

ORIGINAL ARTICLE

Blocking of protease allergens with inhibitors reduces allergic responses in allergic rhinitis and other allergic diseases

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Abstract

Conclusions. Allergic responses specific to the corresponding proteases were reduced by protease inhibitors, suggesting promise as potent treatments for allergic rhinitis and other allergic conditions. **Objective.** Allergic diseases, such as allergic rhinitis, are caused by the overproduction of IgE antibodies to various allergens. Many reported allergens are proteases that are cysteine, serine, aspartic (acid) proteases and metalloproteases. Conjugation of E64 inhibitor with cysteine protease allergens inhibits the IgE response to the same allergens. However, whether inhibitors of the other protease families reduce IgE levels and whether protease inhibitors reduce allergic symptoms remain controversial. Therefore, we compared the abilities of active and inhibitor-blocked inactive forms of proteases to generate IgE and allergic symptoms in this study to evaluate associations between the allergic response and protease inhibitors. **Materials and methods.** We measured levels of IgE, IgG1, IgG2a, and IgG2b enzyme-specific antibodies, and counted frequency of sneezing and nasal rubbing behavior in mice immunized with active or inactive forms of bromelain, chymotrypsin, chymosin and collagenase (a cysteine protease, a serine protease, an aspartic protease and a metalloprotease, respectively). **Results.** All the inhibitors reduced IgE and IgG1 production in response to corresponding enzymes, and a cysteine protease inhibitor, E64, decreased nasal symptoms, such as sneezing and nasal rubbing.

Keywords: Protease, inhibitor, IgE, nasal rubbing

Introduction

About 15% of the population in developed countries is afflicted with allergic conditions such as allergic rhinitis, atopic dermatitis, and asthma. Pollen, fungi, house dust, insects, and mites are major sources of allergens that cause excessive Th2-controlled antibody responses. Allergic components in allergens are often enzymes, and in particular, cysteine, serine and aspartic proteases (acid proteases) as well as metalloproteases. Der p 1 and Der f 1 (Dermatophagoides group 1), major allergens of the house dust mite, exhibit cysteine protease activity [1], and the cysteine protease bromelain causes allergic disease [2]. Dermatophagoides group 6 conserves a primary

structure characteristic for chymotrypsin-like serine proteases [3]. Bla g2, a major allergen of the German cockroach (*Blattella germanica*), is an aspartic protease [4], and the aspartic protease chymosin also induces allergic reactions [5]. IgE antibodies have been detected against metalloproteases from parasites, such as collagenase from *Gymnorhynchus gigas* [6].

Blocking Der p 1 and a cysteine protease of *Leishmania mexicana* with an inhibitor (E64) actually decreases the production of IgE antibodies against themselves [7,8]. These reports raise questions as to whether inhibitors of other protease families reduce IgE levels and whether protease inhibitors reduce

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allergic symptoms. These issues do not appear to have been investigated. Understanding the effects of protease inhibitors on the corresponding allergic response should provide insight into the mechanism of allergic disease onset, and contribute to the development of a new therapeutic approach to treating allergic conditions.

Allergic rhinitis is caused by IgE-mediated activation, which is accompanied by sneezing, nasal itching, rhinorrhea, and nasal obstruction. Many people suffer from allergic rhinitis, and the incidence of allergic rhinitis has been increasing in recent years. In other words, allergic rhinitis is regarded as a very important disorder; however, the relationship between allergic rhinitis, protease allergens, and inhibitors has not been elucidated at all.

We examined the effects of protease inhibitors on IgE antibody production and nasal symptoms in immunized mice to determine the significance of inactive proteases blocked with inhibitors. We found that the protease-blocked inactive forms of proteases are less potent than active forms in generating allergic responses specific to the corresponding protease, suggesting that protease inhibitors will be useful as a treatment modality.

Materials and methods

Proteases and inhibitors

Bromelain from pineapple stem, *trans*-epoxysuccinyl-L-leucylamido (4-guanido)-butane (E64), chymotrypsin from bovine pancreas, chymosin, microbial pepstatin, collagenase from *Clostridium histolyticum*, ethylenediaminetetraacetic acid (EDTA), and ovalbumin (sourced from hen eggs) were purchased from Sigma-Aldrich (St Louis, MO, USA). Diisopropyl fluorophosphate (DFP) was obtained from Wako Co. Ltd (Tokyo, Japan).

Inactivation of proteases and assays of enzymatic activity

The cysteine protease activity of bromelain was measured using casein as the substrate as follows [9]. An equal volume of bromelain (1 mg/ml) in the presence of 250 mM cysteine was incubated with phosphate-buffered saline (PBS) or PBS containing the inhibitor, E64 (10 mM), at 37°C for 90 min and then dialyzed overnight against PBS. Casein (2% in 100 ml of 40 mM sodium hydroxide) was autoclaved at 120°C for 15 min, adjusted to pH 7.4 with 0.5 M phosphate buffer and used as a substrate. An equal volume of the substrate was added to the bromelain mixture and incubated at 37°C for 60 min; then 1 ml of 5% trichloroacetic acid (TCA) was added to stop the reaction. After standing at room temperature for 10 min, the mixture was centrifuged at 1200 g for

20 min and the absorbance of the supernatant was determined at 280 nm.

The serine protease activity of chymotrypsin (100 µg) was determined as follows. Chymotrypsin (100 µg) was dissolved in 990 µl of saline and mixed with 10 µl of isopropanol alone or containing the inhibitor DFP (final concentration 10 mM) and incubated at 37°C for 90 min. The activity of chymotrypsin was determined in the same manner as described for cysteine protease.

The aspartic protease activity of chymosin was determined as follows. Chymosin (100 µg/ml in 0.1 M citrate buffer, pH 4.0) was incubated with or without the inhibitor, pepstatin, (final concentration 1 mM) for 10 min at room temperature and then the protease activity was determined as described previously [10]. Briefly, chymosin (5 µl) with or without pepstatin was incubated with 1 ml of heat-denatured hemoglobin for 10 min at 37°C and then 2 ml of 5% TCA was added. The filtered precipitate (1 ml) was mixed with 2 ml of 0.5 M NaOH followed by 0.6 ml of phenol and absorbance was determined at 650 nm.

The metalloprotease activity of collagenase was determined as follows. Collagenase (5 µg/ml) was incubated with or without the inhibitor EDTA (final concentration 5 mM) for 24 h at 4°C. The enzymatic activity was determined using a collagenase assay kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 100 µl of collagenase (0.05 M Tris-HCl, 0.15 M NaCl), with or without EDTA, was incubated with gelatin from pig skin (1 mg/ml) for 4 h at room temperature, and then the fluorescence was measured using a microplate reader at 490 nm (excitation) and 540 nm (emission).

Immunization protocols

Intraperitoneal immunization. Active or inactive proteases (5 µg) blocked with an inhibitor containing 2 mg Al(OH)₃ as an adjuvant were injected intraperitoneally into 6-week-old male BALB/c mice twice at an interval of 2 weeks. In another experiment, 5 µg of ovalbumin (OVA) with or without E64, DFP, pepstatin or EDTA inhibitors, was similarly injected intraperitoneally. Serum samples were obtained from groups of 10 mice 2 weeks after the last injection.

Aerosol immunization. After intraperitoneal immunization with active or inactive bromelain, 10 mice were challenged intranasally for 30 min daily for 2 weeks with 0.1% active or inactive bromelain, used at the time of immunization. After the last nasal challenge, all the mice were placed into one cage

(32 × 22 × 10 cm), and the numbers of sneezes and nasal rubbing movements were counted for 10 min as described previously [11–14].

Protease biotinylation. Proteases (50 µl; 1 mg/ml) were diluted 10-fold with 0.1 M NaHCO₃ and mixed with 20 µl of NHS-biotin (5 mg/ml in dimethyl sulfoxide) at room temperature for 2 h. The mixtures were then dialyzed overnight against PBS (pH 7.4) to remove free NHS-biotin and stored at –80°C.

Measurement of antigen-specific IgE, IgG1, IgG2a and IgG2b titers

We measured antigen-specific IgE titers in sera using an ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody (1 µg/ml, Biosource International, Camarillo, CA, USA) overnight at 4°C. After blocking, fivefold diluted sera were added to the plates and incubated for 1 h at 37°C. After washing and addition of biotinylated antigens, the plates were further incubated for another hour at 37°C, washed and then incubated with avidin-peroxidase for 1 h at 37°C. After additional washing, the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was applied according to the manufacturer's instructions, and the optical density (OD) was determined at 450 nm. Antigen-specific IgG1, IgG2a and IgG2b titers in serum samples were measured using ELISA with peroxidase-labeled second antibodies to each IgG subclass as follows. ELISA plates were coated with each protease (10 µg/ml) overnight at 4°C. After blocking, 100 µl of serum samples (diluted 10-fold) were added to the plates and incubated for 1 h at 37°C. After washing, peroxidase-labeled anti-mouse IgG1 (200-fold dilution; The Binding Site, San Diego, CA, USA), IgG2a (100-fold dilution; The Binding Site) or IgG2b (100-fold dilution; Southern Biotechnology, Birmingham, AL, USA) was added and the plates were further incubated for 1 h at 37°C. After additional washing, the same substrate was used as described above. The highest OD for each IgE and IgG subclass in serum samples from mice immunized with each protease was defined as 100 U/ml and this value was used to calculate antibody levels.

Data analysis

Student's *t* test was used to assess whether the antibody titers differed between two groups. A *p* value below 0.05 was considered significant.

Results

Effects of protease inhibitors on IgE, IgG1, IgG2a and IgG2b production to the corresponding protease

Figure 1 shows the antibody titers of IgE and IgG subclasses specific to each protease in mice immunized with 5 µg of the corresponding proteases. Mice immunized with the active enzymes produced significantly more of each protease-specific IgE (*p* < 0.05) and IgG1 (*p* < 0.01 for bromelain and chymotrypsin; *p* < 0.05 for other proteases) than mice immunized with the corresponding inactive proteases. On the other hand, the IgG2a antibody response was significantly higher in mice immunized with inactive than with active chymotrypsin, but no other significant differences were apparent.

Effects of protease inhibitors on IgE, IgG1, IgG2a and IgG2b responses to OVA

The levels of IgE, IgG1, IgG2a and IgG2b produced in mice immunized with OVA (5 µg) or with OVA plus E64, DFP, pepstatin or EDTA were essentially similar (Figure 2).

Effects of E64 in mice challenged intranasally with bromelain on sneezing and nasal rubbing behavior

Mice sneezed and rubbed their noses significantly more often when challenged with active, than with inactive bromelain (Figure 3).

Discussion

That many allergens are proteases is notable and thus the roles of their enzymatic activities in generating allergic responses have been argued. Gough et al. demonstrated that the cysteine protease activity of the major mite allergen, Der p 1, selectively accelerates the IgE antibody response [7]. We also demonstrated here that the active form of the cysteine protease, bromelain, up-regulated IgE and IgG1 antibody responses. Moreover, our results suggested that not only the cysteine, but also active serine and aspartic proteases and metalloprotease enhanced IgE and IgG1 responses. Although the proteolytic activity of Der p 1, a cysteine protease, enhances the IgE response to OVA in mice immunized with a mixture of Der p 1 and OVA [15], the inhibitors did not affect antibody production in response to OVA in the present study. Gough et al. [15] also reported similar results with the cysteine protease inhibitor, E64. Considering these findings, the protease inhibitors themselves probably do not directly affect IgE or IgG1 enhancement.

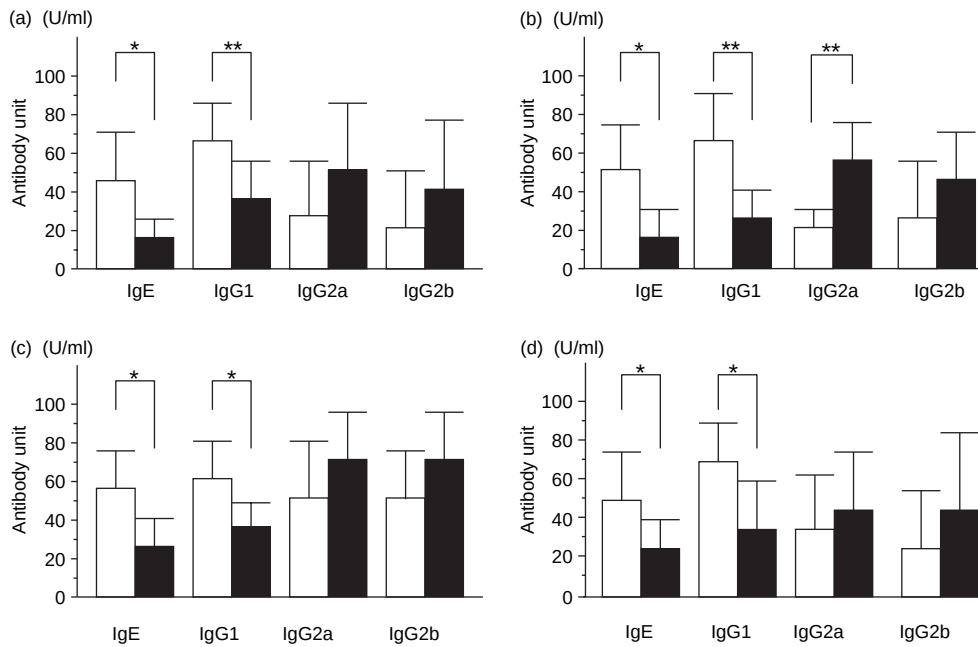


Figure 1. Protease-specific IgE, IgG1, IgG2a and IgG2b levels in sera from mice immunized with active or inactive protease. Bromelain (a), chymotrypsin (b), chymosin (c) or collagenase (d) were injected intraperitoneally twice at an interval of 2 weeks. Columns and vertical bars show means $\pm$ SD. The mean antibody titer and SD were calculated from triplicate experiments for each group and results were compared between immunization with active (white bar) and inactive (black bar) protease. * $p < 0.05$ and ** $p < 0.01$ versus counterpart group of mice immunized with inactive protease.

Evidence of direct enzymatic effects on antibody production is not definite, but several reports suggest that cysteine protease activities do enhance IgE and IgG1. For example, Shulz et al. [16] demonstrated that Der p 1 proteolytically cleaves CD 23 (the low affinity receptor for IgE) from the surface of cultured

human B cells. Given that CD23 is involved in the negative feedback regulation of IgE synthesis in vivo [17], its cleavage by Der p 1 would potentially disrupt the IgE regulatory mechanism, thus causing excessive IgE synthesis. Der p 1 induces mast cell and basophil degranulation, and stimulates IL-4

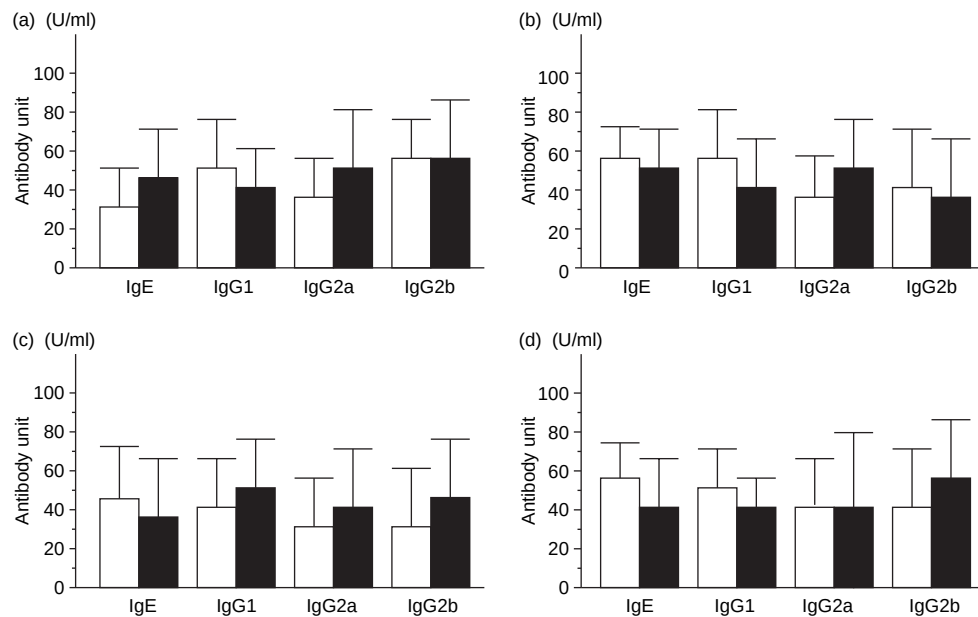


Figure 2. Ovalbumin (OVA)-specific IgE, IgG1, IgG2a and IgG2b levels in sera from mice immunized with OVA or OVA plus inhibitor. OVA with or without E64 (a), DFP (b), pepstatin (c) or EDTA (d) inhibitors, was injected intraperitoneally twice at an interval of 2 weeks. Columns and vertical bars show means $\pm$ SD. The mean antibody titer and SD were calculated from triplicate experiments for each group and results were compared between immunization with OVA only (white bar) and with OVA plus inhibitor (black bar).

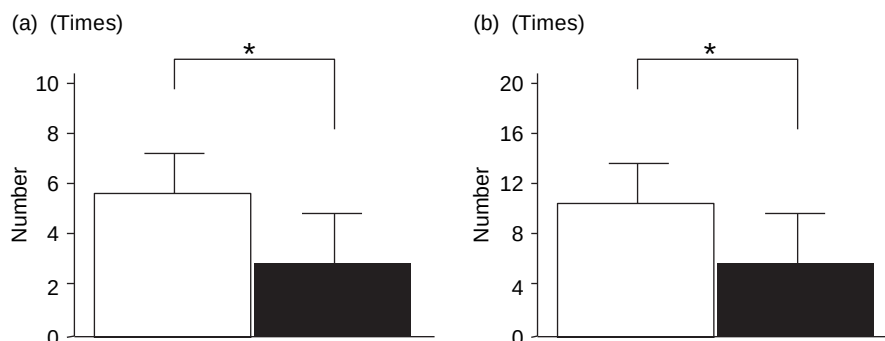


Figure 3. The numbers of sneezes (a) and nasal rubbing movements (b) in 10 mice induced with active (white bar) or inactive (black bar) bromelain. After the last nasal challenge, sneezing and nasal rubbing were evaluated for 10 min. Columns and vertical bars show means $\pm$ SD. * $p < 0.05$ versus counterpart group of mice immunized with inactive protease.

synthesis and secretion in the absence of IgE in vitro [18]. Schulz et al. [19] reported that Der p 1 also cleaves the subunit of the IL-2 receptor (IL-2R or CD25). Since IL-2R is pivotal for the propagation of Th1 cells, its cleavage by Der p 1 might bias the immune response towards Th2. The proteolytic activity of Der p 1 also conditions T cells to produce more IL-4 and less interferon-gamma (IFN- γ) [20]. These facts can explain why protease activity enhances IgE and IgG1 antibodies. Therefore, the enhanced IgE and IgG1 responses here might be attributed to the tested enzyme activities, although other factors can explain this phenomenon, such as three-dimensional conformation or biological stability.

The present study demonstrated that more specific IgE and IgG1 antibodies were generated in response to the corresponding active, rather than inactive forms of proteases, but not all reported allergens are proteases. A major allergen of the German cockroach, Bla g2, is an inactive aspartic protease [4]. These suggest that protease activity is not essential for IgE production and that the enzymatic activities rather enhance IgE and IgG1 antibody production.

Serine proteases cause the activation and release of inflammatory mediators from human eosinophils through protease-activated receptor-2 (PAR-2) [21,22], and an inhibitor of the proteases suppresses eosinophil peroxidase release from activated human eosinophils [23]. Therefore, the present phenomenon might be explained by differential activation of the receptors between active and inactive forms of proteases.

The present study demonstrated significantly more IgG2a production in mice immunized with inactive than with active chymotrypsin. However, inactive forms of the other proteases only tended to increase IgG2a and IgG2b production associated with Th1 responses. Whether inhibitor-blocked inactive proteases induce the Th1 response remains

unknown, although cysteine protease activity induces IL-4 and the Th2 response [16–20]. The inhibitors might decrease only allergen activity.

We used nasal sneezing and rubbing behavior as an index of allergic rhinitis, because sneezes and nasal rubbing behaviors are important symptoms in mice with allergic rhinitis [11–14]. That sneezing and nasal rubbing behavior were decreased in mice immunized with aerosolized inactive, as compared with active bromelain, is notable. The mechanism of this phenomenon is not easily explained, since the enzymatic effects of allergens on nasal symptoms have not been investigated. However, it was reported that Der p 1 induced mast cell and basophil degranulation [18]. This may be one of the possibilities, since mast cell and basophil degranulation induces allergic symptoms. Our observation suggests that inactive allergens could be a useful strategy for desensitizing individuals with allergic rhinitis.

We demonstrated that active proteolytic proteases (cysteine, serine and aspartic proteases and metalloprotease) enhance IgE and IgG1 antibody responses, which might explain why so many allergens are proteases.

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