

The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen

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Summary

Background We have recently demonstrated that immunization of mice with proteolytically active Der p 1, the major dust mite allergen, results in a significant enhancement in total and Der p 1-specific IgE synthesis compared to mice immunized with Der p 1 that has been irreversibly blocked with the cysteine protease inhibitors E-64 and iodoacetamide. Thus, the demonstration that the proteolytic activity of Der p 1 enhances total IgE production, apart from increasing Der p 1-specific IgE, suggests that this allergen may have an IgE-specific adjuvant effect.

Objective To determine if the proteolytic activity of Der p 1 has an IgE-specific adjuvant effect.

Methods We have examined this concept in experiments whereby ovalbumin, used as a bystander antigen, was injected alone or coinjected with either proteolytically active or inactive Der p 1 into groups of mice and IgE and IgG antibody responses were measured.

Results Here we demonstrate for the first time that the proteolytic activity of Der p 1, when given at 10-fold higher concentration, enhances the IgE antibody response to ovalbumin.

Conclusions These findings show that the proteolytic activity of Der p 1 leads to the augmentation of IgE antibody responses to itself and to other allergens present in the microenvironment.

Keywords: allergen, cysteine protease, Der p 1, IgE, mite

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Introduction

The dust mite *Dermatophagoides pteronyssinus* allergen (Der p 1) is considered to be the most immunodominant allergen involved in the expression of IgE-mediated dust mite hypersensitivity [1]. We have recently suggested that the potent IgE-eliciting property of Der p 1 might be due to its cysteine protease activity [2]. For instance, Der p 1 cleaves human CD25, the 55 kDa α subunit of the T cell IL-2 receptor (the high affinity form of which consists of α , β and γ subunits), and as a result these T cells show markedly

diminished proliferation and IFN- γ secretion in response to stimulation by anti-CD3 antibody [3]. Given that IL-2, along with IFN- γ , and IL-4 are considered to be autocrine growth factors for Th1 and Th2 subsets, respectively [4], Der p 1-induced cleavage of CD25 is likely to lead to impaired growth of cells of the Th1 subset and consequent augmentation of those of the Th2 subset. This notion is further underlined by the demonstration that the administration of Der p 1 to mice promotes a Th2 response by modulating the balance between IL-4 and IFN- γ [5].

In keeping with these observations, we have recently demonstrated that Der p 1 cleaves mouse CD25 and that immunization of mice with proteolytically active Der p 1 results in a significant enhancement in total and Der p 1-specific IgE synthesis compared to mice immunized with

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Der p 1 that has been irreversibly blocked with the cysteine protease inhibitors E-64 and iodoacetamide [6]. The demonstration that the proteolytic activity of Der p 1 enhances total IgE production, apart from increasing Der p 1-specific IgE, suggests that this allergen may play a key role in destabilizing the microenvironment within target tissues to one that is pro-allergic, and thus aid in the initiation of IgE antibody responses to other allergens. In other words, the proteolytic activity of Der p 1 may exert an IgE-specific adjuvant effect. We have now examined this concept in a new set of experiments whereby ovalbumin, used as a bystander antigen, was injected alone or coinjected with either proteolytically active or inactive Der p 1 into groups of mice, and IgE and IgG antibody responses were measured. Here we demonstrate that the proteolytic activity of Der p 1 enhances the IgE antibody response to ovalbumin.

Materials and methods

Der p 1 preparation

Der p 1 was isolated from house dust mite fecal pellets (Allergon, Angelholm, Sweden) by our multi-step procedure, 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 fast protein liquid chromatography (FPLC) to remove low molecular weight contaminants [6]. The purity of the preparation was previously 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 preactivation with cysteine and is totally inhibited by E-64 (see below for further details) [6]. 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 preactivated with 5 mM cysteine (Sigma) to regenerate its thiol group which becomes oxidized during purification. 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 [7]. To block the proteolytic activity of cysteine-activated Der p 1, 1000-fold molar excess of E-64 [*L-trans*-epoxysuccinyl-leucylamido (4-guanidino) butane] (Sigma) was used.

Immunization protocol

Groups of 10 CBA/J mice were given 6 weekly intraperitoneal injections of either 1 µg or 10 µg of ovalbumin

(Sigma) alone or with either 10 µg of proteolytically active or inactive (E-64-blocked) Der p 1. All immunizations were given in 200 µg of Al(OH)₃, as adjuvant, per protein. A total bleed was obtained by cardiac puncture one week after the last injection. The proteolytic activity of Der p 1 and its inhibition with E-64 in the immunization mixture were ascertained as described above.

Antibody detection

Serum samples were initially titrated to determine the optimal dilution for testing each antibody isotype and subclass. The optimal dilutions used here were 1/10 for detecting total and ovalbumin-specific IgE and 1/20 000, 1/40 000 and 1/250 for detecting ovalbumin-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. Ovalbumin-specific IgE (measured using samples that have been depleted of IgG on a Protein G column, Pharmacia, St Albans, UK), IgG, IgG1 and IgG2b were detected on microtitre plates coated with a 4 µg/mL solution of ovalbumin and developed with biotinylated (for IgE clone R35-118 and for IgG1 clone A85-1, Pharmingen; for IgG2b clone AB275, The Binding Site, Birmingham, UK) or alkaline phosphatase conjugated (for IgG, Sigma) isotype-specific antibodies. Alkaline phosphatase conjugated Extravidine (Sigma) was used in conjunction with biotinylated antibodies. The unpaired Student *t*-test was used to compare levels of antibody responses between the different immunization groups; *P*-values of less than 0.05 were considered significant.

Results

We have previously confirmed the purity of our Der p 1 preparation by N-terminal sequencing, SDS-PAGE analysis and by demonstrating that its proteolytic activity is completely dependent on preactivation with cysteine and is inhibited by the class-specific inhibitors E-64 and iodoacetamide [6]. Here we have examined the potential adjuvant effect of the proteolytic activity of Der p 1 on antibody responses to ovalbumin, as a bystander antigen. Ovalbumin was injected alone or coinjected with either proteolytically active or inactive Der p 1 over a six-week period into groups of 10 CBA/J mice and IgE, IgG, IgG1 and IgG2b antibody responses to ovalbumin were measured.

Our results show that mice immunized with 1 µg of ovalbumin plus 10 µg of proteolytically active Der p 1 produce significantly more ovalbumin-specific IgE compared to mice immunized with 1 µg of ovalbumin alone

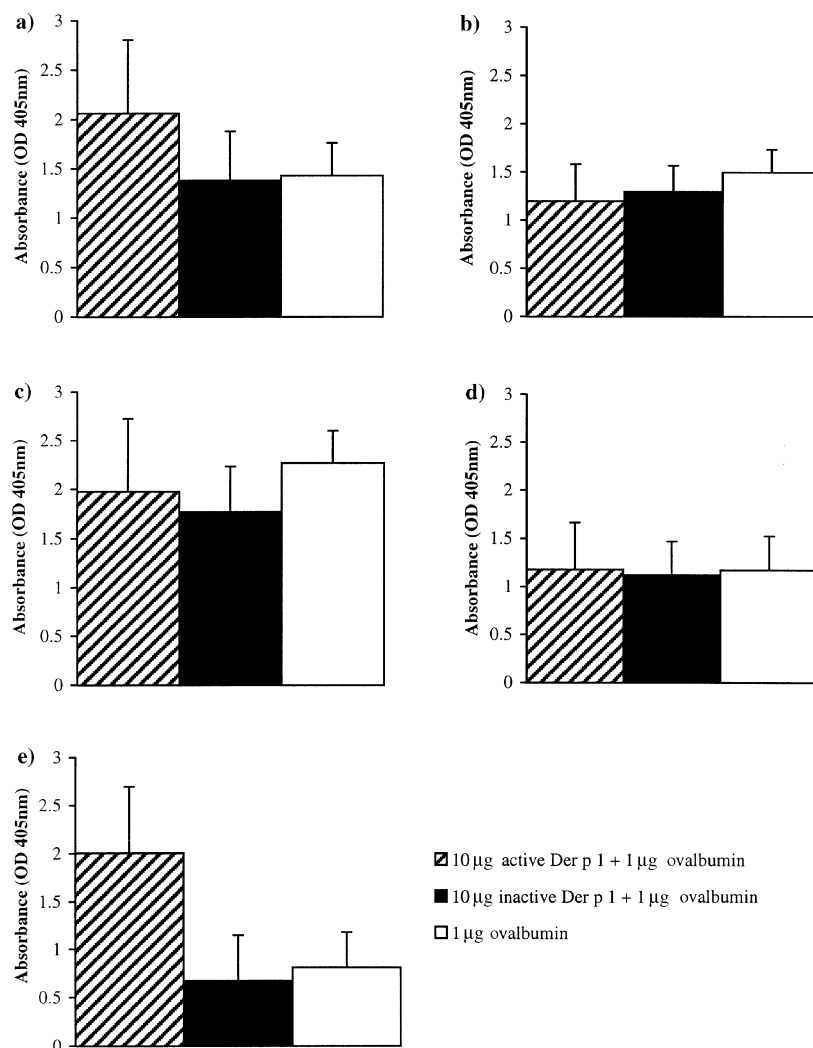


Fig. 1. Immunization of mice with 1 µg of ovalbumin plus 10 µg of proteolytically active Der p 1 produces significantly more ovalbumin-specific IgE compared to mice immunized with 1 µg of ovalbumin alone ($P = 0.02$) or with 1 µg of ovalbumin plus 10 µg of proteolytically inactive (E-64 blocked) Der p 1 ($P = 0.03$) (a). This is an IgE-specific effect, since the measurement of ovalbumin-specific IgG (b), IgG1 (c) and IgG2b (d) showed no such enhancement. The total IgE antibody response follows a similar pattern to that of ovalbumin-specific IgE ($P < 0.01$) (e). There were 10 mice in each group and all animals completed the experiment. Error bars represent 95% confidence intervals.

($P = 0.02$) or with 1 µg of ovalbumin plus 10 µg proteolytically inactive (E-64 blocked) Der p 1 ($P = 0.03$). This is an IgE-specific effect, since the measurement of ovalbumin-specific IgG, IgG1 and IgG2b showed no such enhancement. There was also a significant enhancement in the production of total IgE in mice immunized with 1 µg of ovalbumin plus 10 µg of proteolytically active Der p 1 compared to mice immunized with 1 µg of ovalbumin alone ($P < 0.01$) or with 1 µg of ovalbumin plus 10 µg of proteolytically inactive (E-64 blocked) Der p 1 ($P < 0.01$) (Fig. 1). The latter observation confirms our earlier findings showing that immunization of mice with proteolytically active Der p 1 results in a significant enhancement in total IgE synthesis compared to mice immunized with Der p 1 that has been irreversibly blocked with the cysteine protease inhibitors E-64 and iodoacetamide [6].

We then sought to ascertain whether this effect could

also be demonstrated when mice were immunized with equal amounts of ovalbumin and Der p 1 (i.e. 10 µg of each protein). The results show, rather unexpectedly, that immunization of mice with ovalbumin plus proteolytically inactive Der p 1 suppresses the IgE antibody response to ovalbumin compared to immunization with ovalbumin alone ($P < 0.05$). This is most probably due to intermolecular antigenic competition [8]. Interestingly, a proteolytically active Der p 1 overcomes this suppressive effect. Again, this effect is IgE-specific, since it is not observed in the IgG, IgG1 and IgG2b antibody responses to ovalbumin. The total IgE antibody response followed a similar pattern to that of ovalbumin-specific IgE (Fig. 2).

Discussion

The dust mite *Dermatophagoides pteronyssinus* is a major cause of allergic disease in the western world. Der p 1, one

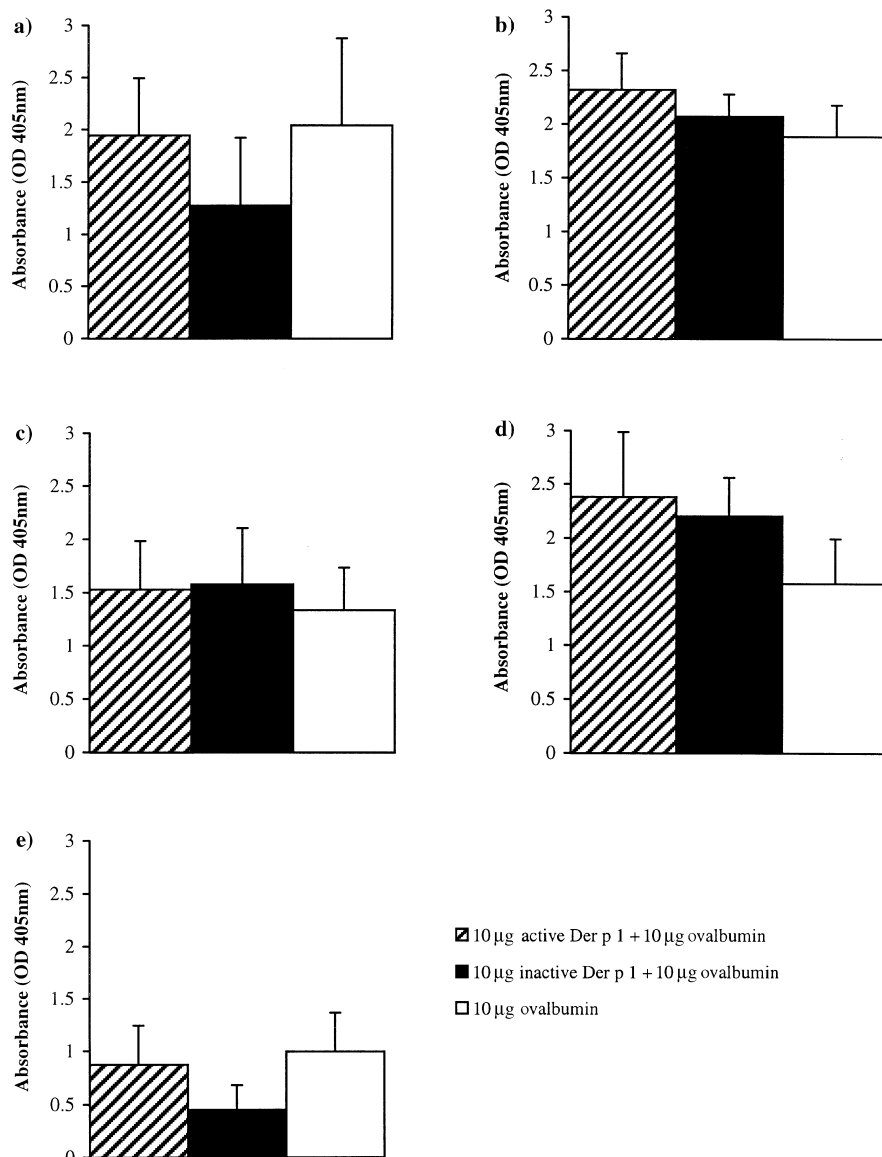


Fig. 2. Immunization of mice with 10 µg of ovalbumin plus 10 µg of proteolytically inactive Der p 1 suppresses the IgE antibody response to ovalbumin compared to immunization with ovalbumin alone ($P < 0.05$), but a proteolytically active Der p 1 overcomes this effect (a); no such effect is seen on IgG (b), IgG1 (c) and IgG2b (d) antibody responses. The total IgE antibody response follows a similar pattern to that of ovalbumin-specific IgE (e). There were 10 mice in each group and all animals completed the experiment. Error bars represent 95% confidence intervals.

of several 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.

Here we demonstrate for the first time that immunization of mice with ovalbumin, as a bystander antigen, plus a 10-fold higher amount of proteolytically active Der p 1 results in a selective and significant enhancement in ovalbumin-specific IgE antibody response. This effect was not due to E-64 (used for inactivating the cysteine protease activity of Der p 1) exerting a direct suppressive influence on IgE production, since we have previously demonstrated that immunization of mice with ovalbumin plus E-64 does not affect their IgE antibody response to this antigen [6]. Our

data suggest that the proteolytic activity of Der p 1 has an IgE-specific adjuvant effect. We are not sure why the IgG1 antibody response did not follow that of IgE, since those two isotypes are considered to be coregulated in mouse. However, the IgE-restricted enhancement seen in response to immunization with ovalbumin, coinjected with proteolytically active Der p 1, does suggest a mechanism that is unique to IgE isotype switching/synthesis.

Thus, these results indicate that the cysteine protease activity of Der p 1 not only enhances IgE antibody responses to itself [6], but also to other allergens present in the microenvironment. Clearly, any extrapolation of these effects to the clinical situation would depend on the expected concentration of Der p 1 in the target organ, namely the lungs. Der p 1 accounts for 15–20% of the total

protein content of mite fecal pellets, and there is no doubt that it reaches cells of the immune system in sufficient concentrations (nanogram range) to elicit IgE antibody responses. In this context, we have shown that Der p 1, at a concentration as low as 10 ng/mL, cleaves the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [7], thereby suggesting that this concentration is sufficient for cleaving *in vivo* protein substrates.

The IgE-specific enhancing effect of Der p 1 is most likely to be due to its ability to cleave CD25, the IL-2 receptor on T cells [3,6], which would be expected to influence T cell responses in an oligoclonal, antigen non-specific fashion. As a result, these T cells show markedly diminished proliferation and IFN- γ secretion in response to antigenic stimulation [3]. Given that IL-2, along with IFN- γ , and IL-4 are considered to be autocrine growth factors for Th1 and Th2 subsets, respectively [4], Der p 1-induced cleavage of CD25 is likely to lead to impaired growth of cells of the Th1 subset and consequent augmentation of those of the Th2 subset. This is consistent with the recently reported ability of Der p 1 to induce a Th2 response in mice by modulating the balance between IL-4 and IFN- γ [5].

Collectively, these findings show that the proteolytic activity of Der p 1 leads to augmentation of IgE antibody responses to itself and to other potential allergens present in the microenvironment. In other words, Der p 1 may play a central role in destabilizing the microenvironment within target tissues to one that is proallergic, and thus aids in the initiation and propagation of the allergic cascade [2].

Acknowledgement

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