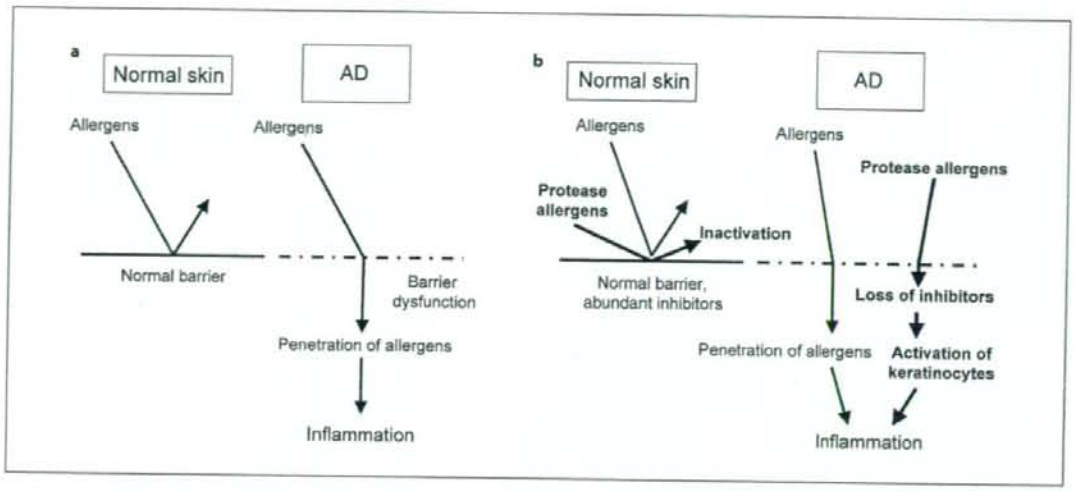

Fig. 6. Physical and biochemical barrier function of the skin. **a** Conventional concept of the 'physical' barrier function of the skin. **b** Protease inhibitors in the skin function as a 'biochemical' barrier against environmental protease allergens.

CSF and IL-8, which could contribute to the initiation and perpetuation of cutaneous inflammation in the microenvironment. Calcium concentration, which is an important factor for differentiation of keratinocytes, seems important in the response of keratinocytes (fig. 2c, d); however, the release of GM-CSF was upregulated at a low calcium concentration in some experiments (fig. 5c and unpublished observations), suggesting that conditions such as cell density and stage of differentiation affect the susceptibility of the cells to stimulation.

Where and how the cysteine protease activity of the mite group 1 allergens can be activated in vivo is still unclear, although Herbert et al. [5] speculated that Der p 1 could be activated by reduction with glutathione present in airway tissue on the basis of their in vitro results. Interestingly, unactivated rDer f 1 could upregulate the release of proinflammatory cytokines (fig. 4, 5c). Its cysteine protease activity was partially regenerated after incubation with keratinocytes (fig. 5b) and capable to cause cell detachment at 400 nM of rDer f 1 (unpublished data). Cystatin A inhibited the upregulation of GM-CSF release from keratinocytes stimulated with the unactivated rDer f 1 (fig. 5c). These results suggest that oxidized catalytic cysteine residue of the mite group 1 allergens could be reduced by interaction with the cells or components in the culture supernatant including cell-derived factors and that cysteine protease activity regenerated after incubation with the cells stimulates the keratinocytes.

Cystatin A was stable (fig. 3) even though it interacted with active rDer p 1 or rDer f 1 (fig. 2a), which can be explained by the high-affinity binding of this inhibitor to the cysteine protease and by the finding that the N-terminal hook of cystatin family inhibitors binds to part of the substrate-binding cleft of cysteine proteases in a substrate-like manner but then turns away from the active site, preventing proteolytic processing [29]. The stability of cystatin A is considered to contribute to a homeostatic role of cystatin A against skin inflammation. Indeed, the cystatin A content is reported to have decreased in the lesional skin of patients with AD [30], and very recently, Vasilopoulos et al. [22] found a significant association of a cystatin A genotype, in which mRNA of cystatin A is unstable, with AD. Thus, a decrease in cystatin A by nongenetic [30] or genetic factors [22] could increase the risk of sensitization through the skin and the perpetuation of AD. The physical barrier dysfunction is a major manifestation of AD, and its involvement with the pathogenesis has been reported (fig. 6a) [25, 31, 32]. Adding to this conventional concept, protease inhibitors such as cystatin A could function as a biochemical skin barrier to prevent the activation of keratinocytes by environmental protease allergens (fig. 6b).

Analysis of interactions between environmental and genetic factors such as those between protease allergens and their inhibitors is important to elucidate the pathogenesis of allergic diseases and to prevent their development [23–25]. Proteolytic activity of rDer f 1 and rDer p 1 upregulates GM-CSF and IL-8 release from keratinocytes in vitro, suggesting possible contributions to sensitization through the skin and the perpetuation of AD, as well as a homeostatic role for cystatin A against inflammation of the skin. An analysis of the mechanism of stimulation of keratinocytes with Der f 1 and Der p 1 should be addressed in a future study.

Acknowledgements

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ORIGINAL ARTICLE

Protease Activity of Allergenic Pollen of Cedar, Cypress, Juniper, Birch and Ragweed

Hendra Gunawan[1,2], Toshiro Takai[1], Shigaku Ikeda[2], Ko Okumura[1,3] and Hideoki Ogawa[1]

ABSTRACT

Background: Pollen is an important trigger of allergic rhinitis, conjunctivitis, and/or asthma, and an exacerbating factor in atopic dermatitis. Although it is proposed that protease activity from allergen sources, such as mites, enhances allergenicity, little information is available on that from relevant allergenic pollens such as Japanese cedar and Japanese cypress pollens, which are the major cause of pollinosis in Japan.

Methods: We analyzed the protease activities derived from allergenic pollen of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, and white birch and short ragweed, using synthetic substrates and class-specific inhibitors.

Results: We found that the pollen of the three members of the *Cupressaceae/Taxodiaceae* family contained serine protease activity, that the pollen of white birch and short ragweed contained not only serine protease activity but also cysteine protease activity, that all five types of pollen tested contained at least one other type of serine protease, whose sensitivity to a serine protease-specific inhibitor was relatively low, and that the content and releasability of the pollen-derived proteases differed according to the plant families.

Conclusions: Clinically relevant allergenic pollens tested in the present study can release serine and/or cysteine endopeptidases. Information on the spectrum of the endopeptidase activities from these allergenic pollen grains will be useful for investigating their contribution to the pathogenesis of allergies.

KEY WORDS

birch, cysteine protease, Japanese cedar, Japanese cypress, juniper, pollen allergy, protease inhibitor, proteolytic activity, ragweed, serine protease

INTRODUCTION

Enzymes,[1-4] lipids,[5-7] and chitin[8] produced by allergen-producing organisms have been suggested to be involved in the pathogenesis of allergic diseases through IgE-independent innate immunity. Proteolytic activity from allergen sources has been one of the candidates proposed to lead to enhanced allergenicity since initial studies.[1-3] Proteases derived from house dust mites have been suggested to be involved in the pathogenesis of allergies in sensitization and/or exacerbation by facilitating the passage of their own and other allergens across tissue barriers, cleaving various molecules, and modulating the functions of various cells and immune responses.[1,3,9-21] Some reports suggested that proteases derived from cockroaches,[22] molds,[23] and pollens[24] also contribute to the pathogenesis of allergies.

Pollen is an important trigger of allergic rhinitis, conjunctivitis, and/or asthma, and an exacerbating factor in atopic dermatitis.[25-27] In Europe and North America, birch of *Betula* species is the most important allergenic tree.[28] One report described the substrate specificity of birch pollen-derived protease activity.[29] Pollen of trees of the *Cupressaceae/Taxodiaceae* family, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtuse*), *Juniperus* species, and *Cupressus* species, are relevant allergens.[25,26,28] In Japan, Japanese cedar pollinosis is a common seasonal allergic disease posing a major public health problem caused by inhalation of pollen of Japanese cedar and Japanese cypress.[25,30] Aller-

[1]Atopy (Allergy) Research Center, [2]Department of Dermatology and [3]Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan.
Correspondence: Toshiro Takai, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.
Email: t-takai@med.juntendo.ac.jp
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gens from Japanese cedar share IgE-epitopes with homologous allergens from Japanese cypress. Almost no information is available on endopeptidases from pollen of the *Cupressaceae/Taxodiaceae* family although a research group reported on purification and characterization of an aminopeptidase from Japanese cedar pollen.[31,32] Ragweed pollen of *Ambrosia* species is one of the most clinically relevant allergenic pollen, and serine proteases have been purified from ragweed pollen and characterized.[33,34] Grass pollen also contain protease activity, which causes detachment of murine airway cells *in vitro*.[24]

Here, we compare the strength and substrate specificity of proteolytic activities derived from allergenic pollen of Japanese cedar, Japanese cypress, Rocky mountain juniper, white birch, and ragweed using synthetic substrates and class-specific inhibitors.

METHODS

POLLEN

Pollen of Japanese cedar (*Cryptomeria japonica*) was purchased from Wako (Osaka, Japan) or kindly provided by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan). Pollen of Japanese cypress (*Chamaecyparis obtusa*) was purchased from Wako. Pollen of Rocky mountain juniper (*Juniperus scopulorum*) and white birch (*Betula alba*) were purchased from Sigma (St. Louis, MO, USA). Pollen of short ragweed (*Ambrosia artemisiifolia*) was purchased from Polysciences (Warrington, PA, USA).

EXTRACTION OF PROTEASES FROM POLLEN GRAINS

Pollen grains were suspended in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS, pH 7.4) (10 mg-grains/1 ml in 1.5 ml-tubes). The suspension was shaken gently at 37℃ for 15 minutes and centrifuged for 5 minutes at 2,000 *g*. The supernatant was collected and filtered (0.22 μm) (Sup 1). The precipitate was resuspended with fresh PBS. The procedure for extraction described above was repeated twice to prepare Sup-2 and Sup-3. The third precipitate was resuspended with fresh PBS. The suspension was sonicated once for 15 minutes (10 cycles of 1 minute of sonication at 30-second intervals) (Olympus, Tokyo, Japan). After centrifugation, the supernatant was filtered (Sup-S). The filtered supernatants were stored at −80℃ until used. These samples or those prepared with the modification of skipping the preparation of Sup-3 were used to obtain the data shown in Figure 1B and C, respectively.

Another procedure for extraction was also applied as follows. Pollen grains were suspended in saline, PBS (pH 7.4), or 50 mM Tris-Cl (pH 9.0) (10 mg-grains/1 ml in 1.5 ml-tubes). The suspension was shaken gently at 37℃ for 2 hours, sonicated twice, and incubated overnight on ice. The next day, the suspension was sonicated twice again. After centrifugation, the supernatant was filtered and stored at −80℃ until used. These samples were used to obtain the data shown in Figure 2B. Samples extracted with the Tris buffer were used to obtain the data shown in Figures 3–5 and Table 1.

MEASUREMENT OF PROTEASE ACTIVITY

Protease activity was measured as previously described[35] with minor modifications as follows. A 5-μl volume of each sample was used for each assay. After incubation with or without 1 mM dithiothreitol (DTT) and further incubation with or without E-64 (50 μM) and/or 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (500 μM), or EDTA (20 mM)in 50 μl, 50 μl of substrate solution was added. Assays were conducted in PBS containing DTT with substrates (0.1 mM) at 37℃ in 100 μl. The final concentrations of E-64, AEBSF, and EDTA were 25 μM, 250 μM, and 10 mM, respectively. The substrates used were butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA), benzoyl-Arg-MCA (Bz-R-MCA), butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA), butyloxycarbonyl-Phe-Ser-Arg-MCA (Boc-FSR-MCA), butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA), succinyl-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA) succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), and succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA). The fluorescence of aminomethylcoumarin released from the substrate was measured on a fluorometer SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, CA, USA).[16] Kinetic data were collected in all the experiments. The data shown are representatives of three or more independent experiments.

PROTEIN ASSAY

Protein concentrations were determined by the Bradford procedure with a protein assay kit (Bio Rad, Richmond, CA, USA), with bovine serum albumin (Bio Rad).

RESULTS

TIME COURSE FOR RELEASE OF PROTEASE ACTIVITY FROM POLLEN GRAINS AND EFFECT OF SONICATION

According to the procedure shown in Figure 1A, three supernatants, Sup-1, Sup-2, and Sup-3, were extracted from pollen grains under mild conditions without sonication. Sup-S was prepared with sonication. Protease activity against a synthetic substrate, Boc-QGR-MCA, in the presence of DTT was tested (Fig. 1B). In Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, Sup-S prepared with sonication showed particularly stronger activity than the supernatants prepared without sonication. Enhancement of the protease activity against Boc-QGR-MCA by sonication was statistically significant (Fig. 1C).

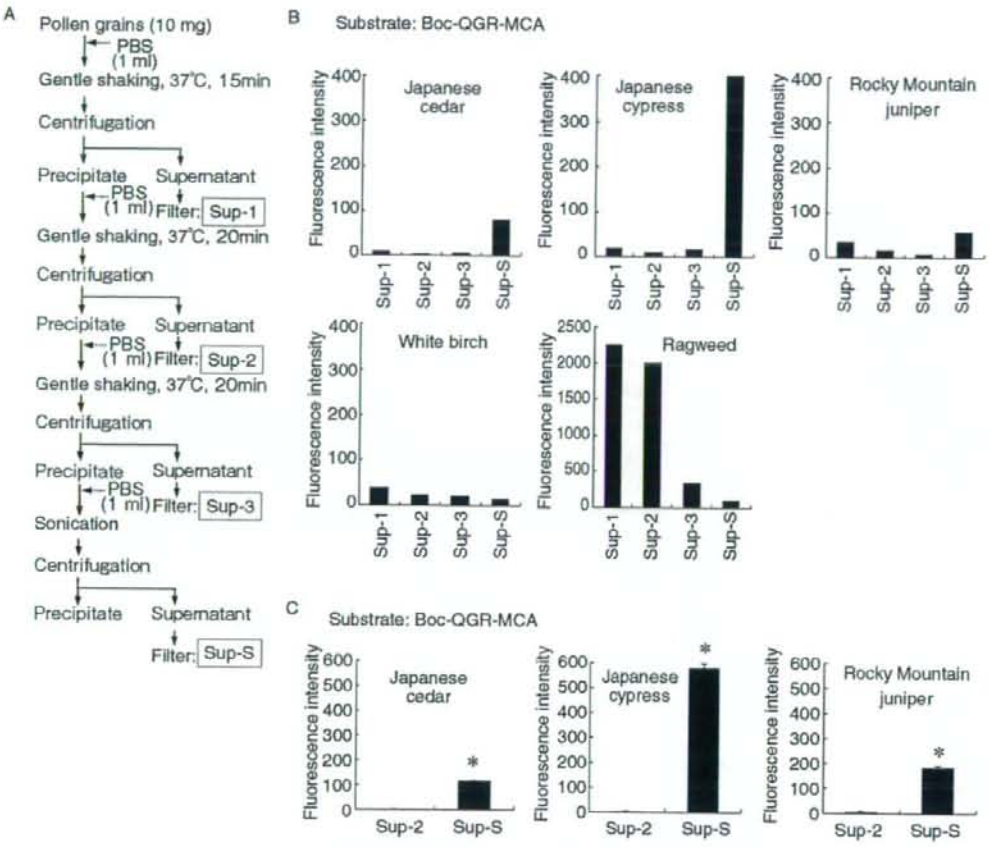

Fig. 1 Time course for release of protease activity from pollen grains and effect of sonication. (**A**) Schematic representation of the procedure used to prepare samples. (**B**) and (**C**) Protease activity against the Boc-QGR-MCA substrate in the presence of DTT. The fluorescence intensity at 120 minutes after the beginning of the protease reaction is shown. The vertical scale is the same among the different types of pollen except for ragweed. In **B**, Sup-S was prepared after the preparation of Sup-3 according to the procedure shown in **A**. In **C**, Sup-S was prepared after the preparation of Sup-2 by skipping the preparation of Sup-3, and means and SD for triplicated wells are shown. * $p < 0.05$ compared with Sup-2 without sonication by unpaired *t*-test (two-tailed) in **C**.

Sup-1 from white birch pollen showed stronger activity than Sup-2, Sup-3, and Sup-S. Ragweed pollen showed the strongest protease activity among the pollens tested, and the majority of its activity was released in Sup-1 and Sup-2 without sonication. These results indicated that all the pollen tested contained protease activity but their content and releasability differed among plant families.

EFFECT OF BUFFERS ON EFFICIENCY OF EXTRACTION OF PROTEASE ACTIVITY

Pollen extracts were prepared with three buffers, saline, PBS (pH 7.4), and Tris buffer (pH 9.0) according to the procedure shown in Figure 2A, which has a longer time for extraction and repeated sonications compared with that shown in Figure 1A. Protease activity against the synthetic substrate Boc-QGR-MCA in the presence of DTT was tested (Fig. 2B). In Japanese cedar, Japanese cypress, and Rocky mountain juniper, PBS and the Tris buffer showed equivalent efficiency but little or no activity was extracted when using saline. In white birch, activity could be extracted with the three buffers, and the ratio of efficiency among the Tris buffer, PBS, and saline was approximately 4 : 2 : 1. In ragweed pollen, PBS and the Tris buffer showed equivalent efficiency, and saline

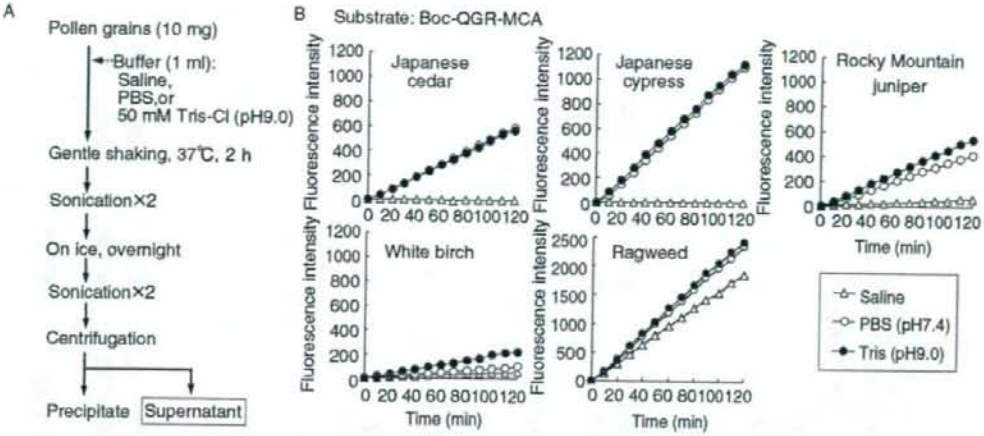

Fig. 2 Effect of buffers on efficiency of extraction of protease activity. (**A**) Schematic representation of the procedure used to prepare samples. (**B**) Protease activity against the Boc-QGR-MCA substrate in the presence of DTT. The vertical scale is the same among the different types of pollen except for ragweed.

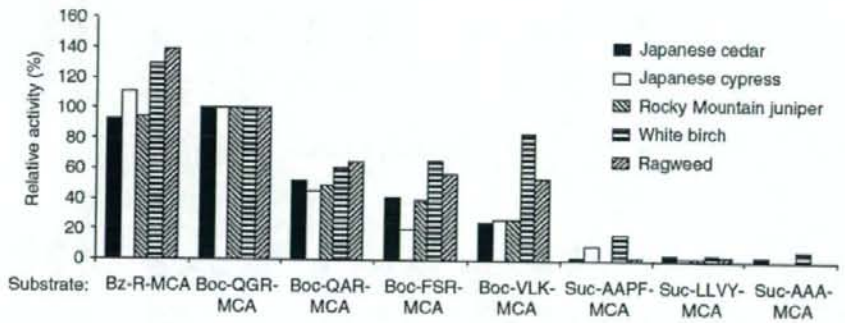

Fig. 3 Substrate specificity of protease activity within pollen extracts obtained with the Tris buffer. The activity measured in the presence of DTT for each substrate is shown as the relative fluorescence intensity to that for the substrate Boc-QGR-MCA at 120 minutes after the beginning of the protease reaction.

showed approximately 80% efficiency compared with the two buffers.

Approximate ranges of pH of the supernatants prepared according to the procedure shown in Figure 2A were estimated with the use of pH test paper to be pH 5.0–5.5 for Japanese cedar, Japanese cypress, and Rocky mountain juniper and pH 5.5–6.0 for white birch and ragweed when extracted with saline, pH 6.5–7.0 for the five types of pollen when extracted with PBS (pH 7.4), and pH 8.5–9.0 for the five types of pollen when extracted with the Tris buffer (pH 9.0) (unpublished data), suggesting that pollen extracts contained acidic materials and that pH affected the efficiency of extraction of protease activity. Additionally, pH in measuring protease activity was considered to be similarly neutral because the assay was conducted after 1/20-dilution of the supernatants. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for all the subsequent assays. Protein concentrations of pollen extracts with the Tris buffer of Japanese cedar, Japanese cypress, Rocky mountain juniper, white birch, and ragweed were 150, 240, 50, 130, and 410 μg/ml, respectively.

SUBSTRATE SPECIFICITY

The substrate specificity of protease activity in the pollen extracts in the presence of DTT was compared (Fig. 3). Although the overall pattern was not so different among the plants, some differences were ob-

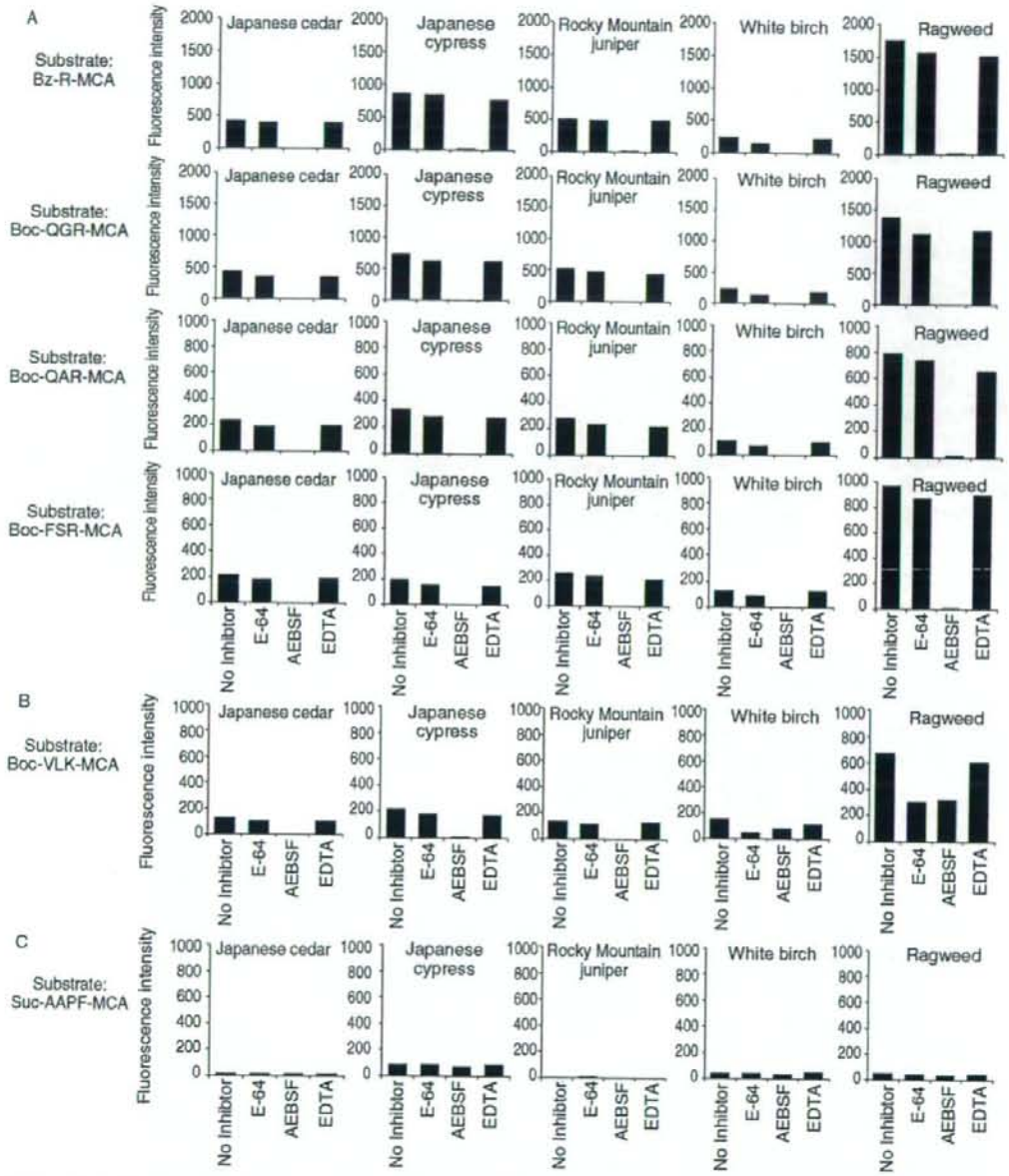

Fig. 4 Inhibition test using class-specific protease inhibitors. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement in the presence of DTT with or without treatment with E-64, AEBSF, or EDTA. The fluorescence intensity at 120 minutes after the beginning of the protease reaction is shown. The vertical scale is the same among the different types of pollen.

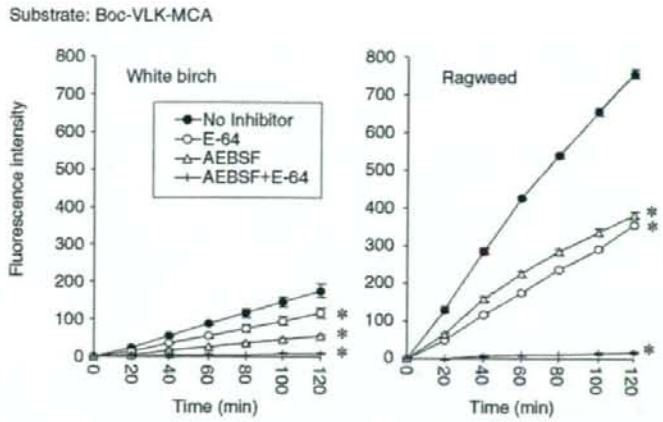

Fig. 5 Combination of E-64 and AEBSF almost completely inhibited protease activity within pollen extracts of white birch and ragweed against the Boc-VLK-MCA substrate. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement in the presence of DTT. Means and SD for triplicated wells are shown. * $p < 0.05$ vs. the other groups (E-64 + AEBSF) and vs. no inhibitor (E-64 or AEBSF) at 120 minutes by the Tukey post hoc test followed by one-way ANOVA.

Table 1 DTT-dependency of protease activity within pollen extracts.

Substrate	Japanese cedar	Japanese cypress	Rocky mountain juniper	White birch	Ragweed
Bz-R-MCA	351 † (347 ‡)	1058 (837)	457 (396)	219 (191)	1785 (1225)
Boc-QGR-MCA	364 (330)	888 (706)	449 (421)	183 (148)	1322 (929)
Boc-QAR-MCA	196 (211)	376 (308)	230 (220)	112 (102)	770 (884)
Boc-FSR-MCA	199 (184)	223 (180)	188 (174)	139 (123)	785 (579)
Boc-VLK-MCA	88 (88)	249 (199)	129 (122)	163 (105)	711 (317)
Suc-AAPF-MCA	16 (9)	104 (133)	3 (10)	38 (47)	29 (209)

Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement. † and ‡ : Fluorescence intensity in the presence and absence of DTT at 120 minutes, respectively.

served. For example, with the Boc-VLK-MCA substrate, the pollen extracts of white birch and ragweed showed greater relative activity than those of Japanese cedar, Japanese cypress, and Rocky mountain juniper.

INHIBITION TESTS USING CLASS-SPECIFIC PROTEASE INHIBITORS

To identify the class of proteases within the pollen extracts, the effects of class-specific protease inhibitors, the cysteine protease-specific inhibitor E-64, the serine protease-specific protease inhibitor AEBSF, and the metalloprotease-specific inhibitor EDTA, were analyzed in the presence of DTT (Fig. 4). Protease activity against substrates containing arginine at the P1 position was almost completely inhibited with AEBSF in all the pollen samples tested (Fig. 4A). Protease activity against the Boc-VLK-MCA substrate was completely inhibited with AEBSF in Japanese cedar, Japanese cypress, and Rocky mountain juniper, and partially inhibited with E-64 and AEBSF in white birch and ragweed (Fig. 4B). The combination of E-64 and AEBSF almost completely inhibited protease activity within extracts of pollen of white birch and ragweed against this substrate (Fig. 5). Protease activity against the Suc-AAPF-MCA substrate was not inhibited at the inhibitor concentrations tested (Fig. 4 C), although a higher concentration of AEBSF inhibited the activity (unpublished data).

These results indicated that pollen extracts of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, contained serine protease, that those of white birch and ragweed contained both serine pro-

tease and cysteine protease, and that the extracts from the five types of pollen contained another serine protease, activity of which was detected with the Suc-AAPF-MCA substrate and the sensitivity of which to AEBSF was lower.

DTT-DEPENDENCY OF PROTEASE ACTIVITY

Protease activities in the presence and absence of DTT were compared (Table 1). For the Boc-VLK-MCA substrate, significant enhancement in the presence of DTT was observed in pollen extracts of white birch and ragweed (Table 1, White birch, Ragweed). For Bz-R-MCA, Boc-QGR-MCA, and Boc-FSR-MCA, enhancement in the presence of DTT was observed in ragweed pollen extract. Protease activity against the Suc-AAPF-MCA substrate was reduced by addition of DTT most extensively in ragweed.

These results provided evidence that significant cysteine protease activity was contained in white birch and ragweed pollen extracts (Fig. 4B, White birch, Ragweed; and Fig. 5) and indicated that addition of DTT partially inactivated protease activity against the Suc-AAPF-MCA substrate in ragweed.

DISCUSSION

Although proteolytic activity from allergen sources is one of the first candidates proposed to lead to enhanced allergenicity,[1-3] little information is available on that from relevant allergenic pollens such as Japanese cedar and Japanese cypress pollen, which are the major cause of pollinosis in Japan. Here, we analyzed the protease activities derived from allergenic pollen of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, and white birch and ragweed at neutral pH, using synthetic substrates and class-specific inhibitors. We demonstrated that (1) the pollen of the three members of the *Cupressaceae/Taxodiaceae* family contain serine protease activity, (2) the pollen of white birch and short ragweed contain not only serine protease activity but also cysteine protease activity, (3) all five types of pollen tested contain at least one other type of serine protease, and (4) the content and releasability of the pollen-derived proteases differ according to the plant families. Information on the spectrum of the endopeptidase activities from these allergenic pollen grains will be useful for investigating their contribution to the pathogenesis of allergies.

Although the ragweed pollen extract exhibited the strongest protease activity, the other pollen extracts also exhibited significant protease activity (Table 1). The releasability of the proteases against Boc-QGR-MCA differs according to the plant family in buffers suitable for extraction (Fig. 2B) and necessity of sonication (Fig. 1B and C). Without sonication, protease activity against this substrate was not effectively released from the pollen of the three members of the *Cupressaceae/Taxodiaceae* family. However, the possibility that the activity is releasable *in vivo* in mucosa or epidermis, where various biochemical active molecules exist, cannot be excluded. The highest protein concentration was detected in ragweed and it could explain the strongest protease activity. The differences in releasability of proteases and proteins might be due to differences in the pollen structure among plant families (unpublished data). The overall pattern of specificity against the synthetic substrates tested was not so different (Fig. 3).

In Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, protease activity against four substrates containing arginine at the P1 position and Boc-VLK-MCA were almost completely inhibited with AEBSF (Figs 4A and 4B). This indicates that the pollen of the *Cupressaceae/Taxodiaceae* family contain serine protease. The serine protease activity detected in the present study is considered to be that of an endopeptidase and not to be derived from an aminopeptidase from Japanese cedar pollen reported by another research group,[31,32] because aminopeptidases hydrolyze at the unblocked N-terminal amino acid residue of substrates and generally cannot hydrolyze the substrates used in the present study, which have a chemically blocked N-terminus.

In white birch and ragweed, protease activity against four substrates containing arginine at the P1 position were almost completely inhibited with AEBSF (Fig. 4A), and activity against Boc-VLK-MCA was partially inhibited with either AEBSF or E-64 (Fig. 4B and Fig. 5) and almost completely inhibited with a combination of the two (Fig. 5). This indicates that the white birch and ragweed pollen contain both serine protease and cysteine protease. The significant decrease in activity against Boc-VLK-MCA in the absence of DTT supports the existence of a cysteine protease within the pollen extracts of white birch and ragweed (Table 1). Protease activity in white birch pollen, possibly of a serine protease, was reported,[29] and trypsin- and chymotrypsin-like serine proteases were purified from ragweed pollen and characterized.[33,34] The detection of cysteine protease activity in white birch and ragweed pollen has been demonstrated in the present study for the first time.

In the pollen of the five species tested, protease activity against Boc-AAPF-MCA in the presence of DTT was not inhibited with AEBSF, E-64, and EDTA at the concentrations tested in the present study (Fig. 4C). As a higher concentration of AEBSF inhibited the activity (unpublished data), the activity is suggested to be from another serine protease, the sensitivity of which to AEBSF was relatively low. Protease activity against this substrate was greater in the absence of DTT in the ragweed pollen extract (Table 1), indicating DTT partially inactivates at least one molecular species exhibiting serine protease activity against this

substrate. In our preliminary experiments, the protease activity against the substrate was released from the pollen of the three members of the *Cupressaceae/Taxodiaceae* family without sonication (unpublished data) unlike that against the Boc-QGR-MCA shown in Figure 1. Comparison of the releasability of the pollen-derived proteases is an important issue to be addressed in a future study, because their releasability could relate to exposure of human tissues to the protease activity in physiological conditions.

House dust mite group 1 allergens are cysteine proteases and group 3, 6, and 9 allergens are serine proteases.[35,36] Protease activities of these mite allergens have been suggested to be involved in the pathogenesis of allergies in sensitization towards IgE production and/or exacerbation by reducing physical and biochemical tissue barriers, cleaving various molecules, and modulating the functions of various cells.[1,3,9-21] Although no proteases have been reported as allergens recognized by patients' IgE for the types of pollen tested in the present study, enzymatic activities of the serine protease and/or cysteine protease detected in the present study might contribute to form a microenvironment with dysfunctional tissue barriers and inflammation toward sensitization or exacerbation as well as pollen-derived lipids,[5-7] and oxidase,[4] which have been suggested to be involved in the pathogenesis of allergic diseases through IgE-independent pathway. The pollen-derived protease activities should be examined for contribution to the pathogenesis of allergic diseases in future studies.

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ORIGINAL PAPER

Development of electron spin resonance radical immunoassay for measurement of airborne orchard grass (*Dactylis glomerata*) pollen antigens

Yuichi Takahashi · Masaaki Aoyama · Etsuko Abe · Takeshi Aita · Shigeto Kawashima · Nobuo Ohta · Masahiro Sakaguchi

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Abstract We have developed a highly sensitive method for the measurement of airborne orchard grass (*Dactylis glomerata*: Dac g) pollen antigens using an electron spin resonance (ESR) radical immunoassay. In this immunoassay, the lowest detectable level of Dac g antigen in a sample is 0.1 arbitrary unit; the amount of Dac g antigen in single pollen grains was found to be as 1.84 units. Thus, Dac g antigens can be detected in amounts of 1/20th of that contained in the grain. This immunoassay enables early detection of grass pollen antigens. Such information may be useful for patients with grass pollinosis, especially for those who show symptoms when only low levels of the pollen antigens are present in air. In this study, minor amounts of Dac g antigen (cross-reactive antigens) were detected in late March, after which the levels gradually increased. The levels were detected to be 10 units/m^3 until the middle of May and then increased after blooming of orchard grass. High levels were maintained until the middle of June. Some patients who suffer from grass pollinosis show symptoms in late April and early May, when the airborne Dac g antigen levels were found to be 5–10 units/m^3.

Keywords Airborne pollen · Allergen · ESR radical immunoassay · Orchard grass · Japanese cedar

Abbreviations

BCA	Bicinchoninic acid
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
Dac g	*Dactylis glomerata*
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal bovine serum
ESR	Electron spin resonance
HRP	Horseradish peroxidase
MONALISA	Monitoring Network of Allergen by Immuno-Sampling

Y. Takahashi · E. Abe · T. Aita
The Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, Japan

M. Aoyama
RADIA Project, Yamagata Promotional Organization for Industrial Technology Institute for Life Support Technology, Yamagata 990-2473, Japan

S. Kawashima
Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

N. Ohta
Department of ENT, School of Medicine, Yamagata University, Yamagata 990-9585, Japan

M. Sakaguchi (✉)
Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan
e-mail: sakagum@azabu-u.ac.jp

NHS	*N*-hydroxysuccinimide
PBS	Phosphate-buffered saline
RSI	Relative signal intensity
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPT	Skin prick test

1 Introduction

The number of airborne pollen grains is typically counted morphologically using microscopy. Pollen information is derived from these data. However, because grass pollen shares similar morphological characteristics, it is difficult to discriminate between species of pollen grains using light-microscopic pollen counts. It is known that species of grass pollen antigenicity differ with each species of grass pollen, and the pollen antigens of some grasses are not cross-reactive with those of other grass species (Esch 1999; Martin et al. 1985; Weber and Nelson 1985). Fortunately, most grasses associated with pollinosis in our region are cross-reactive species belonging Poöideae. In addition, submicron-size particles bearing pollen antigens such as starch granules (Spieksma et al. 1991) exist other than as antigens from the pollen itself. Such submicron-size particles have been isolated from pollen grains under wet conditions (Suphioglu et al. 1992) and are also considered to be the causative agents of pollinosis.

To obtain more information about the pollen, it is desirable to measure antigens that are cross-reactive with grass pollen. We applied an immunochemical technique for the quantification of airborne grass pollen antigens. A major problem in this context is that only small amounts of these antigens are present in air, and a highly sensitive analytical technique is required for such measurements. Recently, an electron spin resonance (ESR) radical immunoassay was developed as a highly sensitive method for detecting hepatitis B surface antigen (Matsuo et al. 1998; Aoki et al. 2002). In our previous study, we have shown that the sensitivity of this immunoassay is 10- to 100-fold higher than that of the conventional enzyme-linked immunosorbent assay (ELISA) (Aoyama and Takahashi 2004; Takahashi et al. 2007).

In this study, we used the ESR radical immunoassay to quantify airborne grass pollen antigen. Orchard grass pollen antigen was chosen as the target, because this species is universally distributed throughout our region and is considered to be the most common species contributing to total regional airborne grass pollen antigens.

2 Materials and methods

2.1 Sampling and antigen extract

A cyclone sampler, CM 90 (Burkard Manufacturing, Rickmansworth, UK) was installed at Iwanami in Yamagata City, and airborne pollen antigens were collected in 1-ml tubes. No large community of grasses is present in any direction within 500 m of the sampling site. The tubes were replaced and samples were collected at 6:00 daily. The antigens in the airborne samples were extracted with 100 μl of 0.125 M ammonium bicarbonate in 0.1% bovine serum albumin (BSA) for 2 h at room temperature. The scratch extract of *Dactylis glomerata* pollen (B3SFV2) (Torii Pharmaceutical, Tokyo, Japan) was used for the standard solution, and we defined the concentration of the scratch extract as 100,000 arbitrary units/ml. Dac g content in single pollen grains was determined by extraction of a reference pollen provided from International Biological (Piedmont, OK) with 300 μl of 0.125 M ammonium bicarbonate overnight at 4°C. After centrifugation, the aliquot was used to measure Dac g antigen, and the pellets were used to count the number of Dac g pollen grains.

2.2 Preparation of horseradish peroxidase (HRP)-conjugated antibody

Antibodies against Dac g antigens were prepared with Japanese white rabbits. Two rabbits were immunized subcutaneously with 400 μg (200 μg each rabbit) Dac g antigen from Greer Laboratories (Lenoir, NC) mixed with Freund's complete adjuvant. Boosts were given at 4, 8 and 12 weeks after the first injection and small bleeds were taken a week after each boost to check the antibody response. A large bleed was taken a week after the final boost (Kane and Banks 2000). Antisera were precipitated with 35% saturated ammonium sulfate, and they were dialyzed with 0.125 M NaCl and 0.05 M phosphate-buffered saline (PBS) at

pH 7.0. The ammonium precipitates were then passed through protein A column to purify IgG fraction. A portion of the IgG fraction was further purified with the antigen-combined column, and the purified samples were eluted with 10 mM glycine-hydrochloric acid at pH 2.0. The eluate was immediately replaced with PBS (pH 7.0). The final protein concentration of the refined antibody against Dac g was 0.5 mg/ml as measured using the BCA protein assay kit (Pierce, Rockford, IL). The antigen-combined column was made using Dac g antigen (Funakoshi, Tokyo, Japan) reacted with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The refined antibody was conjugated with horseradish peroxidase using peroxidase labeling Kit-SH (Dojindo Molecular Technologies, Tokyo, Japan).

2.3 Electron spin resonance (ESR) radical immunoassay for Dac g antigen

Dac g antigen for SPT extract (Torii Pharmaceutical) was diluted 2,000-fold with PBS. The protein concentration of the Dac g antigens measured with BCA protein assay kit (Pierce) was 2.75 mg/ml. One hundred μl of the diluted antigen was put in a 96-well plate (Nunc, Kamstrupvej, Denmark), and the samples were reacted for 6 h at 4°C. After three washes with ultra pure water, 370 μl Stabilguard (SurModics, Eden Prairie, MN) was placed in each well, and the reaction was blocked overnight at 4°C. The plate were washed with ultra-pure water, dried in a desiccator, and kept at 4°C until use. Measurements were carried out as follows. Seven different concentrations of Dac g standard solution (5, 10, 20, 40, 100, 200, and 400 units/ml) were prepared prior for each measurement. One hundred μl of the standard solution or 100 μl of PBS containing 2% BSA was placed in each well of Dac g antigen-coated plate, and then 0.125 M ammonium bicarbonate (30 μl) or an airborne sample (30 μl) was added to each well. Then, 50 μl HRP-conjugated antibody against Dac g diluted with 10% fetal bovine serum (FBS) and 0.1 M PBS was added to each well, and the solutions were mixed thoroughly. Each sample was usually put in a single well, and in some noticeable cases, samples were measured in duplicate. The seven standard solutions were measured each time. The plate was left for 2 h at room temperature or left overnight at 4°C. After several washes in washing solution, 150 μl of 4 mM *p*-acetamidophenol and 0.34 mM 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline 3-oxide and 0.01% hydroperoxide were added to each well and reacted for 1 h at 37°C. The enzyme reaction was stopped with 50 μl sodium azide (100 mM). The amount of nitroxide radical (stable radical) produced as a result of the enzyme reaction was measured with an ESR device (FR30, JEOL, Tokyo, Japan) at a center field of 336.1 ± 5 mT. Details of the ESR measurement technique have been reported elsewhere (Matsuo et al. 1998; Aoki et al. 2002).

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The pollen protein samples were analyzed by SDS-PAGE (12.5% acryl amide concentration; Bio-Rad, Hercules, CA). Prestained protein molecular weight standards-high (Life Technologies, Tokyo, Japan) was used as a molecular weight standard. Electrophoresis was performed at a constant voltage of 12 V for 1 h. After electrophoresis, the gel was placed on a nitrocellulose membrane, and the antigens were transferred onto the membrane. The gels were stained with Coomassie-blue G-250. The membrane was blocked with PBS in 5% BSA overnight at 4°C, then the samples were reacted with 500-fold diluted biotinylated antibody against Dac g for 2 h. Antibody against Dac g was biotinized with long-arm NHS biotin (Vector Laboratories, Burlingame, CA) (Abdul-Ahad and Brett 2000). After three washes with PBS, the samples were reacted with 500-fold diluted alkaline phosphatase conjugated streptavidine (Vector Laboratories). Finally, the Dac g antigen bands were visualized with BCIP/NBT substrate (KPL, Washington, DC).

3 Results

3.1 Dac g reacts with the antibody against Dac g

The Dac g antigen from Greer laboratory that was used for immunization showed five clear bands in SDS-PAGE, with approximate molecular weights of

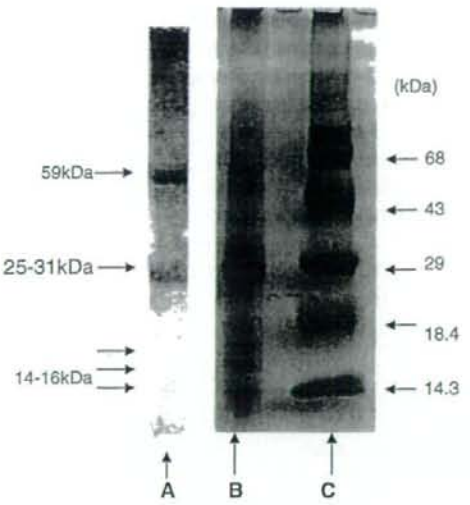

Fig. 1 Determination of molecular components of Dac g extract analyzed by SDS-PAGE and antibody against Dac g specificity by Western blotting. A: Antibody against Dac g analyzed by Western blotting. B: Molecular components of the Dac g extract analyzed by SDS-PAGE. C: Molecular weight markers: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), and albumin (68 kDa)

59, 25–31, 16, 15, and 14 kDa. The 25–31 kDa band was broad and dense. The 59 and 25–31 kDa bands reacted with the antibody against Dac g. Three bands of low molecular weights, 14, 15, and 16 kDa, did not react with the antibody against Dac g (Fig. 1). Therefore, the antibody against Dac g contained antibodies against 59 and 25–31 kDa antigens.

3.2 Standard curve of ESR radical immunoassay

A standard curve was obtained using ESR radical immunoassay, and the results are shown in Fig. 2. The vertical axis indicates the relative signal intensity (RSI) (%); the horizontal axis indicates Dac g concentration expressed as unit/ml. The RSI (%) was calculated from the following equation.

$$\mathrm{RSI}(\%) = \frac{\text{Signal intensity of a standard Dac g}}{\text{Signal intensity of a zero standard (without Dac g)}} \times 100$$

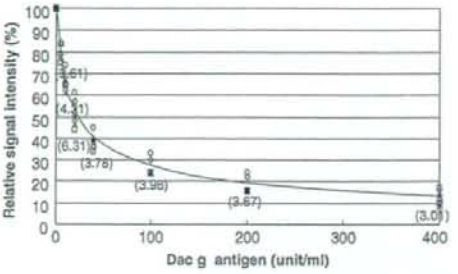

Fig. 2 Relationship between Dac g levels and relative signal intensity determined by ESR radical immunoassay. Seven concentrations (5, 10, 20, 40, 100, 200, and 400 units/ml) of Dac g were measured five times, and the RSI (%) was plotted on the vertical axis. A regression curve is derived from the mean value of the five measurements. Standard errors are shown in parentheses

The standard error of RSI (%) was in the range of 3.0–6.3% (mean 4.1%, $n = 6$) (Fig. 2), and therefore we defined a 90% intercept value of RSI to the zero standard signal intensity as the detection limit in this study. A zero standard means maximum binding capacity of the system in this case. The 90% intercept value of the signal intensity was approximately 3.5 units/ml. On the other hand, the amount of Dac g in single pollen grains was found to be 1.84 units/grain (1.38–2.24 units/grain, $n = 8$) based on a reference pollen obtained commercially.

3.3 Measurement of airborne Dac g antigen

The level of airborne Dac g antigens was examined during the period of 22 March and 30 June in 2006 (Fig. 3). Minor amounts of cross-reactive antigens to Dac g were already detected in late March, and the levels gradually increased thereafter. The levels were found to be 10 units/m^3 until the middle of May. The Dac g levels increased after the blooming of orchard grass, and a high level of Dac g antigen was maintained until the middle of June. Symptoms of some grass pollinosis patients had already begun in

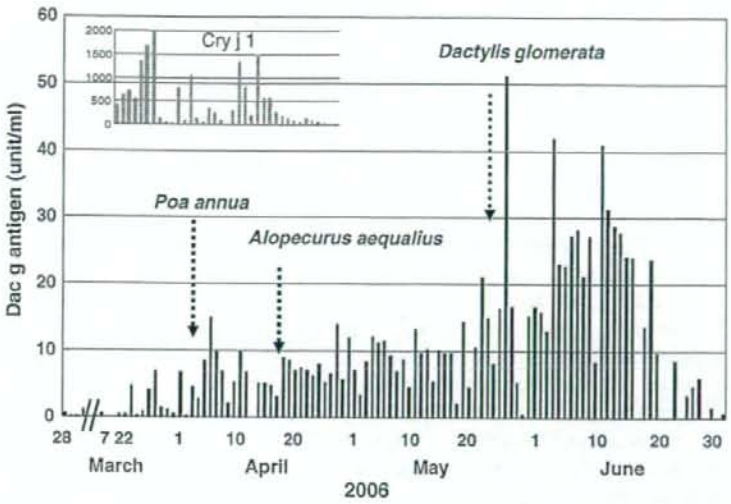

Fig. 3 Daily fluctuation of airborne Dac g measured by ESR radical immunoassay during the period from 7 March to 30 June 2006. Arrows indicate the flowering time of relevant grasses near the sampling site. Daily fluctuation of airborne Cry j 1 level is shown. The horizontal scale is adjusted for comparison

late April, when the airborne Dac g levels were fluctuating between 5 and 10 units/m^3.

The relationship between the daily amount of airborne Cry j 1 (a major pollen allergen from Japanese cedar pollen) and that of Dac g was examined, and no relationship between the two values was observed (between 22 March and 10 May, $r = -0.1828$, $n = 50$).

4 Discussion

We have developed a highly sensitive method for measuring airborne Dac g antigens using ESR radical immunoassay. The 90% intercept value was calculated as 3.5 units/ml as described in Sect. 3. Each measurement needs 30 μl extract; therefore, a level of Dac g in excess of 0.1 unit in the sample is detectable. The Dac g content in single pollen grains of *D. glomerata* was determined as 1.84 units. Thus, the amount of Dac g that could be detected was as low as 1/20th of that contained in single grains. Using this method, the Dac g antigen was detectable during the early stage of the grass pollen season. This approach is expected to provide useful information for grass pollinosis patients, especially for those who show symptoms at times when only a low level of antigen is present in air. Data are available 2½ h after sampling, namely, 30 min extraction (Takahashi et al. 2001), 60 min antibody reaction and 60 min radical reaction. We could use the above treatment times without sensitivity loss on the occasion of the pollen allergen information. It is possible to supply the pollen allergen information to local residence through mass media until evening time on the same day.

Also, we have been providing information about Cry j 1 antigen in airborne pollen during the pollen season since 2005. It is well known that some patients displayed symptoms several weeks before airborne Japanese cedar pollen has been detected by microscopy. No airborne Cry j 1 has been detected using the conventional ELISA during this times. The development of the ESR radical immunoassay has now made it possible to conduct such measurements, and it has been reported that some patients show their symptoms during a period in which airborne Cry j 1 levels fluctuating between 1 and 3 pg/m^3. Such information could be useful for the patients whose symptoms begin early in the flowering season (Takahashi et al. 2007). Now we have been supplying airborne Cry j 1 information through internet and local TV at the pollen season.

Low levels of Dac g were present during the latter half of the Japanese cedar pollen season. Japanese cedar pollinosis is the most common in Japan, and more than 10% of Japanese suffer from it. Some patients who suffer from the pollinosis during this

season think that Japanese cedar pollen is their causative pollen, but it is clarified from this study that Dac g pollen was also detected in air at that time. It has been reported that some grass pollinosis patients show symptoms in late April and early May in our region (Takahashi et al. 1987), and this time corresponds to the period when early flowering species of grasses start to bloom. It is likely that the symptoms of these patients are provoked not only by Japanese cedar pollen, but also by grass pollen. Interestingly, no positive correlation was observed between the amounts of Cry j 1 and Dac g antigen in air, samples of which were collected during Japanese cedar pollen season. Dac g and Cry j 1 do not have any cross-reactive antigens. The comparison was made because we want to examine whether they appear in air simultaneously or independently controlled under different meteorological conditions, and further whether there are more patient symptoms in connection with the appearance of these antigens.

Airborne samples were taken using Burkard Cyclone sampler in this research. The manufacturer of the sampler announced that the collection efficiency of the sampler is 90% in 1-μl range particles; that is to say, allergens existed as minute particles under 1 μl, and gas-shaped particles could not be sampled. In Europe, the MONALISA (Monitoring Network of Allergen by Immuno-Sampling) project was initiated in 2005. The aim of the project is to characterize pollen allergens such as Poaceae, *Betula*, *Ambrosia*, *Artemisia*, *Cupressus*, *Parietaria*, and *Olea* pollens for the benefit of pollinosis patients. Several sampling methods were examined in the MONALISA project. According to Rantio-Lehtimäki of Turk University, samplings into the liquid were diluted, and ELISA results were not reliable (personal communication). The advantage of the Cyclone sampler is that allergens in large volumes of air could be collected in a very small quantity of extraction medium (at least 50 μl). Therefore, we chose the cyclone sampler for the study.

In this research, we used an antibody against Dac g antigen, because *D. glomerata* is the most widely distributed species in our region and is considered to be the main species contributing to the total airborne grass pollen antigens in this region. A number of studies have been conducted to analyze *D. glomerata* pollen antigens (Esch and Klapper 1989; Cuerin-Marchand et al. 1996; Roberts et al. 1993; Leduc-Brodard et al. 1996; van Oort et al. 2001). The extent of cross-antigenicity among grass antigens remains difficult to estimate. The antibody used in this study recognized antigens with molecular weights of 59 and 27–35 kDa. It is likely that 59 kDa antigen is Dac g 4, and 27–35 kDa antigen is Dac g 1 and/or Dac g 5. According to Esch (1999), Dac g 5 has a wide molecular weight distribution and is cross-reactive within pollen antigens from Poöideae. Therefore, Dac g 5 was considered to be one of the suitably defined antigens for our purpose.

As for antibody, several categories of antibodies are available for the airborne antigen measurements, e.g., antibodies created from crude antigen extracts, antibodies created from defined antigens, monoclonal antibodies, and polyclonal antibodies. There are no commercial products for antibodies against grass pollen antigens in Japan. First of all, we must prepare antibodies against grass pollen antigens. Monoclonal antibodies are easy to manage concerning quality control as indicated by the example that the MONALISA project has selected them. However, we prepared polyclonal antibodies because we want to use antibodies that have wide specificities related to local grass pollinosis, and it can be made easily. There is, certainly, a problem with quality control among preparations. However, we do not worry about this matter concerning the standpoint of pollen allergen information, because outstanding sensitivity of the ESR method made it possible to dilute the HRP-conjugated antibody to 10,000-fold. So, we can use the same preparation for many years. Addition to this, we think suitable antibody will differ from place to place as discussed in the next paragraph.

Some patients show symptoms before the flowering of *D. glomerata*. The amounts of airborne allergens causing grass pollinosis can be roughly estimated to quantify the airborne Dac g levels, because the antibody used in the present study reacts not only to Dac g pollen antigens, but also to grass pollen antigens cross-reactive with Dac g from other plants of the Poöideae subfamily. Thus, the airborne grass pollen antigens observed before *D. glomerata* flowering season that bloom until late May (Takahashi et al. 1993) may not have been Dac g itself, but may have been other species having cross-reactive antigens with Dac g antigen. Two early blooming species of grasses in our region are *Alopecurus aequalis* and *Poa annua*. However, early blooming species are not

limited to the above-mentioned species; for example, *Anthoxanthum odoratum* blooms prior to the major grass pollen season in some areas in Japan (Sudo et al. 2005). It would be desirable to investigate antibodies with a broad range of specificity against major allergens related to local grass pollinosis. Moreover, it would be helpful to examine antibodies in immunization with a mixture of grass pollen antigens from different species. Antibodies suitable for such studies will be those against the most important species in a particular region, and we think the suitable antibody will differ from place to place. Further research is still needed to clarify the differences among applied antibodies and to identify the appropriate antibodies for the measurement.

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Identification of *c-kit* mutations-independent neoplastic cell proliferation of canine mast cells

Keitaro Ohmori [a,b,*], Shinpei Kawarai [a], Nobutaka Yasuda [a], Akane Tanaka [b], Hiroshi Matsuda [b], Ryohei Nishimura [c], Nobuo Sasaki [c], Hajime Tsujimoto [a], Kenichi Masuda [d]

[a] *Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan*
[b] *Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*
[c] *Department of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan*
[d] *Laboratory for Vaccine Design, Research Center for Allergy and Immunology, Yokohama Institute, RIKEN, Kanagawa, Japan*

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Abstract

Gain-of-function mutations in the proto-oncogene *c-kit* have been considered the molecular mechanism of neoplastic proliferation of mast cells. However, the importance of *c-kit* gene mutations is not well evaluated in canine mast cell tumors (MCTs). In the present study, we established and characterized a mast cell line, HRMC, derived from a dog with MCT. We also examined *c-kit* mutations in HRMC cells and assessed an inhibitory effect of a tyrosine kinase inhibitor, STI571, on HRMC cells. HRMC cells had cytoplasmic metachromatic granules, chymase and tryptase, and expressed both KIT and FcεRI on the cell surface. HRMC cells contained histamine and released β-hexosaminidase through FcεRI cross-linking and calcium ionophore stimulation. Nucleotide sequence analysis demonstrated no mutations in an open reading frame of *c-kit* cDNA and genomic DNA of the juxtamembrane domain of *c-kit* in HRMC cells. STI571 did not show any inhibitory effects on the proliferation of HRMC cells. These findings clearly demonstrated the existence of *c-kit* mutations-independent neoplastic canine mast cell proliferation. The growth factor-independent mast cell line established in this study might be valuable to explore novel mechanisms of *c-kit* mutations-independent neoplastic proliferation of mast cells in dogs.

Keywords: *c-kit*; Mutations; Mast cell tumor; Dog

1. Introduction

Mast cell tumors (MCTs) are one of the common tumors in dogs, accounting for approximately 20% of cutaneous canine tumors (London and Seguin, 2003). Most of MCTs develop in the dermis and subcutaneous tissue of dogs, while visceral MCTs occasionally occur in the spleen, liver, gastrointestinal organs, and bone marrow (London and Seguin, 2003). MCTs in dogs show various biological behaviors, ranging from benign

* Corresponding author at: Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan. Tel.: +81 42 367 5925; fax: +81 42 367 5916.
E-mail address: k-ohmori@cc.tuat.ac.jp (K. Ohmori).

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