Allergic rhinitis is a global health problem that affects 10 to 20% of the population and whose incidence is increasing year by year. Immunoglobulin E (IgE) plays a central role in the pathogenesis of allergic rhinitis through binding to its high affinity receptor on mast cells and basophils. Many studies suggest that a large part of the IgG that is generated against inhaled aeroallergens in allergic rhinitis is synthesized in the nasal mucosa. Although the concept of local IgE synthesis in allergic rhinitis has been widely recognized for a number of years, the details of its underlying mechanism are still unclear.

Mast cells are well known to be effector cells of type I hypersensitivity reactions. When mast cells are activated by cross-linking of their high affinity IgE receptors via the antigen and antigen-specific IgE interaction, they release pro-inflammatory chemical mediators, cytokines and enzymes and contribute to acute and chronic inflammatory responses. Moreover, it has been proposed that activated mast cells also enhance local IgE synthesis in the nasal mucosa of allergic rhinitis patients by interaction with dendritic cells, T-cells and B cells, via the release of histamine, eicosanoids, tumor necrosis factor (TNF)-alpha, interleukin (IL)-4 and IL-13. Although the concept of local IgE synthesis in allergic rhinitis has been widely recognized for a number of years, the details of its underlying mechanism are still unclear. Mast cells are well known to be effector cells of type I hypersensitivity reactions. When mast cells are activated by cross-linking of their high affinity IgE receptors via the antigen and antigen-specific IgE interaction, they release pro-inflammatory chemical mediators, cytokines and enzymes and contribute to acute and chronic inflammatory responses.

To clarify the effect of mast cell activation in local IgE synthesis in allergic rhinitis, we investigated whether simultaneous co-treatment with C48/80, a mast cell stimulator, enhances synthesis of IgE and immunoglobulin G (IgG) induced by intranasal application of ovalbumin (OVA), and induces allergic rhinitis in mice.

MATERIALS AND METHODS

Animals Five-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were used for all experiments. The mice were housed in conventional animal facilities with a 12/12 h light/dark cycle (lights on at 8 a.m.) and were kept in an air-conditioned room maintained at 23±1°C with humidity of 55±5%. Food and water were given ad libitum. All animal experiments were carried out under approved guidelines provided by the animal use committee at Tokushima Bunri University in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Sensitization Schedule The sensitization schedule is shown in Fig. 1. Mice were sensitized by intranasal application of OVA (Sigma-Aldrich, St. Louis, MO, U.S.A.) with C48/80 (Sigma-Aldrich). In brief, 15 µL of saline or a low (1 µg) or high (10 µg) amount of OVA mixed with either 1, 10 or 100 µg of C48/80 in 15 µL saline were instilled into the bilateral nose cavity on days 0, 7, 14 and 21. The same treatment was then used for intranasal challenge once a day from days 28 to 42.

Key words compound 48/80; allergic rhinitis; local immunoglobulin E (IgE) synthesis
OVA-Specific IgE and IgG Enzyme-Linked Immunoabsorbent Assay (ELISA) Serum OVA-specific IgE or IgG was analyzed on days 28 and 42 (Fig. 1). The experiments were carried out using the method described by Okano et al. with some modification. Briefly, for the determination of OVA-specific IgE or OVA-specific IgG, 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, U.S.A.) were coated overnight at 4°C with 100 μL/well of mouse anti-IgE (1:100; MCA419, AbD Selotec, Oxford, U.K.) or 100 μg/mL OVA, respectively, in carbonate–bicarbonate buffer (pH 9.6). The plates were washed and 400 μL/well of 1% bovine serum albumin (BSA) in 0.1% Tween-20-containing phosphate buffered saline was added for blocking. After incubation for 1 h at room temperature, the plates were washed and 100 μL of diluted serum (1:5 for OVA-specific IgE; 1:30000 for OVA-specific IgG) were added and incubated for 1 h at room temperature. The plates were washed and subsequently 100 μL of an OVA-horseradish peroxidase (HRP) conjugate (1:50; BUF048: AbD Selotec) or a mouse IgG-fragment crystalline (Fc) antibody peroxidase (POD) conjugate (1:50000; A90-131P: BETHYL, Montgomery, TX, U.S.A.) for detection of OVA-specific IgE and OVA-specific IgG, respectively, were added. After incubation for 1 h at room temperature, the plates were washed. Color was developed with 100 μL/well of 1% 3,3′-diaminobenzidine and 0.006% hydrogen peroxide. Digital images were obtained using a light microscope (BZ-9000: KEYENCE, Osaka, Japan).

Colorimetric Staining for OVA-Binding OVA-binding was histologically detected using the method described by KleinJan et al. with some modification. To block endogenous peroxidase, sections were treated with 0.01% hydrogen peroxide and 1% sodium azide in PBS for 30 min at room temperature. The slides were incubated for 10 min with 1% BSA in PBS and then for 1 h with an OVA-HRP conjugate (1:50) and 1% BSA in PBS. Color was developed by reacting the slides with a mixture containing 0.025% 3,3′-diaminobenzidine and 0.006% hydrogen peroxide. Digital images were obtained using a light microscope (BZ-9000: KEYENCE, Osaka, Japan).

Fluorescent Double Staining for OVA-Binding and IgE or IgG To detect OVA-specific IgE, the sections were incubated for 20 min with a Tween-20-containing Tris buffered solution (TBS-T) containing normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.), followed by overnight incubation with rat anti-mouse IgE monoclonal antibody (1:100; sc-69812: Santa Cruz, Dallas, TX, U.S.A.). Thereafter the slides were incubated for 1 h with anti-rat IgG Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, CA, U.S.A.) and Alexa Fluor 647 conjugated OVA (5 mg/mL; Invitrogen). To detect OVA-specific IgG, the sections were incubated for 20 min with TBS-T containing normal goat serum, followed by 60 min incubation with anti-mouse IgG Alexa Fluor 568 (1:500; Invitrogen) and Alexa Fluor 647 conjugated OVA (5 mg/mL; Invitrogen). Finally, the sections were rinsed with TBS-T and covered with VECTASHIELD Hard Set (Vector Laboratories, Burlingame, CA, U.S.A.). Immunofluorescent images were analyzed using a confocal laser-scanning microscope (A1Rsi: Nikon, Tokyo, Japan).

IL-4 ELISA Nasal mucosal IL-4 was analyzed on days 0, 22 and 28 (Fig. 1). The mice were anesthetized with sodium pentobarbital (75 mg/kg intraperitoneally (i.p.)). The head of the mouse was cut off and then the lower jaw of the mouse was removed. The palate was peeled out with forceps from the upper jaw of the mouse and was then placed on a glass slide. The mucosal tissue was collected with scalpels into 1.5 mL plastic tubes. The tissue was homogenized in lysis buffer (0.5% NP-40, 100 mM NaCl, 10 mM N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES), 1.5 mM MgCl₂, 10 mM potassium chloride (KCl), 25% Glycerol) using a pestle mixer. After incubation on ice for 30 min, the samples were centrifuged (15,000 × g, 30 min, 4°C) and the supernatant was collected. Sample protein concentrations were measured using a commercial kit (bicinchoninic acid (BCA) protein assay kit; Thermo Fischer Scientific, Waltham, MA, U.S.A.). IL-4 protein content was measured using commercial ELISA kits (Mouse IL-4 Instant ELISA; ebioscience, Vienna, Austria). ELISA was performed according to the manufacturer’s in-

Histology Histological analysis was performed on day 28 (Fig. 1). The head of the mouse was separated from the rest of the body and the lower jaw was then cut. The skin, muscle and connective tissue on the surface of the skull were removed. The cleaned skulls were fixed with 4% paraformaldehyde containing 0.1 M phosphate buffered saline (PBS) overnight at 4°C. After fixation, the fixed skulls were washed with 0.1 M PBS at 4°C for 1 h and were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA)–2Na containing 0.1 M phosphate buffered saline. Color was developed with 0.025% 3,3′-diaminobenzidine and 0.006% hydrogen peroxide. Digital images were obtained using a light microscope (BZ-9000: KEYENCE, Osaka, Japan).
Nasal Symptom The nasal symptom (sneezing) was observed on day 42 (Fig. 1). Twenty-four hours after the last intranasal sensitization, 15 µL of OVA solution (10 µg/mouse) was bilaterally instilled into the nasal cavities. After challenging with OVA, the mice were immediately placed into a plastic cage (30 × 20 × 10 cm), and the number of sneezes was counted over 5 min.

Statistical Analysis Each value is expressed as the mean±standard error of the mean (S.E.M.). Statistical differences were evaluated by one-way ANOVA with Bonferroni’s correction or by an unpaired Student’s t-test. Differences were considered to be significant at p<0.05.

RESULTS

OVA-Specific IgE or IgG in Serum Following weekly intranasal challenges for four weeks with 1 µg or 10 µg of OVA, Balb/c mice produced detectable but low levels of OVA-specific IgE in serum (Fig. 2A). Simultaneous application of C48/80 with OVA enhanced the production of OVA-specific IgE (Fig. 2A). In particular, 10 µg of C48/80 significantly enhanced the level of serum OVA-specific IgE that was induced by 10 µg of OVA on day 28. However, simultaneous application of 100 µg C48/80 with 10 µg of OVA did not further increase OVA-specific IgE (Fig. 2A). C48/80 also significantly enhanced the production of OVA-specific IgG in serum by 10 µg of OVA on day 28, in a dose-dependent manner (Fig. 2B). We used the combination of 10 µg OVA and 10 µg C48/80 for further analysis because, of the tested doses, this combination was most efficient for induction of OVA-specific IgE.

OVA-Specific IgE or IgG in the Nasal Mucosa To detect OVA-specific immunoglobulins in the nasal mucosa, binding of OVA to cells of paraformaldehyde fixed and decalcified nasal sections were evaluated. On day 28, OVA-binding cells were only found in the nasal sections of the 10 µg OVA + 10 µg C48/80 treated group (Figs. 3C, D). In these sections, large numbers of OVA-binding cells were distributed in the nasal mucosa in close proximity to the nasal mucosa-associated lymphoid tissue (NALT). No OVA-binding cells were found in the nasal sections from the saline treated control group. To identify the immunoglobulin subclasses of the OVA-binding immunoglobulins, double staining with fluorescent dye-labeled OVA and antibody against IgE or IgG was performed. Double
staining of OVA binding and IgE revealed few OVA binding and IgE double-positive cells in the nasal mucosa; most of the OVA binding cells were IgE negative (Figs. 3E, F). In contrast, double staining of OVA binding and IgG showed that most of the OVA binding cells were also anti-IgG positive (Figs. 3G, H). The volume view confocal image revealed that OVA-specific or -nonspecific IgE signals were located in the cytoplasm surrounding the cell nuclei but not on the cell surface (Fig. 3I). These data suggested that at least some of these OVA binding and immunoglobulin positive cells are probably

Fig. 3. Histological Detection of OVA-Specific IgE or IgG

Histological analyses were performed on day 28 (n=6). Colorimetric detection of OVA-binding cells in mouse nasal sections (A–D). (A) The whole-image of a representative coronal nasal section. The red square indicates the area shown in the microphotographs B, C. OVA binding to the nasal mucosa of saline-treated (B) and 10 µg OVA + 10 µg C48/80-treated (C) mice. Representative examples of OVA binding cells are marked with black arrows (×100). NALT, nasal mucosa-associated lymphoid tissue. (D) An enlarged image of OVA-binding cells (×400). Confocal laser scanning microscopic images of double staining of nasal sections for OVA-binding (red), and IgE or IgG (green) (×100) (E–H). Representative examples of double positive cells are marked with white arrows. (I) Representative images of Z-stack confocal laser scanning microscopic images of nasal sections double stained for OVA-binding (red) and IgE (green) (×400). A double positive cell is marked with an asterisk (*), and an IgE-single positive cell is marked with the hash symbol (#).
antibody producing cells or plasma cells.

Nasal Symptoms After weekly application of $10\mu g$ OVA + $10\mu g$ C48/80 for four weeks followed by daily application of $10\mu g$ OVA + $10\mu g$ C48/80 for 2 weeks, OVA-induced sneezing was significantly increased in the $10\mu g$ OVA + $10\mu g$ C48/80 group compared with the saline group on day 42 (Fig. 4).

**IL-4 in the Nasal Mucosa** IL-4 was transiently and significantly increased in the nasal mucosa on day 22 (24 h after the third weekly application of $10\mu g$ OVA + $10\mu g$ C48/80), and the IL-4 level then returned to the basal level on day 28 (Fig. 5).

DISCUSSION

In the present study, we demonstrated that OVA-specific IgE production by intranasal sensitization with OVA was enhanced by co-treatment with C48/80, a non-immunological mast cell stimulator, and that continuous sensitization induced further serum OVA-specific IgE increase and nasal symptoms. These results are consistent with the theory that activated mast cells contribute to local IgE production and ongoing allergic inflammation. In addition to OVA-specific IgE, OVA-specific IgG was also increased in our model. The role of IgG in the pathology of allergic rhinitis is still controversial. Allergen-specific IgG may play a significant role as a blocking antibody in allergen-specific immunotherapy against IgE-mediated allergy. On the other hand, IgG may also play a role via its Fc receptors in the occurrence of anaphylactic events. Further analysis to determine whether enhanced IgG is an effector or suppressor of the progress of rhinitis in this model will be needed.

In the past, most conventional animal models of allergic rhinitis were induced by systemic sensitization of animals with an allergen to produce antigen-specific IgE, followed by later nasal challenge with the same allergen. Since it has been recognized that allergen-specific IgE might be locally synthesized in nasal mucosa in allergic rhinitis, many efforts have been made to establish experimental animal models of allergic rhinitis without systemic sensitization. As a result, some locally sensitized allergic rhinitis models with or without adjuvants have been established. Our present model was designed as a specialized model aimed at elucidating the role of mast cells in the mechanisms of local IgE induction in allergic rhinitis.

Although C48/80 is widely used as an inducer of degranulation and histamine release, C48/80-treated cultured mast cells also release PGD$_2$, leukotriene 4 (LTC$_4$), TNF-alpha and IL-4 as well as histamine$^{7,9,14}$ in vitro. These mediators can promote the maturation, functional activation and migration of dendritic cells, favoring the development of sensitization to additional antigens; promote the recruitment and activation of Th2 cells; and control B cell class switching to IgE producing plasma cells.$^{2,5}$ In this study, we demonstrated that IL-4 was transiently increased in nasal mucosa one day after OVA + C48/80 treatment. Since IL-4 is a typical Th2 cytokine and is essential for the induction of IgE and IgG1 synthesis,$^{2,5}$ it may, at the least, account for the increase in IgE and IgG in this model. Further analysis to determine whether other mediators are involved in antibody synthesis in this model will be needed.

A mucosal adjuvant effect of C48/80 has been reported.$^{15,16}$ In 2008, McLachlan et al. demonstrated that nasal instillation of a vaccine antigen from Bacillus anthracis with C48/80 evoked antigen-specific secretory IgA and serum IgG.$^{15}$ In contrast with our results, they showed that C48/80 did not induce total or antigen-specific IgE. However, the isotype of induced antibodies depends on the property of the antigens and, in general, IgE tends to be induced by parasites or food allergens such as OVA, rather than by bacterial antigens.

In addition, in the present study, we propose a potential histological method to detect OVA-specific binding to nasal mucosa. A few previous studies have histologically shown the presence of antigen-specific IgE through the double staining of the binding of enzyme-labeled antigens and anti-IgE antibody to cells in acetone-fixed nasal biopsy specimens from patients.$^{3,17}$ We used paraformaldehyde-fixed and EDTA-decalcified mouse nasal cryosections and HRP-conjugated or

![Fig. 4. Nasal Symptoms](image)

![Fig. 5. Nasal Mucosal IL-4](image)
fluorescent dye-conjugated OVA. By using this method, we successfully demonstrated OVA-specific binding cells in nasal mucosa, which indicated that the immunoglobulins in the processed sections retained OVA binding ability. Moreover, double staining with anti-IgE or anti-IgG demonstrated the existence of OVA-specific IgE and IgG in the nasal mucosa, and the result of confocal microscopic analysis suggested that OVA-specific IgE- or IgG-producing cells are present in the sensitized nasal mucosa. To our knowledge, this is the first report of histological detection of OVA-specific IgE and IgG in fixed and decalcified mouse tissue. However, further analysis to characterize the responsible cell type using specific antibody against plasma cells or antigen producing cells will be needed. We consider that our method should be a useful tool for determining the distribution of antigen-specific antibody in various tissues including hard tissue.

In conclusion, we demonstrated that simultaneous intranasal application of OVA and C48/80, a mast cell stimulator, enhanced antigen-specific IgE and IgG synthesis and induced nasal hypersensitivity in mice. Our results provide additional evidence that mast cells contribute to local IgE synthesis in allergic rhinitis. We believe that the present animal model will contribute to elucidation of the role of mast cells in local IgE synthesis in allergic rhinitis.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


