feasibility of this approach in primates

MATERIALS AND METHODS

Test Materials. Transgenic rice strain 7crp#10, which accumulates a hybrid peptide composed of seven human T-cell epitopes (7Crp) derived from Japanese cedar (*Cryptomeria japonica*) pollen allergens in its seed (*24*), and its corresponding parental wild-type strain cv. Kitaake were cultivated in a contained field of the National Institute for Agrobiological Sciences (Tsukuba, Japan) in 2004. Cv. Kitaake served as a control. The harvested seeds of these strains were polished, microwave steamed, and packaged aseptically by Satake Corp. (Higashihiroshima, Japan).

Characterization of Test Materials. Transgenic 7crp#10 rice was generated by *Agrobacterium*-mediated transformation (*24*). Levels of 7Crp accumulated in the field-grown 7crp#10 seeds at generation T_7 were confirmed by immunoblotting analysis using a rabbit 7Crp-specific antibody (*24*).

The nutrient composition of brown rice of 7crp#10 and cv. Kitaake strains was examined according to the standard methods established for analysis of foods by Japan Food Research Laboratories (Tokyo, Japan), as described elsewhere (*26*). Proximates (moisture, crude protein, crude fat, total ash, total sugars) were determined (**Table 1**). The crude protein content was calculated from total nitrogen content using a conversion factor of 5.95.

Animals, Housing, and Animal Care. All animal experiments were conducted at Mitsubishi Chemical Safety Institute Ltd. (Tokyo, Japan), with the approval of the institutional animal ethics committee of Mitsubishi Chemical Safety Institute Ltd. Eleven male and 11 female cynomolgus (crab-eating or long-tailed) macaques (*Macaca fascicularis*) 4−5 years old were purchased from Japan Laboratory Animals Inc. (Tokyo, Japan). These macaques were bred by Nafovanny Co. (Vietnam).

Monkeys were maintained under the following conditions: temperature was kept at 22.7−28.6 °C and relative humidity at 44.2−90.3% with a 12 h light/dark cycle. Throughout the study, animals were fed 100 g/day of commercial solid food (CMK-2, Clea Japan Inc., Tokyo, Japan) in the morning after oral administration of rice. After about a 12 month quarantine period, 9 cynomolgus macaques of each gender were used in this safety study. The monkeys were divided into three groups of three monkeys of each gender and administered (1) a high-dose of transgenic rice, (2) a low-dose transgenic rice, or (3) nontransgenic rice.

Table 1. Proximate Composition of Brown Rice Materials from Cv. Kitaake and Transgenic Rice 7crp#10[a]

component	Kitaake	7crp#10
moisture content (g/100 g)	11.3	11.7
crude protein (g/100 g)	7.3	6.7
crude fat (g/100 g)	3.7	3.0
total ash (g/100 g)	1.8	1.7
total sugars (g/100 g)	73.0	73.8

[a] Values are the average of two independent measurements.

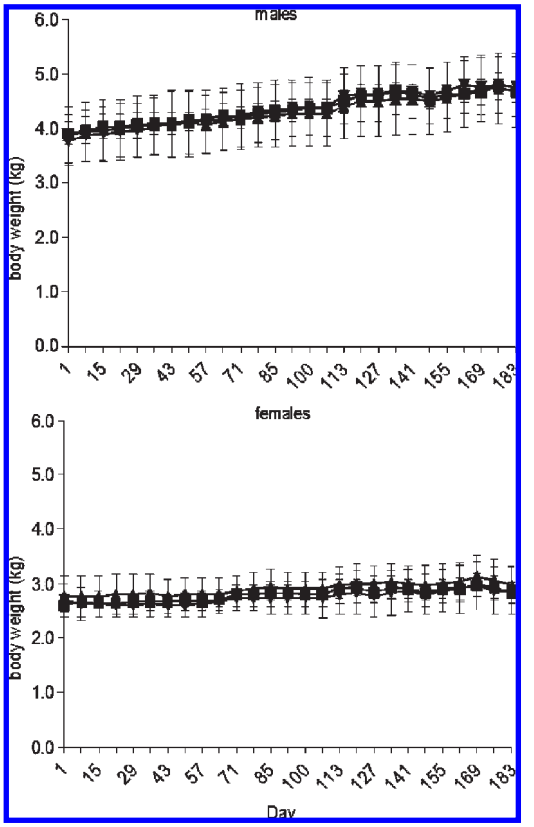

Figure 1. Body weight changes of macaques administered cv. Kitaake and 7crp#10 rice suspensions for 26 weeks: (■) cv. Kitaake (6 g/kg); (▼) 7crp#10 (3 g/kg); (▲) 7crp#10 (6 g/kg). The curves show group means based on three macaques/sex/group. Error bars on data points represent standard errors.

Administration of Test Materials. Aseptically packed 7crp#10 and corresponding wild-type steamed rice were mashed with distilled water to obtain steamed rice at 40% w/v before oral administration via gavage at 15 mL/kg of body weight (high-dose group, 6 g/kg/day) or 7.5 mL/kg body weight (low-dose group, 3 g/kg) for 182 days. The dose level in high-dose groups (6 g/kg/day) would correspond to the maximum dose in a single oral administration to macaques.

Subchronic 26-Week Oral Toxicity Study in Macaques. Routine clinical observations, body weight (**Figure 1**), and food consumption were measured throughout the study period. Hematology and blood biochemistry data were evaluated at the 1st, 13th, and 26th weeks (**Tables 2** and **3**). Fresh urine was collected at 13 and 26 weeks after oral administration and tested for pH, specific gravity, protein, glucose, ketone bodies, bilirubin, occult blood, and urobilinogen. The next day after the 26 week treatment

Table 2. Main Hematological Data of Macaques Administered Cv. Kitaake and 7crp#10 Steamed Rice Suspensions for Weeks 1 and 26[a]

	Kitaake, 6 g/kg		7crp#10, 3 g/kg		7crp#10, 6 g/kg	
	week 1	week 26	week 1	week 26	week 1	week 26
			Males			
RBC count ($\times 10^6/\mu$L)	5.53 ± 0.48	5.36 ± 0.31	5.57 ± 0.05	5.43 ± 0.14	5.96 ± 0.74	5.71 ± 0.55
Hb concn (g/dL)	13.50 ± 0.46	13.10 ± 0.10	14.23 ± 0.51	14.10 ± 0.79	14.47 ± 1.86	14.07 ± 1.50
HCT (%)	43.60 ± 0.75	43.77 ± 0.64	46.47 ± 0.83	46.90 ± 2.00	41.73 ± 1.38	44.80 ± 3.58
WBC count ($\times 10^4/\mu$L)	10.05 ± 3.76	9.63 ± 3.29	7.56 ± 1.00	8.45 ± 0.91	7.26 ± 1.92	5.88 ± 0.41
platelet count ($\times 10^3/\mu$L)	368.67 ± 66.94	380.00 ± 33.60	332.67 ± 74.20	310.67 ± 70.22	316.00 ± 119.05	309.00 ± 46.81
			Females			
RBC count ($\times 10^6/\mu$L)	5.20 ± 0.51	5.06 ± 0.51	5.55 ± 0.75	5.60 ± 0.68	5.28 ± 0.42	5.15 ± 0.46
Hb concn (g/dL)	12.80 ± 0.98	12.63 ± 1.37	13.53 ± 0.78	14.17 ± 0.92	12.93 ± 1.65	12.60 ± 1.71
HCT (%)	41.23 ± 2.03	40.70 ± 2.75	43.90 ± 3.67	45.33 ± 3.44	41.43 ± 5.11	41.17 ± 5.35
WBC count ($\times 10^4/\mu$L)	9.70 ± 2.85	10.75 ± 2.31	9.95 ± 2.88	7.93 ± 1.92	7.91 ± 0.43	8.31 ± 1.49
platelet count ($\times 10^3/\mu$L)	362.67 ± 56.89	348.00 ± 20.42	360.33 ± 40.22	367.33 ± 42.02	308.00 ± 89.20	327.33 ± 118.37

[a] Parameters hematology: red blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), and white blood cell count (WBC). Values are means ± SD (n = 3/group). Data not shown for mean cell volume, mean hemoglobin concentration, mean corpuscular hemoglobin concentration, reticulocyte ratio, prothrombin time, and activated partial thromboplastin time.

Table 3. Blood Chemistry Values of Macaques Administered Cv. Kitaake and 7crp#10 Steamed Rice Suspensions for 26 Weeks[a]

	Kitaake, 6 g/kg	7crp#10, 3 g/kg	7crp#10, 6 g/kg
		Males	
GOT (U/L)	30.3 ± 8.5	26.0 ± 4.0	32.3 ± 10.7
GPT (U/L)	48.3 ± 19.7	31.7 ± 4.0	49.7 ± 23.3
γGT (U/L)	123.3 ± 24.3	126.3 ± 11.1	107.3 ± 27.0
ALP (U/L)	1836.7 ± 107.6	1661.0 ± 773.0	1115.7 ± 240.8
TBiL (mg/dL)	0.10 ± 0.10	0.17 ± 0.06	0.13 ± 0.06
BUN (mg/dL)	24.23 ± 8.52	15.93 ± 3.13	18.40 ± 1.77
creatinine (mg/dL)	0.83 ± 0.06	0.90 ± 0.17	0.93 ± 0.15
glucose (mg/dL)	70.7 ± 3.8	77.0 ± 11.5	61.0 ± 13.5
total protein (g/dL)	8.13 ± 0.25	7.93 ± 0.21	8.10 ± 0.10
A/G ratio	1.44 ± 0.10	1.45 ± 0.14	1.57 ± 0.20
albumin	4.77 ± 0.15	4.70 ± 0.26	4.90 ± 0.26
globulin α1	2.33 ± 0.32	2.27 ± 0.38	2.63 ± 0.42
total cholesterol (mg/dL)	117.0 ± 27.2	109.7 ± 4.7	104.7 ± 38.4
triglyceride (mg/dL)	46.3 ± 8.4	32.0 ± 10.0	31.0 ± 5.6
		Females	
GOT (U/L)	34.7 ± 10.7	26.7 ± 14.2	26.7 ± 3.1
GPT (U/L)	60.3 ± 16.5	42.3 ± 13.0	41.0 ± 11.5
γGT (U/L)	137.0 ± 130.8	73.7 ± 9.6	68.7 ± 8.1
ALP (U/L)	737.3 ± 180.1	656.7 ± 163.4	603.0 ± 51.5
TBiL (mg/dL)	0.23 ± 0.06	0.13 ± 0.06	0.17 ± 0.06
BUN (mg/dL)	17.77 ± 1.71	13.63 ± 3.29	18.27 ± 2.35
creatinine (mg/dL)	0.70 ± 0.10	0.63 ± 0.15	0.70 ± 0.00
glucose (mg/dL)	62.7 ± 2.1	67.3 ± 8.5	61.3 ± 8.5
total protein (g/dL)	7.90 ± 0.36	7.70 ± 0.36	7.83 ± 0.29
A/G ratio	1.43 ± 0.12	1.18 ± 0.16	1.23 ± 0.04
albumin	4.63 ± 0.06	4.13 ± 0.23*	4.33 ± 0.12
globulin α1	2.43 ± 0.25	3.10 ± 0.17*	2.53 ± 0.15
total cholesterol (mg/dL)	98.3 ± 18.9	104.3 ± 24.9	110.0 ± 3.0
triglyceride (mg/dL)	49.0 ± 26.9	81.0 ± 84.2	33.3 ± 16.6

[a] Parameters blood biochemistry: glutamic oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), γ-glutamyl-transpeptidase (γGT), alkaline phosphatase (ALP), total bilirubin (TBiL), blood urea nitrogen (BUN), albumin and globulin (A/G). An asterisk indicates significant difference between groups at $P < 0.05$. Data not shown for globulin α2, globulin β, globulin γ, phospholipid, calcium, sodium, potassium, chloride, and inorganic phosphorus.

period, all animals were killed by intravenous injection of thiopental sodium together with bleeding from the common carotid and axillary artery and vein. All organs (11 from males and 10 from females) were examined for pathological changes, and liver, kidney, pituitary, adrenal, ovary, testis, prostate, thymus, spleen, brain, lung, and heart were weighed and examined histopathologically.

Statistical Analysis. All statistical calculations on data obtained from the toxicity study were carried out using the MiTOX statistical system (Mistui Zosen Systems Research Inc., Chiba, Japan). The homogeneity of variances was evaluated using Bartlett's test. In the case of homogeneous data, a one-way analysis of variance (ANOVA) was performed. When significant differences were observed, the Kruskal−Wallis test was performed. When significant differences between groups were found as a result of these analyses, Dunnett's multiple-comparison test for homogeneous variance or Dunnett-type multiple-comparison tests for nonhomogeneous variance were performed to compare the control and treatment groups. The significance levels were set to 5% for Bartlett's test, ANOVA, and the Kruskal−Wallis test. When Dunnett's and Dunnett-type multiple-comparison tests were conducted, the 5 and 1% levels of significance were used, respectively.

RESULTS

Compositional Analyses of 7crp#10 Rice and Cv. Kitaake Rice. The approximate nutritional compositions of unpolished seeds of 7crp#10 transgenic rice and cv. Kitaake nontransgenic rice are shown in **Table 1**. Moisture content, crude protein, crude fat, total ash, and total sugars of 7crp#10 were all essentially identical to those of the parental strain.

Food Consumption, Body Weight, and Clinical Observations. Food consumption was about 100 g of solid food/animal/day throughout the study period, and there were no significant differences between groups (data not shown).

Monkey body weight increased during the study. The growth curves for males and females are shown in **Figure 1**. There were no significant differences in the body weights between groups administered transgenic and nontransgenic rice.

The monkeys were checked every day for clinical signs. Survival was 100% in all groups during the study, and no adverse effects on general behavior of animals were observed. Loose stool was infrequently observed in females and diarrhea infrequently in males administered control nontransgenic rice. Overall, there were no abnormal clinical signs attributable to administration of this rice in any group.

Hematology, Blood Biochemistry, and Urinalysis. The main hematological data are shown in **Table 2**. There were few differences in hematological values between groups administered transgenic or nontransgenic rice. The main blood biochemistry values 26 weeks after starting administration are shown in **Table 3**.

Table 4. Absolute and Relative Organ Weights at Necroscopy of Animals Administered Cv. Kitaake and 7crp#10 Steamed Rice Suspensions[a]

	males			females		
	Kitaake, 6 g/kg	7crp#10, 3 g/kg	7crp#10, 6 g/kg	Kitaake, 6 g/kg	7crp#10, 3 g/kg	7crp#10, 6 g/kg
			Absolute Weights			
final body wt (kg)	4.67 ± 0.32	4.77 ± 0.95	4.70 ± 1.18	2.83 ± 0.32	2.87 ± 0.76	2.97 ± 0.60
brain (g)	71.2 ± 3.7	64.4 ± 6.8	66.5 ± 6.3	61.7 ± 4.9	56.9 ± 4.0	61.6 ± 6.7
pituitary (mg)	90.0 ± 22.5	74.0 ± 24.8	67.7 ± 8.0	64.7 ± 16.6	66.7 ± 14.4	64.0 ± 10.8
thymus (g)	2.4 ± 0.5	2.5 ± 0.9	1.0 ± 0.1	2.3 ± 1.4	2.4 ± 1.2	2.2 ± 0.7
lungs (g)	24.3 ± 0.5	20.4 ± 6.1	21.0 ± 6.4	12.8 ± 1.6	13.9 ± 0.9	14.5 ± 2.8
heart (g)	16.6 ± 1.8	14.9 ± 1.7	15.1 ± 3.6	10.4 ± 0.6	9.7 ± 2.3	10.6 ± 1.4
liver (g)	73.4 ± 5.2	65.4 ± 8.7	65.0 ± 17.1	47.0 ± 5.4	48.4 ± 15.1	51.6 ± 3.0
spleen (g)	3.8 ± 1.5	3.3 ± 0.5	4.3 ± 2.4	2.6 ± 1.0	2.6 ± 0.8	1.9 ± 0.3
kidney left (g)	7.6 ± 0.7	6.8 ± 1.2	7.3 ± 1.2	5.3 ± 1.0	5.5 ± 0.7	5.3 ± 1.3
kidney right (g)	7.8 ± 0.7	6.7 ± 0.9	7.1 ± 0.9	5.3 ± 0.9	5.4 ± 0.8	5.1 ± 1.2
kidneys (g)	15.3 ± 1.4	13.5 ± 2.1	14.4 ± 2.1	10.6 ± 1.8	10.9 ± 1.5	10.5 ± 2.5
adrenal left (mg)	274.0 ± 41.2	303.3 ± 122.7	233.7 ± 31.6	204.3 ± 53.2	236.7 ± 48.4	212.3 ± 16.2
adrenal right (mg)	218.3 ± 57.7	233.0 ± 31.3	204.3 ± 18.6	164.0 ± 41.2	190.7 ± 26.8	172.0 ± 17.8
adrenals (mg)	492.3 ± 98.6	536.3 ± 153.9	438.0 ± 30.4	368.3 ± 92.7	427.3 ± 73.2	384.3 ± 33.7
testis left (g)	8.4 ± 5.3	9.0 ± 9.5	9.8 ± 4.7			
testis right (g)	9.6 ± 4.6	9.1 ± 9.5	10.3 ± 4.3			
testes (g)	18.0 ± 9.9	18.10 ± 19.0	20.2 ± 9.0			
prostate (g)	2.9 ± 1.3	2.1 ± 1.7	2.2 ± 0.8			
ovary left (mg)				145.7 ± 72.3	98.3 ± 28.7	174.7 ± 76.3
ovary right (mg)				165.3 ± 32.9	193.0 ± 115.5	209.7 ± 136.6
ovaries (mg)				311.0 ± 58.2	291.3 ± 140.4	384.3 ± 204.2
			Relative Weights			
brain (%)	1.53 ± 0.16	1.37 ± 0.14	1.46 ± 0.26	2.18 ± 0.10	2.07 ± 0.53	2.13 ± 0.49
pituitary ($\times 10^{-3}$%)	1.9 ± 0.4	1.5 ± 0.2	1.5 ± 0.3	2.3 ± 0.7	2.5 ± 1.1	2.3 ± 0.8
thymus ($\times 10^{-3}$%)	51.9 ± 7.8	54.5 ± 25.0	23.1 ± 6.2	79.7 ± 40.0	81.3 ± 19.2	77.5 ± 25.5
lungs (%)	0.52 ± 0.05	0.42 ± 0.04	0.44 ± 0.08	0.45 ± 0.02	0.50 ± 0.10	0.49 ± 0.02
heart (%)	0.36 ± 0.02	0.32 ± 0.04	0.32 ± 0.02	0.37 ± 0.03	0.34 ± 0.02	0.36 ± 0.03
liver (%)	1.57 ± 0.10	1.39 ± 0.19	1.38 ± 0.03	1.66 ± 0.11	1.68 ± 0.10	1.77 ± 0.28
spleen (%)	0.08 ± 0.03	0.07 ± 0.02	0.09 ± 0.03	0.09 ± 0.02	0.10 ± 0.04	0.07 ± 0.01
kidney left (%)	0.16 ± 0.01	0.14 ± 0.00	0.16 ± 0.03	0.19 ± 0.02	0.20 ± 0.04	0.18 ± 0.02
kidney right (%)	0.17 ± 0.00	0.14 ± 0.01	0.16 ± 0.03	0.19 ± 0.02	0.20 ± 0.04	0.17 ± 0.01
kidneys (%)	0.33 ± 0.00	0.28 ± 0.01	0.31 ± 0.06	0.37 ± 0.03	0.39 ± 0.09	0.35 ± 0.03
adrenal left ($\times 10^{-3}$%)	5.9 ± 0.6	6.3 ± 1.5	5.4 ± 2.3	7.2 ± 1.6	8.9 ± 3.6	7.3 ± 1.1
adrenal right ($\times 10^{-3}$%)	4.7 ± 1.0	5.0 ± 0.6	4.5 ± 1.2	5.8 ± 1.5	7.1 ± 2.7	5.9 ± 0.6
adrenals ($\times 10^{-3}$%)	10.5 ± 1.6	11.2 ± 1.7	9.9 ± 3.5	13.0 ± 3.0	16.0 ± 6.2	13.2 ± 1.7
testis left ($\times 10^{-3}$%)	182.2 ± 112.0	174.5 ± 155.1	211.0 ± 84.9			
testis right ($\times 10^{-3}$%)	206.3 ± 97.9	175.7 ± 156.3	222.7 ± 79.7			
testes ($\times 10^{-3}$%)	388.4 ± 209.8	350.1 ± 311.2	433.7 ± 164.4			
prostate ($\times 10^{-3}$%)	62.4 ± 25.7	41.7 ± 26.4	48.5 ± 14.4			
ovary left ($\times 10^{-3}$%)				5.3 ± 3.1	3.5 ± 1.1	5.8 ± 1.7
ovary right ($\times 10^{-3}$%)				5.8 ± 0.5	6.5 ± 3.6	6.6 ± 3.1
ovaries ($\times 10^{-3}$%)				11.1 ± 2.9	10.0 ± 4.4	12.5 ± 4.1

[a] Values are means ± SD (n = 3/group).

Overall, no dose-dependent changes in blood biochemistry values were observed. There was one exception, namely, when 3 g/kg transgenic rice was administered, a decrease in albumin and an increase in globulin α1 in female monkeys was statistically significant at the 5% level (**Table 3**). There were no significant differences in urinalysis between groups after 13 or 26 weeks of administration (data not shown).

Gross Necropsy, Histopathology, and Absolute and Relative Organ Weights. No gross abnormalities were detected for monkeys in any group at autopsy. Some non-dose-dependent changes in the organs occurred sporadically. Histopathology also revealed no dose-dependent changes in the organs. Thus, there were no particular histopathological findings observed in any monkeys fed either transgenic or nontransgenic rice. There were also no significant differences between groups in organ weights in any of the animals (**Table 4**).

DISCUSSION

The major constituents (moisture, crude protein, crude fat, total ash, total sugars) of unpolished seeds from the transgenic and nontransgenic rice plants were comparable (**Table 1**). The macronutrient composition of unpolished seeds of the field-grown transgenic rice strain 7crp#10 was very similar to that of its corresponding parental wild-type strain cv. Kitaake, as previously reported (*24*). According to the OECD's consensus document on compositional considerations in rice (*27*), variation in nutrient and biochemical composition should be within the range reported in the nontransgenic rice varieties. GM foods should be comparable with analogous conventional foods in terms of nutritional qualities, toxicity, and other characteristics. The transgenic rice containing 7Crp was considered to be essentially equivalent in nutritional and biochemical

characteristics to the nontransgenic control except for the presence of 7Crp.

Cynomolgus macaques are well-established as a primate model for safety assessment studies. The 26-week subchronic safety study reported here was designed to evaluate the toxicity of field-grown transgenic rice strain 7crp#10. The results revealed no apparent adverse effects due to long-term administration of the 7crp#10 transgenic rice. Clinical signs were normal over the 26 weeks, food consumption was not significantly different between groups, and body weight increased normally in all. The infrequent loose stools in females and diarrhea in males were not repeatedly observed. No differences attributable to administered dose of 7Crp#10 seeds were seen for any assessed parameters. Therefore, these dose-independent clinical signs were considered to be unrelated to treatment.

The hematological indices—red blood cell counts, hemoglobin, hematocrit, white blood cell, and platelet counts—were not significantly different between groups and remained within the range of normal reference values for cynomolgus monkeys (*28*) after 1 and 26 weeks of oral administration. The group given 3 g/kg transgenic rice showed a slight but statistically significant decrease in albumin and an increase in globulin α1, but only in females. In general, decreases in serum albumin levels could possibly indicate hepatic or renal dysfunction, or malnutrition, even though neither gross necropsy nor histopathology of the liver and kidney showed any sign of dysfunction. The decrease in albumin and increase in globulin α1 was not seen in the same individuals within a group and was not observed in animals receiving 6 g/kg transgenic rice or the same dose of nontransgenic rice. These abnormalities of blood biochemical values were sporadic and not dose-dependent and were therefore not attributable to the administration of the transgenic rice.

The weights of the thymi in the male group given 6 g/kg transgenic rice were somewhat lower than in the other male groups. For this parameter, assumption of equal variance among the three groups was rejected by the Bartlett test; therefore, the nonparametric Kruskal−Wallis test was conducted to analyze variance. This showed no significant difference between groups ($p = 0.0665$). Thymic histopathology in the male group given 6 g/kg of 7crp#10 revealed focal lymphocyte infiltration and macrophage infiltration in one animal. The absolute thymus weight of that animal was similar to those of the other two males in the same dosage group whose thymi appeared to be normal by gross necropsy and histopathology. Thus, the cellular infiltration in the one animal appeared to be unrelated to thymic weight loss and is considered to be insignificant.

The 7crp#10 transgenic rice specifically expresses major human T-cell epitopes from Japanese cedar pollen allergens in mature seed. It is notable that 7Crp accumulated to a high level (about 60 μg/grain), which is estimated to be sufficient to induce immune tolerance against Japanese cedar pollen allergens were it to be included in the daily diet of humans as a staple food in the form of steamed rice (*29*). In the present study, the potential allergenicity of the 7Crp was not evaluated. The 7Crp is composed of major human T-cell epitopes derived from the Japanese cedar pollen allergens Cry j 1 and Cry j 2, which are known not to be bound by Japanese cedar pollen allergen-specific IgE antibodies from 48 Japanese cedar pollinosis patients (*23*). Furthermore, 7Crp could induce higher T-cell proliferative responses (representing immunogenicity) than a mixture of the seven individual T-cell epitopes (*23*). Evidence for only a low potential risk of allergenicity for the 7Crp was also obtained from the finding that it was readily degraded in an in vitro pepsin and pancreatin digestion assay (*24*). Taken together, the 7Crp is expected to improve the safety and efficacy of immunotherapy relative to the intact native allergens on the basis of its higher immunogenicity, as required for immune reactions, while lacking allergenicity.

This study is the first oral and long-term safety assessment of transgenic plant products containing 7Crp using nonhuman primates and is aimed toward eventual induction of human specific oral immune tolerance against Japanese cedar pollen allergens. The transgenic rice seed contained high concentrations (about 60 μg/grain) of 7Crp. This is in marked contrast with the risk assessment carried out in first-generation GM crops grown with insect resistance or herbicide tolerance (*2*−*4*). In this context, the idea of "the principle of substantial equivalence", which was developed by Codex (*19*), will also work as a starting point of safety evaluation of biopharmaceutical transgenic crops in the measure of nutritional and antinutritional components. However, different from first-generation GM crops, biopharmaceutical transgenic crops are designed to accumulate target substances to as high a level as possible in the harvested parts of the plant, and in some cases, not enough scientific knowledge on the substance property is available. Therefore, properly designed experimental toxicological evaluation of bioactive gene products should be conducted as a part of the risk analysis procedure. Risk analyses of specific biopharmaceutical transgenic crops such as oral vaccines that are directly administered as foods have been limited to date because biopharmaceuticals are usually used as purified products extracted from the transgenic plants. Plants containing biopharmaceuticals have to be cultivated and produced according to accepted standard production criteria such as Good Manufacturing Practices (GMP). Biopharmaceuticals including plant-made vaccines should in principle be developed according to the same rules for other biopharmaceutical products. Furthermore, to gain pharmaceutical product approval, extensive risk assessment including toxicity studies, pharmacokinetics, and efficacy studies, etc., must be completed as part of the process of development.

In the present study, serial administration of steamed transgenic rice for 182 days had no adverse effects on cynomolgus macaques monitored for clinical signs, body weight, necropsy findings, histopathological findings, hematological data, blood biochemical data, and urinalysis. Thus, these results demonstrate the safety of oral administration of transgenic rice 7crp#10 to cynomolgus macaques, and we conclude that the 7crp#10 steamed rice products are safe even when eaten every day. Further data on validation and quality testing, etc., will be required for evaluating safety and efficacy.

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