Mast cell–derived proteases control allergic inflammation through cleavage of IgE

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Background: Cross-linking of mast cell–bound IgE releases proinflammatory mediators, cytokines, and proteolytic enzymes and is a key event in allergic inflammation.

Objective: We sought to study the effect of proteases released on effector cell activation on receptor-bound IgE and their possible role in the regulation of allergic inflammation.

Methods: Using molar ratios of purified recombinant tryptase and human IgE, we studied whether tryptase can cleave IgE. Similar experiments were performed with mast cell lysates in the presence or absence of protease inhibitors. IgE cleavage products were detected in supernatants of allergen cross-linked, cultivated mast cells and in tissue fluids collected from patients’ skin after IgE-mediated degranulation. The effects of protamine, an inhibitor of heparin-dependent proteases on IgE-mediated allergic in vivo skin inflammation in human subjects were studied.

Results: We show that β-tryptase, a major protease released during mast cell activation, cleaves IgE. IgE degradation products were detected in tryptase-containing tissue fluids collected from sites of allergic inflammation.

The biologic significance of this mechanism is demonstrated by in vivo experiments showing that protease inhibition enhances allergic skin inflammation.

Conclusion: We suggest that IgE cleavage by effector cell proteases is a natural mechanism for controlling allergic inflammation.

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Key words: Allergy, mast cells/basophils, allergic inflammation, proteases

The prevalence of allergic diseases has dramatically increased during the last decades. Complex genetic and environmental factors seem to be implicated in the development of IgE responses against per se harmless antigens (ie, allergens). IgE is the least abundant class of immunoglobulin but can elicit severe inflammatory reactions through the activation of various immune cells. The most common and characteristic manifestations of allergic disease are caused by IgE-mediated activation of mast cells in mucosal tissues. They include allergic rhinitis and conjunctivitis (commonly termed hay fever), asthma, and gastrointestinal symptoms ranging from oral swelling to diarrhea and anaphylactic shock. The mucosal manifestations of allergy are mainly elicited by intruding allergens that cross-link IgE antibodies bound through FceRI to mast cells. The bridging of IgE molecules by allergens and their aggregation transmits a cascade of signaling events that ultimately leads to the release of inflammatory mediators, cytokines, and proteases through the intracellular portions of FceRI. These events are responsible for acute allergic tissue inflammation. The IgE-mediated response is inappropriate with respect to the fact that it is directed against harmless antigens, but it serves its principal purpose, namely defense and elimination of its target. In this context it should be mentioned that there is evidence for a role of IgE-mediated responses in defense against parasites.

Although the mechanisms leading to IgE-mediated mast cell activation have been investigated in detail, much less information is available about whether there are natural mechanisms for controlling allergic inflammation. Unlimited mast cell activation and systemic release of mast cell products would eventually lead to a severe and life-threatening condition, anaphylactic shock, and complete degranulation of mast cells. Fortunately, most allergic reactions are only of a local nature and quickly cease after elimination of the causative allergen. Furthermore, mast cells withstand a limited degranulation process, survive, regranulate, and can be activated again.

Here we investigated whether tryptase, an abundant mast cell protein that is specifically released in large quantities from activated mast cells, can regulate mast cell activation by cleaving IgE and, if so, whether mast cell proteases can control overwhelming allergic reactions.
Methods

Materials and reagents

Human monoclonal IgE specific for the major birch allergen Bet v 1 and anti-human IgE antibody mAb12 recognizing FcεRI-bound IgE were purified, as previously described. Recombinant human skin β-tryptase was obtained from Promega (Madison, Wis). Recombinant pollen allergens were from Novartis Research Institute (Vienna, Austria). Rabbit antibodies specific for Bet v 1 were raised against purified recombinant Bet v 1.

Tryptase-mediated protein cleavage and ELISA detection of components

In a typical reaction, components (ie, purified human IgE, FcεRI α-chain, and IgE/α-chain complexes) were incubated with tryptase (tryptase:IgE molar ratio = 1:40) and, for control purposes without tryptase, in 20 μL of H₂O or PBS at 37°C for 2 hours. Reactions were either immediately processed for ELISA analysis or, for immunoblot analysis, stopped by adding SDS sample buffer containing β-mercaptoethanol and by boiling. Components were coated in triplicate on ELISA plates (Nunc-Immuno, Maxisorp, Roskilde, Denmark) in 0.1 M sodium bicarbonate, pH 9.6, at 4°C overnight. Plates were washed twice with TRIS-buffered saline with Tween (TBST) containing 0.5% wt/vol BSA and blocked with TBST containing 3% wt/vol BSA at 37°C for 3 hours. Reactants diluted in TBST were allowed to bind overnight at 4°C. Between incubations, plates were washed 5 times with TBST. Bound reactants were detected by using enzymatically labeled tracers for IgE (AP-coupled anti-human IgE [PharMingen], mAb12, or LE27, followed by horseradish peroxidase [HRP]-coupled anti-mouse IgG antibodies [Amersham, United Kingdom]; biotinylated mAb12 followed by HRP-streptavidin conjugates [Amersham]) or rabbit anti-Bet v 1 immunoglobulin (HRP-coupled donkey anti-rabbit immunoglobulin [Amersham]). OD values correspond to bound antibodies and represent means ± SD of triplicate determinations.

Effect of protamine on late phase allergic skin inflammation in vivo

To study whether inhibition of heparin-dependent proteases by protamine affects IgE-mediated allergic skin inflammation, a clinical study was performed in human subjects. Eleven patients with grass pollen allergy,

FIG 1. Tryptase cleaves IgE and abolishes binding of IgE to allergen. A, Nitrocellulose-blotted human IgE treated with (+) or without (−) tryptase was stained with anti-human IgE antibodies. Molecular weights (in kilodaltons) are displayed. B and C, Birch pollen allergen (Bet v 1)-specific human IgE was treated with (+) or without (−) tryptase (molar ratios: solid column, tryptase:IgE = 1:20; open column, tryptase:IgE = 1:40) and reacted with ELISA plate–bound Bet v 1 or the grass pollen allergen Phl p 5. OD values (y-axis) correspond to bound IgE detected with 2 different monoclonal anti-IgE antibodies (Fig 1, B; Pharmingen; Fig 1, C; LE-27). D, Plate-bound Bet v 1–specific IgE treated with (IgE +) or without (IgE −) tryptase and tryptase alone were incubated with Bet v 1, and bound Bet v 1 was detected with rabbit anti-Bet v 1 antibodies. OD values (y-axis) correspond to bound Bet v 1–specific rabbit antibodies.

Mast cell culture and cellular experiments

Human cord blood was obtained from healthy female donors taking no medication. Cord blood mast cells were generated by culturing human cord blood mononuclear cells in the presence of stem cell factor and IL-6. Cord blood mast cells (typically 10⁶ cells of >90% purity) were washed with protein-free piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (PIPES, 25 mmol/L; NaCl, 110 mmol/L; KCl, 5 mmol/L; CaCl₂, 2 mmol/L; and glucose, 1 g/L [pH 7.35]) and either lysed in water by means of freeze-thawing or used for activation experiments. Purified IgE was exposed to cell lysates in the presence or absence of the serine protease inhibitor leupeptin (Sigma-Aldrich, Vienna, Austria) and analyzed by means of immunoblotting with iodine 125–labeled anti-human IgE (Phadia).

The effects of tryptase release on mast cell–bound IgE was studied by loading mast cells (10⁶/well) in duplicate wells with Bet v 1–specific IgE (10 μg/mL) for 4 hours at 37°C. Unbound IgE was washed away with PIPES buffer, and cells were either activated with Ca²⁺ ionophore (Sigma-Aldrich) or not. Supernatants and cellular extracts were analyzed by means of immunoblotting regarding IgE. Tryptase levels were measured in lysates and supernatants by means of RIA (Phadia).
1 patient with birch pollen allergy, and 1 nonallergic subject were subjected to skin testing with grass pollen or birch pollen allergens (Allergopharma, Reinbek, Germany). In addition, immediate-type skin reactions were induced in 2 nonallergic subjects with a monoclonal anti-IgE antibody (concentration = 64 μg/mL) that cross-links receptor-bound IgE. Non–IgE-mediated skin reactions were induced with codeine, and NaCl served as a negative control (Stallergenes, Antony, France). Skin sites at the volunteers’ forearms had been pretreated by means of intracutaneous injection with 25 μL of 10 IU of protamine (ICN-Pharmaceuticals, Salzburg, Austria) or sterile 0.9% NaCl. Two hours after intracutaneous injections, skin prick testing was performed in duplicate, and late-phase reactions were recorded after 6 to 10 hours with a ballpoint pen and transferred with a transparent tape to paper. Calculation of the size of the late-phase reaction was performed by means of digital planimetry. Statistical analysis was done with the SPSS program (SPSS, Inc, Chicago, Ill). Experiments in human subjects have been approved by the local ethics committee and the Austrian Ministry of Health, and informed consent was obtained from all subjects.

Collection and analysis of suction blister fluids

After approval of the study protocol by the local ethics committee and obtaining informed consent from patients, skin prick tests with rPhl p 1 (20 μg/mL) and PBS were performed at the volar forearms of an allergic and a nonallergic person in quadruplicate. Fifteen minutes later, a sterile 8-well skin suction chamber was attached to cover the 4 allergen-challenged and 4 PBS-challenged skin areas. A vacuum of 400 mm Hg was applied with an aspirator pump (PTC 3300 V AC; Innokas Medical, Kempele, Finland) for 1.5 hours, as described previously.23 A heating pad warmed to 45°C was wrapped around the chamber to promote blister formation. Blisters were punctured, blister fluid was aspirated with a tuberculin syringe, and samples were immediately stored at −80°C until analysis. Total tryptase concentrations in the samples were measured with FEIA ImmunoCAP (Phadia). Blister fluids were analyzed by means of immunoblotting for the presence of IgE.

RESULTS

Tryptase cleaves IgE and abolishes binding of IgE to allergens and FcRI

In a first set of experiments, we demonstrate that purified recombinant tryptase cleaves human IgE at even lower molar ratios (tryptase:IgE = 1:40) than those at which they occur on mast cell surfaces after cell activation (Fig 1, A). Next we investigated whether tryptase-mediated IgE cleavage affects IgE binding to allergens. Binding of human IgE specific for the major birch pollen allergen Bet v 1 to the allergen was lost after tryptase treatment and could not be detected with monoclonal anti-IgE antibodies specific for 2 epitopes on the IgE constant region (Fig 1, B and C). Untreated Bet v 1–specific IgE reacted with Bet v 1 but not with an unrelated grass pollen allergen, Phl p 5 (Fig 1, B and C). Cleavage occurred not only in the constant region but also in the variable region of IgE because tryptase-treated IgE immobilized to ELISA plates failed to react with allergen (Fig 1, D). The first set of experiments thus demonstrates that tryptase cleavage abolishes IgE reactivity to allergens.

In another set of experiments, we studied the effects of tryptase on IgE binding to the extracellular portion of FcRI. No binding of tryptase-treated IgE to the α-chain could be detected, whereas untreated IgE bound to the α-chain and could be traced with a monoclonal anti-IgE antibody recognizing receptor-bound IgE (Fig 2, A). The α-chain seemed to be more resistant than IgE against tryptase cleavage because it still reacted with IgE, despite the fact that it had been treated with tryptase under conditions that had caused degradation of IgE (Fig 2, B). Binding of IgE to the α-chain did not preserve IgE from cleavage by tryptase because IgE was degraded even when it was complexed with the α-chain (Fig 2, C). The second set of experiments thus showed that tryptase cleaves FcεRI-bound IgE. They also indicate that the α-chain might be more resistant against tryptase than IgE.

Mast cell lysates cleave IgE

In the next set of experiments, we investigated the effects of products from purified human mast cells on IgE. Fig 3, A, shows that lysates from mast cells containing high amounts of tryptase (1.6 mg/L) cleaved IgE, even after 10 minutes of exposure (Fig 3, A, lane 3), and that this process could be inhibited with the pro tease inhibitor leupeptin (Fig 3, A, lane 4). To mimic more closely in vivo conditions, we loaded purified mast cells with IgE and induced degranulation of the cells using calcium ionophore (Fig 3, B). We found degraded IgE (45 kd) in the supernatant fractions of activated mast cells (Fig 3, B, lanes 1 and 2) but not in
supernatants from nonactivated mast cells (Fig 3, B, lanes 3 and 4). Degraded IgE in cellular fractions of activated and nonactivated cells might represent endocytosed and intracellularly degraded IgE (Fig 3, B, lanes 5-8).

**Protamine augments IgE-dependent allergic inflammation in vivo**

It has been demonstrated that protamine, an inhibitor of heparin-dependent proteases, such as tryptase, augments IgE-dependent but not IgE-independent histamine release from basophil granulocytes. Assuming that protamine can inhibit the cleavage of IgE by effector cell–derived proteases and thus increase their degranulation, we investigated whether inhibition of effector cell–derived proteases can increase allergic inflammation in vivo. We studied the effects of protamine on allergic skin inflammation in human subjects. Intracutaneous injections of protamine (Fig 4, A) or NaCl (Fig 4, A) were given to human subjects. Two hours later, IgE-mediated allergic skin inflammation was induced by skin prick testing with pollen allergens in 12 allergic patients and with a monoclonal anti-IgE antibody (Fig 4, A) in 2 nonallergic persons. The effects of protamine on non–IgE-mediated mast cell degranulation were studied by challenge with codeine (Fig 4, A). Codeine stimulation induces IgE-independent activation of human mast cells and, as recently shown, activation of subsequent chemokine production.

Pretreatment with protamine led to strong increases of IgE-mediated late-phase allergic reactions caused by allergen or anti-IgE, but almost no late-phase inflammatory reactions were observed after pretreatment with NaCl (Fig 4, A). No late-phase skin reactions were observed when mast cell degranulation was induced through a non–IgE-dependent mechanism using codeine, regardless of whether skin sites had been pretreated with protamine or with NaCl (Fig 4, A).

No allergic skin inflammation was observed under any of the above conditions when a nonallergic individual was skin tested with allergens (data not shown).

If IgE was indeed cleaved by tryptase from activated mast cells in vivo, we assumed that it might be possible to detect cleaved IgE in tissue fluids from the sites of allergic inflammation. We therefore induced allergic skin reactions by means of skin prick testing in a patient with grass pollen allergy with the major grass pollen allergen Phl p 1 and obtained tissue fluids from the sites of allergic skin inflammation by using the skin suction blister technique. IgE degradation products could be detected in these tissue fluids (concentration$_{tryptase}$ 260 µg/L), but not in fluids obtained from a noninflamed site of the allergic patient who was challenged only with PBS (concentration$_{tryptase}$ 79.4 µg/L) or in tissue fluids obtained from a nonallergic patient who was treated with Phl p 1 (concentration$_{tryptase}$ 20.8 µg/L) and PBS (concentration$_{tryptase}$ 14.7 µg/L; Fig 4, B).

**DISCUSSION**

Our results suggest that proteases released from allergic effector cells (ie, mast cells and basophils) in the course of IgE-mediated degranulation cleave IgE and thus can control allergic inflammation. Our hypothesis that IgE cleavage by effector cell–derived proteases is a natural mechanism for controlling allergic reactions is supported by experiments performed with purified proteins, as well as by cellular in vitro and in vivo data. First, we used purified molecules to demonstrate that tryptase cleaves IgE in the constant and variable region and thus affects IgE binding of allergens, as well as binding to FcεRI. Furthermore, we show that FcεRI-bound IgE is cleaved by tryptase, whereas the α-chain of FcεRI seemed rather resistant to tryptase. These results were obtained in in vitro experiments, and the molar ratios chosen for IgE and tryptase took into consideration calculations regarding the number of IgE molecules and the amount of tryptase
released per mast cell.\textsuperscript{11,24} We have shown by means of \textit{in vitro} experiments with purified mast cells, as well as by means of \textit{in vivo} experiments, that tryptase-mediated cleavage of IgE occurs on activated mast cells. IgE cleavage products were detected in supernatants of activated mast cells.

Support for the biologic relevance of our findings comes from the fact that protamine, an inhibitor of heparin-dependent effector cell proteases, augmented IgE-dependent, but not IgE-independent, effector cell degranulation \textit{in vitro} (data not shown) and allergic skin inflammation \textit{in vivo}.

Although tryptase is the most abundant mast cell protease and can be found in all mast cell types,\textsuperscript{9-11} it is possible that other mast cell–derived proteases also contribute to the degradation of IgE. Taking into consideration that other mast cell–derived proteases might be involved in the cleavage of IgE, it could be difficult, if not impossible, to determine the contribution of tryptase to the

\textbf{FIG 4.} Protamine augments IgE-dependent allergic inflammation \textit{in vivo}. \textbf{A,} Box plot representation of late-phase allergic skin inflammation (y-axis, in square centimeters) in a group of 12 allergic patients challenged with pollen allergens and 2 nonallergic persons challenged with anti-IgE after pretreatment with protamine (a) or NaCl (b). Non–IgE-dependent late-phase reactions (LPR) after challenge with codeine after pretreatment with protamine (c) or NaCl (d) in the same group of individuals are also shown. Mean values and outliers are indicated. \textbf{B,} An allergic patient and a nonallergic subjects underwent skin prick tests with allergen (+) or PBS (−). Nitrocellulose-blotted skin blister fluids obtained from the application sites were analyzed by means of Western blotting for the presence of IgE. Molecular weights (in kilodaltons) are shown.
cleavage of IgE by using effector cell–derived proteases through the generation of trypsin-deficient mice. Furthermore, it needs to be considered that other mechanism might influence allergic effector cell activation.

In addition to the trypsin/protease-mediated cleavage of IgE suggested by us as a mechanism for the termination of IgE-mediated mast cell activation, it has been proposed that mast cell activation can be downregulated through co-cross-linking of FcεRI and FcγRIIb.27 However, it is not clear whether human mast cells express enough FcγRIIb in vivo28 and whether all allergen-specific IgG antibodies capable of co-cross-linking to control allergen-induced inflammation. Another possible mechanism for termination of mast cell activation might be endocytosis of IgE, which has been observed for rat basophils,29 but no such data exist for human mast cells.

Recently, we have found that effector cell–derived proteases cleave allergens to a varying degree and, depending on the degree of cleavage, can influence their allergenic activity.30 This mechanism might also contribute to the prolongation of allergic inflammation. However, in the case of allergen cleavage by trypsin, we have observed that not all allergens are cleaved equally well, and in the case of certain allergens, it is possible that proteolytic cleavage might liberate even highly allergenic allergen fragments.30 Furthermore, we found that IgE is more readily cleaved by trypsin than most of the allergens that represent rather stable proteins that can survive in the environment (data not shown). The fact that proteolytic enzyme in the absence of antigen specific IgE antibodies in mouse model of type I allergy: identification and characterization of a mononaphilic anti-human IgE antibody fragment that blocks the IgE-FcepsilonRI interaction and reacts with receptor-bound IgE. J Allergy Clin Immunol 2001;108:409-16.


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