In Vivo Analysis of the Anti-allergic Activities of *Camellia japonica* Extract and Okicamelliaside, a Degranulation Inhibitor

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Based on the previous finding of okicamelliaside (OCS), a highly potent anti-degranulation ellagic acid glucoside, in the leaves of *Camellia japonica* (*C. japonica*), we evaluated an extract of these leaves and OCS itself for their potential to suppress allergic reactions *in vivo*. Two conventional animal allergy models were used. In the allergic conjunctivitis model, male S.D. rats were stimulated with anti-ovalbumin (OVA) serum and challenged with OVA/Evans blue mixture. Oral administration of extracts from *C. japonica* at 1000 mg/kg for 10 days significantly reduced the vascular permeability of conjunctivas. In the second model, male BALB/c mice were stimulated with a Japanese cedar pollen extract and challenged by nasal instillation of the antigen. The sneezing frequency during the 10 min immediately after the challenge tended to decrease by intraperitoneal administration of 0.2 mg/kg of OCS for 24 days. These results suggest that *C. japonica* extracts (CJE) and OCS prepared from them could be useful to alleviate the symptoms of an immediate-type allergy.

Key words —— anti-allergic activity, *Camellia japonica*, okicamelliaside, degranulation inhibitor, conjunctivitis, pollinosis

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INTRODUCTION

Allergic diseases are on the increase in developed countries, including Japan, where about 30% of the population reportedly suffers from allergies. Thus it is highly desirable to find appropriate measures to mitigate these diseases. According to Coombs & Gell, allergic reactions can be classified into four types.[1] In type I allergy, mast cells preactivated with a foreign antigen degranulate upon binding with an allergen-specific immunoglobulin E (IgE) antibody and release chemical mediators such as histamine, serotonin, and leukotriene that further lead to the immediate-type reaction. Because many important allergic diseases belong to the type I allergy group and are triggered by degranulation of mast cells, we screened Okinawan plants for degranulation inhibitors using RBL-2H3 cells. In *Camellia japonica* (*C. japonica*) leaves, we found three ellagic acids, one new and two known, that inhibited degranulation at very low concentrations.[2,3] The principal and new compound, named okicamelliaside (OCS), exhibited 12000 times the potency of the anti-allergic drug ketotifen fumarate (KF). This prominent *in vitro* potency prompted us further to explore its *in vivo* activities using two animal models: an allergic conjunctivitis model in rats and a Japanese cedar pollinosis model in mice. Here we report that the extract of *C. japonica* and OCS itself showed anti-allergic effects in *in vivo* experiments.

MATERIALS AND METHODS

Animals —— Male S.D. rats at 4 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan) and male BALB/c mice at 6 weeks of age from Charles River Japan (Tsukuba, Japan). They were housed individually in air-conditioned rooms maintained at 23 ± 3°C with a relative humidity of 55 ± 15% under a 12 hr light-dark cycle. The animals had *ad libitum* access to laboratory rodent chow (Oriental Yeast, Tsukuba, Japan) and tap water. Experiments were performed according to the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.

Reagents —— KF was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Zaditen ophthalmic solution, a commercial product containing 0.05% KF, was obtained from Novartis Pharma (Tokyo, Japan). Cedar Pollen Extract-Cj, used as an antigen, was obtained from Cosmo Bio Co. Ltd. (Tokyo, Japan). Extracts: The *C. japonica* extract (CJE) was prepared by immersing dried and powdered leaves in 70% methanol at room temperature overnight with subsequent filtration, condensation under reduced pressure, and lyophilization. One gram of CJE was equivalent to 13.5 g of dried *Camellia* leaves and contained OCS at a concentration of 304.8 ± 28 μg/g. For comparison of the anti-allergic activity, we used a hot water (80°C) extract of "tien-cha", the dried powder of the plant *Rubus suavissimu* with known anti-allergic activity.[4] This extract was abbreviated to *R. suavissimu* extract (RSE).

Rat Model for Allergic Conjunctivitis[5] —— The experiment involving allergic conjunctivitis was consigned to Nihon Bioresearch Inc. (Gifu, Japan). Quarantine and acclimatization periods before use were 5 and 8 days, respectively. At 6 weeks of age, the rats were divided on the basis of their mean body weight into 4 groups of 10 rats each: the control, CJE, RSE, and KF groups. For the CJE and RSE groups, CJE or RSE suspended in 0.5% (w/v) methyl cellulose (MC) solution was orally administered with a feeding needle at 1000 mg/kg body weight once/day for 10 days. Based on the OCS content in CJE, the OCS dose administered to the rats was 305 μg/kg body weight/day. The rats in the control group were administered 0.5% MC solution at 10 ml/kg body weight so as to equalize the MC dose between the control and experimental groups. The rats in the KF group received a topical application into each eye of a 5-μl portion of Zaditen ophthalmic solution before stimulation of anaphylaxis by the following method.

On the eighth day, rats were sensitized by injecting 5 μl of a rat anti-OVA serum diluted in saline into the palpebral conjunctivas of individual rats. At 48 hr after sensitization, an OVA/saline solution (2 mg/ml) containing 1% (w/v) Evans blue was injected into the tail vein. Thirty minutes later, the rats were exsanguinated from the aorta abdominalis, and the palpebral conjunctivas were enucleated. The conjunctivas were extracted with formamide and Evans blue was quantified by measuring the absorption at 620 nm.

Mouse Model for Japanese Cedar Pollinosis[6, 7] —— Male BALB/c mice at 6 weeks of age were acclimatized for 8 days before use and were divided on the basis of their mean body weight into 3 groups: the control (n = 10), OCS (n = 8), and KF (n = 8) groups. For the OCS and KF groups, OCS or KF dissolved in phosphate-buffered saline (PBS) was intraperitoneally administered at 0.2 mg/kg (OCS) or 5 mg/kg (KF) every other day. The antigen (1 mg Cedar Pollen Extract-Cj/ml PBS) and adjuvant solutions (Imject Alum, Thermo Fisher Scientific Inc., Rockford, Illinois, U.S.A.) were mixed in a 1 : 1 ratio (v/v) and stirred at room temperature for 30 min. The mice were intraperitoneally immunized on the 6th and 13th day, each time with 0.3 ml of the mixture. One week after the second immunization, 10 μl of the antigen solution (100 μg/ml PBS) was instilled into the bilateral nasal cavities once/day for 5 days. OCS or KF was injected 1 hr before the nasal instillation of the antigen on each day.

On the fifth day of antigen challenge, the sneezing frequency was measured for 10 min immediately after treatment. The mice were sacrificed under anesthesia and blood samples taken from the heart were used for IgE measurements.[6]

Statistical Analysis —— Data are expressed as mean ± standard error. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with Statistical Analysis System software (ver. 9.1, SAS Institute Japan Ltd., Tokyo, Japan). In all cases, probability (p) values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Anti-allergic Effect of CJE in Rat Conjunctivitis Model

The allergic conjunctivitis model with rats was designed to evaluate the suppression of excessive vascular permeability, one of the fastest reactions to occur in acute inflammation.[8] The body weights of rats in the CJE, RSE, and KF groups are shown in Fig. 1. No difference was observed between the control and KF group. The weight gain in the CJE group appeared slightly lower than that of the control group but the difference was statistically insignificant. The body weights of the RSE group were significantly lower than those of the control group on the 10th day. However, no abnormalities

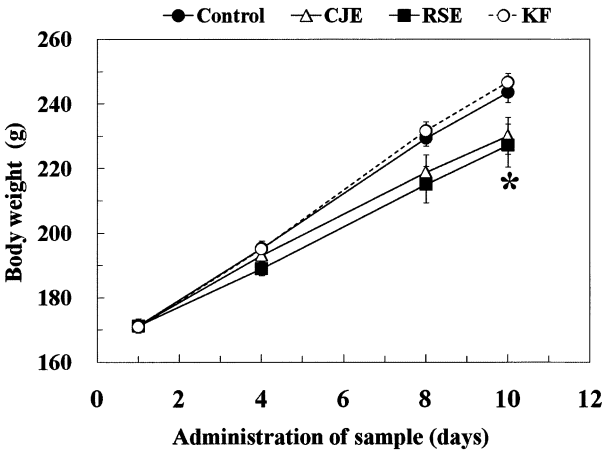

Fig. 1. Body Weights of Rats During the Experimental Period in the Allergic-conjunctivitis Model

●, Control group; △, CJE group; ■, RSE group; ○, KF group. *Body weight was significantly lower in the RSE group than in the control and KF groups ($p < 0.05$).

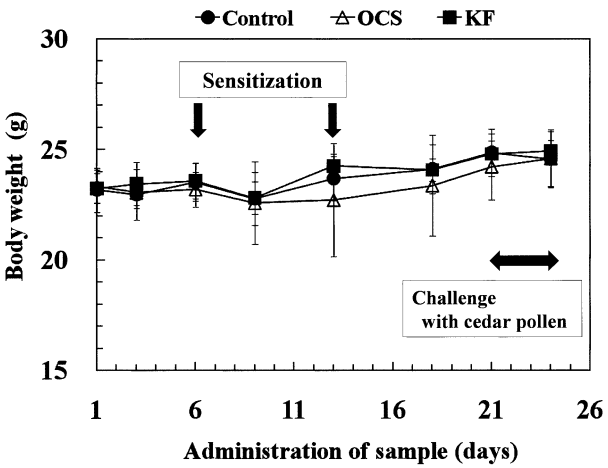

Fig. 3. The Body Weights of Mice During the Experimental Period in the Pollinosis Model

●, Control group; △, OCS group; ■, KF group.

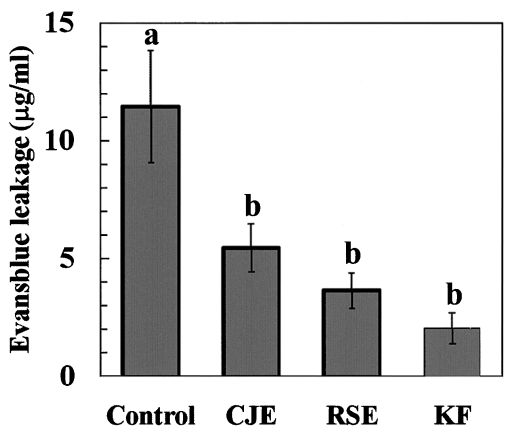

Fig. 2. Evans Blue Leakage in the Allergic-conjunctivitis Rat Model after Antigen Stimulation

CJE and RSE were orally administrated to rats at 1000 mg/kg. A 0.5% methyl cellulose solution was used to dissolve CJE and RSE, and as the reference vehicle in the control group. The effect of KF was examined by topically applying a 5-μl portion of Zaditen ophthalmic solution to a rat eye 30 min before stimulation. The letters of the alphabet denote significant differences ($p < 0.05$).

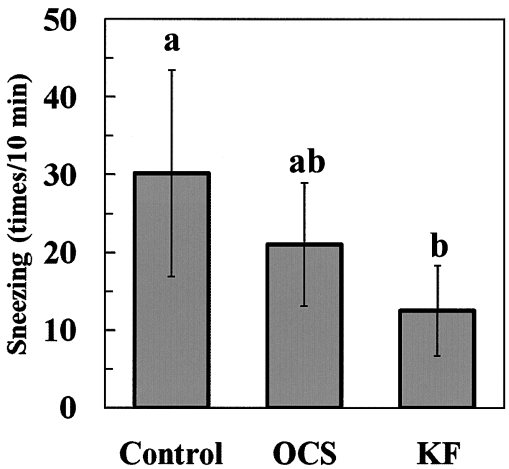

Fig. 4. Effects of OCS or KF on Sneezing Induced by Japanese Cedar Pollen Antigen

OCS (0.2 mg/kg) or KF (5 mg/kg) was intraperitoneally administered every other day. During the stimulation period (5 days), OCS or KF was injected 1 hr before nasal instillation of the antigen every day. The letters of the alphabet express significant differences ($p < 0.05$).

were observed in the appearance or behavior of the rats.

As shown in Fig. 2, vascular permeability was significantly ($p < 0.05$) suppressed by oral intake of CJE, RSE, or KF.

Effects of OCS in Cedar Pollinosis Model with Mice

The mice body weights in the OCS and KF groups appeared to decrease slightly for a brief period after the first sensitization with the antigen/adjuvant mixture but the difference was statistically insignificant (Fig. 3).

The effects of OCS and KF on sneezing frequency during the 10 min immediately after antigen instillation on the 5th day are shown in Fig. 4. In this cedar pollinosis model, the sneezing frequency varied widely from individual to individual in all three groups, making it difficult to draw a clear conclusion by statistical analysis of the data. The sneeze-suppressing effect of KF was judged significant but that of OCS, though apparently effective, was not significant.

The serum IgE concentrations in the control,

OCS, and KF groups rose from 1.64 ± 0.34 μg/ml to 7.17 ± 1.44, 9.19 ± 1.48, and 8.21 ± 1.21 μg/ml, respectively, when exposed to the cedar pollen antigen. However, no significant differences in IgE levels were observed among the three groups, suggesting that KF and OCS might exert their anti-allergic activity by suppressing degranulation, not by enhancing IgE production.

OCS, 3,4-dioxoloellagic acid 4′-glucoside, was extremely potent when tested *in vitro* on RBL-2H3 cells.[2, 3] The IC_{50} value determined for OCS was 14 nM, indicating that it was > 10000 times more potent than KF (IC_{50} = 169 μM). In addition to the potent *in vitro* activity, *in vivo* ability of CJE and OCS to suppress allergic reactions by oral intake was indicated in the present study. For oral administration of CJE, we chose the dose 1000 mg/kg because this was that used to verify the anti-inflammatory effect of RSE in rats.[4] Because of the very limited availability to prepare CJE and OCS, we were unable to evaluate the effects of CJE and OCS at various doses. For utilization of CJE as a health food, optimization of the orally effective dose would be required.

By taxonomy *C. japonica* is closely related to *C. sinensis*, which is widely used to prepare a tea drunk for its excellent taste as well as potential health benefits. Although green tea also exhibits anti-allergic activity, OCS was not detected in the leaves of *C. sinensis* in our previous study.[9] According to Tachibana *et al.,* catechins, especially methylated catechin [(−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate], are the major anti-allergic constituents in green tea.[10] The anti-allergic activity of RSE is attributable to the presence of ellagitannin.[11] Therefore *Camellia* leaves would provide health benefits by entirely different constituents. Although less tasty than tea, the hot-water extract of *C. japonica* leaves would make a good candidate for a health drink to mitigate allergic reactions. We have already confirmed in S.D. rats that oral intake of CJE at 500 and 2000 mg/kg has no adverse effects on body weight and produces no undesirable changes in clinical parameters, as determined by repetitive oral toxicity tests for 28 days.[12] Further effort is being made to elucidate the mechanism of action of OCS with increased amounts of OCS and with sophisticated methods such as microarray analysis.

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