Inhibition of an Allergen–Antibody Reaction Related to Japanese Cedar Pollinosis Using DNA Aptamers Against the Cry j 2 Allergen

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Japanese cedar pollinosis is one of the most prevalent allergies in Japan. Reducing the allergen content of pollen plays a major role in the alleviation of allergy symptoms. Aptamers, oligonucleotides with an affinity for specific molecules, have great potential for reducing allergic activity. In this study, we report that the anti-Cry j 2 aptamers, CJ2-04 and CJ2-08, inhibited allergen–antibody reactions between Cry j 2, one of the major allergens in Japanese cedar pollen, and immunoglobulin E in serum collected from a patient with Japanese cedar pollinosis. In addition, the suppression of Ca^{2+} mobilization in basophils, which is related to degranulation, was observed in samples preincubated with either of these DNA aptamers. This study indicates that anti-Cry j 2 aptamers may inhibit allergen–antibody reactions and suppress the induction of Japanese cedar pollinosis, possibly leading to a novel external defense against this and other types of allergens.

Introduction

THE POLLEN OF JAPANESE CEDAR, *Cryptomeria japonica*, causes pollinosis during the pollen dispersal season, which occurs annually between February and April in Japan. Japanese cedar pollinosis has recently become one of the most prevalent disorders in Japan, afflicting ~26.5% of the population [1]. The Japanese cedar pollen exists both outdoors and indoors during the pollen dispersal season [2]. Although complete avoidance of pollen is impossible, many people use eyeglasses, face masks, and pollen forecast information to avoid pollen exposure and minimize symptoms [3]. Thus, personal avoidance of pollen has become commonplace in Japan; however, little action has been taken to avoid allergen exposure. Wang *et al.* showed that information regarding Japanese cedar allergens in the air would be helpful for the prevention of pollinosis because allergen particles were observed even when pollen dispersal was low [4].

Cry j 2, which shows polymethylgalacturonase activity, has been identified as one of the primary allergens derived from Japanese cedar pollen [5]. Cry j 2 is found inside starch granules in the cytoplasm of pollen grains [6] and is released into the environment when pollen grains rupture when exposed to water during rain events. More than 90% of patients with Japanese cedar pollinosis have immunoglobulin E (IgE)

antibodies specific to Cry j 2 [7], indicating that Cry j 2 is directly linked to Japanese cedar pollinosis.

The reaction between an allergen and allergen-specific IgE plays a crucial role in the induction of allergic symptoms. Miyaji *et al.* reported that a major sequential IgE epitope (including ¹²⁴KWVNGREI¹³¹) on Cry j 2, which reacted with IgE from 71% patients with Japanese cedar pollinosis, overlapped with the binding site of the mouse monoclonal antibody (mAb) T27 [8]. Binding of an allergen to receptor-bound IgE creates a cross-link with the high-affinity IgE receptor (FcεRI) expressed in basophils and mast cells [9], causing an increase in intracellular free Ca^{2+} , thereby leading to degranulation, which is essential for the release of inflammatory mediators such as histamine [10].

The application of anti-IgE antibody therapy for the alleviation of Japanese cedar pollinosis has been suggested [11]; however, it is not popular because Japanese cedar pollinosis is not life-threatening and the cost of the antibody used for this therapy is high. Antibodies could also be applied to the inhibition of FcεRI signaling in targeted indoor locations where allergens are expected to be present (ie, curtains and coats brought in from outdoors). By binding inhibitory molecules to allergens in advance, FcεRI signaling would be suppressed even if allergens invade the body.

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We recently suggested that anti-Cry j 2 aptamers could be used in allergen recognition as an alternative to antibodies [12]. Aptamers are oligonucleotides with specific binding ability comparable with antibodies. Aptamers can be easily synthesized by chemical processes and are well suited for large-scale production, saving considerable time and cost compared with antibody production [13]. In addition, aptamers are stable across a wide range of temperatures and have low immunogenicity [14]. Because of these characteristics, aptamers would be well suited for the development of new preventive measures against Japanese cedar pollinosis.

In this study, we evaluated whether eight aptamers previously identified as Cry j 2 ligands [12] inhibit the allergen-antibody reaction between Cry j 2 and IgE collected from a patient with Japanese cedar pollinosis. Although several anti-allergen aptamers have been reported for use in sensing techniques, aptamers that can regulate immune response to allergens have not been developed to date [15–18]. To the best of our knowledge, this is the first report to demonstrate the inhibition of an allergen-antibody reaction using aptamers.

Materials and Methods

Preparation of antiserum derived from a patient with Japanese cedar pollinosis

Human antiserum from a patient with Japanese cedar pollinosis (class 4, CAP-RAST) was prepared as a source of Cry j 2-specific IgE. Before blood sampling, verbal and written informed consent was obtained from the patient. The experimental procedure was approved by the ethics committee of Tokyo University of Agriculture and Technology.

Anti-Cry j 2 aptamers

Anti-Cry j 2 aptamers [12] were purchased from Fasmac or Eurofins Genomics as unmodified 24-mer DNA oligonucleotides. These aptamers were heat treated before use, according to a previous report [12].

Evaluation of inhibitory effects in an allergen-antibody reaction

Inhibitory effects of aptamers in an allergen-antibody reaction were investigated by dot blotting. The aptamers were incubated with immobilized Cry j 2 (100 ng; Hayashibara) at room temperature (RT) for 1 h before incubation with antiserum or anti-Cry j 2 mAb T27 (Hayashibara). A 24-mer poly-thymine (poly dT) was used as a control. Data were normalized using the value without oligonucleotides.

Dot blotting

Cry j 2 was immobilized on a nitrocellulose membrane (GE Healthcare) and blocked with 4% (w/v) fat-free milk in TBST [10 mM Tris/HCl, 150 mM NaCl, 5 mM KCl, and 0.05% (v/v) Tween 20; pH 7.4]. The membrane was then incubated with antiserum (1:100), mouse anti-Cry j 2 mAb T27 (10 ng/mL; Hayashibara), or anti-Cry j 2 polyclonal antibody (pAb, 50 ng/mL; Hayashibara) diluted in 2% (w/v) fat-free milk in TBST, followed by incubation with HRP-conjugated anti-human IgE antibody (100 ng/mL; Abcam), HRP-conjugated anti-mouse IgG (1:1,000; Dako A/S), or

HRP-conjugated anti-rabbit IgG (1:2,000; Dako A/S). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore). All incubation steps were performed at RT for 1 h. The signal was quantified using ImageQuant TL (GE Healthcare) and ImageJ (NIH).

Aptamer blotting

Aptamer blotting was performed according to a previous report [12]. In brief, Cry j 2 immobilized on a nitrocellulose membrane was blocked with 2% (w/v) bovine serum albumin in TBS (10 mM Tris/HCl, 150 mM NaCl, and 5 mM KCl; pH 7.4). After incubation with biotinylated aptamers, the membrane was incubated with HRP-conjugated NeutrAvidin (Thermo Fisher Scientific). The signal was detected in the same manner as dot blotting.

Denaturation of Cry j 2

Cry j 2 was heated at 95°C for 4 min in PBS (–) (Nissui) containing 2% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich) and 5% (v/v) 2-mercaptoethanol (Sigma-Aldrich).

Sample preparation for immunoblotting

KU812-F cells (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were maintained with RPMI 1640 medium (Life Technologies) containing 10% (v/v) fetal bovine serum (HyClone Laboratories) as a culture medium in humidified (5% CO₂) air at 37°C and precultured in culture medium with 1% patient antiserum for 16 h. Whole cell samples were prepared using KU812-F cells dissolved and sonicated in SDS-PAGE sample buffer. Cell membrane proteins were prepared using the ProteoExtract® Native Membrane Protein Extraction Kit (Merck Millipore) and dissolved in SDS-PAGE sample buffer. These samples were heated at 95°C for 5 min before use.

Immunoblotting

The samples, equivalent to 2×10^3 KU812-F cells, were separated using Any kD™ Mini-PROTEAN® TGX™ Gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 4% (w/v) fat-free milk in TBST and incubated with anti-human FcεRI antibody (CRA1; Bio Academia) or anti-GAPDH antibody (6C5; Merck Millipore), followed by HRP-conjugated anti-mouse IgG (GE Healthcare). Detection was performed using ECL western blotting detection reagents (GE Healthcare).

Intracellular Ca²⁺ inhibition assay using a human basophilic leukemia cell line

KU812-F cells were seeded in 24-well cell culture plates (BD Falcon) at 1×10^5 cells/well under the same conditions as precultures in immunoblotting. KU812-F cells were then incubated in culture medium containing 2 μM Fluo-3/AM (Life Technologies) at 37°C with 5% CO₂ for 1 h and washed in RPMI 1640. The aptamers were diluted at 1 μM in the culture medium and incubated with Cry j 2 (40 ng/mL) at room temperature for 1 h. Cry j 2 (final concentration: 20 ng/mL) was added, alone or preincubated with each aptamer or poly dT, to the Fluo-3/AM-treated KU812-F cells, and

fluorescence was observed using a microscope IX71 (Olympus). For each sample, a video file was recorded for 120 s, with the stimulation of Cry j 2 occurring 10 s after the start. The file was analyzed for the average fluorescence of each pixel using ImageJ (NIH). The positive signal was defined as explained in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/nat).

Results and Discussion

To use an antiserum derived from a patient with Japanese cedar pollinosis for experiments, the reactivity between IgEs in the antiserum and Cry j 2 was preliminarily investigated by dot blotting. As a result, signals of Cry j 2 were detected in a dose-dependent manner using the antiserum (Fig. 1A), indicating that the antiserum contains sufficient Cry j 2-specific IgEs for dot blotting. We then performed dot blotting assays for Cry j 2 treated with each of several anti-Cry j 2 aptamers before incubation with the antiserum for identifying aptamers having an inhibitory effect on the allergen–antibody reaction between Cry j 2 and IgE. Eight aptamers that had been obtained previously [12] were screened. Two of these aptamers, CJ2-04 (5′-GGCGAGGGAAAGGGGGG CGGACGA-3′) and CJ2-08 (5′-GGTAGGGAAAGGGGT GTGGCCTGG-3′), inhibited allergen–antibody binding compared with poly dT ($P < 0.05$, t -test) (Fig. 1B). In addition, CJ2-04 and CJ2-08 exhibited dose-dependent inhibition

against the binding of IgE to Cry j 2 ($P < 0.01$ and $P < 0.05$, respectively; one-way ANOVA) (Fig. 1C). These results suggest that CJ2-04 and CJ2-08 disrupted the antigen–antibody reaction between Cry j 2 and IgE by binding to Cry j 2 before IgE recognition. With regard to the epitope on Cry j 2, it was reported that anti-Cry j 2 mAb T27 binds to Cry j 2, competing for the antibodies binding to a major epitope, including $^{124}\text{KWVNGREI}^{131}$ [8]. We preliminarily confirmed that the antiserum used in this study contained IgE that bound to Cry j 2 competitively with anti-Cry j 2 mAb T27 using the method of Miyaji *et al.* with slight modification (Data not shown) [8]. Therefore, the antiserum used here is considered to be analogous to the standard antiserum derived from patients with Japanese cedar pollinosis, although it was obtained from a single patient. Subsequently, we performed dot blotting assays for Cry j 2 treated with the anti-Cry j 2 aptamers, CJ2-04 and CJ2-08, before incubation with mAb T27 for evaluating the inhibition of binding to the site equivalent to the major IgE epitope on Cry j 2. As a result, CJ2-04 showed significant inhibition of allergen–antibody binding compared with poly dT ($P < 0.05$, t -test), whereas CJ2-08 did not show inhibition of allergen–antibody binding ($P > 0.05$, t -test) (Fig. 1D). These results indicate that CJ2-04 has the potential to inhibit the major epitope, including $^{124}\text{KWVNGREI}^{131}$, on Cry j 2. However, synergistic inhibition of allergen–antibody binding was not observed by coapplication of both CJ2-04 and CJ2-08 (data not

FIG. 1. Inhibitory effects of an allergen–antibody reaction between Cry j 2 and anti-Cry j 2 antibodies as demonstrated by dot blotting assays. (A) Cry j 2 immobilized on a nitrocellulose membrane detected using immunoglobulin E (IgE) in human serum. (B) Anti-Cry j 2 aptamers with an inhibitory effect on binding of human IgE to Cry j 2 were identified by dot blotting. The aptamer concentration used here was 500 nM. Data are presented as mean $\pm$ SEM ($n = 3$ –5). (C) Dose-dependent inhibition of allergen–antibody binding between Cry j 2 and human IgE from patient serum by CJ2-04 or CJ2-08 was evaluated by dot blotting. Data are presented as mean $\pm$ SD ($n = 3$ –5). (D) Inhibition of allergen–antibody reaction between Cry j 2 and mouse anti-Cry j 2 monoclonal antibody T27 using CJ2-04 and CJ2-08. The aptamer concentration used here was 500 nM. Data are presented as mean $\pm$ SD ($n = 4$ –5). * $P < 0.05$ compared to poly dT.

FIG. 2. Recognition of nondenatured and denatured Cry j 2. (A) Aptamer blotting using CJ2-04 and CJ2-08. (B) Dot blotting using anti-Cry j 2 mAb and pAb.

shown). There is a possibility that CJ2-04 and CJ2-08 inhibit the same IgE clone.

Furthermore, the stability of the binding site for the aptamers, CJ2-04 and CJ2-08, was evaluated by comparison with that of anti-Cry j 2 antibodies. The signal against denatured Cry j 2 was lower than that against nondenatured Cry j 2 in the aptamer blotting assay for both aptamers (Fig. 2A), whereas the opposite behavior was observed in the dot blotting assay using anti-Cry j 2 antibodies (Fig. 2B). Given that anti-Cry j 2 mAb T27 has been considered to recognize sequential epitopes [8], the lack of reduction of the signal upon denaturation of Cry j 2, as observed in Fig. 2B, was easily predicted. In other words, the reduction observed in Fig. 2A implies that the conformation of nondenatured Cry j 2 is important for recognition by the aptamers. Consequently, CJ2-04 and CJ2-08 may recognize the conformation of Cry j 2, leading to the inhibition of binding to IgE against Cry j 2.

Subsequently, we investigated whether CJ2-04 and CJ2-08 could inhibit Ca^{2+} mobilization induced by Cry j 2 through cross-linking of IgE in KU812-F cells. First, we confirmed the presence of the FcεRI α subunit (FcεRIα), which binds to the Fc region of IgE [19], in KU812-F cells by immunoblotting. FcεRIα was detected in whole cell (WC) and membranous protein fractions (Fig. 3A), while glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme mainly present in the cytosol, was detected in WC and non-membranous protein fractions (Fig. 3A). These results indicate that human IgE possibly binds to the surface of KU812-F cells through FcεRIα even if the KU812-F cells are in a prebasophilic state.

Ca^{2+} mobilization was then evaluated using KU812-F cells. Ca^{2+} mobilization induced by Cry j 2 was observed only in KU812-F cells preincubated with antiserum (data not shown), indicating that Ca^{2+} mobilization is induced by Cry j 2 through IgE cross-linkage in KU812-F cells. We evaluated the effects of aptamers using this cellular response as an indicator of degranulation. In the KU812-F cells incubated

with CJ2-04 or CJ2-08, we observed an apparent decrease in Ca^{2+} mobilization induced by Cry j 2 ($P < 0.05$, *t*-test, vs. Cry j 2 only) (Fig. 3B). No significant decrease was observed using aptamer or poly dT without Cry j 2 (data not shown). These results suggest that CJ2-04 and CJ2-08 inhibit primary

FIG. 3. Suppression of Ca^{2+} mobilization in KU812-F cells using anti-Cry j 2 aptamers. (A) FcεRIα in KU812-F cells evaluated by immunoblotting. MP, membranous protein; NMP, nonmembranous protein; WC, whole cells. (B) Intracellular Ca^{2+} levels in KU812-F cells preincubated with patient serum. The values are presented as mean $\pm$ SD ($n = 3-4$). AU, arbitrary unit.

immune signaling and reduce the degranulation of basophils and mast cells to block allergy symptoms.

In the present study, we identified aptamers that had an inhibitory effect on allergen–antibody binding between Cry j 2 and IgE in human serum collected from a patient with Japanese cedar pollinosis. We observed in a human basophilic leukemia cell line (KU812-F) that aptamers, CJ2-04 and CJ2-08, suppressed Ca^{2+} mobilization, which is related to degranulation and induction of allergy symptoms. These results indicate that the CJ2-04 and CJ2-08 aptamers have a great potential to reduce the induction of Japanese cedar pollinosis if applied to the Cry j 2 allergen before it invades the body. In particular, CJ2-04 may be applicable to many patients with Japanese cedar pollinosis because the inhibition of the reaction between Cry j 2 and anti-Cry j 2 mAb T27, which recognizes the major epitope for IgE on Cry j 2, was observed. In the future, inhibition of degranulation using the aptamers should be evaluated by monitoring histamine release as an indicator of allergic symptoms. Furthermore, it would be of interest to evaluate the relationship between aptamer sequence or structure and inhibitory effect. A common 11 nucleotide sequence (5′-AGGGAAAGGGG-3′) is shared by five of the eight aptamers screened here for inhibitory capacity (CJ2-02, CJ2-04, CJ2-05, CJ2-06, and CJ2-08); however, the sequence or structural elements responsible for differences in binding to Cry j 2 have not been addressed yet.

We propose the use of aptamers as a new approach for preventing allergy symptoms. In the case of Japanese cedar pollinosis, we know that these allergens are present indoors. Takahashi *et al.* reported that most pollen enters homes through ventilation systems and on textile products brought inside during the pollen dispersal season and suggested that paying special attention to these routes would lead to improved self-care for pollinosis [2]. Applying aptamers (ie, spraying them in locations in the home where Cry j 2 may be present) would be a novel preventive measure against Japanese cedar pollinosis. The binding and inhibitory properties of chemically modified aptamers may be more effective in preventing pollinosis than those of the nonmodified aptamers described herein and should thus be addressed in future studies.

This approach could also be applied to other allergens present in the environment and could play an important role in helping those who suffer from allergy symptoms.

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Author Disclosure Statement

No competing financial interests exist.

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