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Safety evaluation of standardized allergen extract of Japanese cedar pollen for sublingual immunotherapy

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ABSTRACT

Japanese cedar (JC) pollinosis is caused by Japanese cedar pollen (JCP) and most common seasonal allergic disease in Japan. Subcutaneous immunotherapy (SCIT) with allergen extract of JCP (JCP-allergen extract) is well established for JC pollinosis treatment with improvement of symptoms. However, major drawbacks for SCIT are repeated painful injections, frequent hospital visits and anaphylactic risk. Currently, sublingual immunotherapy (SLIT) has received much attention as an advanced alternative application with lower incidence of systemic reactions because the liquid or tablet form of allergen is placed under the tongue. The aim of this study was safety evaluation of standardized JCP-allergen extract currently developed for SLIT in JC pollinosis. JCP-allergen extract showed no potential genotoxicity. No systemic effects were observed in rats administered JCP-allergen extract orally for 26 weeks followed by 4-week recovery period. Mild local reactions such as hyperplasia and increased globule leukocytes resulting from vehicle (glycerin)-induced irritation were observed in stomach. No-observed-adverse-effect level was greater than 10,000 JAU/kg/day for systemic toxicity, equivalent to 300-fold the human dose. No local irritation was found in rabbits oral mucosae by 7-day sublingual administration. These results demonstrate the safe profile of standardized JCP-allergen extract, suggesting it is suitable for SLIT in JC pollinosis.

1. Introduction

Seasonal allergic rhinitis, also named Japanese cedar (JC) pollinosis, is caused by Japanese cedar pollen (JCP). It is a type-I allergic disease and is the most common disease in Japan and thus is considered a national affliction (Kaneko et al., 2005; Okubo et al., 2011; Yamada et al., 2014). The main symptoms are sneezing, watery rhinorrhea, nasal blockage and itching of eyes. More than one third of the Japanese population has JC pollinosis, and this number has significantly increased in the last 2 decades (Okubo et al., 2011; Yamada et al., 2014). In addition, many JC pollinosis patients have also been sensitized to cypress pollen scattered after the JCP season by IgE cross-reactivity (Di Felice et al., 2001), which causes a long-term symptom onset.

Allergen-specific immunotherapy is the practice of administering gradually increasing quantities of allergen extracts to ameliorate the symptoms associated with the subsequent exposure to the causative allergen in order to induce a state of tolerance (Malling and Weeke, 1993; Bousquet et al., 1998; Cappella and Durham, 2012; Eifan et al., 2013; Canonica et al., 2014). It is considered to be a cure for allergic disease although symptomatic treatment such as antihistamines and nasal corticosteroids allows tentative relief from symptoms (Bousquet et al., 1998). Subcutaneous immunotherapy (SCIT) has been used to treat JC pollinosis but with limited usage because of inconvenience such as injection site pain and frequent hospital visits, and risk of severe side effect such as systemic allergic reaction including rarely occurred anaphylaxis (Bernstein et al., 2004; Cox et al., 2010) . Allergen-specific

Abbreviations: JAU, Japanese allergy units; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCA, passive cutaneous anaphylaxis; %MNPCE, ratio of micronucleated polychromatic erythrocyte; %RET, ratio of reticulocytes; JC, Japanese cedar; JCP, Japanese cedar pollen; SLIT, sublingual immunotherapy; SCIT, Subcutaneous immunotherapy; NOAEL, no-observed-adverse-effect level; GLP, Good Laboratory Practice; 2AA, 2-aminoanthracene; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; s.c., sub-cutaneous; p.o., per os; PBS, phosphate buffered saline; DMN, dimethylnitrosamine; MMC, mitomycin C; SPF, specific pathogen-free; HE, hematoxylin and eosin.

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sublingual immunotherapy (SLIT) is considered to be a safe and efficient treatment for respiratory allergy, and has been introduced as an alternative to SCIT (Didier et al., 2011; Nelson et al., 2011; Durham et al., 2012; Cox et al., 2012). SLIT reduces the burden on patients compared with SCIT because it can be administered at home and is associated with fewer severe side effects. In SLIT, the allergen extract is placed under the tongue for 1 or 2 min and then swallowed (SLIT-swallow) or spat out (SLIT-spit). Based on clinical results and pharmacokinetic considerations, only SLIT-swallow is currently performed (Canonica and Passalacqua, 2003).

A standardized allergen extract of JCP has been used for SCIT treatment of the JC pollinosis patients for 15 years. The potency of allergen extract of JCP (JCP-allergen extract) is expressed as Japanese allergy units (JAU) as defined by the Japanese Society of Allergology based on skin tests of allergy patients. A liquid containing 200 or 2000 JAU/mL is formulated by dilution of the standardized allergen of JCP original solution (10,000 JAU/mL; Torii Pharmaceutical Co., Ltd., Tokyo, Japan) containing the major allergen Cry j 1 (Yasueda et al., 1983) at 7.3–21.0 μg/mL (Yasueda et al., 1996) to provide the indicated potency.

Several clinical studies using small patient populations have shown that SLIT might be effective for the treatment of patients with JC pollinosis (Horiguchi et al., 2008; Okubo et al., 2008; Fujimura et al., 2011), in which SCIT products of standardized allergen extract of JCP were used for sublingual administration by SLIT. A modified liquid formulation of the SCIT product of standardized JCP-allergen extract has been developed (CEDARTOLEN®, Torii Pharmaceutical Co., Ltd.) for SLIT clinical applications.

In Japan, there is no specific regulatory guideline for allergen-specific immunotherapy drugs in Japanese new drug application process. Thereby, nonclinical data in allergen-specific immunotherapy drug should be applied to the regulatory requirements as well as a small molecule compound drug. Here, we investigated the safety of standardized JCP-allergen extract to evaluate its clinical application to SLIT. Genotoxicity studies, a repeated 26-week oral toxicity study and a repeated 7-day sublingual dose oral mucosal irritation study were conducted under Good Laboratory Practice (GLP) compliance.

2. Materials and methods

2.1. Standardized allergen extract of Japanese cedar pollen

Test liquids of 200 and 2000 JAU/mL (CEDARTOLEN®) were formulated by dilution of standardized JCP-allergen extract original solution (10,000 JAU/mL) containing 7.3–21.0 μg/mL Cry j 1 (Yasueda et al., 1996) to provide the indicated potency, respectively (Torii Pharmaceutical Co., Ltd.). In addition, 50% glycerin-containing sodium chloride was used for the vehicle control group. In the genotoxicity study (Ames bacterial reverse mutation test, *in vitro* chromosomal aberration test and *in vivo* micronucleus test), 10,000 JAU/mL of standardized JCP-allergen extract and its diluted solutions were used. Doses of 12,500, 25,000 and 50,000 JAU/kg were administered subcutaneously to rats in the bone marrow micronucleus test. In a repeated 26-week oral toxicity study, 200, 2000 and 10,000 JAU/mL of standardized JCP-allergen extract were used for oral administration. For the oral mucosal irritation study, 2000 JAU/mL of standardized JCP-allergen extract was administered sublingually, as the maximum dose used in the maintenance phase of SLIT in JC pollinosis.

The protein profile of JCP-allergen extract was assessed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis. Two major allergens (Cry j 1 and Cry j 2) were identified (Fig. 1A).

The allergen profile of JCP-allergen extract was confirmed by measuring immune responses in animals. Levels of the JCP specific-antibody in serum was significantly increased following i.p. administration with alum compared with naïve serum (data not shown). In addition, allergic specific reactions were evaluated using PCA (titer = 128, Fig. 1B).

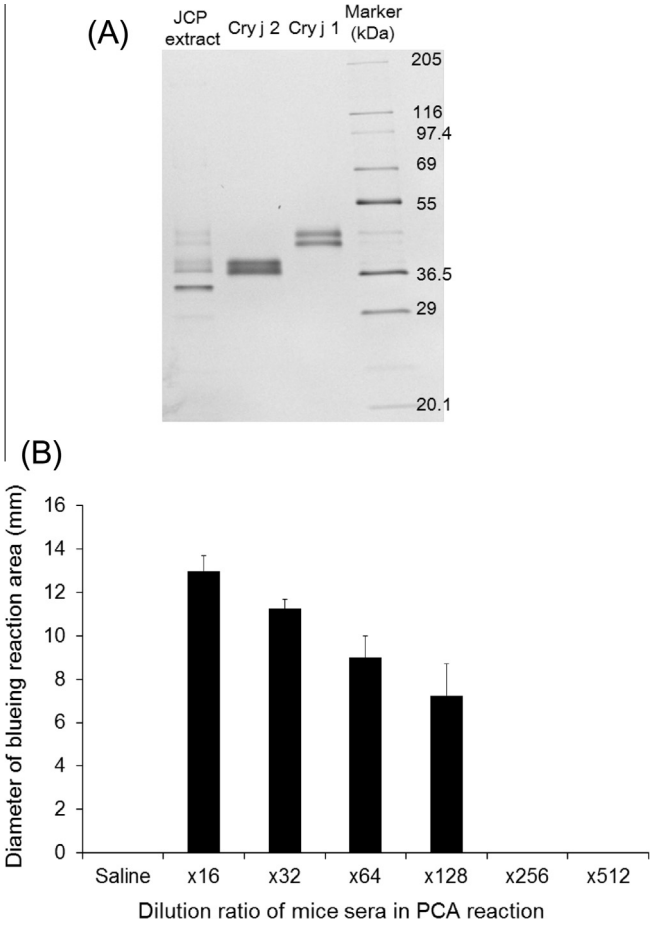

Fig. 1. SDS–PAGE and PCA reaction of JCP-allergen extract. JCP-allergen extract was assessed by SDS–PAGE analysis under non-reduction conditions followed by staining with silver (A). Rats were intradermally injected with serial dilutions of mouse anti-JCP-allergen extract serum. After 48 h, PCA reactions were elicited by intravenous injection of JCP-allergen extract. Values are expressed as mean ± SD of 4 sites in two animals (B).

2.2. Characterization of JCP-allergen extract

2.2.1. SDS–PAGE

JCP-allergen extract (2000 JAU/mL) was boiled in Tris–Glycine SDS sample buffer (TEFCO, Tokyo, Japan) and separated by PAGE on a Mini-Protean TGX Precast Gel (4–20% gradient; BIO-RAD, Hercules, CA). The gel was stained using a Silver Stain Kit (Wako Pure Chemical Industries, Osaka, Japan). Purified Cry j 1 (provided by Torii Pharmaceutical Co., Ltd.) and purified Cry j 2 (Hayashibara Co., Ltd., Okayama, Japan) were used as controls of the two major allergens in JCP.

2.2.2. Passive cutaneous anaphylaxis (PCA) reaction

All procedures used in the experiments were approved by the Animal Ethics Committee of Nihon Bioresearch Center, Gifu, Japan, in accordance with laboratory animal welfare guidelines. Two male Crl:CD (Sprague–Dawley) rats were used (9 weeks old, Charles River, Kanagawa, Japan). Serial dilutions of mouse anti-JCP-allergen extract sera were intradermally injected into shaved dorsal skin. The PCA reaction was measured after a 48-h sensitization period

Table 1
Design and implementation on safety evaluation studies of JCP-allergen extract.

Study	Species	Number of animals (scheduled)	Route	Test article	Dosage
Genotoxicity					
Ames test	–	–	–	Glycerin solution	0 JAU/plate
				10,000 JAU solution	31.25–1000 JAU/plate
Chromosomal aberration test	–	–	–	PBS	0 JAU/mL
				10,000 JAU solution	625–2500 JAU/mL
In vivo micronucleus test	Rats (male)	5	s.c.	Glycerin solution	0 JAU/kg
				10,000 JAU solution	12,500 JAU/kg
					25,000 JAU/kg
					50,000 JAU/kg
Repeated toxicology (26 weeks)	Rats (male/female)	12	p.o.	Glycerin solution	0 JAU/kg
				200 JAU solution	200 JAU/kg
				2000 JAU solution	2000 JAU/kg
				10,000 JAU solution	10,000 JAU/kg
Local irritation	Rabbits (male)	3	Sublingual	Glycerin solution	0 JAU/head
				2000 JAU solution	400 JAU/head

Treatment groups are compared with vehicle control group (0 JAU/plate and 0 JAU/kg). Vehicle group (0 JAU/plate and 0 JAU/kg) are compared with control group; distilled water for Ames test, chromosomal aberration test and *in vivo* micronucleus test; saline for repeated toxicology and local irritation. JAU, Japanese allergy units; s.c., subcutaneous; p.o., per os; PBS, phosphate buffered saline.

followed by intravenous administration of 10 mg (protein content) standardized JCP-allergen extract original solution (10,000 JAU/mL) in 1 mL/kg of 0.5% Evans blue. After 30 min, the diameter of blueing reaction in the dorsal skin area was expressed as a mean value (mm) of the major and minor diameters in two animals. The PCA titer was expressed by the reciprocal of the final dilution giving a positive (>5 mm) blueing reaction.

2.3. Genotoxicity studies

All studies were conducted in accordance with the Guidelines for Genotoxicity Studies in compliance with the GLP Regulations specified by the Japanese Ordinance.

2.3.1. Bacterial reverse mutation test (Ames test)

The mutagenic potential of JCP-allergen extract was evaluated in a bacterial reverse mutation test using four strains of *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and one strain of *Escherichia coli* WP2*uvrA*. The test solutions were prepared by dilution of standardized JCP-allergen extract original solution (10,000 JAU/mL) with 50% glycerin-containing sodium chloride and treated using the pre-incubation method with or without metabolic activation with S9 mix (Aroclor™ 1254-induced rat liver S9, Oriental Yeast Co., Ltd., Tokyo, Japan). Two plates per treatment were used in the dose-range finding test and main test. The plates were incubated at 37 °C for 48 h and the revertant colonies were counted and examined. The results were judged positive if the mean number of revertant colonies showed a dose dependent increase that was twofold greater than that of vehicle control at one or more concentrations.

A dose-range finding test was performed on all of strains both with and without S9 mix at the following doses of standardized JCP-allergen extract original solution (10,000 JAU/mL). The 100% standardized JCP-allergen extract original solution (10,000 JAU/mL) was selected as the highest dose and lower dose levels, 25.0%, 6.25%, 1.56%, 0.391%, 0.0977% and 0.0244% were set at a geometric ratio of 4. Distilled water was used as a negative control. The 50% glycerin solution containing sodium chloride was used as a vehicle control. Positive controls consisted of 0.01 μg/plate of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for TA100 and WP2*uvrA* without S9 mix activation; 0.1 μg/plate of AF-2 for TA98 without S9 mix activation; 0.5 μg/plate of sodium-azide for TA1535 without S9 mix activation; 80 μg/plate of 9-aminoacridine hydrochloride for TA1537 without S9 mix activation; 1, 2, 10, 0.5, and 2 μg/plate of 2-aminoanthracene (2AA) for TA100, TA1535, WP2*uvrA*, TA98, and TA1537, respectively with S9 mix activation.

Based on a dose-range finding study, the test solutions for the definitive test were selected as 3.125%, 6.25%, 12.5%, 25%, 50% and 100% of the standardized JCP-allergen extract original solution 10,000 JAU/mL (31.25, 62.5, 125, 250, 500 and 1000 JAU/plate) with or without S9 mix (Table 1).

2.3.2. In vitro chromosomal aberration test

The potential for JCP-allergen extract to induce chromosomal aberrations in cultured mammalian cells was evaluated using a fibroblast cell line from the lung of a Chinese hamster (CHL/IU; DS Pharma Biomedical, Osaka, Japan), either with or without S9 mix activation. The test solutions were prepared by dilution of standardized JCP-allergen extract original solution (10,000 JAU/mL) with distilled water and treated with or without metabolic activation with S9 mix (Aroclor™ 1254-induced rat liver S9, Oriental Yeast).

For short-time treatments, the treatment time was 6 h and viable cell counts and preparation of chromosome specimens were conducted 18 h after completion of treatment. For continuous treatment, the treatment time was 24 h and viable cell counts and preparation of chromosome specimens were conducted at completion of treatment. Two dishes were used per dose, condition (with or without S9 mix) and exposure time. The positive control articles were diluted with culture media to dimethylnitrosamine (DMN; Wako Pure Chemical Industries, Japan) set at a concentration of 500 μg/mL for the 6- and 24-h treatments without S9 mix and with mitomycin C (MMC; Kyowa Hakko Kirin, Tokyo, Japan) at a concentration of 0.1 μg/mL for the 6- and 24-h treatment without S9 mix. One hundred well-spread metaphases per dish were examined microscopically for structural and numerical chromosomal aberrations. The value of the incidence of cells with chromosomal aberrations was recorded according to the dish and the mean for each dose level was calculated. For each dose level, the mean incidences were evaluated as <5%: negative, ⩾5% and <10%: equivocal and ⩾10%: positive, with no statistical comparisons being made.

A dose-range finding study was conducted with vehicle (50% glycerin containing sodium chloride) at 12.5%, 25%, 50% and 100%. Test solutions in the definitive test were selected as 6.25%, 12.5% and 25% of the standardized JCP-allergen extract original solution (10,000 JAU/mL) for the 6-h treatment with or without S9 mix and 24-h treatment without S9 mix. Thus, the final doses

Table 2
Results of bacterial reverse mutation test (Ames test) conducted on JCP-allergen extract.

Concentration (JAU/plate)	Reverse colonies per plate (mean ± SD)									
	TA100		TA1535		WP2*uvrA*		TA98		TA1537	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Negative control (distilled water)	138	142	11	7	31	40	19	32	14	19
0	151	143	11	8	32	32	18	35	10	20
31.25	NT	154	NT	9	NT	26	NT	34	7	18
62.5	147	154	12	7	29	41	21	26	11	16
125	131	144	8	8	37	37	19	29	8	17
250	166	149	10	7	31	36	18	31	11	20
500	139	162	10	8	22	29	19	38	12	17
1000	156	NT	9	NT	36	NT	13	NT	10[c]	NT
Positive controls[a,b]	538	1068	478	283	132	1092	481	380	401	191

NT: not tested; JAU, Japanese allergy units.
[a] Positive controls −S9: 0.01 μg/plate 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for TA100 and WP2*uvrA*, 0.1 μg/plate AF-2 for TA98, 0.5 μg/plate sodium-azide for TA1535, and 80 μg/plate 9-aminoacridine hydrochloride for TA1537.
[b] Positive controls +S9: 0.5 1 and 10 μg/plate 2-aminoanthracene (2AA) for TA98, TA100 and WP2*uvrA* , respectively, and 2 μg/plate 2AA for TA1535 and TA1537.
[c] Bacterial growth inhibition was observed.

of JCP-allergen extract in the definitive test were 625, 1250 and 2500 JAU/mL (Table 2).

2.3.3. In vivo bone marrow micronucleus test

All procedures employed in the experiments were approved by the Animal Ethics Committee of Nihon Bioresearch Center, Japan in accordance with laboratory animal welfare guidelines.

Male Crl:CD (Sprague–Dawley) rats (Charles River, Japan), aged 5 weeks, with body weights of 135–148 g for a preliminary dose-finding study and 123–146 g for the definitive micronucleus study were housed in a 12-h light–dark cycle at a temperature of 21.7–24.5 °C, with *ad libitum* access to food (certified diet CRF-1, Oriental Yeast Co., Ltd.) and water. The 31 rats were stratified by body weight and randomly assigned to one of six groups (five rats/group) on the first day (day 1) prior to dosing. In the preliminary dose-finding study using doses of 12.5%, 25%, 50% and 100% standardized JCP-allergen extract, no cytotoxicity was observed for all doses. The 100% dose was set as the highest dose, and doses of 50% and 25% were selected as middle and low doses, respectively. Animals were dosed twice subcutaneously with 5 mL/kg at 24 h-intervals. The doses of JCP-allergen extract were 12,500, 25,000 and 50,000 JAU/kg (Table 1). A 50% glycerin solution containing sodium chloride and distilled water were administered subcutaneously as vehicle control and negative control, respectively. MMC (Kyowa Hakko Kirin) was used as a positive control and administered at a dose of 2 mg/kg/day by intraperitoneal administration.

All animals were observed for clinical signs, mortality and body weights were measured prior to and after dosing on days 1 and 2, and just prior the preparation of specimens. All animals were euthanized by 20 v/v% isoflurane anesthesia exposure 24 h after the final dosing. The femur was excised for the preparation of bone marrow cell suspensions. Slides coated with acridine orange were prepared for incidence of micronucleated polychromatic erythrocyte (%MNPCE) analysis among 2000 polychromatic erythrocytes per animal. To assess bone marrow cell proliferation, the ratio of reticulocytes (%RET) in 1000 erythrocytes was analyzed using slides coated with new methylene blue for Giemsa staining.

Statistical analyses were performed between the negative control group and vehicle control group, negative control group and positive control group, and between vehicle control group and test article treated groups. The conditional binomial test (Kastenbaum–Bowman test) was performed on %MNPCE and the Student's *t*-test was performed on %RET.

2.4. Repeated 26-week oral toxicity study

This study was conducted according to the Guidelines for Repeated-dose Toxicity Studies in compliance with GLP Regulations specified by the Japanese Ordinance. All procedures employed in the experiments were approved by the Animal Ethics Committee of Mitsubishi Chemical Medience Corporation, Kumamoto, Japan, in accordance with laboratory animal welfare guidelines.

2.4.1. Animals and husbandry

Specific pathogen-free (SPF) Crl:CD (Sprague–Dawley) rats were supplied by Charles River Laboratories (Shiga, Japan) at 4 weeks of age. On arrival, body weights of 92 male and female rats were measured and were within the range of 70.1–90.8 g and 67.5–82.8 g, respectively. All animals were acclimatized to the testing environment for 12–14 days. Dosing of the test articles was initiated at 6 weeks of age. Animals were housed in stainless-steel cages under controlled environmental conditions with temperatures between 22.1 and 24.5 °C, relative humidity of 21.1–77.3%, air ventilation of 10–20 times/h, illumination 12-h per day (light on at 7:00 a.m. and off at 7:00 p.m.), and feed (certified diet CRF-1, Oriental Yeast Co., Ltd.) and water were available *ad libitum*.

2.4.2. Treatment of test articles

Dose levels were selected based on the repeated 2-week oral toxicity study (non-GLP, unpublished) conducted with the test article. In this study, rats (12/[sex group]) received daily oral doses of 200, 2000 and 10,000 JAU/mL of standardized JCP-allergen extract by gastric gavage at a constant dosing volume of 1 mL/kg for 26 weeks, resulting in 0 (vehicle control group), 200, 2000, and 10,000 JAU/kg/day (Table 1). Saline was used for control group. The 50% glycerin solution containing sodium chloride was used for vehicle control group indicating as 0 JAU/kg/day.

Oral administration was selected for administration, as this is the route used in the clinic (droplets is placed under the tongue, maintained for 2 min and then swallowed). In this study, 200 JAU/mL, 2000 JAU/mL and standardized JCP-allergen extract original solution 10,000 JAU/mL (Torii Pharmaceutical Co., Ltd.) were used. The vehicle consisted of JCP-allergen extract with 50% glycerin containing sodium chloride. The day of administration was defined as 'day 1'. The week of administration was defined as 'week 1'.

2.4.3. Clinical observations, body weights and food consumption

All animals were observed twice daily (before dosing and after dosing) for clinical signs during the dosing period and once daily during the recovery period. Body weights and food consumption were measured immediately before the initiation of dosing on day 1, once a week during the dosing period and the recovery period. A feeding vessel containing food was weighed and set in the cage. The remaining diet was weighed at 24 h to calculate food consumption.

2.4.4. Ophthalmoscopy

Ophthalmoscopy was conducted on all animals from each group before the initiation of dosing: $n = 12$ animals in week 13 and 26 before dosing per group, and all animals during the recovery period. External eye observations were performed and followed by examination for light reflex using direct ophthalmoscope (Heine Optotechnik, Herrsching, Germany). After observation of the cornea, iris, conjunctiva, lens, and vitreous body using a slit-lamp (SL-15, Kowa Company, Nagoya, Japan), the ocular fundus was examined using binocular indirect ophthalmoscope (Heine Optotechnik).

2.4.5. Urinalysis

Urinalysis was performed for 12 animals per group at weeks 13 and 26 of the dosing period, and for all animals in the recovery period. Fresh urine was collected in the morning before dosing and the following parameters were examined using dipstick analysis: pH, protein, glucose, ketone bodies, bilirubin, occult blood and urobilinogen. Accumulated urine was collected overnight for 24 h and the following parameters were examined: volume, color, osmotic pressure (Osmomat 030-D-RS, Genotec, Berlin, Germany), sodium, potassium and chloride ion concentration (EA07, A&T Corporation, Yokohama, Japan). Urine sediments were microscopically examined for epithelial cells, erythrocytes, leukocytes, casts and crystals.

2.4.6. Hematology and blood chemistry

Hematology was conducted using blood and plasma obtained at necropsy. The animals were fasted for 16–23 h before blood sampling. Ten hematological parameters were measured: numbers of leukocytes and erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte counts, platelets and differential leukocyte counts, using a hematology system (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL). Two hematological parameters – prothrombin time and activated partial thromboplastin time – were determined by an autocoagulometer (Sysmex CA-5000, Sysmex Corporation, Kobe, Japan).

Blood chemistry was conducted using sera obtained at necropsy. Estimations of various biochemical parameters: total protein, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides, phospholipids, glucose, blood urea nitrogen, creatinine, inorganic phosphorus and calcium (Ca), were performed using an autoanalyzer (7170, Hitachi Ltd., Tokyo, Japan). Serum protein fractions (total protein × ratio), albumin and albumin/globulin ratio (A/G ratio) were estimated by an electrophoresis system (AES320, Beckman Coulter, Brea, CA. Sodium (Na), potassium (K) and chloride (Cl) were estimated by an electrolyte analyzer (EA07, A&T).

2.4.7. Necropsy, organ weights and histopathology

Following euthanasia by exsanguination from the abdominal inferior vena cava under pentobarbital anesthesia, all animals were examined in detail for gross lesions. Submandibular glands (with sublingual glands), liver, lungs (with bronchi), thymus, spleen, heart, kidneys, prostate gland, seminal vesicle, testes, epididymis, ovaries, uterus, pituitary, thyroids (with parathyroids), adrenals and brain were weighed after macroscopic examination, and the relative weight of each organ to the final body weight was calculated. For histopathology, after processing sections and staining with hematoxylin and eosin (HE) for light microscopy, the tongue, esophagus, stomach, duodenum, jejunum, ileum (with Peyer's patch), cecum, colon, rectum, parotid gland, pancreas, trachea, lower jaw lymph nodes, mesentery lymph node, aorta (chest), bladder, vagina, mammary gland, spinal cord (cervical region), optic nerves, sciatic nerve, eyeballs, Harderian glands, biceps femoris muscle, sternum (with bone marrow), femoral fracture (with bone marrow), skin (lower abdomen) and the organs and tissues above that were examined macroscopically were analyzed histopathologically. Organ weights were expressed as relative weights versus body weights (100 g) measured at necropsy day.

2.4.8. Statistical analysis

Statistical analysis was performed for body weight, food consumption, urinalysis hematology, blood chemistry and organ weights using the MiTOX-PPL system (Mitsui Zosen Systems Research Inc., Chiba, Japan). For multiple comparisons, mean values and standard deviations were calculated for vehicle control group and treatment groups. The homogeneity of variances was evaluated using Bartlett's test (significance level: 5%). In the case of homogeneous data, Dunnett's multiple-comparison test was performed to compare with the vehicle control group. In the case of heterogeneous data, Steel's multiple-comparison test was performed to compare with the vehicle control group. In cases of multiple comparisons, the two-sided test was used ($P < 0.05$, $P < 0.01$). For comparisons between two groups, mean values and standard deviations (SD) were calculated for control and vehicle control groups. The homogeneity of variances was evaluated using the *F*-test (significance level: 5%). For homogeneous data, the Student's *t*-test was used to compare with the control group. For heterogeneous data, Welch's test was performed to compare with the control group. For comparison between the two groups, a two-sided test was used ($P < 0.05$, $P < 0.01$).

2.5. Repeated 7-day sublingual dose oral mucosal irritation study

This study was conducted under GLP compliance with approval of the Animal Ethics Committee of Mitsubishi Chemical Medience Corporation in accordance with laboratory animal welfare guidelines.

2.5.1. Animals and husbandry

SPF male Kbs: New Zealand White rabbits of 17 weeks of age were supplied by Kitayama Labes Co., Ltd. (Nagano, Japan). Upon arrival, ten male rabbits were weighed (range of 2829–3315 g). All animals were acclimatized to the testing environment for 6 days. Dosing of the test article was initiated at 18 weeks of age. The body weight range at initiation of dosing was 3056–3485 g. Animals were housed in stainless-steel cages under controlled environmental conditions with temperatures between of 23.1 and 24.4 °C, relative humidity of 47.6–61.6%, air ventilation of 10–20 times/h, illumination 12-h per day (light on at 7:00 a.m. and off at 7:00 p.m.), and feed (LRC6, Oriental Yeast Co., Ltd.) and water were available *ad libitum*.

2.5.2. Treatment of test article

Three male rabbits per group received daily sublingual administration for 7 days under sedation by intramuscular injection of medetomidine, with saline (control group), 50% glycerin solution containing sodium chloride (vehicle control group) or JCP-allergen extract (2000 JAU/mL). The 0.2 mL test solution was administered to the sublingual area for 20 min followed by washing with

30 mL of saline. The 2000 JAU/mL dose was used in this study as it was the maximum dose in the maintenance phase of SLIT in JC pollinosis. The dosing volume of 0.2 mL/head was the maximum technical dose for sublingual administration to rabbits, resulting in 400 JAU/head (Table 1).

Sublingual administration was selected as it is the intended clinical route.

2.5.3. Clinical observations and body weights

All animals were observed twice daily (before dosing and after dosing) for clinical signs during the dosing period (from days 1 to 7) and once at day 8. Body weights were measured immediately before the initiation of dosing on day 1 and on the last day of dosing (day 8).

2.5.4. Macroscopic observation of administration site

Macroscopic observation of the administration site was performed under sedation before administration from days 1 to 8. The administration site in the oral cavity was estimated for irritative scores according to Ohbayashi's criteria in rabbits (Ohbayashi et al., 1998) as follows: 0: No reaction; 1: Slight erythema (barely perceptible); 2: Clear erythema (well defined erythema); 3: Slight white fur (barely perceptible); 4: Mild white fur (well defined white fur); 5: Moderate white fur (thick white fur raised less than one half); 6: Severe white fur (thick white fur raised more than one-half).

2.5.5. Histopathology of administration site

Histopathology was conducted at the administration site in the oral area of the rabbits. At day 8 after clinical observation, all rabbits were anesthetized with intravenous injection of sodium pentobarbital (30 mg/kg) followed by macroscopic observation of administration site, and euthanasia was performed. Oral mucosal tissues including tongue, buccal and mouth floor were removed and fixed in 10 vol% neutral buffered formalin. The mucosa tissues were embedded in paraffin, sectioned, stained with HE and examined microscopically.

3. Results

3.1. Genotoxicity studies

3.1.1. Bacterial reverse mutation test (Ames test)

In the dose-finding test, inhibition of bacterial growth was observed on the plates at 100% of JCP-allergen extract for TA1537 without S9 mix. However, no inhibition of bacterial growth inhibition was observed at any dose tested for TA98, TA100, TA1535, TA1537 and WP2*uvrA* with or without S9 mix and TA 1537 with S9 mix. No precipitation of the test article was observed at the start or end of the treatment in all conditions. The test article did not increase the number of revertant colonies of any strain $\geqslant$twofold when compared with the vehicle control with or without S9 mix (data not shown).

The definitive test was performed using the test solutions of 3.125%, 6.25%, 12.5%, 25%, 50% and 100% of JCP-allergen extract on all strains without S9 mix based on the results of the dose-finding test. Thus, the final amounts of JCP-allergen extract were 31.25, 62.5, 125, 250, 500 and 1000 JAU/plate. In the definitive test, inhibition of bacterial growth was observed on the plates at 1000 JAU/plate of JCP-allergen extract for TA1537 without S9 mix. However, no inhibition of bacterial growth inhibition was observed at any dose tested for TA98, TA100, TA1535, TA1537 and WP2*uvrA* with or without S9 mix and TA1537 with S9 mix. The test article did not increase the number of revertant colonies of any strain by $\geqslant$two-fold compared with the vehicle control with or without S9 mix (Table 2). No precipitation of the test article was observed either at the start or end of the treatments under all the conditions.

3.1.2. In vitro chromosomal aberration test

The dose-finding test of the chromosomal aberration test was conducted with vehicle (50% glycerin containing sodium chloride) at doses of 12.5%, 25%, 50% and 100%. The incidence of cells with numerical chromosomal aberrations was less than 5% under all conditions. The 50% and 100% vehicle in the short treatment without S9 mix was equivocal and 100% of vehicle for continuous treatment was positive for the incidence of structural chromosomal aberrations of 5%, 8% and 16%, respectively (data not shown). Cell viability was >90% with no cytotoxicity under all conditions in the dose-finding test. No precipitation of the test article was observed at the start or end of treatments under all the conditions in the dose-finding test.

In the definitive test, test solutions were 6.25%, 12.5% and 25% of JCP-allergen extract, thus the final doses of treatment were 625, 1250 and 2500 JAU/mL. The incidence of cells with numerical and structural chromosomal aberrations was less than 5% in all JCP treatment group with short treatment or continuous treatment (Table 3). The negative control induced no chromosomal aberrations as expected. The positive control agents increased the percentage of cells with numerical or structural chromosomal aberrations. Thus, JCP-allergen extract was not considered clastogenic in this test system. No precipitation of the test article was observed at the start or end of the treatments in all the conditions.

3.1.3. In vivo bone marrow micronucleus test

In the *in vivo* micronucleus test, no mortality was observed following two administrations of JCP-allergen extract at 12,500, 25,000 or 50,000 JAU/kg. No clinical signs or change in body weight was observed when compared with the vehicle control group. The incidence of MNPCE and ratio of RET are shown in Table 4.

%MNPCE in the JCP treatment group showed no significant difference compared with the vehicle control group and no dose-dependent change. No significant difference was observed for %RET compared with the vehicle control group. Administration of the positive control significantly increased the incidence of micronucleated cells as MNPCE and resulted in a significant decrease in the ratio of immature erythrocytes as RET compared with the negative control group. There was no possibility of the vehicle having an effect on the test system because there was no significant difference in the incidence of MNPCE and %RET compared with the negative control. Thus, it was concluded that JCP-allergen extract did not induce micronuclei in PCE of bone marrow of rats treated with up to 50,000 JAU/kg by subcutaneous administration.

3.2. Repeated 26-week oral toxicity study in rats

3.2.1. Clinical observations, body weights and food consumption

No test article related mortality was observed in any JCP-allergen extract treatment group and there were no treatment related changes in clinical observations, body weights (Fig. 2) and food consumption (Fig. 3) during the 26-week dosing period and 4-week recovery period. Non-treatment related changes, callus in hind limb, were observed in one male each from the vehicle control group and 2000 JAU/kg group.

3.2.2. Ophthalmoscopy and urinalysis

Ophthalmologic examinations did not reveal treatment-related ocular lesions in any of the animals tested (data not shown). Urinalysis and microscopic examination of sediment in the vehicle control group indicated increased sodium ions and chloride ions compared with the control group at 13 and 26 weeks, but the findings were not observed in the recovery period. No changes were

Table 3
Results of *in vitro* mammalian chromosomal aberration test conducted on JCP-allergen extract.

Concentration (JAU/mL)	Numerical aberration	Structural aberration							
	Number of polyploid cells (%)	Types and numbers (cumulative)[a]						Number of cells with chromosomal aberration (%)[b]	
		gap	ctb	csb	cte	cse	frg	+g	−g
Short-time treatment, −S9									
Negative control (distilled water)	1 (0.5)	0	0	0	1	0	0	1 (0.5)	1 (0.5)
625	2(1.0)	0	1	0	1	1	0	3 (1.5)	3 (1.5)
1250	3 (1.5)	0	1	0	0	0	0	1 (0.5)	1 (0.5)
2500	1 (0.5)	0	0	0	0	0	0	0 (0)	0 (0)
Positive control (0.1 μg/mL mitomycin C)	1 (0.5)	0	63	2	69	1	0	108 (54.0)	108 (54.0)
Short-time treatment, +S9									
Negative control (distilled water)	1 (0.5)	0	1	0	1	0	0	2 (1.0)	2 (1.0)
625	2 (1.0)	0	1	0	1	0	0	2 (1.0)	2 (1.0)
1250	1 (0.5)	0	0	0	0	0	0	0 (0)	0 (0)
2500	3 (1.5)	0	0	0	0	0	0	0 (0)	0 (0)
Positive control (500 μg/mL dimethylnitrosamine)	1 (0.5)	0	52	2	85	0	0	115 (57.5)	115 (57.5)
Continuous treatment									
Negative control (distilled water)	1 (0.5)	0	2	0	0	0	0	2 (1.0)	2 (1.0)
625	0 (0)	0	0	0	0	0	0	0 (0)	0 (0)
1250	2 (1.0)	0	0	0	0	0	0	0 (0)	0 (0)
2500	2 (1.0)	0	0	0	1	0	0	1 (0.5)	1 (0.5)
Positive control (0.05 μg/mL mitomycin C)	0 (0)	0	45	2	36	0	0	81 (40.5)	81 (40.5)

−S9, without metabolic activation; +S9, with metabolic activation; JAU, Japanese allergy units; a: ctb, chromatid break; csb, chromosome break; cte: chromatid exchange; cse: chromosome exchange; frg, fragmentation; b: (+g): total aberrant cells including the gap; (−g): total aberrant cells excluding the gap.

Table 4
Results of *in vivo* mammalian erythrocyte micronucleus test in rats administered JCP-allergen extract.

Dosage (JAU/kg)	%MNPCE	%RET
Negative control (distilled water)	0.09 ± 0.04	76.7 ± 0.54
0	0.08 ± 0.03	76.1 ± 0.84
12,500	0.12 ± 0.07	76.3 ± 0.97
25,000	0.13 ± 0.04	76.8 ± 0.95
50,000	0.07 ± 0.06	76.7 ± 2.30
Positive control (2 mg/kg mitomycin C)	3.40 ± 0.34**	66.3 ± 3.02##

Animals were dosed twice subcutaneously with JCP-allergen extract and negative control at a 24-h interval. Positive control was dosing by intraperitoneal administration. Values are expressed as mean ± SD of 5 animals in each group. **$p < 0.01$ (significantly different from negative control); ##$p < 0.01$ (significantly different from negative control). %MNPCE, micronucleated polychromatic erythrocyte; %RET, ratio of reticulocytes; JAU, Japanese allergy units.

observed in the JCP-allergen extract treatment groups compared with the vehicle control group at 13 weeks, 26 weeks and the recovery period (data not shown).

3.2.3. Hematology and blood chemistry

Results for hematology are shown in Table 5. Treatment-related changes were not observed in the JCP-allergen extract treatment group compared with the vehicle control group. The mean platelet counts was significantly increased in 10,000 JAU/kg group male rats but was not considered treatment related because the individual counts of rats ($n = 12$) were within the range measured in the control group. The mean lymphocyte ratio was significantly increased in 10,000 JAU/kg group male rats and the mean of monocyte ratio was significantly decreased in 200 JAU/kg, 2000 JAU/kg and 10,000 JAU/kg group male rats. These were not considered treatment related changes because no changes were seen in counts of leucocytes and monocytes. Increased numbers of erythrocytes observed in 200 JAU/kg male rats was not dose-dependent.

Results of blood chemistry are shown in Table 6. Treatment-related changes were not observed in JCP treatment groups compared with the vehicle control group. The following changes were not dose-dependent: decreased Cl in 200 JAU/kg females, increased γ-globulin ratio in 2,000 JAU/kg males and decreased

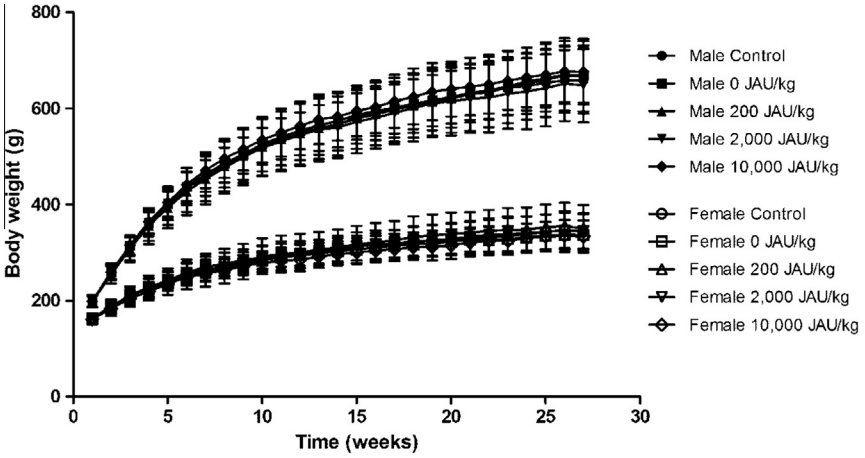

Fig. 2. Body weights of rats following oral administration of JCP-allergen extract. Rats were administered 200, 2000 or 10,000 JAU/kg of JCP-allergen extract, vehicle (0 JAU/kg) or saline (control) daily for 26 weeks. Values are expressed as mean ± SD of 18 animals for all groups except for female control group where $n = 17$ and 200 JAU/kg and 2000 JAU/kg groups where $n = 12$.

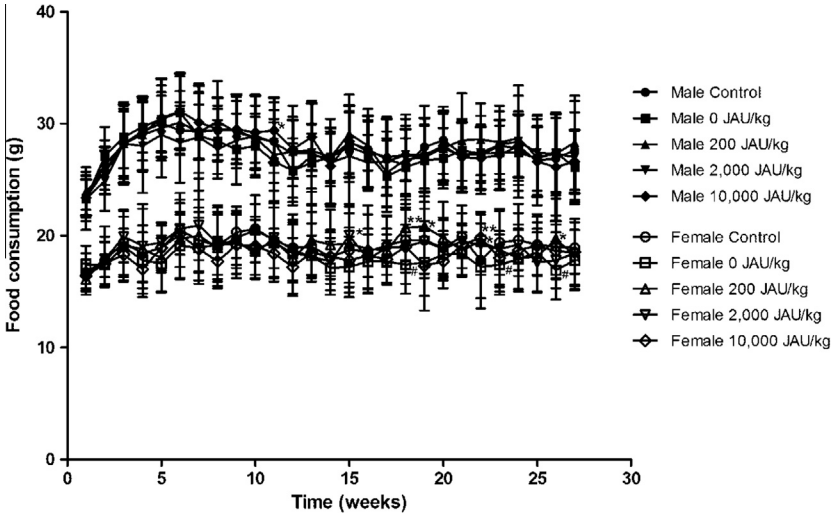

Fig. 3. Food consumption of rats following administration of JCP-allergen extract. Rats were orally administered 200, 2000 and 10,000 JAU/kg of JCP-allergen extract, vehicle (0 JAU/kg) or saline (control) daily for 26 weeks. Values are expressed as mean ± SD of 18 animals in all groups except female controls where $n = 17$ and 200 JAU/kg and 2000 JAU/kg groups where $n = 12$. $^{\#}p < 0.05$ (significantly different from control); $^{*}p < 0.05$, $^{**}p < 0.01$ (significantly different from 0 JAU/kg).

Table 5
Hematological findings of rats following 26 weeks oral administration of JCP-allergen extract.

Dosage (JAU/kg)	Male					Female				
	Control	0	200	2000	10,000	Control	0	200	2000	10,000
Leukocytes ($10^3/\mu L$)	9.49 ± 3.43	8.71 ± 2.51	9.36 ± 2.05	9.09 ± 1.32	8.09 ± 1.31	5.57 ± 1.22	4.64$^{\#}$ ± 0.88	4.56 ± 0.80	4.82 ± 1.25	4.95 ± 1.04
Erythrocytes ($10^4/\mu L$)	840 ± 47	846 ± 24	876* ± 21	849 ± 38	849 ± 54	757 ± 22	757 ± 32	762 ± 24	745 ± 30	759 ± 43
Hemoglobin (g/dL)	14.8 ± 0.8	14.7 ± 0.5	15.0 ± 0.5	14.8 ± 0.5	14.7 ± 0.8	14.3 ± 0.4	14.4 ± 0.8	14.5 ± 0.6	14.3 ± 0.3	14.4 ± 0.6
Hematocrit (%)	42.4 ± 2.2	41.9 ± 1.3	42.9 ± 1.0	42.8 ± 1.6	42.0 ± 2.0	40.4 ± 1.1	40.8 ± 2.0	41.0 ± 1.3	40.2 ± 0.9	40.6 ± 1.8
Mean corpuscular volume (fL)	50.5 ± 1.5	49.5 ± 1.0	49.1 ± 1.1	50.4 ± 1.5	49.6 ± 2.4	53.4 ± 1.6	53.9 ± 1.6	53.8 ± 2.0	54.0 ± 1.9	53.5 ± 1.7
Mean corpuscular hemoglobin: MCH (pg)	17.6 ± 0.6	17.4 ± 0.4	17.1 ± 0.6	17.5 ± 0.6	17.3 ± 0.9	18.8 ± 0.7	19.1 ± 0.5	19.1 ± 1.0	19.1 ± 0.7	19.0 ± 0.6
MCH concentration (g/dL)	34.9 ± 0.6	35.1 ± 0.3	34.8 ± 0.6	34.7 ± 0.4	34.9 ± 0.5	35.3 ± 0.5	35.4 ± 0.6	35.4 ± 0.8	35.5 ± 0.8	35.4 ± 0.5
Reticulocytes (%)	2.1 ± 1.1	1.8 ± 0.2	1.7 ± 0.3	1.8 ± 0.5	2.2 ± 1.9	1.9 ± 0.4	1.9 ± 0.3	1.8 ± 0.4	1.9 ± 0.2	1.9 ± 0.2
Platelets ($10^4/\mu L$)	104.9 ± 15.5	95.0 ± 6.8	97.4 ± 13.1	101.8 ± 9.0	105.7* ± 12.0	92.9 ± 10.5	94.9 ± 8.5	88.4 ± 7.2	96.7 ± 11.5	98.6 ± 7.2
Prothrombin time (sec)	11.5 ± 1.2	11.5 ± 1.5	11.6 ± 1.3	11.2 ± 0.5	11.0 ± 0.4	9.5 ± 0.2	9.3 ± 0.2	9.3 ± 0.2	9.4 ± 0.3	9.3 ± 0.2
Activated partial thromboplastin time (sec)	20.8 ± 1.7	20.9 ± 2.2	21.2 ± 1.4	21.3 ± 1.0	21.2 ± 1.4	18.4 ± 1.3	18.6 ± 1.0	18.5 ± 0.8	19.2 ± 0.8	18.7 ± 1.6
Eosinophils (%)	1.9 ± 1.0	1.9 ± 0.5	1.8 ± 0.4	1.7 ± 0.6	1.9 ± 0.6	2.2 ± 1.1	2.1 ± 0.7	2.0 ± 0.6	1.8 ± 0.5	1.8 ± 0.8
Neutrophils (%)	29.3 ± 13.5	24.9 ± 8.6	20.2 ± 7.5	19.4 ± 3.3	18.8 ± 4.4	20.3 ± 9.4	18.2 ± 4.4	19 ± 9.4	26.4 ± 10.8	17.9 ± 7.4
Lymphocytes (%)	64.7 ± 14.2	68.9 ± 8.6	74.6 ± 7.7	75.5 ± 4.1	75.6*±5.2	73.2 ± 10.0	75.8 ± 4.6	75.0 ± 10.2	67.3 ± 12.0	76.7 ± 8.5
Basophils (%)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
Monocytes (%)	2.7 ± 0.7	3.2 ± 0.9	2.3* ± 0.5	2.1** ± 0.6	2.3* ± 0.8	3.0 ± 0.7	2.5 ± 0.5	2.7 ± 0.7	3.0 ± 1.2	2.6 ± 0.9
Large unstained cells (%)	1.0 ± 0.6	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.5	1.0 ± 0.5	0.9 ± 0.5	0.9 ± 0.6	0.9 ± 0.5	1.0 ± 0.7	0.7 ± 0.4

Values are expressed as mean ± SD of 12 animals in each group. $^{\#}p < 0.05$ (significantly different from control). $^{*}p < 0.05$, $^{**}p < 0.01$ (significantly different from 0 JAU/kg).

A/G ratio, albumin ratio and total bilirubin in 2000 JAU/kg females. At the recovery period, no treatment related changes in hematology and blood chemistry were observed in the vehicle control group compared with the control group and between the JCP treatment group and vehicle control group (data not shown).

3.2.4. Necropsy, organ weight and histopathology

No gross abnormalities were detected for rats in any group by necropsy at the treatment period and recovery period (data not shown). Calluses in hind limbs were observed in one male each from the vehicle control group and 2000 JAU/kg group corresponding to the clinical observations. A yellow–brown neoplasm was observed in the abdominal cavity of a male rat in the control group.

There were no treatment-related differences in organ weights between the vehicle control and 10,000 JAU/kg groups in male (Fig. 4A) and female (Fig. 4B). A decrease in relative organ weight of the thymus was observed in 200 JAU/kg group females and increased relative organ weight of the kidney (left) was observed in 2000 JAU/mL males. These were not dose-dependent changes.

Results for histopathology are shown in Table 7. Histopathology revealed no systemic toxicological changes in the organs of vehicle and 10,000 JAU/kg groups, although some changes in organs occurred sporadically (Table 7). Except for the stomach findings, these changes were considered incidental because they occurred in the control or vehicle control groups, or with a comparable frequency and similar level to the control group. The stomach is the locally affected area when drugs are administered by oral treatment. Hyperplasia in the forestomach and increased globule leukocytes in the glandular stomach were observed at comparable levels between the vehicle control group and 10,000 JAU/kg group. The stomach findings disappeared during the recovery period suggesting they were temporary. Histopathology of the stomach at the recovery period indicated no changes (data not shown).

3.3. Repeated 7-days sublingual dose oral mucosal irritation study in rabbits

Oral mucosal irritation was evaluated using a repeated sublingual dose study in rabbits. In a preliminary study, induction of

Table 6
Blood chemical findings of rats following 26-week oral administration of JCP-allergen extract.

Dosage (JAU/kg)	Males					Females				
	Control	0	200	2000	10,000	Control	0	200	2000	10,000
Total protein (g/dL)	5.7 ± 0.2	5.8 ± 0.3	6.0 ± 0.3	5.9 ± 0.2	5.9 ± 0.2	6.4 ± 0.5	6.4 ± 0.4	6.5 ± 0.3	6.5 ± 0.4	6.6 ± 0.3
Albumin (g/dL)	2.69 ± 0.17	2.76 ± 0.14	2.83 ± 0.17	2.75 ± 0.11	2.8 ± 0.16	3.65 ± 0.40	3.68 ± 0.33	3.78 ± 0.23	3.61 ± 0.33	3.79 ± 0.20
A/G ratio	0.89 ± 0.09	0.92 ± 0.07	0.91 ± 0.08	0.87 ± 0.06	0.89 ± 0.08	1.33 ± 0.13	1.38 ± 0.13	1.4 ± 0.13	1.25* ± 0.16	1.36 ± 0.09
α1-Globulin (%)	24.2 ± 2.0	25.1 ± 1.8	24.7 ± 2.3	24.6 ± 1.8	25.2 ± 2.1	17.2 ± 1.1	16.5 ± 1.9	16.4 ± 1.4	17.7 ± 1.1	16.9 ± 1.4
α2-Globulin (%)	5.4 ± 0.8	5.1 ± 0.5	5.0 ± 0.4	5.2 ± 0.5	4.9 ± 0.6	4.9 ± 0.5	4.8 ± 0.6	4.9 ± 0.6	5.0 ± 0.6	4.6 ± 0.6
β-Globulin (%)	17.5 ± 1.9	17.1 ± 0.9	17.2 ± 0.8	17.5 ± 1.1	17.3 ± 0.9	14.5 ± 1.8	14.2 ± 1.0	14.1 ± 1.2	15.0 ± 1.9	14.3 ± 1.0
γ-Globulin (%)	5.9 ± 1.6	5.0 ± 1.0	5.5 ± 0.8	6.2* ± 1.1	5.5 ± 1.0	6.5 ± 1.0	6.6 ± 1.3	6.4 ± 0.7	7.1 ± 1.1	6.7 ± 0.8
Albumin (%)	47.0 ± 2.6	47.7 ± 2.0	47.6 ± 2.2	46.6 ± 1.9	47.1 ± 2.1	56.9 ± 2.5	57.9 ± 2.3	58.2 ± 2.2	55.2* ± 3.1	57.6 ± 1.6
Total bilirubin (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0* ± 0.0	0.1 ± 0.1
Aspartate aminotransferase (IU/L)	90 ± 31	83 ± 10	79 ± 17	81 ± 26	78 ± 8	103 ± 58	103 ± 36	148 ± 159	146 ± 142	106 ± 56
Alanine aminotransferase (IU/L)	29 ± 16	21 ± 4	23 ± 6	26 ± 19	21 ± 4	36 ± 21	35 ± 18	53 ± 57	56 ± 64	40 ± 24
Alkaline phosphatase (IU/L)	138 ± 33	123 ± 13	124 ± 24	120 ± 24	130 ± 33	53 ± 12	51 ± 12	52 ± 11	54 ± 21	51 ± 12
Total cholesterol (mg/dL)	85 ± 19	78 ± 14	87 ± 21	76 ± 11	89 ± 16	94 ± 25	86 ± 12	98 ± 19	100 ± 20	91 ± 9
Triglycerides (mg/dL)	78 ± 39	76 ± 31	92 ± 33	67 ± 25	87 ± 47	39 ± 18	41 ± 21	43 ± 15	43 ± 28	45 ± 27
Phospholipids (mg/dL)	140 ± 24	131 ± 15	143 ± 23	127 ± 15	142 ± 22	182 ± 39	172 ± 25	189 ± 30	190 ± 35	182 ± 21
Glucose (mg/dL)	125 ± 13	128 ± 12	126 ± 9	123 ± 8	126 ± 23	119 ± 6	117 ± 13	116 ± 6	117 ± 6	120 ± 9
Blood urea nitrogen (mg/dL)	16.2 ± 3.7	16.5 ± 3.2	14.8 ± 2.0	15.8 ± 2.1	16.9 ± 1.4	16.1 ± 2.4	17.8 ± 2.9	16.3 ± 2.4	15.3 ± 1.7	17.1 ± 3.4
Creatinine (mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.1
Inorganic phosphorus (mg/dL)	6 ± 0.7	5.9 ± 0.7	5.7 ± 0.6	6.0 ± 0.4	5.9 ± 0.6	4.5 ± 0.7	4.6 ± 0.9	4.9 ± 0.8	4.8 ± 0.9	4.9 ± 1.0
Ca (mg/dL)	10.2 ± 0.3	10.2 ± 0.3	10.4 ± 0.3	10.2 ± 0.3	10.4 ± 0.3	10.5 ± 0.3	10.4 ± 0.4	10.6 ± 0.3	10.5 ± 0.2	10.5 ± 0.2
Na (mEq/L)	146 ± 0.8	145.9 ± 0.7	145.8 ± 0.9	146.4 ± 0.9	145.9 ± 1.0	144.4 ± 0.7	144.6 ± 0.9	143.6 ± 1.3	144.6 ± 1.1	144.9 ± 0.9
K (mEq/L)	4.51 ± 0.18	4.53 ± 0.28	4.42 ± 0.16	4.57 ± 0.17	4.67 ± 0.21	3.89 ± 0.21	4.05 ± 0.21	3.95 ± 0.21	4.0 ± 0.23	3.99 ± 0.32
Cl (mEq/L)	106.4 ± 1.4	106.1 ± 1.5	105.9 ± 1.1	106.4 ± 1.3	106 ± 1.1	106.8 ± 1.5	107.6 ± 1.5	106.2* ± 1.1	107.2 ± 0.8	107.4 ± 1.1

Values are expressed as mean ± SD of 12 animals in each group. *$p < 0.05$ (significantly different from 0 JAU/kg). JAU, Japanese allergy units.

irritative response was observed by positive control (35% sodium lauryl sulfate) in the oral mucosa of rabbits (data not shown). In this study, there were no treatment-related changes in the clinical observations and body weight of treated rabbits during treatment with 2000 JAU/mL of JCP-allergen extract (Table 8).

The macroscopic observation of the administration site revealed no irritation in the sublingual mucosa of animals throughout the dosing period. In addition, histopathology of oral mucosal tissues including tongue, buccal and mouth floor revealed no abnormalities in the treatment groups, although there was mild lymphocytic infiltration in the mucosa of the mouth floor of one animal in the control group (data not shown).

4. Discussion

A position paper of SLIT (Canonica et al., 2014) reported the mechanism of allergen specific immunotherapy involves changes in the induction pattern of immunoglobulins such as IgG4, inhibition of effector cells such as mast cells and basophils, as well as modification of T cell responses to allergens. Although changes in specific immunoglobulins is an important factor when determining immunotherapy mechanisms, JCP-specific immunoglobulins were not discussed in this report because the purpose of the toxicological studies was to assess the physical properties of the drug using normal animals. Characterization of the JCP-allergen extract identified the major allergens Cry j 1(Yasueda et al., 1983) and Cry j 2 (Sakaguchi et al., 1990) (Fig. 1A) and immune reactions were confirmed by PCA reaction in rats (Fig. 1B).

In genotoxicity studies, a series of tests were conducted including the Ames test to assess potential mutagenic properties, an *in vitro* mammalian chromosomal aberration test to evaluate the potential for induction of structural or numerical chromosomal aberrations in cultured CHL/IU cells and an *in vivo* mammalian erythrocyte micronucleus test to determine the potential to induce micronuclei. All tests indicated no potential of JCP-allergen extract to induce genotoxicity. Regarding the safety margin, the highest dose tested in this study, 50,000 JAU/kg/day, corresponds to approximately a 1500-fold greater level than the intended clinical dose (2000 JAU/subject/day) based on body weight. Therefore, the genotoxicity study suggested the potential for genotoxicity in clinical use is very low. This is supported by the absence of adverse genotoxic or carcinogenic effects during the 15 years of clinical experience with JCP-allergen extracts as SCIT products.

A 26-week repeated oral dose toxicity study in rats was conducted with standardized JCP-allergen extract to evaluate its safety for application to SLIT. The route of dosing was selected based on the human therapeutic route of JCP-allergen extract that is SLIT-swallow. Rats were administered JCP extract at doses of 200, 2000 and 10,000 JAU/kg-day by body weight. The effect of JCP-allergen extract was compared with a vehicle control group (50% glycerin containing sodium chloride) to evaluate the test substance and the vehicle control group was compared with control group (saline) to evaluate the vehicle. In this study, no treatment-related mortality or systemic toxicity was found. Regarding the safety margin, the highest dose tested in this study, 10,000 JAU/kg/day, corresponds to an approximately 300-fold higher dose than the intended clinical dose level of 2000 JAU/subject/day based on human weight of 60 kg. Regarding toxicokinetics, the amounts of Cry j 1, the major JCP allergen, were measured in the serum from rats treated with 10,000 JAU/mL at week 1, week 13 and week 26 before administration and 1, 2, 4, 8 and 24 h after administration. Cry j 1 protein was not detected in the sera at all time points tested (data not shown), indicating proteins in the JCP-allergen extract administered orally were degraded immediately in the stomach. Urinalysis indicated that sodium chloride in the vehicle of JCP-allergen extract increased sodium and chloride ion levels compared with the control group. Indeed the finding was observed in both the JCP treatment group and vehicle control group compared with the control group (saline) during the treatment period but not in the recovery period. This suggested that increases of sodium and chloride ions by urinalysis have no toxicological significance. There were no JCP-allergen extract related toxicologically relevant changes in clinical signs, body weight change, food consumption, ophthalmoscopy, urinalysis, hematology, blood chemistry, necropsy, organ weights and histopathology although slight local changes caused by irritation from the vehicle were observed at the administration site.

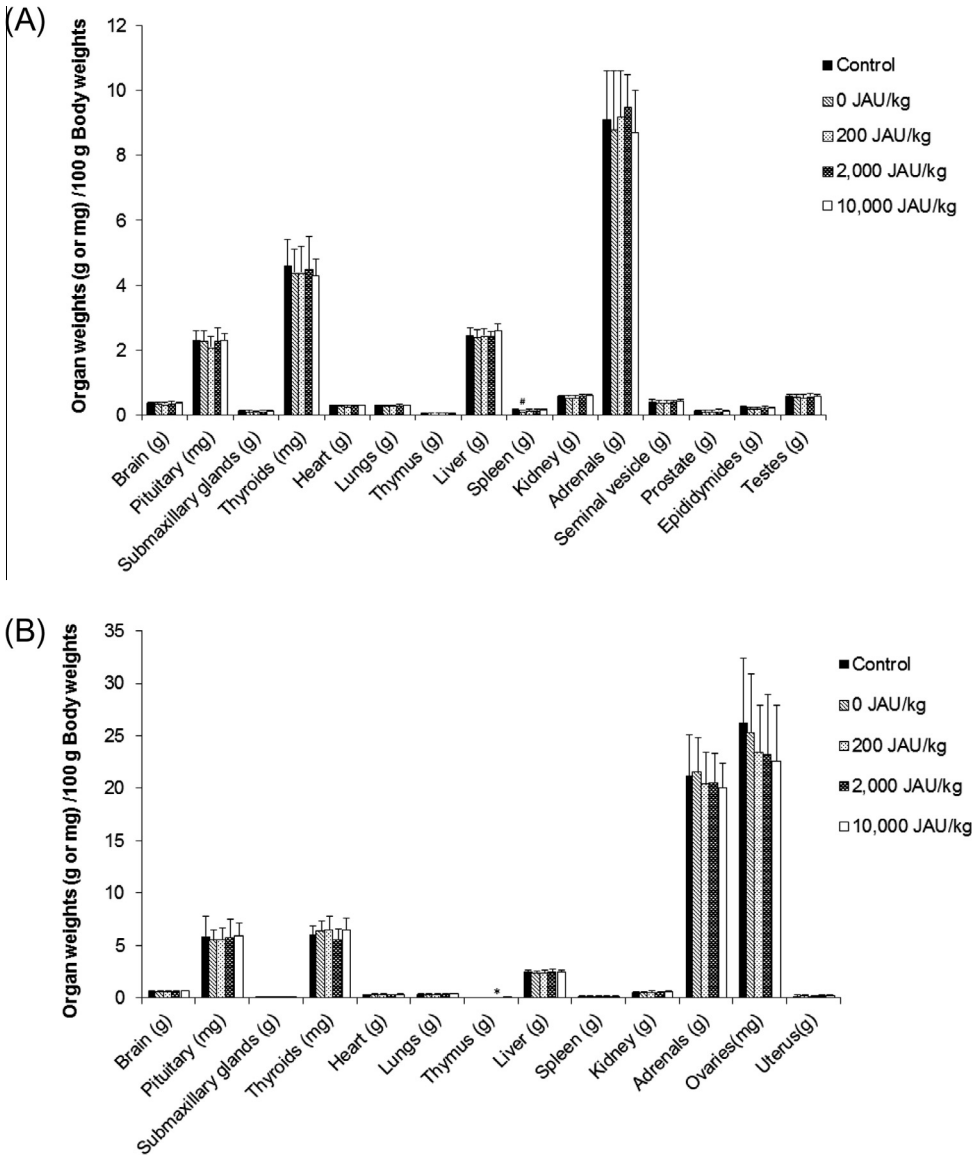

Fig. 4. Organ weights of rats following 26-week oral administration of JCP-allergen extract. Rats were orally administered 200, 2000 and 10,000 JAU/kg of JCP-allergen extract, vehicle (0 JAU/kg) or saline (control) daily for 26 weeks. Values are expressed as mean ± SD of 12 animals in each group of males (A) and females (B). $^{\#}p < 0.05$ (significantly different from control); $^{*}p < 0.05$ (significantly different from 0 JAU/kg).

Local changes were observed microscopically in the stomach, the first organ to come into contact with orally-administered JCP-allergen extract. Hyperplasia was induced slightly in the squamous epithelium of the limiting ridge of the forestomach. This might be caused by irritation from glycerin (Staples et al., 1967) in the vehicle solution because it was not observed in the control group administered saline and the frequency and grade of hyperplasia were comparable between vehicle and 10,000 JAU/mL treated group. Of note, it is not appropriate to extrapolate the findings observed in the forestomach, a unique organ in rodents, to potential local changes in the stomach of humans. The globule leukocyte is a large intra-epithelial cell, which is thought to be derived from a common precursor of mast cells (Ikeda and Yamashita, 1993), arising during intestinal nematode infections in rodents and ruminants and with granulated lymphocytes (Huntley, 1992). The mild increase of globule leukocytes in the glandular stomach observed in this study might be caused by irritation from glycerin in the vehicle solution. In addition, increased numbers of globule leukocytes are associated with the presence of lesions in the gastrointestinal tract (Narama et al., 1999) .These findings occurred in the stomach of rats of the repeated 26-week oral toxicity study were toxicologically mild and were not observed after the 4-week recovery period in the control and JCP-allergen extract treatment groups. As mentioned above, NOAEL for male and female rats was 10,000 JAU/kg/day with regard to systemic toxicity.

Oral irritative reactions are distinctive adverse events in SLIT with allergic patients that include stomatitis and throat irritation induced in the early stages of treatment, in response to the allergen, which activates the immune system. Stomatitis and throat irritation were reported as adverse events in a clinical trial using a sublingual tablet of timothy pollen allergen extract in Europe (Nelson et al., 2011). Irritation study is required as one of the toxicological studies in the study package for new drug application to authority. The purpose of the study is to evaluate a physical property of the drug its self, so normal animals were used for the investigation.

The oral mucosal irritation study in rabbits used 2000 JAU/mL, which was the maximum dose used in the clinic and the optimal

Table 7
Selected histopathological findings of rats following 26-week oral administration of JCP-allergen extract.

Organ/tissue	Finding	Dose (JAU/kg/day)					
		Male			Female		
		Control (Saline)	0	10,000	Control (Saline)	0	10,000
Digestive system							
Stomach	Hyperplasia, squamous epithelium, forestomach, limiting ridge	0	3	4	0	4	3
	Erosion, glandular stomach	0	1	1	0	1	0
	Globule leukocyte, increased, glandular stomach	0	3	5	0	2	1
Parotid gland	Cellular infiltration, lymphocyte	3	2	2	1	1	1
Liver	Cellular infiltration, mononuclear cell	7	5	7	2	2	2
Pancreas	Atrophy, acinus	3	0	3	0	0	0
	Deposit, pigment, yellow–brown	2	0	2	0	0	0
	Cellular infiltration, eosinophil	0	1	1	1	0	0
	Cellular infiltration, lymphocyte	4	5	1	2	5	4
	Fibrosis, islet	2	1	3	0	0	0
Respiratory system							
Lung	Metaplasia, osseous	2	0	1	0	0	0
	Accumulation, foam cell	1	2	3	2	2	1
	Mineralization, artery	1	2	2	2	3	4
Hematopoietic system							
Spleen	Hematopoiesis, extramedullary	1	0	2	0	0	0
Cardiovascular system							
Heart	Cellular infiltration, mononuclear cell	3	3	0	0	1	0
Urinary system							
Kidney	Tubule, basophilic	3	5	3	–	–	–
	Deposit, pigment, tubule, yellow–brown	1	0	2	–	–	–
	Cast, proteinaceous	2	3	3	–	–	–
	Cellular infiltration, pelvis, inflammatory	1	1	0	0	0	1
	Cellular infiltration, lymphocyte	6	5	7	0	1	3
	Cyst				1	1	0
	Mineralization	3	1	2	1	1	0
Genital system							
Prostate	Cellular infiltration, lymphocyte	7	2	7	–	–	–
Ovary	Cyst	–	–	–	4	3	5
Endocrine system							
	Cyst	0	1	0	2	0	0
Thyroid	Remnant, ultimobranchial body	1	1	2	1	4	2
Special sense organs							
Eye	Mineralization, cornea	1	1	2	0	0	0
Harderian gland	Cellular infiltration, lymphocyte	2	1	2	4	5	1

Groups contained 12 rats. The organs and tissues of the 200 JAU/mL and 2000 JAU/mL groups were not examined. The grades of all findings were mild. No findings indicating systemic toxicity were observed in any organs or tissues tested. Findings observed in more than 3 male or female animals were selected. JAU, Japanese allergy units.

Table 8
Macroscopic observations of the sublingual/buccal cavity of rabbits administered JCP-allergen extract in a local irritation study.

	Body weights (g)		Scoring of oral mucosa	
	Day 1	Day 8	Day 1	Day 8
Control (Saline)	3197 ± 185	3206 ± 254	0 ± 0	0 ± 0
Vehicle	3295 ± 219	3244 ± 200	0 ± 0	0 ± 0
JCP	3195 ± 152	3132 ± 86	0 ± 0	0 ± 0

Values are expressed as mean ± SD of three animals in each group. JCP, Japanese cedar pollen.

volume of administration (0.2 mL) was determined in a preliminary study to ensure the maximum volume did not leak from the mouth or accidentally be ingested. The administration time to the sublingual area of rabbits was 20 min. This was longer than the 2 min used for human clinical application because the increased grade of lesion is dependent on administration time in oral mucosal irritation studies using rabbits (Ohbayashi et al., 1998). No local irritation of the sublingual/buccal mucosa occurred in healthy rabbits after 1 week repeated sublingual treatment with JCP-allergen extract drug product with 10-times longer application than for clinical use. Further study is required in JCP-allergen extract-sensitized animals to evaluate the local irritation that appeared during SLIT in patients with JC pollinosis.

Recently, Okamoto et al. conducted a placebo-controlled double-blind randomized clinical trial with 531 JC pollinosis patients to investigate the efficacy and safety of SLIT using a modified drug of standardized JCP-allergen extract from SCIT product for two consecutive pollen seasons. They demonstrated improvement of the nasal and ocular symptoms of JC pollinosis, allowing a reduced usage of relief medication (in preparation). The results from the current *in vitro* and *in vivo* studies support the safety profile of standardized JCP-allergen extract applied to SLIT in JC pollinosis patients.

5. Conclusion

Based on the non-clinical studies of standardized JCP-allergen extract, the safety profile was consistent with the clinical experience of SCIT products, and these results support the safe use of standardized JCP-allergen extract for SLIT in JC pollinosis patients.

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