

Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model

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Summary

Background We have recently demonstrated that intraperitoneal immunization of mice with proteolytically active Der p 1, the major house dust mite allergen, results in a significant and selective enhancement of total and Der p 1-specific IgE synthesis compared to mice immunized with proteolytically inactive Der p 1.

Objective To investigate whether the proteolytic activity of Der p 1 would lead to enhanced inflammatory cellular infiltration of the lungs and systemic IgE production when administered through the respiratory system, which is the natural route of entry for this allergen.

Methods Groups of mice were initially sensitized with proteolytically active Der p 1 through the intraperitoneal and the subcutaneous routes and subsequently exposed intranasally to either proteolytically active Der p 1, inactive Der p 1 or PBS. The extent of cellular infiltration of the lungs and systemic IgE production in the three animal groups were then compared.

Results Here, we show for the first time that the administration of proteolytically active Der p 1 to mice through the intranasal route leads to significant inflammatory cellular infiltration of the lungs and systemic production of IgE.

Conclusions These data underline the important role of the proteolytic activity of Der p 1 in driving the allergic response in the lungs.

Keywords allergenicity, cysteine protease, Der p 1

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Introduction

One of the most important properties of allergens is their ability to induce Th2 responses culminating in IgE antibody production and allergic disease. This is best exemplified by allergens from the house dust mite (HDM) *Dermatophagoides pteronyssinus*, which are a major cause of allergic disease in the western world. Der p 1, one of several such mite allergens, elicits IgE antibody responses in over 80% of patients who are sensitive to *D. pteronyssinus*, and is considered to be the most immunodominant allergen involved in the expression of IgE-mediated dust mite hypersensitivity [1].

We and others have previously shown that the potent IgE-eliciting property of Der p 1 is due to its ability to cleave proteolytically cell surface molecules involved in the homeostatic control of IgE synthesis [2], namely CD40 on dendritic cells [3], CD25 on T cells [4, 5] and CD23 on B cells [6, 7]. Recent human *in vitro* studies have demonstrated that the proteolytic activity of Der p 1 conditions dendritic cells to express more CD86 [8] and less CD40 [3], thereby enabling them to stimulate the differentiation of naïve T cells towards

the Th2 phenotype [3]. The proteolytic activity of Der p 1 was also shown to have a direct effect on human Th cell subset development. For example, Der p 1-induced cleavage of CD25 from the surface of T cells resulted in Th cells that produce low levels of IFN- γ and high levels of IL-4, a cytokine profile characteristic of Th2 cells [5, 9]. Such Th cells were then shown to be able to support autologous B cells to produce high levels of IgE [10].

The ability of the proteolytic activity of Der p 1 to enhance IgE synthesis has been previously confirmed in appropriate mouse models. For instance, we have demonstrated that intraperitoneal immunization of mice with proteolytically active Der p 1 results in a significant and selective enhancement of total IgE and Der p 1-specific IgE synthesis compared to animals immunized with Der p 1 that has been irreversibly blocked with the cysteine protease inhibitor E-64 [L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane] [11]. We have also demonstrated that the proteolytic activity of Der p 1 significantly enhances the IgE antibody response to ovalbumin, as a bystander antigen [12]. These studies are in line with a previous investigation demonstrating that a catalytically active bee venom phospholipase A2 allergen enhances IgE antibody responses in mice [13].

Given that our previous *in vivo* experiments [11, 12] were conducted using systemically administered Der p 1, they still leave open the important question of whether the proteolytic

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activity of Der p 1 would lead to enhanced cellular infiltration of the lungs and systemic IgE production when given through the respiratory system, which is the natural route of entry for this allergen. In this study, we show that the administration of proteolytically active Der p 1 to mice through the intranasal route leads to significant inflammatory cellular infiltration of the lungs and systemic production of IgE.

Materials and methods

Der p 1 preparation

Der p 1 was isolated from HDM fecal pellets (Allergon, Angelholm, Sweden) by a multi-step procedure [11] involving immunoaffinity chromatography on immobilized monoclonal anti-Der p 1 antibody (clone 4C1, Indoor Biotechnologies, Manchester, UK), removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (Sigma, Poole, UK), and finally FPLC to remove low molecular weight contaminants. The purity of the preparation was confirmed by N-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Foster City, CA, USA), SDS-PAGE analysis (15% gel) and by demonstrating that enzymatic activity is completely dependent on pre-activation with cysteine and is totally inhibited by E-64 (see below for further details). Protein concentration was determined using a BCA microtitre plate assay and confirmed spectrophotometrically using the empirical absorption coefficient value for Der p 1 of $E^{1\%}_{280\text{nm}} = 16.4$.

Before use, Der p 1 was pre-activated with 5 mM cysteine (Sigma) to regenerate its thiol group that becomes oxidized during purification. This concentration of cysteine is unlikely to result in free thiols, since Der p 1 does not become fully activated until this level is reached. The catalytic activity of Der p 1 was ascertained in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [11]. To block the proteolytic activity of cysteine-activated Der p 1, an equimolar quantity of E-64 (Sigma) was used.

Immunization protocol

Three groups of eight female Balb/c mice were sensitized with 100 µL of proteolytically active Der p 1 through both the intraperitoneal (10 µg) and the subcutaneous (10 µg) routes in

200 µg of Al(OH)₃ as adjuvant (day 0). Two weeks later (day 14), all animals were given a 50 µL intranasal boost of 1 µg of proteolytically active Der p 1, 1 µg of E-64-blocked Der p 1 or PBS, under Halothane anaesthesia. Finally, the animals were given three 50 µL intranasal challenges on alternate days (days 17, 19 and 21) of either 2 µg of proteolytically active Der p 1, 2 µg of E-64-blocked Der p 1 or PBS (Table 1), again under halothane anaesthesia. At 24 h after the third intranasal challenge (day 22), the animals were killed by an overdose of sagatal and blood samples were taken following exsanguination.

Cell counting

The trachea was also cannulated and a bronchoalveolar lavage (BAL) was carried out by flushing the lungs and airways three times with 0.4 mL PBS. The BAL cells were counted and 1×10^5 cells were spun onto glass slides. The slides were stained with Diff-Quik (Baxter, Deerfield, IL, USA) according to the manufacturer's instructions. Percentages of macrophages, lymphocytes, eosinophils and neutrophils were determined microscopically using standard histological criteria [14].

Antibody detection

Serum samples were initially titrated to determine the optimal dilution for testing each antibody isotype and subclass [11]. The optimal dilutions used here were 1:10 for detecting total IgE and Der p 1-specific IgE, and 1:20 000, 1:30 000 and 1:250 for detecting Der p 1-specific IgG, IgG1 and IgG2b, respectively. Total IgE was detected by a sandwich ELISA using one monoclonal anti-mouse IgE (clone R35-72, Pharmingen, San Diego, CA, USA) as a capture antibody and a second biotinylated monoclonal anti-mouse IgE (clone R35-118, Pharmingen) as a detection antibody. Der p 1-specific IgE (measured using samples that have been depleted of immunoglobulin G (IgG) on a Protein G column, Pharmacia, St Albans, UK) and Der p 1-specific IgG, IgG1 and IgG2b were detected on microtitre plates coated with a 4 µg/mL solution of Der p 1 and developed with either biotinylated (for IgE clone R35-118 and for IgG1 clone A85-1, Pharmingen; for IgG2b clone AB275, The Binding Site,

Table 1. Sensitization, boost and challenge protocol for the triple (chronic) inhalation mouse model

		Challenge			
Sensitization Day 0	Boost Day 14	Day 17	Day 19	Day 21	Killing Day 22
Active Der p 1					
10-µg i.p./s.c. active Der p 1	1-µg i.n. active Der p 1	2-µg i.n. active Der p 1	2-µg i.n. active Der p 1	2-µg i.n. active Der p 1	24 h post-final challenge
Inactive Der p 1					
10-µg i.p./s.c. active Der p 1	1-µg i.n. inactive Der p 1	2-µg i.n. inactive Der p 1	2-µg i.n. inactive Der p 1	2-µg i.n. inactive Der p 1	24 h post-final challenge
PBS control					
10-µg i.p./s.c. active Der p 1	i.n. PBS	i.n. PBS	i.n. PBS	i.n. PBS	24 h post-final challenge

All three mice groups were sensitized with proteolytically active Der p 1 through both the intraperitoneal (i.p.) and the subcutaneous (s.c.) routes and then boosted and subsequently challenged intranasally (i.n.) with either proteolytically active or inactive (E-64 blocked) Der p 1 or PBS.

Birmingham, UK) or alkaline phosphatase conjugated (for IgG, Sigma) isotype-specific antibodies. Alkaline phosphatase conjugated extravidine (Sigma) was used in conjunction with biotinylated antibodies.

Statistical analysis

The unpaired Student's *t*-test was used to compare levels of cellular infiltration and antibody responses between the different immunization groups; *P*-values less than 0.05 were considered significant.

Results

We have purified Der p 1 from mite fecal pellets using a multi-step procedure and confirmed its identity/purity by N-terminal sequencing, SDS-PAGE analysis [11] and by demonstrating that enzymatic activity is completely dependent on pre-activation with cysteine and is totally inhibited by the class-specific inhibitor E-64 (Fig. 1).

Groups of mice were initially sensitized with proteolytically active Der p 1 and subsequently exposed intranasally to either

proteolytically active Der p 1, inactive Der p 1 or PBS (Table 1). The extent of cellular infiltration of the lungs and systemic IgE production in the three animal groups were then compared. The lungs of mice that have been exposed to proteolytically active Der p 1 showed increased cellular infiltration compared to those that have been exposed to proteolytically inactive Der p 1 ($P < 0.03$) or to PBS ($P < 0.001$). Much of this increase in the influx of cells into the lungs, under the influence of the proteolytic activity of Der p 1, was due to macrophages ($P < 0.01$) and, to a lesser extent, neutrophils, eosinophils and lymphocytes (Fig. 2).

Mice that have been intranasally exposed to proteolytically active Der p 1 produced significantly higher levels of circulating total IgE compared to those that have been exposed to proteolytically inactive Der p 1 ($P < 0.05$) or to PBS ($P < 0.005$). This increase in total IgE levels was accompanied by a similar significant increase in Der p 1-specific IgE in mice exposed to proteolytically active Der p 1 compared to those exposed to proteolytically inactive Der p 1 ($P < 0.02$) or to PBS ($P < 0.001$) (Fig. 3). No such differences were observed for Der p 1-specific IgG and IgG1 between mice exposed to proteolytically active or inactive Der p 1 (Fig. 4).

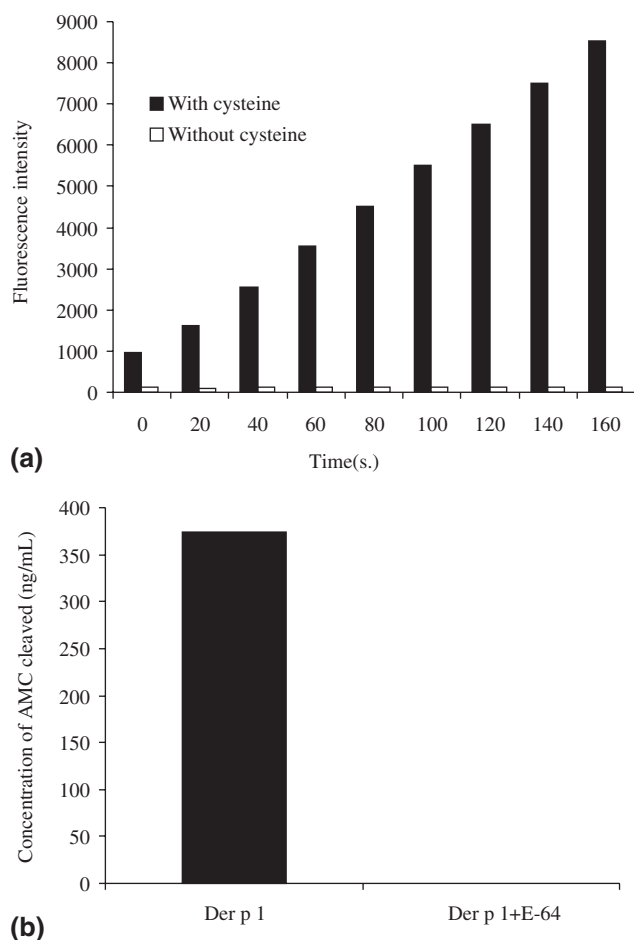


Fig. 1. (a) Catalytic activity of Der p 1, measured in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC, with or without pre-activation with cysteine. (b) The addition of E-64 results in 100% inhibition of the enzymatic activity of Der p 1. Data presented in (a) and (b) are the means of duplicate experiments; the standard error was less than 5%.

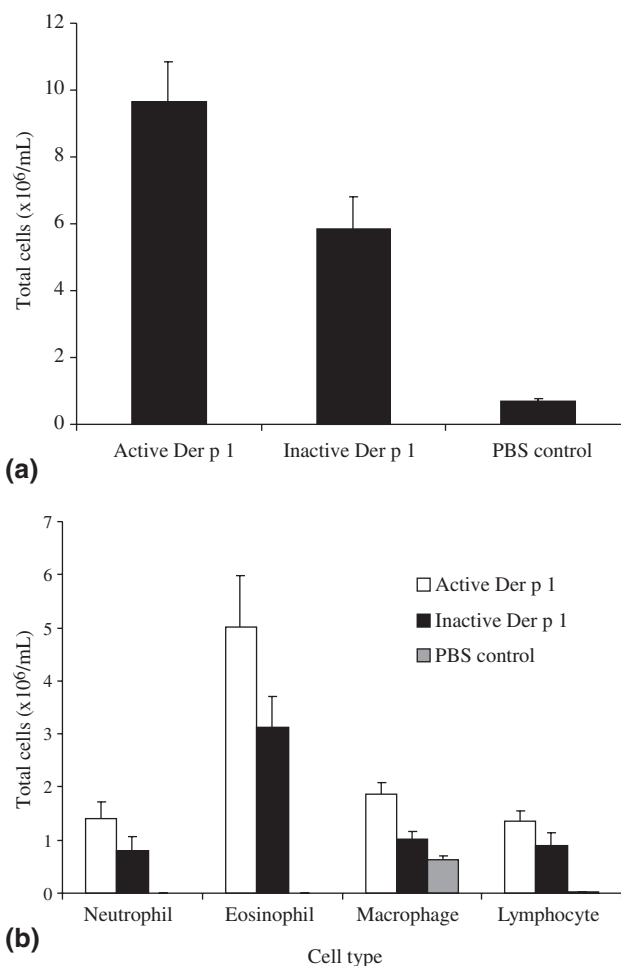


Fig. 2. Cell counts in BAL fluid of groups of mice challenged intranasally with either proteolytically active Der p 1, inactive Der p 1 or PBS: (a) total cell count; (b) neutrophil, eosinophil, macrophage and lymphocyte counts.

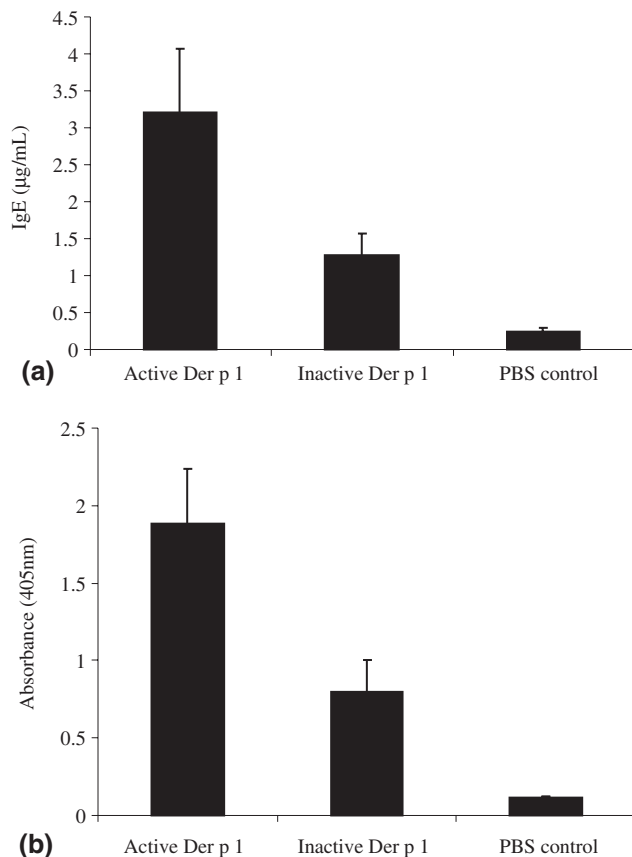


Fig. 3. (a) Total IgE and (b) Der p 1-specific IgE levels in blood samples (diluted 1:10) obtained from groups of mice challenged intranasally with either proteolytically active Der p 1, inactive Der p 1 or PBS. For Der p 1-specific IgE, an absorbance value of 1.0 is approximately equivalent to 0.22 μg/mL. Error bars represent 95% confidence intervals.

Discussion

We have recently suggested that non-immunological properties, such as enzymatic activity, of some allergens might contribute to their allergenicity [2]. This is best exemplified by the major HDM *D. pteronyssinus* allergen Der p 1, which we have shown, in addition to acting as immunogen, has the intrinsic ability to subvert the regulatory process controlling human IgE synthesis, thereby favouring an allergic outcome [3–10]. These *in vitro* findings are in line with our *in vivo* work which showed that immunization of mice with proteolytically active Der p 1 results in a significant and selective enhancement in total and specific IgE antibody responses against itself [11] and other potential bystander allergens [12].

Because of their small size (10–20 μm), mite fecal pellets become easily airborne and inhaled, thereby delivering Der p 1 to the respiratory mucosa, the natural route of exposure of this allergen in allergic asthma [15]. Thus, within the context of asthma, any pro-allergic effects that the proteolytic activity of Der p 1 might have should ideally be demonstrated in experimental animals by administration through the inhalation route. Here, we demonstrate that Balb/c mice that have been intranasally exposed to proteolytically active Der p 1 show significantly higher levels of inflammatory cellular infiltration of the lungs and circulating total and Der p 1-

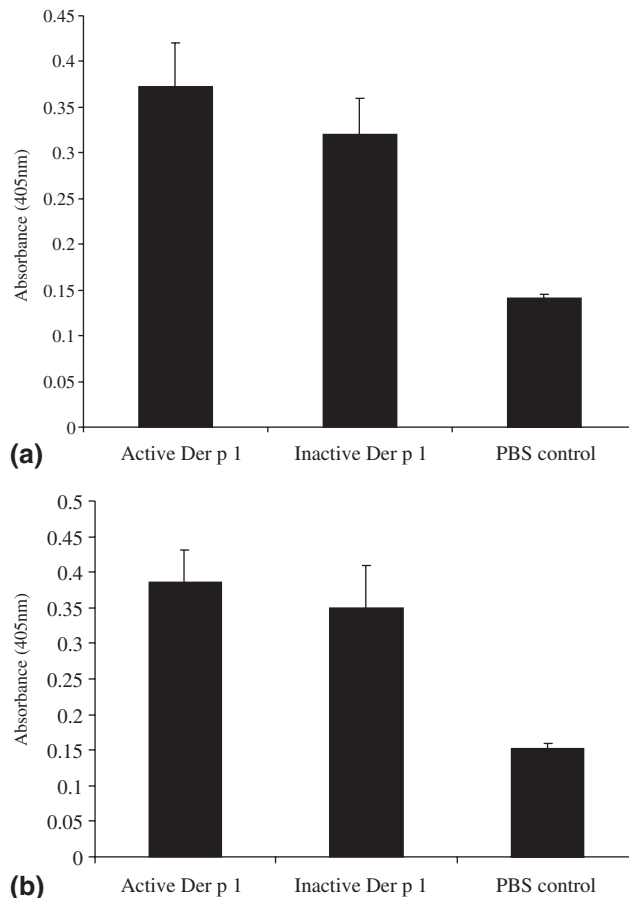


Fig. 4. Der p 1-specific (a) IgG (diluted 1:20 000) and (b) IgG1 (diluted 1:30 000) levels in blood samples obtained from groups of mice challenged intranasally with either proteolytically active Der p 1, inactive Der p 1 or PBS. Der p 1-specific IgG2b was not detectable. For Der p 1-specific IgG and IgG1, an absorbance value of 1.0 is approximately equivalent to 0.06 μg/mL. Error bars represent 95% confidence intervals.

specific IgE compared to those that have been exposed to proteolytically inactive Der p 1. This was an IgE-specific effect, because the measurement of Der p 1-specific IgG and IgG1 showed no such enhancement.

The above observation is in line with our previous *in vivo* mice experiments, whereby Der p 1 was administered through the intraperitoneal route [11, 12]. We are not sure why the IgG1 response did not follow that of IgE, since those two isotypes are considered to be co-regulated in mouse. However, the IgE-restricted enhancement seen in response to intranasally administered proteolytically active Der p 1 does suggest a mechanism that is unique to IgE isotype switching/synthesis. Furthermore, we have previously demonstrated that this IgE-specific effect is not due to E-64 exerting a suppressive influence on IgE production by a mechanism that is independent of its binding to the Der p 1 enzyme active site [11]. For instance, we have shown that the IgE antibody response to ovalbumin, a proteolytically inactive antigen, is not suppressed when mice are immunized with ovalbumin plus E-64.

The fact that the PBS control mice, which incidentally were also sensitized with proteolytically active Der p 1 through the intraperitoneal and the subcutaneous routes, did not show

any measurable cellular infiltration of the lungs, or indeed any increase in circulating IgE levels, does demonstrate that these effects were driven by intranasally administered Der p 1. Furthermore, the observation that proteolytically inactive Der p 1 also elicits cellular infiltration of the lungs and systemic IgE antibody production, suggests that other additional features (i.e. other than proteolytic activity) must contribute to allergenicity. Clearly, any extrapolation of these effects to the human situation would partly depend on the expected concentration of Der p 1 in the target organ, namely the lungs. Der p 1 accounts for about 20% of the total protein content of mite fecal pellets (i.e. 0.1 ng/pellet) [1], and it is possible that chronic exposure would result in the cumulative build-up of high concentrations in the lungs.

Der p 1 has been shown to detach cultured epithelial cell monolayers from both artificial and natural biopolymer substrata and cause visible disruption of epithelial architecture, thereby increasing the permeability of the bronchial mucosa to macromolecules, such as serum albumin [16, 17]. Furthermore, we [6] and others [18] have shown that Der p 1 completely cleaves human α 1-anti-trypsin, an anti-protease whose function is to protect the respiratory mucosa against serine proteases. Therefore, cleavage of α 1-anti-trypsin by Der p 1 might potentially upset the local protease-anti-protease balance and enhance the activity of endogenous serine proteases, especially those from neutrophils (elastase, cathepsin G), thereby leading to further tissue damage.

These observations provide a plausible mechanism whereby Der p 1 would facilitate its own passage (along with other allergens) across the epithelium and gains access to cells of the immune system. The systemic stimulation of IgE production that follows, as shown here, would result in the sensitization of lung mast cells with allergen-specific IgE. Further exposure to Der p 1 would result in the cross-linking of mast cell receptor-bound IgE leading to cellular degranulation and the active release of mediators, which in this study is manifested by the observed significant inflammatory cellular infiltration. In this context, Der p 1 has recently been shown to also directly induce the release of pro-inflammatory cytokines from the respiratory epithelium and this is mediated by the activation of protease-activated receptor (PAR)-2 [19].

Therefore, the present data further illustrate the important role of the proteolytic activity of Der p 1 in driving the allergic response in the target organ, namely the lungs.

Acknowledgements

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