



Topical ocular treatment with monoclonal antibody Fab fragments targeting Japanese cedar pollen Cry j 1 inhibits Japanese cedar pollen-induced allergic conjunctivitis in mice

Nobuaki Mizutani^{a,*}, Takeshi Nabe^b, Shin Yoshino^a

^a Department of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada, Kobe 658-8558, Japan

^b Laboratory of Immunopharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

ARTICLE INFO

Keywords:

Fabs
Allergic conjunctivitis
Japanese cedar pollen
Cry j 1
Eosinophils

ABSTRACT

Fab fragments (Fabs) of antibodies having the ability only to bind to specific allergens lack effector functions due to the absence of the Fc portion. In the present study, we examined whether IgG1 monoclonal antibody (mAb) Fabs targeting Japanese cedar pollen (JCP) Cry j 1 were able to regulate JCP-induced allergic conjunctivitis in mice. BALB/c mice actively sensitized with JCP were repeatedly challenged by topical administration of JCP eye drops. Fabs prepared by the digestion of anti-JCP IgG1 mAbs (P1-3 and P1-8) with papain were applied to the eye 15 min before the JCP challenges followed by measurement of the clinical conjunctivitis score. In the *in vitro* experiments, P1-3 and P1-8 showed specific binding to JCP Cry j 1. Furthermore, intact P1-3 binding to Cry j 1 was inhibited by P1-3 Fabs, but not P1-8 Fabs; additionally, P1-8 Fabs, but not P1-3 Fabs, suppressed the intact P1-8 binding, suggesting that the epitopes of Cry j 1 recognized by P1-3 and P1-8 were different. Topical ocular treatment with P1-3 Fabs or P1-8 Fabs was followed by marked suppression of JCP-induced conjunctivitis ($P < 0.01$). In histological evaluation, P1-8 Fabs showed a reduction in eosinophil infiltration in the conjunctiva ($P < 0.01$). These results demonstrated that topical ocular treatment with IgG1 mAb Fabs to Cry j 1 was effective in suppressing JCP-induced allergic conjunctivitis in mice. Furthermore, it suggests the possibility that some epitopes recognized by Fabs could be used as a tool to regulate allergic conjunctivitis.

1. Introduction

Allergic conjunctivitis is a type I allergic disease with a high prevalence (Friedlaender, 1991; Bielory and Friedlaender, 2008). The disease is characterized by early and late phase responses after exposure to the allergen. The early response involves itching, hyperemia, and oedema; the infiltration by inflammatory cells including eosinophils into the conjunctiva is recognized in the late phase (Hingorani et al., 1998). Furthermore, in the therapies for allergic conjunctivitis, not only several drugs including anti-histamines, vasoconstrictors, mast cell stabilizers, and glucocorticoids (Bisca, 1997), but also subcutaneous and sublingual allergen immunotherapies (Dretzke et al., 2013; Casale and Stokes, 2014) are used.

Fab fragments (Fabs) of antibodies maintain the ability to bind specific allergens although the lack of an effector function associated with the absence of Fc portion is recognized. Clinically, cetrolizumab is a monoclonal antibody (mAb) Fab targeting TNF- α and used in the

treatment of patients with rheumatoid arthritis or Chron's disease (Nesbitt et al., 2007; Deeks, 2013; Da et al., 2013). Furthermore, we have reported that the topical application of allergen-specific IgG1 mAb Fabs, but not the intact IgG1 mAb, suppressed allergen-induced asthma (Yoshino et al., 2014), rhinitis (Matsuoka et al., 2014), and dermatitis in mice (Sae-Wong et al., 2016). On the basis of the information, Fabs may be useful for the allergen-specific immunotherapy of allergic diseases; the treatment is expected to show immediate effects compared with subcutaneous and sublingual allergen immunotherapies, requiring long-term treatment (Abramson et al., 1995; Pipet et al., 2009; Keles et al., 2011; Passalacqua and Canonica, 2011). However, whether allergen-specific IgG1 mAb Fabs suppress allergen-induced allergic conjunctivitis in mice is unknown.

In the present study, we found that P1-3 and P1-8 showing Japanese cedar pollen (JCP) Cry j 1-specific IgG1 mAbs recognized different epitopes of Cry j 1. These data prompted us to investigate whether both Fabs suppress JCP-induced allergic conjunctivitis.

Abbreviations: H & E, hematoxylin and eosin; OVA, ovalbumin; JCP, Japanese cedar pollen; mAb, monoclonal antibody; P1-3 and P1-8, Cry j 1-specific IgG1 mAb

* Corresponding author.

E-mail address: mizutani@kobepharm-u.ac.jp (N. Mizutani).

<http://dx.doi.org/10.1016/j.ejphar.2017.01.028>

Received 6 September 2016; Received in revised form 20 January 2017; Accepted 20 January 2017
0014-2999/ © 2017 Elsevier B.V. All rights reserved.

Therefore, we examined the effects of topical ocular treatment with the two IgG1 mAb Fabs (P1-3 Fabs and P1-8 Fabs) on JCP-induced allergic conjunctivitis in mice.

2. Materials and methods

2.1. Animals

Male 7-week-old BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). These mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University.

2.2. Production and purification of anti-JCP IgG1 mAb (P1-3 and P1-8) and Fabs

Mice were sensitized with JCP (Biostir, Hyogo, Japan) emulsified with complete Freund's adjuvant (Sigma-Aldrich Fine Chemicals, MI, USA) and the spleen cells were fused with NS-1 myeloma cells, followed by screening and cloning hybridomas that produced anti-JCP mAbs, as previously described (Terato et al., 1992). Hybridomas producing anti-JCP IgG1mAbs including P1-3 and P1-8 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin. To investigate the level of antibodies produced from the hybridomas (P1-3, P1-5, P1-6, P1-7, P1-8, and P1-9), the supernatants were incubated with JCP (100 µg/mL) coated on 96-well plates. After washing, this was followed by addition of an alkaline phosphatase-conjugated anti-Fc of mouse IgG1. The plate was developed with p-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate enzyme-linked immunosorbent assay (ELISA) reader. As a result, the two hybridomas (P1-3 and P1-8) showed high absorbance compared with P1-5, P1-6, P1-7, and P1-9 (Table 1). Furthermore, P1-3 and P1-8 had the ability to specifically bind to IgG1 as shown by using a Mouse Monoclonal Sub-Isotyping Kit (American Qualex International Inc., La Mirada, CA, USA) (Table 2); therefore, we used two anti-JCP IgG1 mAbs (P1-3 and P1-8) for the in vivo experiments.

P1-3- and P1-8-producing hybridomas were grown in the CELLLine CL1000 with a BD-Cell-Mab medium (BD Biosciences, San Diego, CA, USA) supplemented with 20% heat-inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin. To prepare P1-3 Fabs and P1-8 Fabs, the mAbs purified using a protein G column (GE Healthcare UK Ltd., Little Chalfont, UK) were digested with agarose-linked papain (Sigma-Aldrich, St. Louis, MO, USA) according to the methods described previously (Katpally et al., 2008). P1-3 Fabs and P1-8 Fabs were separated using a protein G column.

2.3. Allergenic specificity of P1-3 and P1-8

To investigate the allergenic specificity of P1-3 and P1-8, the mAbs (1 and 10 µg/mL) were incubated with 2 µg/mL of various allergens including Cry j 1, Cry j 2, ovalbumin (OVA), haemocyanin, collagen,

Table 1

The binding ability to Japanese cedar pollen in IgG1 mAbs produced by hybridomas.

	P1-3	P1-5	P1-6	P1-7	P1-8	P1-9
OD (405 nm)	1.907 ± 0.020	0.905 ± 0.016	0.283 ± 0.072	0.187 ± 0.009	1.692 ± 0.031	0.166 ± 0.056

The supernatants of cultured hybridomas were incubated with 100 µg/mL of JCP coated on the plate. This was followed by addition of alkaline phosphatase-conjugated anti-Fc of mouse IgG1. The plate was developed with p-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate ELISA reader.

Table 2

The P1-3 and P1-8 ability to specifically bind to IgG1.

	OD (405 nm)					
	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
P1-3	0.463 ± 0.042	0.012 ± 0.001	0.020 ± 0.008	0.007 ± 0.003	0.010 ± 0.003	0.009 ± 0.001
P1-8	0.450 ± 0.027	0.015 ± 0.004	0.007 ± 0.005	0.006 ± 0.003	0.005 ± 0.003	0.010 ± 0.002

The P1-3 and P1-8 ability to specifically bind to IgG1 was investigated by using a Mouse Monoclonal Sub-Isotyping Kit.

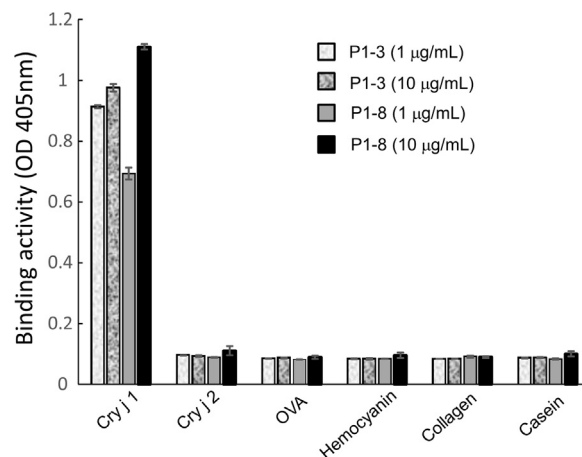


Fig. 1. P1-3 and P1-8 specifically bind to Cry j 1. The indicated amounts of P1-3 and P1-8 were added to various allergens including Cry j 1, Cry j 2, OVA, haemocyanin, collagen, and casein coated on the plate. This was followed by the further addition of an alkaline phosphatase-conjugated anti-Fc of mouse IgG1. Then, p-nitrophenyl phosphate was added to the plates before measuring absorbance at 405 nm using an ELISA reader.

and casein coated on 96-well plates. After washing, the alkaline phosphatase-conjugated anti-Fc of mouse IgG1 was added. Each plate was developed with p-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate ELISA reader.

2.4. Analysis of epitope specificity of P1-3 and P1-8

To investigate epitope specificities of P1-3 and P1-8, P1-3 and P1-8 Fabs (1–30 µg/mL) were incubated with Cry j 1 (2 µg/mL) coated 96-well plates. After washing, intact P1-3 or P1-8 (0.5 µg/mL) was added. To measure the intact P1-3 or P1-8 binding to Cry j 1, an alkaline phosphatase-conjugated anti-Fc of mouse IgG1 was added; the plate was developed with p-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate ELISA reader. Additionally, P1-3 Fabs or P1-8 Fabs only were prepared as a negative control.

2.5. Induction of allergic conjunctivitis

As shown in Fig. 3, sensitization was performed by i.p. injection of JCP adsorbed to alum (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) (100 µg JCP/5 mg alum/0.5 mL/mouse) on days 0 and 14. The sensitized mice were challenged by topical administration of eye drops of JCP (100 µg/4 µL/mouse) under anaesthesia with isoflurane (WAKO Pure Chemical Industries) on days 28, 29, 30, and 35. The occurrence of conjunctivitis was examined 10, 20, and 30 min after each antigen challenge. Four clinical signs were observed: conjunctival oedema; palpebral oedema; conjunctival hyperemia, and lacrimation. A scale of 0–4 with increments of 0.5 was used for each sign. Thus, each animal was given a total clinical score between 0 and 16.

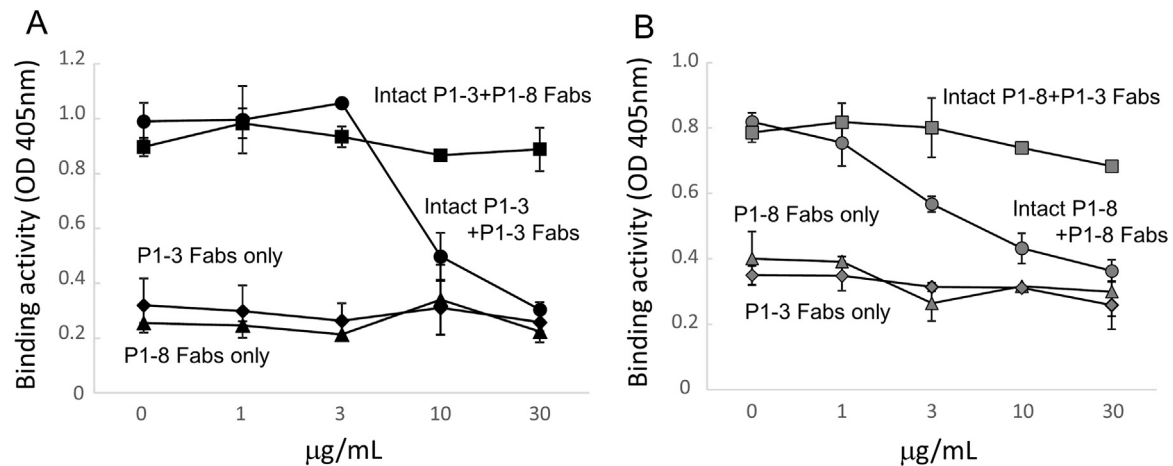


Fig. 2. P1-3 and P1-8 recognize different epitopes of Cry j 1. P1-3 Fabs or P1-8 Fabs were incubated with Cry j 1 coated on the plates. This was followed by the addition of intact P1-3 (A) or P1-8 (B) to the plates followed by the alkaline phosphatase-conjugated anti-Fc of mouse IgG1. The plate was developed with p-nitrophenyl phosphatase, and measurements were made at 405 nm using a microplate ELISA reader.

Animals: Balb/c mice

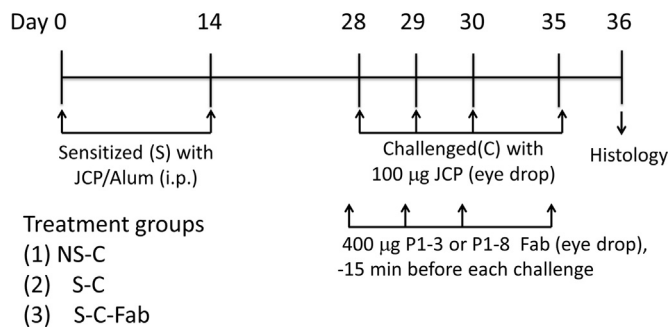


Fig. 3. Experimental protocols. To induce allergic conjunctivitis, mice were i.p. sensitized with JCP plus alum on days 0 and 14 followed by topical application of the same pollen by eye drops on days 28, 29, 30, and 35 (S-C group). The P1-3 Fabs or P1-8 Fabs were administered 15 min before each JCP challenge by eye drops (S-C-Fab group). JCP was administered by eye drops to non-sensitized mice as a negative control (NS-C group).

2.6. Administration of P1-3 Fabs or P1-8 Fabs

P1-3 Fabs or P1-8 Fabs (400 µg/4 µL/mouse) dissolved in PBS were administered via eye drops 15 min before each challenge with JCP on days 28, 29, 30, and 35. As a control, the Fabs of IgG purified from naïve mouse serum (Rockland Immunochemicals, Gilbertsville, PA, USA) were used. Meanwhile, in our previous study using a murine model of allergic rhinitis, when P1-8 Fabs (400 µg/mouse) and JCP (100 µg/mouse) were administered, P1-8 Fabs reduced allergic rhinitis symptoms after the JCP challenge (Yoshino and Mizutani, 2016). Based on these data, in the present study we administered P1-8 Fabs (400 µg/mouse) against JCP (100 µg/mouse) to allergic conjunctivitis mice.

2.7. Measurement of total IgE, JCP-specific IgE, and JCP-specific IgG1 antibodies in serum

The level of total IgE antibodies in serum 24 h after the fourth antigen challenge was measured using quantitative colormetric sandwich ELISA kits (BioLegend, San Diego, CA, USA).

Levels of JCP-specific IgE and IgG1 antibodies in serum 24 h after the fourth antigen challenge were measured using ELISA modified by methods described previously (Matsuoka et al., 2014). JCP-specific IgE or IgG1 were detected by using plates coated with JCP; biotin-labeled anti-mouse IgE or alkaline phosphatase-conjugated anti-mouse IgG1

were added, respectively. In the IgE plate, streptavidin-HRP was added and the plate was developed with 3,3',5,5'-tetramethylbenzidine solution followed by stopping the reaction with sulfuric acid. The IgG1 plate was developed with p-nitrophenyl phosphate. Measurements of the IgE or IgG1 plates were made at 450 or 405 nm using a microplate reader. Values for serum JCP-specific IgE or IgG1 were expressed as absorbance units.

2.8. Histological analysis

Mice were killed by isoflurane inhalation on day 36, and their heads were fixed in 4% neutral-buffered formalin. The skulls remained in EDTA for decalcification for 21 days before embedding in paraffin and sectioning at 4 µm, as described previously (Yoshino and Mizutani, 2016). Sections were stained with haematoxylin and eosin (H & E) to stain the eosinophils, neutrophils, and lymphocytes. Mast cells were stained by toluidine blue. In terms of the number of inflammatory cells, the mean of 3 randomly selected areas per 0.01 mm² was calculated for each group.

2.9. Statistical analysis

Data are shown as the mean ± S.E.M. A one-way analysis of variance (ANOVA) was performed. If significant differences were detected, individual group differences were determined by Bonferroni's comparison test. A probability value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. P1-3 and P1-8 show Cry j 1-specific binding

First, we examined whether P1-3 and P1-8 showed selective binding to Cry j 1, which was mainly contained in JCP as an allergen. P1-3 or P1-8 was incubated in vitro with various allergens including Cry j 1, Cry j 2, haemocyanin, OVA, Type II collagen, and casein. The results indicated that P1-3 and P1-8 bound to only Cry j 1, but not to other allergens (Fig. 1).

3.2. P1-3 and P1-8 bind to different epitopes of Cry j 1

The in vitro preincubation of P1-3 Fabs, but not P1-8 Fabs, with Cry j 1 resulted in the blockage of binding of intact P1-3 to Cry j 1 (Fig. 2A). Furthermore, the binding of intact P1-8 to Cry j 1 was suppressed by P1-8 Fabs, but not P1-3 Fabs (Fig. 2B). Thus, P1-3 and P1-8 recognized

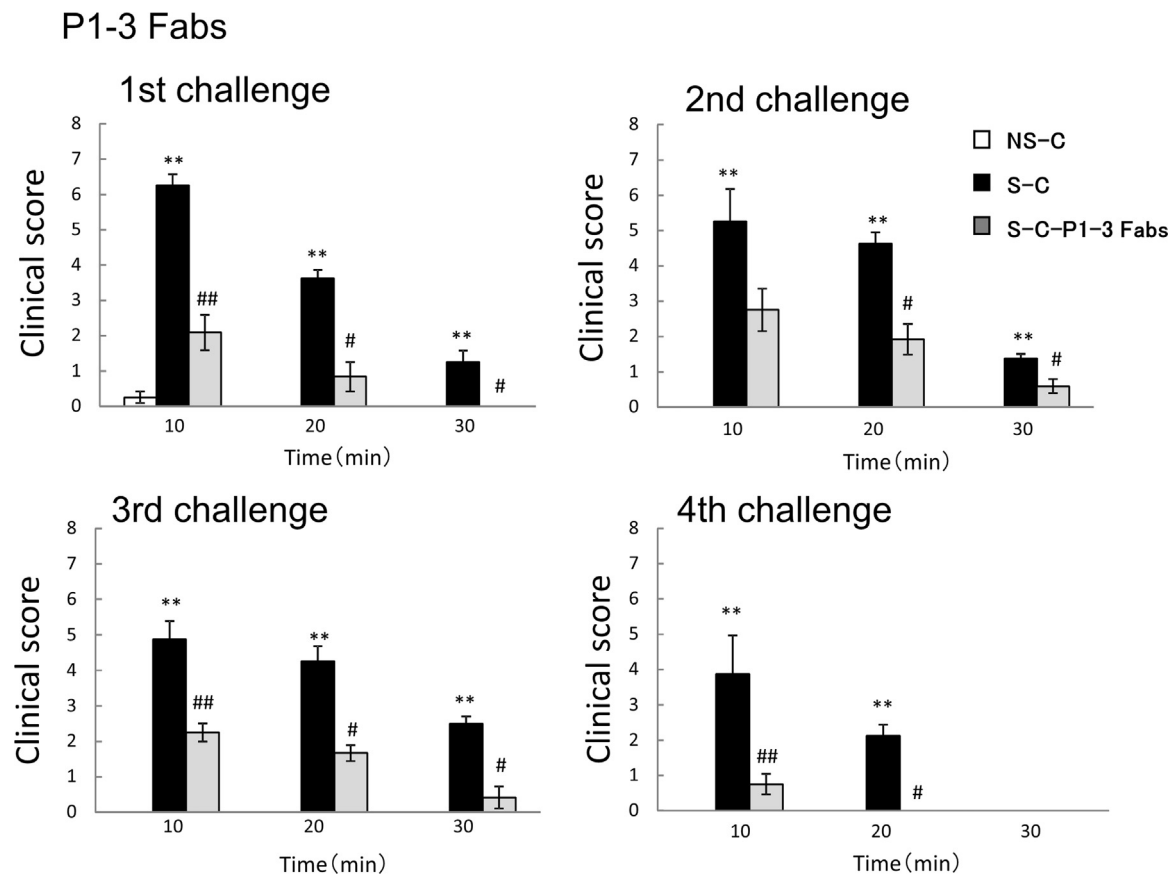


Fig. 4. Topical ocular treatment with P1-3 Fabs suppresses JCP-induced allergic conjunctivitis in mice. P1-3 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-3 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Each value is the mean $\pm$ S.E.M. of 5 animals. ** $P < 0.01$; or # $P < 0.05$, ## $P < 0.01$ compared with the NS-C or S-C group, respectively.

different Cry j 1 epitopes

3.3. Treatment with P1-3 Fabs or P1-8 Fabs suppresses JCP-induced allergic conjunctivitis

Subsequently, we examined whether the two Fabs of P1-3 and P1-8 recognizing different epitopes of Cry j 1 inhibit JCP-induced allergic conjunctivitis. As shown in Fig. 4, a significantly increased clinical score as an indicator of allergic conjunctivitis was observed following each JCP challenge in the S-C group, compared with that in the NS-C group ($P < 0.01$, 10 min after the 1st to 4th challenges). When P1-3 Fabs were administered, animals showed a marked decrease in clinical score in the S-C-Fab group compared with the S-C group during the 1st to 4th challenges ($P < 0.01$, 10 min after the 1st, 3rd, and 4th challenges). Meanwhile, the effect of P1-8 Fabs on allergic conjunctivitis was similar to that in P1-3 Fabs, indicating the inhibitory effect of P1-8 Fabs ($P < 0.01$, 10 min after the 1st to 4th challenges) (Fig. 5).

3.4. Treatment with P1-8 Fabs suppresses the increased level of JCP-specific IgE antibody in serum

Based on the above data, we investigated the effect of P1-8 Fabs on the increased level of JCP-specific IgE antibodies related to the development of allergic conjunctivitis. As shown Table 3, the levels of total IgE and JCP-specific IgE antibodies 24 h after the fourth antigen challenge in the S-C group were increased compared with those in the NS-C group ($P < 0.01$). The treatment with P1-8 Fabs reduced the levels of total IgE and JCP-specific IgE antibodies ($P < 0.05$). However, the increased level of the JCP-specific IgG1 antibody was not inhibited by the treatment.

3.5. Treatment with P1-8 suppresses JCP-induced inflammatory cell infiltration in conjunctiva

It is generally known that mast cells are related to the development of allergic conjunctivitis. In a histological examination, we investigated the effect of P1-8 Fabs on the infiltration by mast cells in the conjunctiva. As shown Fig. 6, there was a significant difference in the infiltration by mast cells 24 h after the fourth challenge in the S-C group compared with that in NS-C group ($P < 0.01$). Treatment with P1-8 Fabs significantly inhibited the infiltration ($P < 0.01$).

Furthermore, the effect of P1-8 Fabs on the infiltration by eosinophils in the conjunctiva was investigated because it has been reported that patients with allergic conjunctivitis showed infiltration by eosinophils in the conjunctiva (Hingorani M, 1998). As shown in Fig. 7, there was a significant difference in the infiltration by eosinophils in the S-C groups 24 h after the 4th challenge compared with that in the NS-C group ($P < 0.01$). When P1-8 Fabs were administered, the infiltration by eosinophils was significantly suppressed ($P < 0.01$). Meanwhile, as shown Table 4, the infiltration by neutrophils in the S-C group was increased compared with that in the NS-C group ($P < 0.01$); however, treatment with P1-8 Fabs failed to suppress the infiltration. Furthermore, infiltration by lymphocytes did not show any differences between the NS-C, S-C, and S-C-P1-8 Fabs groups.

4. Discussion

In the present study, we showed that topical ocular treatment with IgG1 mAb Fabs targeting JCP Cry j 1 (P1-3 and P1-8) resulted in marked suppression of the JCP-increased clinical score as an indicator of allergic conjunctivitis in mice, suggesting the efficacy of allergen-

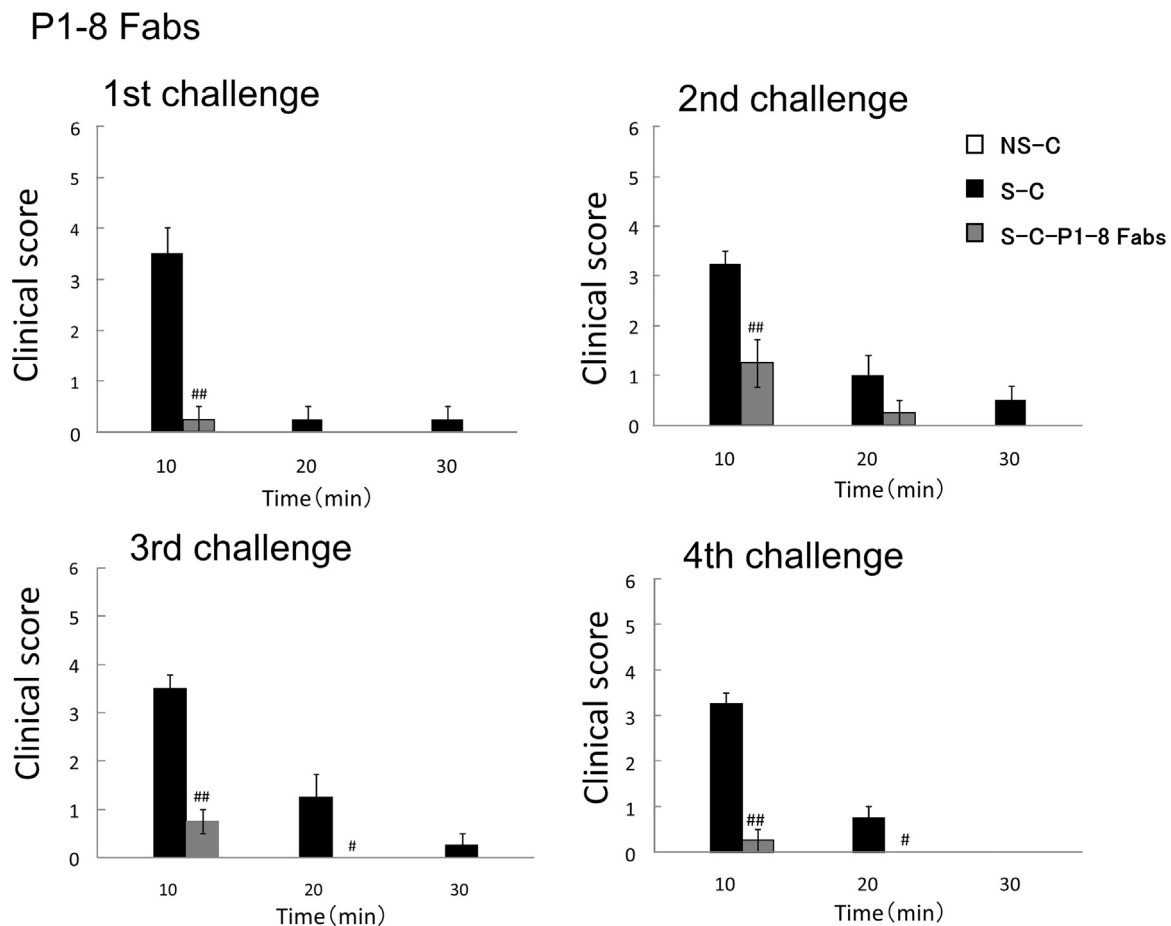


Fig. 5. Topical ocular treatment with P1-8 Fabs suppresses JCP-induced allergic conjunctivitis in mice. P1-8 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-8 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Each value is the mean $\pm$ S.E.M. of 5 animals. # $P < 0.05$, ## $P < 0.01$ compared with the S-C group.

Table 3

Effect of P1-8 Fabs on levels of total IgE, JCP-specific IgE, and JCP-specific IgG1 antibodies in the serum of mice with JCP-induced allergic conjunctivitis.

	$\mu\text{g/mL}$ Total IgE	OD450nm JCP-specific IgE	OD405nm JCP-specific IgG1
NS-C	0.980 ± 0.175	0.087 ± 0.002	0.089 ± 0.002
S-C	8.096 ± 1.042^a	0.167 ± 0.014^a	0.985 ± 0.214^a
S-C-P1-8 Fabs	4.701 ± 0.673^b	0.121 ± 0.009^b	0.980 ± 0.046

P1-8 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-8 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Each value is the mean $\pm$ S.E.M. of 5 animals.

^a $P < 0.01$ versus NS-C group.

^b $P < 0.05$ versus S-C group.

specific IgG1 mAb Fabs against allergic conjunctivitis.

We established two hybridomas showing the high-level production of IgG1 mAbs (P1-3 and P1-8), which bind specifically to Cry j 1; P1-3 and P1-8 recognized different epitopes of Cry j 1. Previously, we reported that P1-8 Fabs suppressed JCP-induced allergic rhinitis in mice (Yoshino and Mizutani, 2016); however, it is unknown whether the epitopes recognized by Fabs of anti-Cry j 1 IgG1 mAb are important for the suppression of JCP-induced allergic responses. Therefore, we examined the effect of not only P1-8, but also P1-3, on JCP-induced allergic conjunctivitis in mice because the two IgG1 mAbs recognized different epitopes of Cry j 1. As a result, both Fabs of P1-3 and P1-8 inhibited allergic conjunctivitis, suggesting the possibility that some

epitopes recognized by Fabs could be used as a tool to regulate allergic conjunctivitis.

It has been generally accepted that the interaction with JCP and JCP-specific IgE binding mast cells is important for the induction of allergic conjunctivitis. Therefore, we examined the effect of P1-8 Fabs on the increased level of total IgE or JCP-specific IgE antibodies and the infiltration by mast cells in the model of allergic conjunctivitis. As a result, these factors were suppressed by the treatment with P1-8 Fabs, suggesting that treatment with Cry j 1-specific IgG1 mAb Fabs decreased the allergic conjunctivitis via suppressing the increased level of the JCP-specific IgE antibody and the infiltration by mast cells.

In patients with allergic conjunctivitis, eosinophils, which are recognized as the hallmark of allergic diseases, are found in tears and tissues (Hingorani et al., 1998), and the clinical severity is significantly correlated with the increased number of eosinophils (Leonardi et al., 2003). In this allergic conjunctivitis model, there was an increased number of eosinophils in the conjunctiva compared with normal mice. Therefore, we examined the effect of P1-8 Fabs on the infiltration by eosinophils in the histological evaluation. In addition to the suppression of the increased clinical score by P1-8 Fabs, the infiltration by eosinophils was inhibited by P1-8 Fabs, further suggesting that topical ocular treatment with allergen-specific IgG1 mAb Fabs was effective in suppressing allergic conjunctivitis.

Why was JCP-induced conjunctivitis suppressed by IgG1 mAb Fabs recognizing only one epitope of Cry j 1? In the mouse allergic models using OVA as an allergen, we reported that treatment with O1-10 (OVA-specific IgG1 mAb) Fabs before OVA challenges suppressed OVA-induced asthma (Yoshino et al., 2014), rhinitis (Matsuoka et al.,

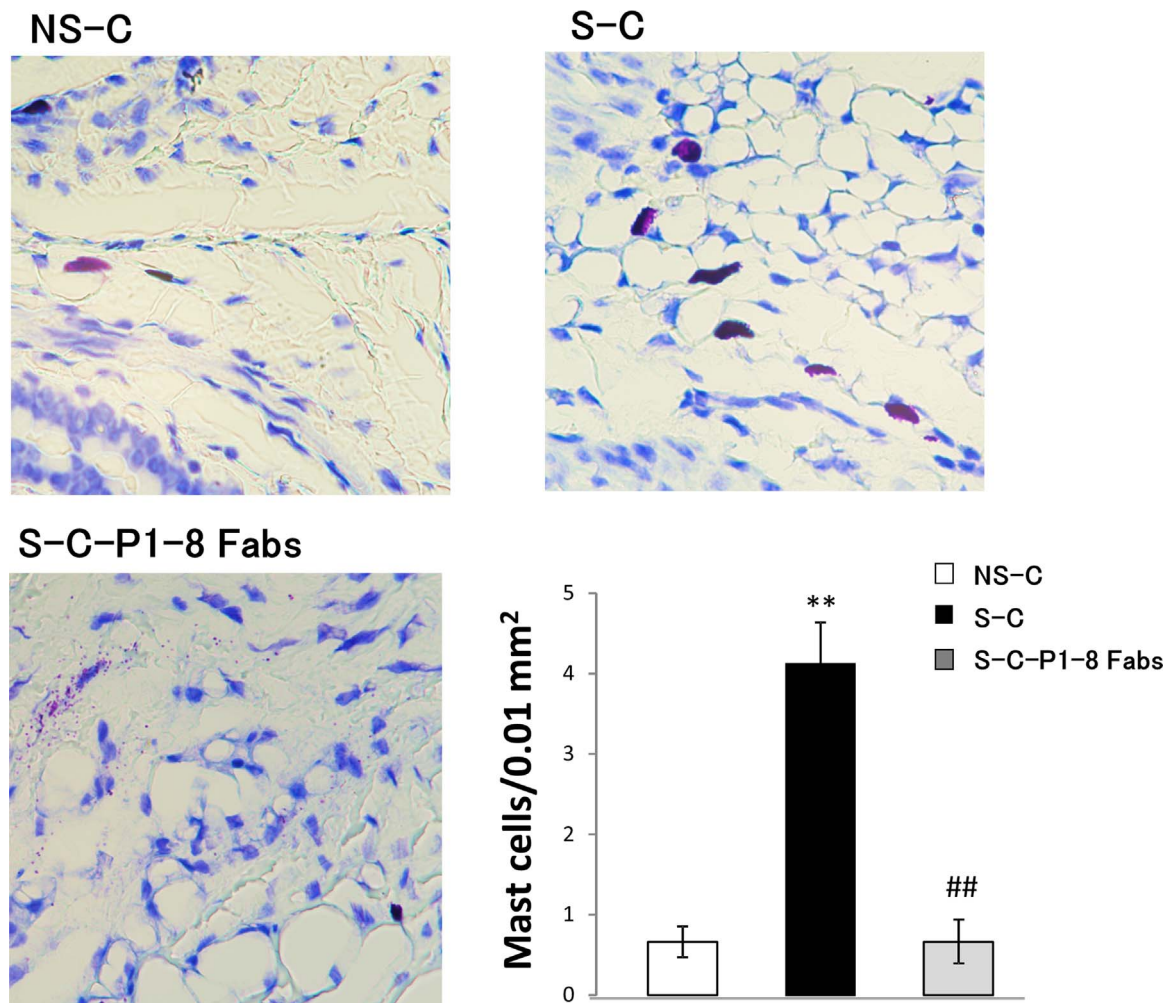


Fig. 6. Topical ocular treatment with P1-8 Fabs suppresses the infiltration by mast cells in the conjunctiva of mice with JCP-induced allergic conjunctivitis. P1-8 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-8 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Mast cells in the conjunctiva were stained with H & E on day 36 and counted in the NS-C, S-C, and S-C-P1-8 Fabs groups. Each value is the mean $\pm$ S.E.M. of 5 animals. ** $P < 0.01$; or ## $P < 0.01$ compared with the NS-C or S-C group, respectively.

2014), and dermatitis (Sae-Wong et al., 2016); furthermore, O1-10 Fabs can reduce the binding of anti-OVA polyclonal antibodies to OVA in in vitro experiments (Yoshino et al., 2014). These data suggest the possibility that mAb Fabs including P1-3 and P1-8 inhibit the interaction of polyclonal antibodies with allergens, and allergic conjunctivitis symptoms are reduced by suppressing the degranulation of mast cells and/or basophils. Meanwhile, we have reported that topical skin treatment with O1-10 Fabs suppressed the increased level of OVA in serum after the repeated application of OVA to the skin in sensitized mice (Sae-Wong et al., 2016), suggesting that anti-Cry j 1 mAb Fabs may suppress allergic conjunctivitis symptoms by reducing the uptake of Cry j 1 into the conjunctiva from the epithelium.

In the present study, JCP-induced allergic conjunctivitis was suppressed by the topical ocular treatment with P1-3 Fabs or P1-8 Fabs as a Cry j 1 allergen-specific therapy. It is generally known that antigen-specific immunotherapies using subcutaneous and sublingual administrations of specific antigens have the possibility to induce life-threatening anaphylactic reactions (Calamita et al., 2006; Eifan et al., 2010; Mungan et al., 1999). However, the frequency of side effects by the local administration of allergen-specific mAb Fab may be lower compared with subcutaneous and sublingual immunotherapies of allergic diseases in terms of allergen-specific therapies.

5. Conclusion

In conclusion, these data demonstrated that topical ocular treatment with IgG1 mAb Fabs targeting JCP Cry j 1 before JCP challenges reduced JCP-induced allergic conjunctivitis in sensitized mice. Therefore, patients with allergic conjunctivitis may be specifically regulated by topical treatment with Fabs of pathogenic allergen-specific IgG1 mAb.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgements

Part of this work was supported by Takeda Science Foundation (to NM) and JSPS KAKENHI Grant Number 16K08289 (to NM). We are grateful to Dr. Kouya Yamaki (Kobe Pharmaceutical University) for establishing the hybridomas. We thank Risa Takeuchi, Tatsuya Zento, Teshyu Ohnita, Yoshifumi Watai, Eriko Hiasa, Shouhei Hasegawa, Hikaru Goto, Sato Nakamura, Naoko Fujimoto, Maho Murata, Takashi Hirouchi, Junpei Yonemura, Shinsaku Araki (Kobe Pharmaceutical University) for their assistance with sample measurements.

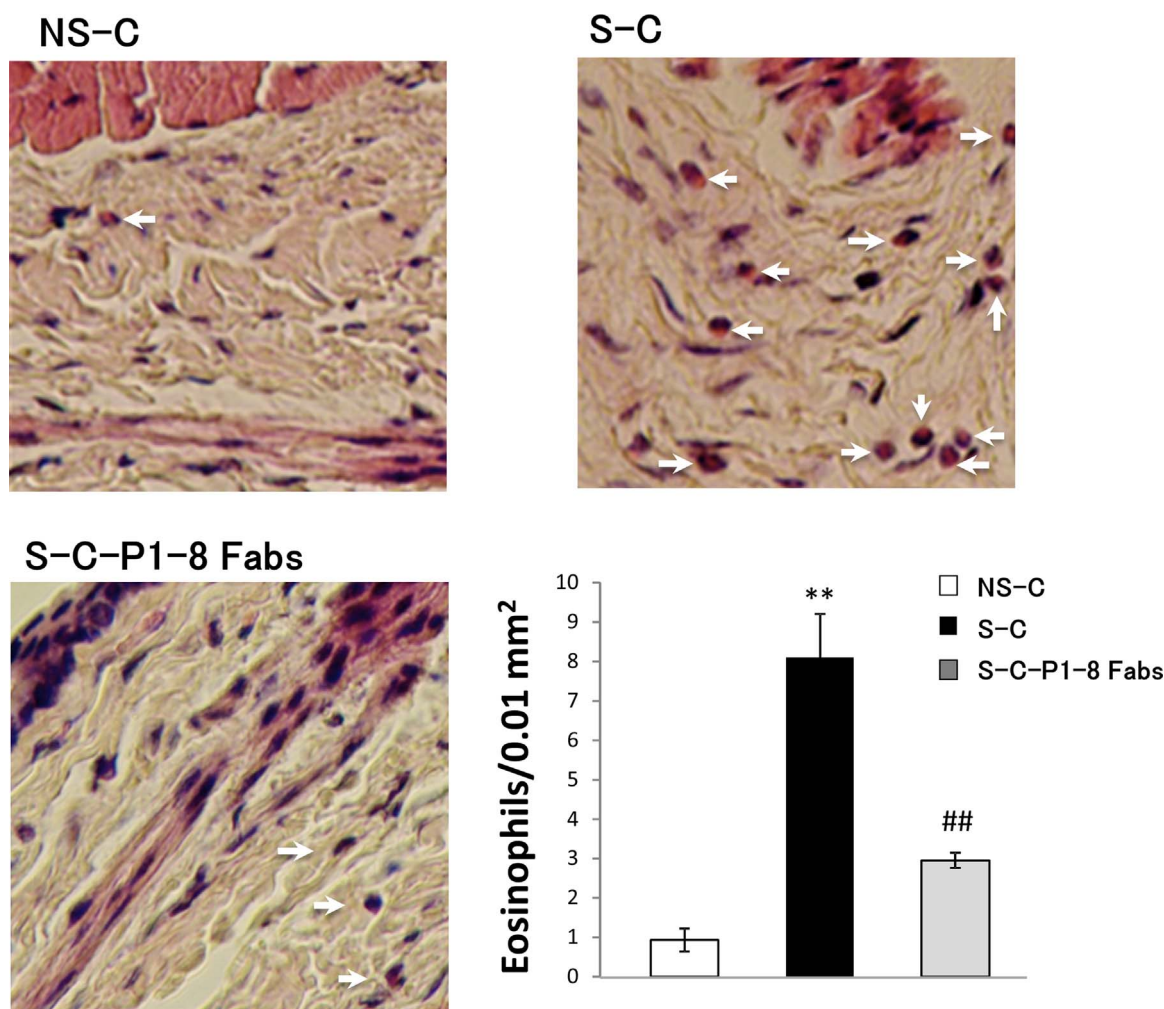


Fig. 7. Topical ocular treatment with P1-8 Fabs suppresses the infiltration by eosinophils in the conjunctiva of mice with JCP-induced allergic conjunctivitis. P1-8 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-8 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Eosinophils in the conjunctiva were stained with H & E on day 36 and counted in the NS-C, S-C, and S-C-P1-8 Fabs groups. Each value is the mean $\pm$ S.E.M. of 5 animals. ** $P < 0.01$; or ## $P < 0.01$ compared with the NS-C or S-C group, respectively.

Table 4

Effect of P1-8 Fabs on the infiltration by neutrophils and lymphocytes in the conjunctiva of mice with JCP-induced allergic conjunctivitis.

	Neutrophils/0.01 mm ²	Lymphocytes/0.01 mm ²
NS-C	0.2 $\pm$ 0.1	12.1 $\pm$ 1.3
S-C	1.8 $\pm$ 0.2 ^a	11.9 $\pm$ 1.0
S-C-P1-8 Fabs	1.2 $\pm$ 0.3	10.0 $\pm$ 1.0

P1-8 Fabs were administered 15 min before each JCP challenge by eye drops on days 28, 29, 30, and 35 in mice sensitized with JCP on days 0 and 14 (S-C-P1-8 Fabs). Control IgG Fabs were also administered by eye drops in mice sensitized and challenged with JCP as a positive control (S-C group). As a negative control, JCP was administered to non-sensitized mice (NS-C). Neutrophils and lymphocytes in the conjunctiva were stained with H & E on day 36 and counted in the NS-C, S-C, and S-C-P1-8 Fabs groups. Each value is the mean $\pm$ S.E.M. of 5 animals.

^a $P < 0.01$ versus NS-C group.

References

- Abramson, M.J., Puy, R.M., Weiner, J.M., 1995. Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am. J. Respir. Crit. Care Med.* 151, 969–974.
- Bielory, L., Friedlaender, M.H., 2008. Allergic conjunctivitis. *Immunol. Allergy Clin. North Am.* 28, 43–58, (vi).
- Bisca, M., 1997. Current therapy of allergic conjunctivitis. *Curr. Ther. Res.* 58, 828–841.
- Calamita, Z., Saconato, H., Pela, A.B., Atallah, A.N., 2006. Efficacy of sublingual

- immunotherapy in asthma: systematic review of randomized-clinical trials using the Cochrane Collaboration method. *Allergy* 61, 1162–1172.
- Casale, T.B., Stokes, J.R., 2014. Immunotherapy: what lies beyond. *J. Allergy Clin. Immunol.* 133, 612–619.
- Da, W., Zhu, J., Wang, L., Lu, Y., 2013. Efficacy and safety of certolizumab pegol for Crohn's disease: a systematic review and meta-analysis. *Adv. Ther.* 30, 541–553.
- Deeks, E.D., 2013. Certolizumab pegol: a review of its use in the management of rheumatoid arthritis. *Drugs* 73, 75–97.
- Dretzke, J., Meadows, A., Novielli, N., Huissoon, A., Fry-Smith, A., Meads, C., 2013. Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: a systematic review and indirect comparison. *J. Allergy Clin. Immunol.* 131, 1361–1366.
- Eifan, A.O., Akkoc, T., Yildiz, A., Keles, S., Ozdemir, C., Bahceciler, N.N., Barlan, I.B., 2010. Clinical efficacy and immunological mechanisms of sublingual and subcutaneous immunotherapy in asthmatic/rhinitis children sensitized to house dust mite: an open randomized controlled trial. *Clin. Exp. Allergy* 40, 922–932.
- Friedlaender, M.H., 1991. Current concepts in ocular allergy. *Ann. Allergy* 67, 5–10, (13).
- Hingorani, M., Calder, V., Jolly, G., Buckley, R.J., Lightman, S.L., 1998. Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases. *J. Allergy Clin. Immunol.* 102, 821–830.
- Katpally, U., Wobus, C.E., Dryden, K., Virgin, H.W., 4th, Smith, T.J., 2008. Structure of antibody-neutralized murine norovirus and unexpected differences from viruslike particles. *J. Virol.* 82, 2079–2088.
- Keles, S., Karakoc-Aydiner, E., Ozen, A., Izgi, A.G., Tevetoglu, A., Akkoc, T., Bahceciler, N.N., Barlan, I., 2011. A novel approach in allergen-specific immunotherapy: combination of sublingual and subcutaneous routes. *J. Allergy Clin. Immunol.* 128, 808–815.
- Leonardi, A., Jose, P.J., Zhan, H., Calder, V.L., 2003. Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis. *Ophthalmology* 110, 487–492.
- Matsuoka, D., Mizutani, N., Sae-Wong, C., Yoshino, S., 2014. Allergen-specific regulation

- of allergic rhinitis in mice by intranasal exposure to IgG1 monoclonal antibody Fab fragments against pathogenic allergen. *Immunol. Lett.* 161, 149–156.
- Mungan, D., Misirligil, Z., Gurbuz, L., 1999. Comparison of the efficacy of subcutaneous and sublingual immunotherapy in mite-sensitive patients with rhinitis and asthma – a placebo controlled study. *Ann. Allergy Asthma Immunol.* 82, 485–490.
- Nesbitt, A., Fossati, G., Bergin, M., Stephens, P., Stephens, S., Foulkes, R., Brown, D., Robinson, M., Bourne, T., 2007. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. *Inflamm. Bowel Dis.* 13, 1323–1332.
- Passalacqua, G., Canonica, G.W., 2011. Specific immunotherapy in asthma: efficacy and safety. *Clin. Exp. Allergy* 41, 1247–1255.
- Pipet, A., Botturi, K., Pinot, D., Vervloet, D., Magnan, A., 2009. Allergen-specific immunotherapy in allergic rhinitis and asthma. Mechanisms and proof of efficacy. *Respir. Med.* 103, 800–812.
- Sae-Wong, C., Mizutani, N., Kangsanant, S., Yoshino, S., 2016. Topical skin treatment with Fab fragments of an allergen-specific IgG1 monoclonal antibody suppresses allergen-induced atopic dermatitis-like skin lesions in mice. *Eur. J. Pharmacol.* 779, 131–137.
- Terato, K., Hasty, K.A., Reife, R.A., Cremer, M.A., Kang, A.H., Stuart, J.M., 1992. Induction of arthritis with monoclonal antibodies to collagen. *J. Immunol.* 148, 2103–2210.
- Yoshino, S., Mizutani, N., Matsuoka, D., Sae-Wong, C., 2014. Intratracheal exposure to Fab fragments of allergen specific monoclonal antibody regulates asthmatic responses in mice. *Immunology* 141, 617–627.
- Yoshino, S., Mizutani, N., 2016. Intranasal exposure to monoclonal antibody Fab fragments to Japanese cedar pollen Cry j1 suppresses Japanese cedar pollen-induced allergic rhinitis. *Br. J. Pharmacol.* 173, 1629–1638.