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Japanese cedar pollen upregulates the effector functions of eosinophils

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ABSTRACT

Background: Symptoms of rhinitis and asthma can be exacerbated during Japanese cedar pollen (JCP)-scattering season, even in subjects who are not sensitized to JCP, suggesting that innate immune responses may contribute to this process. We previously reported that house dust mite directly activates the effector functions of eosinophils. Similar mechanisms may play roles in the JCP-related aggravation of allergic diseases.

Objective: To investigate whether JCP or Cry j 1, a major allergen of JCP, can modify the effector functions of eosinophils.

Methods: Eosinophils isolated from the peripheral blood of healthy donors were stimulated with either JCP or Cry j 1, and their adhesion to human intercellular adhesion molecule-1 was measured using eosinophil peroxidase assays. The generation of eosinophil superoxide anion (O_2^-) was measured based on the superoxide dismutase-inhibitable reduction of cytochrome C. Concentrations of eosinophil-derived neurotoxin in the cell media were measured by enzyme-linked immunosorbent assay as a marker of degranulation.

Results: Both JCP and Cry j 1 directly induced eosinophil adhesiveness, generation of O_2^-, and release of eosinophil-derived neurotoxin. Both anti-αM and anti-β2 integrin antibodies blocked all of these eosinophil functions induced by JCP and Cry j 1. Similarly, PAR-2 antagonists also partially suppressed all of these effector functions induced by JCP and Cry j 1.

Conclusion: JCP and Cry j 1 directly activate the functions of eosinophils, and both αMβ2 integrin and partly PAR-2 are contributed to this activation. Therefore, JCP-induced eosinophil activation may play a role in the aggravation of allergic airway diseases in nonsensitized patients as well as in JCP-sensitized patients.

Keywords: Cry j 1; Eosinophils; Integrin; Japanese cedar pollen; PAR-2

INTRODUCTION

Japanese cedar pollen (JCP) is widely scattered during springtime, mainly between the months of February and April in Japan. The prevalence of the seasonal allergic rhinitis (SAR) by JCP (SAR-JCP) has reached 26.5% in our country, and has increased by almost 10% during 10 years [1, 2]. Even people living in the city have this pollinosis, because JCP can scatter from forest areas over long distances. Therefore, SAR-JCP is a serious health issue for many people in Japan. Until now, 8 allergens in JCP have been identified, and their characteristics have

been elucidated [2]. Among them, Cry j 1, a basic glycoprotein homologous to pectate lyase, is a major allergen of JCP [2]. In fact, most patients suffering from SAR-JCP have specific immunoglobulin (Ig) E to Cry j 1 [3]. Approximately 35 mg of Cry j 1 is routinely extracted per 100 g of JCP [2], although individual and genetic variations of Cry j 1 content in JCP have also been reported [4].

Several reports suggested that JCP can exacerbate asthma like other pollens [5-7]. The level of asthma control deteriorated in the JCP-scattering season in asthmatics with SAR-JCP in urban areas, although 84% of the patients received treatment for rhinitis [5]. We previously reported that the pulmonary function of asthmatics with SAR-JCP worsened in the JCP-scattering season, and that the deterioration was associated with an increased generation of cysteinyl leukotrienes in the airways [6]. Furthermore, we reported that sublingual immunotherapy for SAR-JCP completely suppresses the development of asthma exacerbation in JCP season [7], confirming the role of JCP in the seasonal exacerbation of asthma.

Although the mechanisms that contribute to asthma exacerbation by JCP is still unknown, possible mechanisms have been suggested [8]. For example, orbicules (about 1 μm in size) on JCP can reach the airways and directly worsen the asthma control. Furthermore, nasal obstruction, local release of mediators in the upper airways, and systemic production of cytokines including type-2 cytokines may play roles in the JCP-induced asthma exacerbation [8]. In patients who are sensitized to JCP, exposure to JCP results in the aggravation of allergic diseases through the activation of mast cells or basophils by IgE crosslinking and the subsequent activation of T cells. However, clinically, pollen or house dust mite (HDM) exposure induces allergic symptoms, even in patients who are not sensitized to those specific allergens [9, 10]. Although its mechanism is still unknown, one would postulate that innate immune responses might play roles in the pathogenesis of airway inflammation via a non-IgE-specific fashion.

Eosinophils have pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and protease-activated receptors (PARs) [11, 12]. PAR-2 is associated with the inflammatory response to some infections and microbial proteases [11]. We previously reported that HDM directly activates the functions of eosinophils obtained from the peripheral blood of nonallergic healthy subjects partly through PAR-2 [13]. Therefore, similar mechanisms may play roles in the JCP-related exacerbation of allergic diseases. However, the effects of JCP and Cry j 1 on eosinophil functions have not yet been fully clarified.

Here, we examined whether JCP or Cry j 1 can modify the functions of eosinophils. We found that both JCP and Cry j 1 induced eosinophil adhesiveness, superoxide anion (O_2^-) generation, and release of a specific granule protein, Furthermore, αMβ2 integrin and partly PAR-2 were involved in JCP or Cry j 1-induced eosinophil activation. Therefore, the direct activation of eosinophils by JCP may contribute to the aggravation of allergic diseases.

MATERIALS AND METHODS

Preparation of eosinophils and JCP
Eosinophils were isolated from the peripheral blood collected from nonallergic healthy volunteers with a peripheral blood differential eosinophil count of <5%. Healthy volunteers were defined as subjects with no history of allergic disease, such as asthma or rhinitis, and

no symptoms associated with allergic disease, such as coughing or sneezing. This study was approved by the Ethical Committee of Saitama Medical University (Institutional Review Board permission number: 781-III), and written informed consent was obtained from the subjects before the collection of each blood sample. Eosinophils were isolated by the combination of Percoll density gradient centrifugation and negative selection using anti-CD16 Ab-coated magnetic beads (Miltenyi Biotec, Auburn, CA, USA), as previously described [13-18]. Over 98% of the cells were eosinophils, as determined by morphological criteria using May-Grünwald-Giemsa staining. Eosinophil viability was >99%, as determined by Trypan blue dye exclusion. Eosinophils were resuspended in Hank's balanced salt solution (HBSS) supplemented with gelatin to a final concentration of 0.1% (HBSS/gel). The JCP extract (161-µg protein/mg of dry extract) [19] and purified Cry j 1 were kind gifts from Torii Pharmaceutical Co., Ltd. (Tokyo, Japan).

Eosinophil adhesion

The effects of JCP and Cry j 1 on eosinophil adhesion to recombinant human (rh) intercellular adhesion molecule (ICAM)-1-coated plates were assessed based on the residual eosinophil peroxidase (EPO) activity of the adherent eosinophils, as previously described [13-18]. Briefly, eosinophils (100 µL of 1×10^5 cells/mL in HBSS/gel) from nonallergic volunteers were incubated in the presence or absence of JCP (0.1 to 10 µg/mL) or Cry j 1 (1 to 100 pg/mL) in rh-ICAM-1 (10 µg/mL; R&D Systems, Minneapolis, MN, USA)-coated Costar cell culture plates (Corning, Inc., Corning, NY, USA) at 37℃ for 20 minutes. Corresponding control wells were coated with HBSS/gel. The plates were washed with HBSS, and 100 µL of HBSS/gel was then added to the wells. Standards comprised of serially diluted cell suspensions (1×10^3, 3×10^3, 1×10^4, 3×10^4, and 1×10^5 cells/mL; 100 µL each were added to the empty wells. An EPO substrate (1 mM o-phenylenediamine, 1 mM H_2O_2, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells, and the plates were incubated for 30 minutes at room temperature. The reaction was stopped by adding 20 µL of 4 M H_2SO_4, and the absorbance was measured at 490 nm. In some experiments, suspended eosinophils were pre-incubated with an isotype-matched control mouse IgG1 (3 µg/mL; clone MOPC-21, Becton Dickinson, Franklin Lakes, NJ, USA), anti-αM integrin monoclonal Ab (mAb; 3 µg/mL; clone 2LPM19c, Pierce, Rockford, IL, USA), anti-β2 integrin mAb (3 µg/mL; clone L130, Becton Dickinson), or a PAR-2 antagonist (10 µM FSLLRY-NH2 [13, 20-22], R&D Systems, or 10 µM ENMD-1068 [13, 23-25], Enzo Life Sciences, Farmingdale, NY, USA) for 20 minutes before addition to the wells. Each experiment was performed in quadruplicate using eosinophils from a single donor, and the percentage of eosinophil adhesion was determined from the mean values that were calculated from log-dose response curves. The eosinophil viability after incubation was >98%, as determined by Trypan blue dye exclusion.

Eosinophil O$_2^-$ generation

Eosinophil O_2^- generation was measured in 96-well enzyme-linked immunosorbent assay (ELISA) plates, as previously described, based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C [13-18]. We initially added SOD (0.2 mg/mL in HBSS/gel; 20 µL) to SOD control wells, then added HBSS/gel to all wells of the rh-ICAM-1-coated plates (10 µg/mL) to bring the final volume to 100 µl/well. The eosinophil density was adjusted to 1.25×10^6 cells/mL in HBSS/gel mixed in a 4:1 ratio with cytochrome C (12 mg/mL of HBSS/gel), and 100 µL of the eosinophil suspension was then added to all wells. Immediately after adding JCP (0.1 to 10 µg/mL) or Cry j 1 (1 to 100 pg/mL) to the eosinophils, the absorbance of the cell suspensions in the wells was measured at 550 nm in an Immuno-Mini (NJ-2300; Japan Intermed Co., Tokyo, Japan), followed by repeated measurements over the next 240

minutes. In some experiments, suspended eosinophils were pre-incubated with 3 µg/mL of mouse IgG1, anti-αM integrin mAb, anti-β2 integrin mAb, or 10 µM of a PAR-2 antagonist (FSLLRY-NH2 or ENMD-1068) for 20 minutes before addition to the wells. Each reaction was evaluated in duplicate against the control reaction in wells containing 20 µg/mL of SOD. The results were adjusted for a 1-mL reaction volume, and O_2^- generation was calculated with an extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ as the nanomoles of cytochrome C reduced per 1.0 × 10^6 cells/mL minus the SOD control. The maximum value observed over the incubation period was determined for the evaluation of the effects of various factors on eosinophil O_2^- generation. The cell viability, as determined by Trypan blue exclusion at the end of each experiment, remained at 95% after 240 minutes of incubation.

Release of an eosinophil-specific granule protein
Eosinophils (1 × 10^6 cells/mL) in 96-well plates were incubated for the 240 minutes that were required for the measurement of O_2^- generation, and were then immediately centrifuged (1,500 rpm) at 4°C for 10 minutes. The recovered cell-free supernatants were used for the measurements of eosinophil-derived neurotoxin (EDN), as described previously [13-18]. Concentrations of EDN were quantified using ELISA kits (Medical and Biological Laboratory Co., Ltd., Nagoya, Japan).

Statistical analysis
Values are expressed as the means ± standard error. Results were compared using 1-way analysis of variance followed by the Tukey-Kramer test when differences were significant, or a paired t test for the analysis of differences between the 2 groups. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Effects of JCP and Cry j 1 on eosinophil adhesion
We first examined the effect of JCP on eosinophil adhesion. Eosinophils were incubated with JCP (0.1 to 10 µg/mL) in rh-ICAM-1-coated plate, and eosinophil adhesion to ICAM-1 was measured. JCP at a concentration of more than 1 µg/mL significantly increased eosinophil adhesion when compared to the control (**Fig. 1A**). We then investigated whether Cry j 1, a major allergen of JCP, could also modify the eosinophil adhesion. Cry j 1 at a concentration of more than 10 pg/mL increased eosinophil adhesion when compared to the control (**Fig. 1B**).

Effects of JCP and Cry j 1 on eosinophil O_2^- generation and EDN release
We then examined whether JCP and Cry j 1 could modify eosinophil O_2^- generation. JCP at a concentration of 10 µg/mL induced O_2^- generation of eosinophils in ICAM-1-coated plate when compared to the control (**Fig. 1C**). Furthermore, Cry j 1 at a concentration of more than 1 pg/mL induced O_2^- generation of eosinophils when compared to the control (**Fig. 1D**). We then investigated the effects of JCP and Cry j 1 on eosinophil degranulation. Compared to the control, JCP at a concentration of more than 0.1 µg/mL induced the release of EDN (**Fig. 1E**). Cry j 1 at a concentration of more than 1 pg/mL also induced the release of EDN (**Fig. 1F**).

Effects of anti-integrin mAbs on the eosinophil adhesion, O_2^- generation, and EDN release induced by JCP and Cry j 1
We previously observed that the HDM-activated functions of eosinophils were β2 integrin-dependent [13]. Consequently, to examine the eosinophil integrin(s) involved in JCP-induced
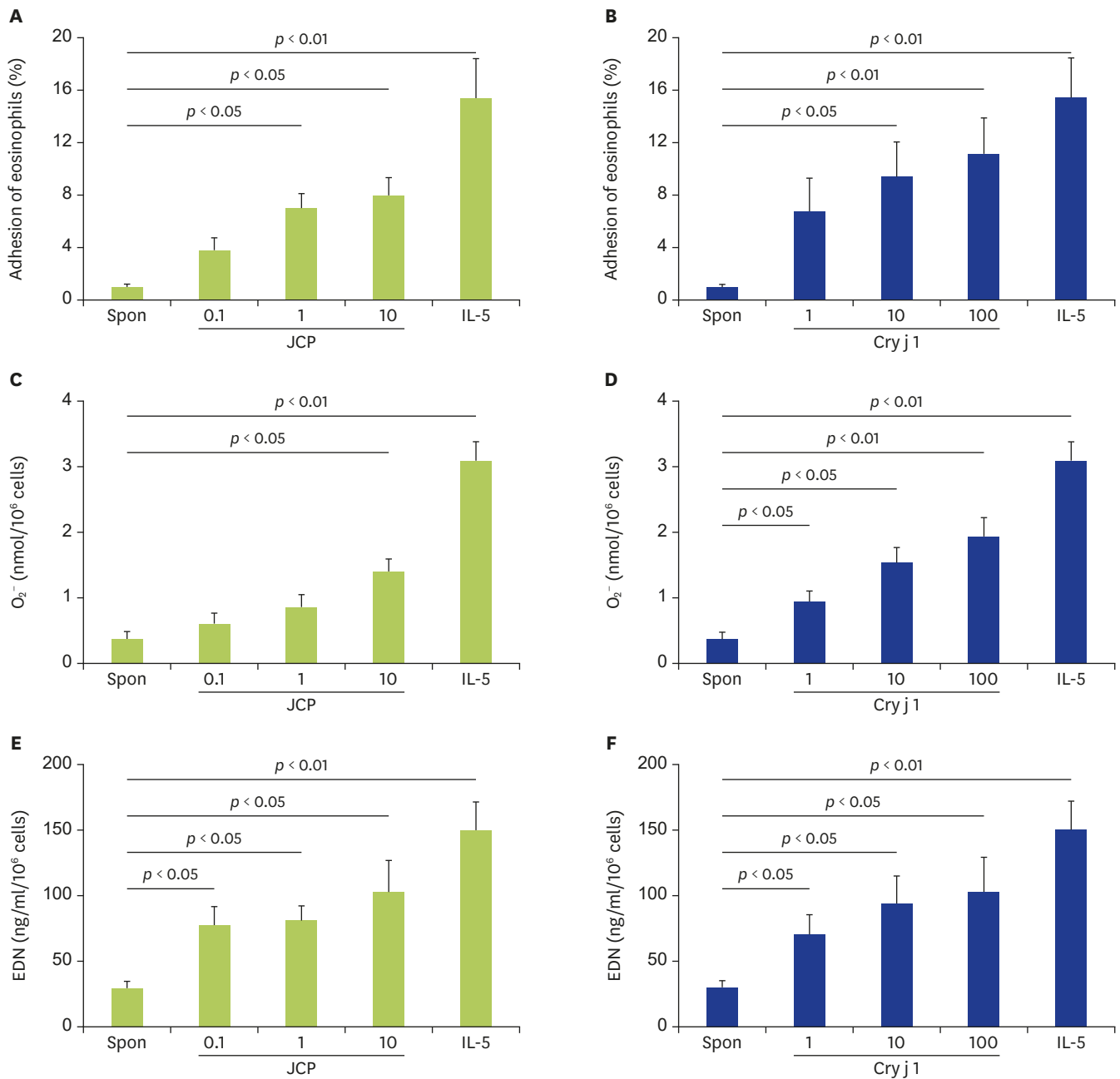
Fig. 1. Effects of Japanese cedar pollen (JCP) and Cry j 1 on eosinophil functions. (A) JCP increases eosinophil adhesion to recombinant human (rh) intercellular adhesion molecule (ICAM)-1-coated plates. Eosinophils (100 μL of 1×10^5 cells/mL in Hank's balanced salt solution [HBSS]/gel) obtained from the blood of healthy donors were incubated with JCP (0.1 to 10 μg/mL) or interleukin (IL)-5 (100 pM) as a positive control in rh-ICAM-1-coated plates. The adhesiveness of the eosinophils was then assessed by an assay of the residual eosinophil peroxidase activity. Data are shown as the means ± standard error of five experiments using cells from different donors. Spon, spontaneous adhesion. (B) Cry j 1 increases eosinophil adhesion to rh-ICAM-1-coated plates. Eosinophils from the blood of healthy donors were incubated with Cry j 1 (1 to 100 pg/mL) or IL-5 (100 pM), then eosinophil adhesion to ICAM-1 was measured (n = 5). Spon, spontaneous adhesion. (C, D) Effect of JCP and Cry j 1 on eosinophil O_2^- generation. The eosinophil cell density was adjusted to 1.25×10^6 cells/mL in HBSS/gel mixed in a 4:1 ratio with cytochrome C, then the eosinophil suspension was added to ICAM-1-coated 96-well plates. Immediately after adding (C) JCP (0.1 to 10 μg/mL) or (D) Cry j 1 (1 to 100 pg/mL), eosinophil O_2^- generation was measured based on the SOD-inhibitable reduction of cytochrome C, followed by repeated measurements over the next 240 minutes. The maximum values during the incubation period are shown as the means ± standard error of 6 experiments using cells from different donors. Spon, spontaneous O_2^- generation. (E, F) Effects of JCP and Cry j 1 on eosinophil eosinophil-derived neurotoxin (EDN) release. Eosinophils (1×10^6 cells/mL) in 96-well plates were incubated with (E) JCP (0.1 to 10 μg/mL) or (F) Cry j 1 (1 to 100 pg/mL) for 240 minutes. The concentration of EDN in the cell-free supernatants was then quantified using enzyme-linked immunosorbent assay. Data are shown as the means ± standard error of 6 experiments using cells from different donors. Spon, spontaneous EDN release.

and Cry j 1-induced eosinophil adhesion, eosinophils were pre-incubated with anti-αM integrin or anti-β2 integrin mAb, or control mouse IgG1, then eosinophil adhesion to ICAM-1 was examined. The enhancement of eosinophil adhesion by JCP (1 μg/mL) was suppressed by anti-αM integrin Ab as well as anti-β2 integrin Ab (**Fig. 2A**). Similarly, the enhancement of eosinophil adhesion by Cry j 1 (10 pg/mL) was also suppressed by anti-αM integrin Ab as well as anti-β2 integrin Ab (**Fig. 2B**). Anti-α4 integrin mAb did not affect the JCP-enhanced and Cry j 1-enhanced eosinophil adhesion (data not shown). Neither anti-αM integrin nor anti-β2 integrin mAb suppressed the spontaneous adhesion of eosinophils (data not shown).

We next examined the effects of anti-αM integrin or anti-β2 integrin Ab on JCP-induced and Cry j 1-induced eosinophil O_2^- generation and EDN release. The eosinophil O_2^- generation induced by JCP (10 μg/mL) and Cry j 1 (100 pg/mL) was suppressed by anti-αM integrin Ab as well as by anti-β2 integrin Ab (**Fig. 2C, D**). Anti-αM integrin and anti-β2 integrin mAbs did not modify the spontaneous O_2^- generation by eosinophils (data not shown). Finally, anti-αM integrin and anti-β2 integrin mAbs inhibited the EDN release induced by JCP (10 μg/mL; **Fig. 2E**) and Cry j 1 (100 pg/mL; **Fig. 2F**).

Effects of PAR-2 antagonists on the eosinophil adhesion, O_2^- generation, and EDN release induced by JCP and Cry J 1

We further evaluated whether PAR-2 was involved in the JCP-induced and Cry j 1-induced eosinophil adhesion. PAR-2 antagonists (FSLLRY-NH2 and ENMD-1068) suppressed the enhanced eosinophil adhesion by JCP (1 μg/mL; **Fig. 3A**). PAR-2 antagonists (FSLLRY-NH2 and ENMD-1068) suppressed the enhanced eosinophil adhesion by Cry j 1 (10 pg/mL; **Fig. 3B**). Therefore, JCP and Cry j 1 appear to upregulate eosinophil adhesion, at least in part, via a PAR-2-dependent mechanism.

We next examined the effects of PAR-2 antagonists on JCP-induced and Cry j 1-induced eosinophil O_2^- generation and EDN release. The eosinophil O_2^- generation induced by JCP (10 μg/mL) and Cry j 1 (100 pg/mL) was inhibited by PAR-2 antagonists (**Fig. 3C, D**). PAR-2 antagonists did not affect the spontaneous O_2^- generation by eosinophils (data not shown). Furthermore, the PAR-2 antagonists significantly inhibited the EDN release induced by JCP (10 μg/mL; **Fig. 3E**) and Cry j 1 (100 pg/mL; **Fig. 3F**).

DISCUSSION

In this study, we found that JCP and Cry j directly induced eosinophil adhesiveness, O_2^- generation, and release of EDN. Anti-αM integrin as well as anti-β2 integrin mAb inhibited the JCP-induced and Cry j 1-induced eosinophil adhesion, O_2^- generation, and EDN release, indicating that the effects of JCP and Cry j 1 involve eosinophil αMβ2 integrin. Moreover, PAR-2 antagonists inhibited the eosinophil activation induced by JCP and Cry j 1. These results suggest that eosinophil activation by JCP may contribute to the aggravation of allergic airway diseases.

JCP and Cry j 1 significantly increased eosinophil adhesion in nonsensitized healthy donors (**Fig. 1A, B**), which were consistent with the findings that allergens, such as pollen, exacerbate allergic diseases, even in nonsensitized patients [9, 10]. One possible mechanism for this may be the involvement of local IgE responses [26, 27]. Another possibility is the involvement of innate immune responses. We recently confirmed that HDM can directly
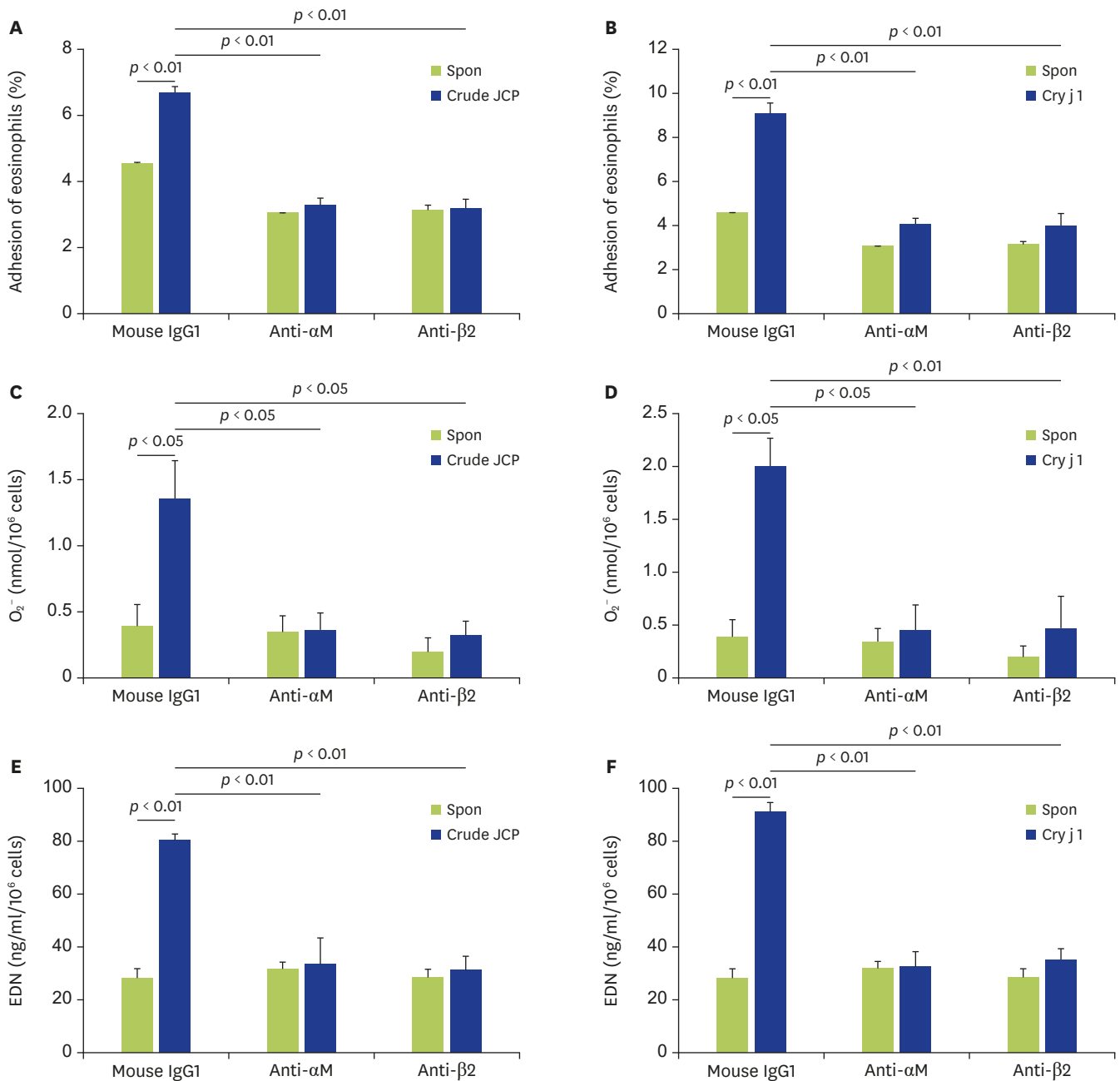
Fig. 2. Effects of anti-integrin mAbs on Japanese cedar pollen (JCP)-induced and Cry j 1-induced eosinophil activation. (A, B) JCP-induced and Cry j 1-induced eosinophil adhesion is inhibited by both anti-αM and anti-β2 integrin mAbs. Eosinophils were pre-incubated with anti-αM integrin mAb, anti-β2 integrin mAb, or control IgG (3 μg/mL each) for 20 minutes prior to the analysis of adhesion to intercellular adhesion molecule-1 in the presence or absence of JCP (1 μg/mL; n = 6) (A) or Cry j 1 (10 pg/ml; n = 6) (B). Spon, spontaneous adhesion. (C, D) JCP-induced and Cry j 1-induced eosinophil O_2^- generation is inhibited by both anti-αM and anti-β2 integrin mAbs. Eosinophils were pre-incubated with anti-αM integrin mAb, anti-β2 integrin mAb, or control IgG (3 μg/mL each) for 20 minutes prior to the analysis of O_2^- generation from eosinophils in the presence or absence of JCP (10 μg/mL; n = 5) (C) or Cry j 1 (100 pg/mL; n = 6) (D). Spon, spontaneous O_2^- generation. (E, F) JCP-induced and Cry j 1-induced eosinophil eosinophil-derived neurotoxin (EDN) release is inhibited by both anti-αM and anti-β2 integrin mAbs. Eosinophils were pre-incubated with anti-αM integrin mAb, anti-β2 integrin mAb, or control IgG (3 μg/mL each) for 20 minutes prior to the analysis of EDN release from eosinophils in the presence or absence of JCP (10 μg/mL; n = 4) (E) or Cry j 1 (100 pg/mL; n = 4) (F). Spon, spontaneous EDN release.

activate the functions of eosinophils mainly through PAR-2 [13]. Collectively, nonspecific activation through PRRs, such as TLRs or PARs, may also play a role in the JCP-related aggravation of allergic diseases.
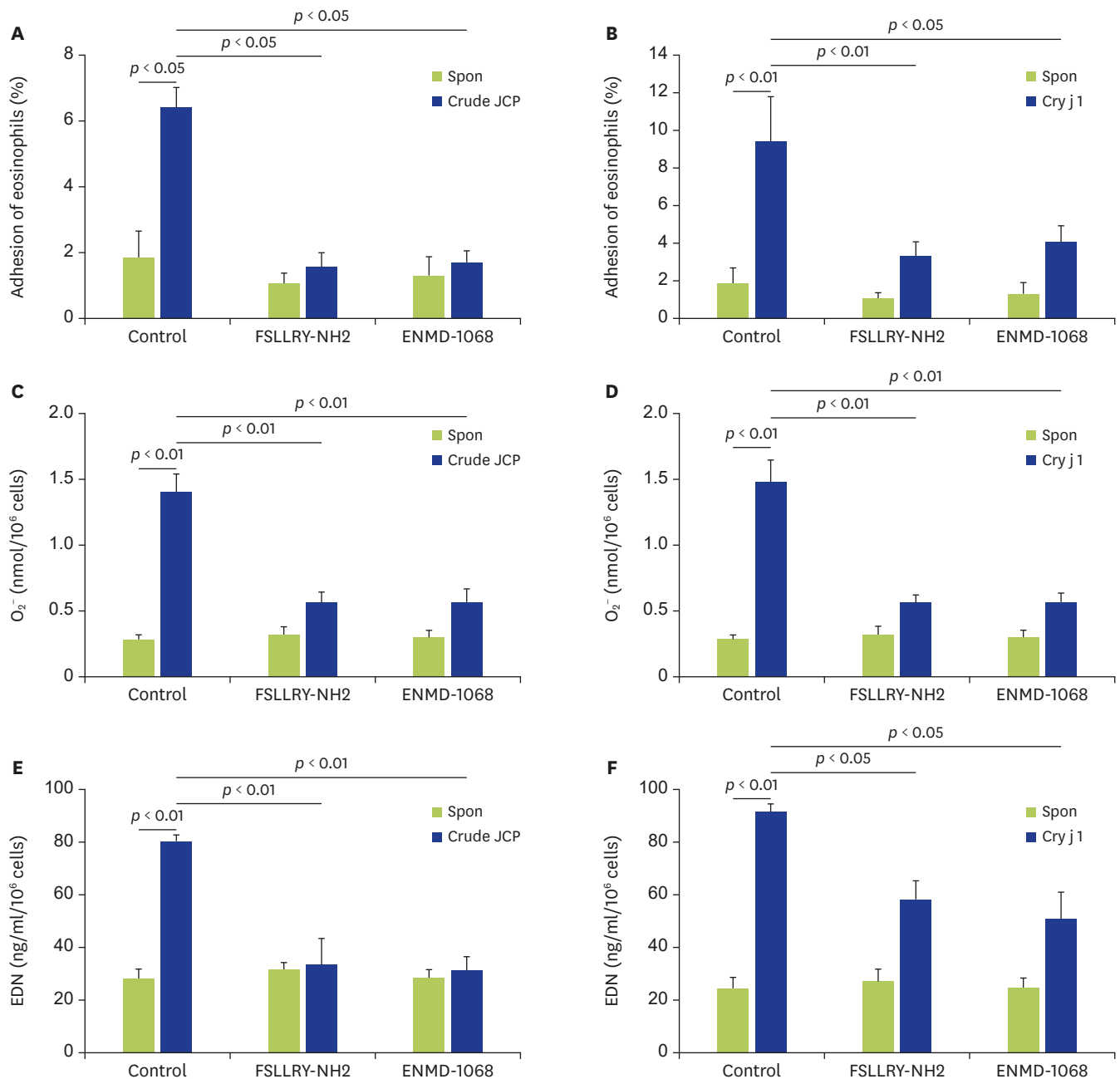
Fig. 3. Effects of PAR-2 antagonists on Japanese cedar pollen (JCP)-induced and Cry j 1-induced eosinophil activation. (A, B) Effects of PAR-2 antagonists on JCP-induced and Cry j 1-induced eosinophil adhesion. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLLRY-NH2 or ENMD-1068; 10 μM each) for 20 minutes prior to the analysis of adhesion to intercellular adhesion molecule-1 in the presence or absence of JCP (1 μg/mL; n = 6) (A) or (B) Cry j 1 (10 pg/mL; n = 6). Spon, spontaneous adhesion. (C, D) Effects of PAR-2 antagonists on JCP-induced and Cry j 1-induced eosinophil O_2^- generation. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLLRY-NH2 or ENMD-1068; 10 μM each) for 20 minutes prior to the analysis of O_2^- generation from eosinophils in the presence or absence of JCP (10 μg/mL; n = 5) (C) or Cry j 1 (100 pg/mL; n = 5) (D). Spon, spontaneous O_2^- generation. (E, F) Effects of PAR-2 antagonists on JCP-induced and Cry j 1-induced eosinophil eosinophil-derived neurotoxin (EDN) release. Eosinophils were pre-incubated with or without a PAR-2 antagonist (FSLLRY-NH2 or ENMD-1068; 10 μM each) for 20 minutes prior to the analysis of EDN release from eosinophils in the presence or absence of JCP (10 μg/mL; n = 5) (E) or Cry j 1 (100 pg/mL; n = 5) (F). Spon, spontaneous EDN release.

In the present study, JCP-induced and Cry j 1-induced eosinophil activation was inhibited by PAR-2 antagonists (**Fig. 3**). PARs are expressed in various type of cells and activated by specific proteases [11]. There are 4 types of PARs (PAR-1 to PAR-4) in humans. Among these,

PAR-2 has been confirmed to play a role in the pathogenesis of allergic inflammation [11, 28-32]. For example, the activation of PAR-2 induces the production of proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor [28-31]. The deletion of the gene encoding PAR-2 and treatment with a PAR-2 antagonist have both been shown to suppress eosinophilic airway inflammation [32]. Furthermore, human eosinophils express PAR-2 protein and *PAR-2* mRNA [33]. However, the involvement of PAR-2 in JCP-mediated and Cry j 1-mediated cell activation has not yet been fully elucidated.

JCP has protease activity [34, 35], e.g., the serine protease CPA9 [2], and PAR-2 may therefore play a role in the JCP-induced cell activation. However, whether Cry j 1 has protease activity remains unclear [36, 37]. For example, Kouzaki et al. [36] reported that JCP-induced IL-25 production from airway epithelial cells through PAR-2-dependent mechanisms [36]. However, Cry j 1 did not induce IL-25 production, suggesting that PAR-2 is unlikely to be involved in the Cry j 1-induced cell activation. Kumamoto et al. [37] reported that Cry j1 itself did not have protease activity; however, Cry j1 increased the protease activity transiently in the medium of cultured keratinocytes, and this increase was inhibited by PAR-2 antagonists [37]. It was speculated that the interaction of Cry j1 with epidermal keratinocytes induces the PAR-2 activation [37]. A similar mechanism may contribute to the Cry j 1-induced PAR-2-mediated eosinophil activation observed in this study, and should be examined in the future. Furthermore, other PARs including PAR-3, the mRNA of which is transcribed in human eosinophils [33], and other PPRs including TLRs, may be involved. We examined the effects of anti-TLR4 mAb on JCP-induced and Cry j 1-induced eosinophil adhesion as JCP may be contaminated with lipopolysaccharide; however, the anti-TLR4 mAb did not inhibit eosinophil adhesion (data not shown). Furthermore, as eosinophils constitutively express functional TLR7 [38], we examined the effects of TLR7-signal blocking on JCP-induced and Cry j 1-induced eosinophil adhesion using synthetic oligodeoxynucleotides with immuno-regulatory sequences that specifically block signaling via TLR7 [39]; however, no suppression of eosinophil adhesion was observed (data not shown).

Pollen, including JCP, has nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [40, 41], which can generate reactive oxygen species. The NADPH oxidase activity of pollen grains differs between species [40]. The activity of JCP was found to be localized at the inner surface and cytoplasm, but not on the outer wall of pollen, which is sloughed off after rupture [40]. Although the NADPH oxidase activity seems to be lower in JCP than in ragweed or birch, it may be involved in the JCP-induced eosinophil O_2^- generation observed in this study. Additionally, pollen has eicosanoid-like lipids [42, 43] that may play a role in JCP-induced eosinophil activation. Indeed, cysteinyl leukotrienes and prostaglandin D_2 directly activate eosinophils [44, 45].

Recently, Kanno et al. [46] reported that β-D-glucan in JCP acts as an immunological adjuvant for allergic inflammation, and may be associated with exacerbated sneezing in a mouse model. β-D-glucan was detected in the exine and cell wall of the generative cell and tube cell of the grain of JCP [46]. In that study, β-D-glucan in the exine stimulated the production of tumor necrosis factor α and IL-6 in bone marrow-derived dendritic cells through dectin-1 [46]. As for eosinophils, the expression of dectin-1 remains controversial [12, 47, 48]. For example, Ahrén et al. [47] reported that non-typeable *Haemophilus influenzae* activates human eosinophils through dectin-1. Therefore, there is a possibility that β-D-glucan in JCP directly activates eosinophils through dectin-1, although we did not examine the role of dectin-1 in this study. On the other hand, Yoon et al. [48] reported that eosinophils do

not express dectin-1, and that β-D-glucan activates eosinophils through αM integrin (CD11b). We found in the present study that the JCP-induced eosinophil adhesion, O_2^- generation, and EDN release were suppressed by anti-αM integrin Ab (**Fig. 3**). Therefore, if β-D-glucan in JCP activates eosinophils through αM integrin, our results regarding the role of αM integrin in JCP-induced eosinophil activation are consistent with the results of Yoon et al. [48].

A limitation of this study is that we did not examine the role of NADPH oxidase or dectin-1 in JCP-induced eosinophil activation, as was described above. Dectin-1 may contribute to the development of allergic inflammation [49]. Another limitation is that there is little information available regarding PAR-2 antagonists such as the optimal concentrations, specificity, and so on [13, 20-25]. Moreover, we used PAR-2 antagonists only for investigation of the role of PAR-2 in the JCP-induced and Cry j 1-induced eosinophil activation, and other approaches including the transfection of small-interfering RNAs should be considered to confirm the role of PAR-2.

In conclusion, JCP and Cry j 1 activate the functions of eosinophils through αMβ2 integrin and in part through PAR-2-dependent mechanisms. Therefore, eosinophil activation by JCP may be involved in the aggravation of allergic airway diseases, even in nonsensitized individuals.

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