

Altered antigenicity of M-177, a 177-kDa allergen from the house dust mite *Dermatophagoides farinae*, in stored extract

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Summary

Background A high molecular weight allergen, M-177 (177 kDa) was isolated from *Dermatophagoides farinae* using a specific antibody raised to an allergenic clone Mag 3, which was obtained by immunoscreening a mite cDNA library. The potent IgE reactivity of M-177 is comparable with that of Der f 2.

Objective The aim of this study was to analyse the molecular characteristics and the allergenic activity of M-177 in stored mite extracts.

Methods Antigens were analysed by immunoblotting and enzyme-linked immunosorbent assay (ELISA; inhibition). Allergenic activity was estimated from IgE reactivity and the results of a histamine release assay.

Results The intact M-177 molecule was present in high concentrations in fresh extract obtained from purified mite bodies, but was only detected in small amounts in stored extracts. Instead of the intact molecule, anti-Mag 3 antibody detected various cross-reactive antigens in the stored preparations. Studies of a stored liquid extract showed that these cross-reactive antigens were produced by the degradation of M-177, and that this change was suppressed by the addition of protease inhibitors. Interestingly, the allergenic activity of the fragmented M-177 (sM-177) isolated from the stored extracts was greater than that of the intact antigen. Specific IgE reacted with sM-177 in 84.2% of 38 sera samples from patients allergic to mites, while 65.8% were positive for M-177-specific IgE. Similarly, the histamine release test showed that sM-177 had greater allergenic activity *in vitro*. ELISA inhibition indicated that the increased allergenic activity resulted from alteration of the antigenicity with the degradation of M-177.

Conclusions M-177 is a protease-sensitive allergen. The breakdown products of M-177 provoked higher allergenic activity than the intact allergen.

Keywords: M-177, Mag 3, recombinant, *Dermatophagoides farinae*, ELISA, IgE, histamine, storage, cross-reactivity, protease, protease inhibitor, high molecular weight allergens

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Introduction

House dust mites (*Dermatophagoides* sp.) are the major causative agents of allergic diseases such as bronchial

asthma, perennial rhinitis and atopic dermatitis [1]. Immunoblotting analysis using patients' IgE has detected a large number of antigenic components with a wide range of molecular masses in two house dust mite species, *D. farinae* and *D. pteronyssinus*.

The number of reported antigens in the respective species ranges from four to 26 and from five to 19 in mite bodies and faeces [2,3]. It has been proposed that the observed

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heterogeneity of crude mite antigens is derived from differences between the mite species, culture conditions, extraction procedures, storage conditions and so on. However, the molecular mechanism producing the heterogeneity remains unknown [4–6].

Recently, the authors made a discovery that has helped to illuminate how this heterogeneity arises from a high-molecular weight allergen. A mite allergen clone from *D. farinae*, mag 3, was obtained by immunoscreening a cDNA library with rabbit antisera raised to crude mite antigens and patients' IgE. The natural Mag 3, a 177-kDa molecule, was then isolated by using a purified polyclonal antibody in recombinant Mag 3-immobilized columns. The natural allergen (called M-177 in this paper) reacted with patients' IgE in $\approx 70\%$ of 23 sera samples (comparable with the reactivity of Der f 2) and was localized in the peripheral tissues of the mite's internal organs by immunohistochemical staining [7]. In subsequent experiments, it was discovered that the immunostaining patterns of mite extracts using anti-Mag 3 antibody changed markedly according to the length of time that the extracts had been stored. Therefore, the authors focused on this observation as a way of explaining the heterogeneity of crude mite antigens.

In this paper, the authors show that immunoblotting stored extracts and commercial products with anti-Mag 3 antibody detected a large variety of cross-reactive antigens, instead of small amounts of the 177-kDa molecule, and that M-177 was degraded into these varied fragments during storage of the extract. Then, the authors focused on the allergenic and antigenic activity of fragmented M-177 in stored extracts. The fragmented antigens were isolated by using an anti-Mag 3 antibody-immobilized column and the intact M-177 molecule was examined by using patients' IgE. Interestingly, enzyme-linked immunosorbent assay (ELISA) showed that the allergenic activity of the fragmented antigens was higher than that of the intact allergen. ELISA inhibition suggested that specific IgE binding to the antigenic structures produced by the degradation of M-177 occurred in the sera of patients allergic to mites.

Materials and methods

Mites and mite extracts

Cultures of the house dust mite, *Dermatophagoides farinae*, were supplied by Fumakilla Ltd (Hiroshima, Japan). Mite bodies (Df-I) were separated from the culture media by centrifugation with NaCl-saturated solution [8]. Purified mite bodies (Df-II) were prepared from Df-I using an improved procedure (submitted for publication). Briefly, Df-I was further purified by centrifugation with 30% EtOH at 6000 *g* for 15 min. The precipitate was then centrifuged with NaCl-saturated solution at 6000 *g* for

15 min and the floating particles containing mite bodies were collected on a filter. Df-I and Df-II were ground with fine quartz powder and then filtered with 0.22 μm membranes (Millipore Corporation, Bedford, MA, USA). Two mite extracts, Dfb and Dfb-II, were extracted from the respective preparations with 20 mmol/L PBS, pH 7.3 in the presence or absence of protease inhibitors. The protease inhibitors were 0.1 mmol/L phenylmethylsulphonylfluoride (PMSF), 5 mmol/L di-sodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA), 1 mmol/L monoiodoacetic acid, and 5 mmol/L 1,2-epoxy-3-(*p*-nitro-phenoxy) propane (EPNP). Dfb-I is equivalent to Dfb described above and sDfb-I is freeze-dried, stored Dfb-I [8]. Commercial *D. farinae* extract was purchased from Torii & Co. Ltd, Tokyo, Japan.

Antigen and antibodies

Rabbit polyclonal antibody to recombinant Mag 3 and anti-Mag 3 antibody-immobilized columns were used as described previously [7]. M-177 and sM-177 were isolated from Dfb-II and sDfb-I, respectively, by using the anti-Mag 3-immobilized column, according to previously described methods. Der f 1, Der f 2 and the specific rabbit sera for these antigens were kindly provided by Dr H. Yasueda of the National Sagami Hospital, Kanagawa, Japan. Rabbit antiserum to Dfb-I was the same as rabbit antiserum to Dfb. M-177 degI and M-177 degII were M-177 digested with Der f 1 and papain-immobilized agarose (Sigma, St Louis, MO, USA), respectively. Der f 1 (1 μg) was added to 1 mL of 200 $\mu\text{g}/\text{mL}$ M-177 in 20 mmol/L PBS, pH 7.3, containing 1 mmol/L 2-mercaptoethanol and then incubated overnight at 37°C. Subsequently, the Der f 1 was removed by incubating the mixture twice with 500 μL of anti-Der f 1 antibody-immobilized beads for 30 min at room temperature. The level of Der f 1 remaining was estimated to be $<2 \text{ ng}/\text{mL}$ by ELISA (data not shown). Anti-Der f 1 beads were prepared with rabbit antisera to Der f 1, using the ImmunoPure[®] IgG Orientation Kit (Pierce, Bockford, IL, USA) according to the manufacturer's instructions. Before use, papain beads were washed with 0.1 mol/L glycine-HCl, pH 2.3, containing 0.5 mol/L NaCl, then with 10 mmol/L PBS, pH 7.3, containing 0.05% Triton X-100, and finally with 10 mmol/L PBS, pH 7.3. Then 2.5 units of papain beads were added to 1 mL of 200 $\mu\text{g}/\text{mL}$ M-177 in 20 mmol/L PBS, pH 7.3, and then incubated for 3 h at 30°C, after which the beads were removed by centrifugation at 9000 *g* for 5 min.

Human blood

Mite-allergic sera were collected from 38 newly diagnosed asthmatic patients who had a positive skin test to mites (erythema $>20 \text{ mm}$, Torii & Co., Ltd). Control sera were

collected from four healthy volunteers (erythema < 9 mm). For the histamine release test, peripheral blood cells were prepared from six new patients and two healthy donors, whose profiles were determined in the same way.

Histamine release test

Histamine release test has been described previously [9]. Briefly, peripheral blood cells were collected from heparinized peripheral blood by centrifugation at 1500 g for 10 min. The cells were suspended in Hanks' buffer and incubated with a series of diluted antigen at 37°C for 30 min. The histamine released in the supernatant was analysed by HPLC, after deproteinization with perchloric acid [10]. Total and control histamine release were determined for perchloric acid lysis of cell aliquots and cells incubated in Hanks' buffer, respectively.

Results were calculated as follows: proportion histamine release (%) = (sample–control)/(total–control) × 100.

SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed under reducing conditions according to the method of Laemmli [11], and immunoblotting of the antigens was performed on a PVDF membrane, immobilon-P (Millipore). The membrane was blocked with 2% skim milk and 0.05% Tween 20 and reacted with rabbit anti-Mag 3 antibody (1 µg/mL), rabbit anti-Der f 1 antisera (× 10 000), or anti-Dfb-I antisera (× 3000) for 1 h. Reactivity was detected by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (× 3000, Cappel, West Chester, PA, USA) for 1 h, followed by staining with 3,3-diaminobenzidine tetrahydrochloride (DAB) and H₂O₂ within 10 min.

ELISA and ELISA inhibition

IgE reactivity of M-177 and sM-177 were analysed by ELISA. The experiment was performed at 37°C without specific attention. Each antigen was coated onto 96-well microtiter plates (Falcon 3912, Becton Dickinson & Co., Oxnard, CA, USA; M-177 [0.1 µg], or sM-177 [0.1 µg]) and blocked with 3% dextran (Amersham Pharmacia biotech, Uppsala, Sweden) and 0.05% Tween 20. The plates were incubated overnight at 4°C with human sera (× 50), and detection was performed with biotin-conjugated goat anti-human IgE antibody (× 1000, for 3–5 h; BioSource, Camarillo, CA, USA), alkaline phosphatase-conjugated streptavidine (× 1000, 2 h; BioSource) and substrate, AttoPhosTM (within 24 h; Boehringer Mannheim, Mannheim, Germany). Fluorescence intensity was estimated using a CytoFluorIITM (PerSeptive Biosystems, Framingham, MA, USA). The amount of specific IgE was estimated in 'units'

from a standard IgE curve on the same plate described previously [7]. One unit is equivalent to 1 ng/mL of standard IgE (Yamasa Shoyu Co. Ltd, Chiba, Japan).

The antigenic potency of Dfb-II, and a comparison of human IgE epitopes with M-177 or sM-177 were analysed by ELISA inhibition. ELISA plates were coated with M-177 (0.1 µg) and sM-177 (0.1 µg). Rabbit anti-Mag 3 antibody (1 µg/mL) or rabbit anti-Der f 1 antisera (× 10 000) was pre-incubated for 30 min with 100 µg/mL of Dfb-II series and detection was performed with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (× 3000 for 1 h; Cappel) and AttoPhosTM (within 15 min) in the same manner. For human IgE analysis, human sera (× 50) was pre-incubated for 30 min with an antigen dilution series, and then analysed using the same procedures described above.

Results

Electrophoretic behaviour of M-177 in mite extracts

The electrophoretic behaviour of the high molecular weight allergen M-177 (177 kDa) in four mite extract preparations were examined by SDS-PAGE and immunoblotting analysis using anti-Mag 3 antibody (Figs 1a and 2b). The amount of M-177 was very low or absent in the stored extracts (sDfb-I) and in one commercial product (Lot b), moderate in the fresh extract (Dfb-I) of the second commercial product (Lot a), and high in the fresh preparation of purified mites (Dfb-II). In addition, anti-Mag 3 detected cross-reactive antigens smaller than 177 kDa in every extract. These showed very different size patterns in each extract. In contrast, when stained with anti-Der f 1, a common binding pattern to a 28-kDa molecule was observed in all extracts (Fig. 1c). These findings suggest two possibilities: either the M-177 was broken down into various antigenic fragments, or a variety of cross-reactive molecules was present in every extract.

Degradation of M-177 in mite extract

To distinguish between these two possibilities, the mite extract Dfb-II was stored for different lengths of time and then examined for alteration of the M-177 molecule. SDS-PAGE and immunoblotting analysis of a series of incubated extracts using anti-Mag 3 showed that almost all the M-177 disappeared from the liquid extract within 10 days, while increasing numbers of other anti-Mag 3 reactive antigens were observed, including many smaller molecules (Figs 2a,b). These changes were completely suppressed by the addition of protease inhibitors (Fig. 2c). ELISA inhibition analysis demonstrated that the antigenic activity of anti-Mag 3 against the intact M-177 molecule was maintained, even though the molecule itself disappeared (Fig. 2b,

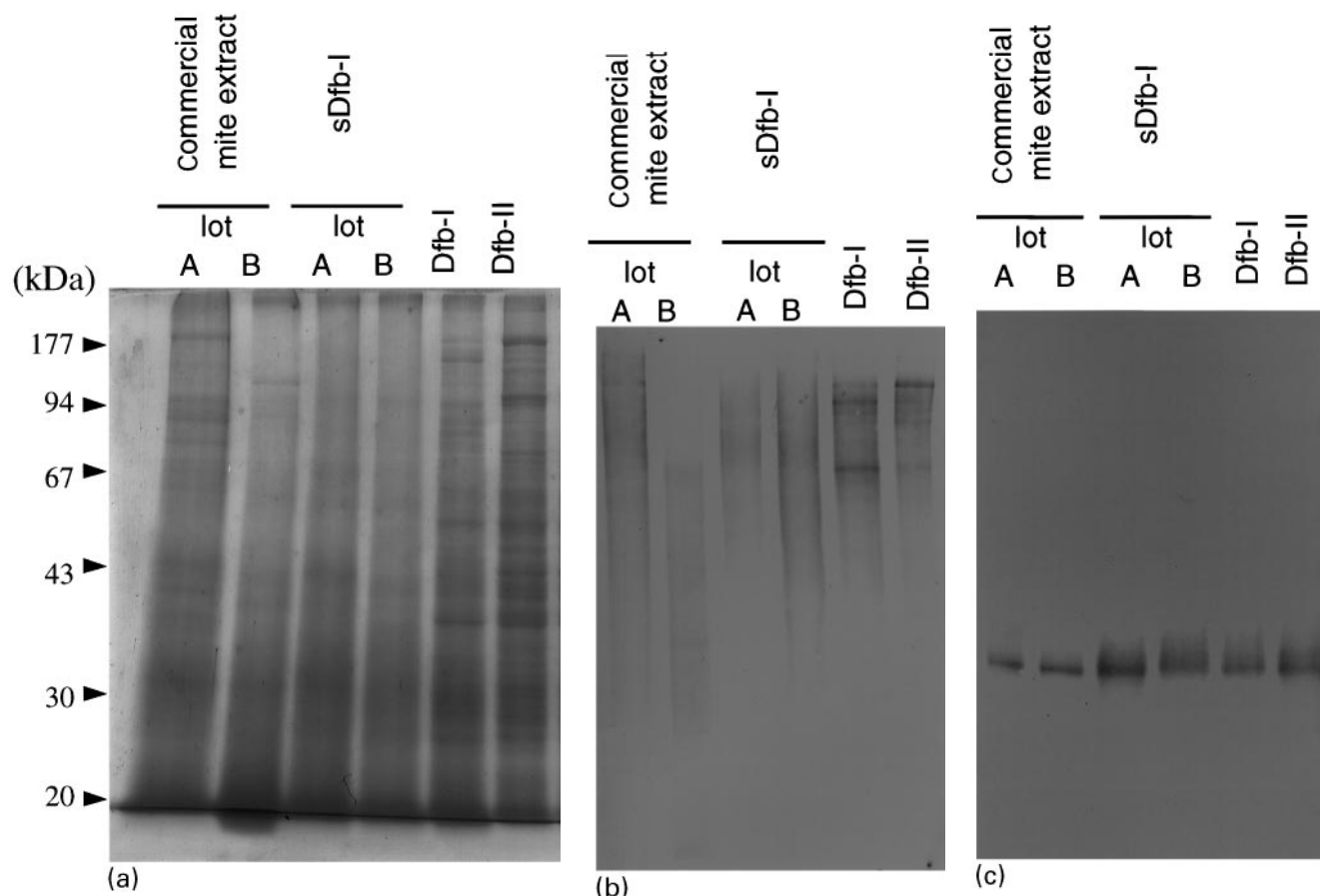


Fig. 1. SDS-PAGE and immunoblotting analysis of mite extracts. Mite extracts (Dfb-II, Dfb-I and two lots of sDfb-I) and two commercial crude mite antigens were separated on 10% SDS-polyacrylamide gels, followed by (a) CBB staining (100 µg), (b) immunoblotting with anti-Mag 3 antibody (20 µg), and (c) anti-Der f 1 antiserum (20 µg). The commercial antigens Lot a and Lot b corresponded with the manufacturer's numbers (DL17L179) and (QSAP), respectively. Both lots of sDfb-I had been stored for 2 years.

upper). Furthermore, the antigenicity of Der f 1 remained constant for 10 days in both analyses (Fig. 2d). Der f 1, which is a cysteine protease, was used to test whether M-177 can be degraded into various fragments by mite proteases. The addition of Der f 1 degraded M-177 into many fragments of various sizes, while the control sample only changed slightly (Fig. 3). These results suggest that the cross-reactive antigens to anti-Mag 3 antibody are not independent molecules. Rather, they are fragments of M-177 produced by the degradation of that molecule by proteases in extracts.

Allergenic activity of fragmented M-177 in stored mite extracts

Next, the allergenic activity of the fragmented M-177 from stored extracts was studied. sDfb-I was incubated with an anti-Mag 3-immobilized column. After washing, the bound antigens were eluted with a low-pH buffer and called sM-177. SDS-PAGE and immunoblotting analysis identified the

major components of sM-177 as intact M-177 (the 177 kDa molecule) and 144 kDa, 125 kDa, and 52 kDa fragments (Fig. 4a). Using rabbit antisera raised to sDfb-I, it was confirmed that every antigenic component in sM-177 was derived from M-177 by immunoblotting analysis. Specific antibodies binding to both sM-177 and M-177 were completely absorbed with mixtures of M-177 and the fragments produced by digestion with papain (M-177 degII) (Fig. 4b,c).

The amount of specific IgE in patients' sera binding to sM-177 and M-177 were estimated using ELISA and ELISA inhibition. There was individual variation in the binding of specific IgE to the two kinds of M-177, and 92.1% (35/38) of the serum samples showed higher IgE binding to sM-177 than to M-177 (Fig. 5a). For the 38 patient sera samples, the amount of specific IgE and the frequency of positive reactions to sM-177 were 736 m units and 84.2% (32/38), respectively, higher than corresponding values for M-177 (411.3 m units and 65.8% (25/38), respectively). ELISA

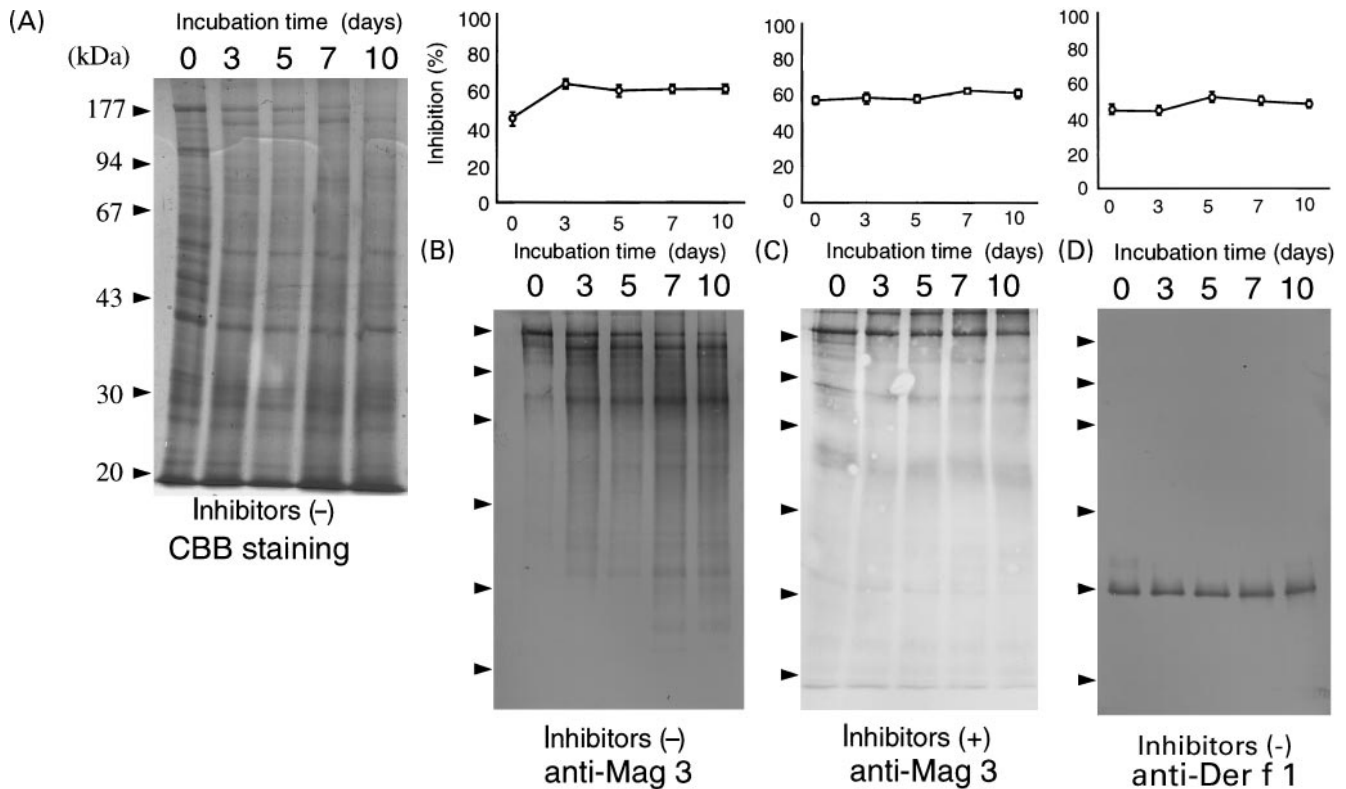


Fig. 2. Electrophoretic behaviour and reactivity of antigens in mite extracts. Dfb-II prepared in the absence (a, b and d) or presence (c) of protease inhibitors was incubated at 37°C for 0, 3, 5, 7 or 10 days. The samples were separated on 10% SDS-polyacrylamide gels, followed by (a) CBB staining (100 µg), or (b),(c) immunoblotting with anti-Mag 3 antibody (20 µg) or (d) anti-Der f 1 antiserum (20 µg). The upper graphs show ELISA inhibition of Dfb-II for anti-Mag 3 antibody binding to M-177 (b), (c) or anti-Der f 1 antiserum (d). Values represent mean $\pm$ SEM of the two experiments.

inhibition analysis using double-positive pooled sera ($n = 10$, in order of reactivity) was used to examine the increased IgE reactivity of sM-177. The inhibition curves showed that both M-177 and sM-177 suppressed IgE binding to M-177 by more than 90% (Fig. 5b), but that M-177 suppressed IgE binding to sM-177 by only 60% (Fig. 5c). These results suggest that the IgE of patients recognizes the distinctive epitope structures present in sM-177 in addition to those of intact M-177. This was further examined using two artificially fragmented M-177 preparations. M-177 digI and M-177 digII were able to suppress the IgE binding to sM-177 to the same degree as sM-177 (Fig. 5b,c). This result suggests that the novel IgE epitopes in sM-177 are not derived from contaminant antigens; rather they result from alteration of the antigenicity of M-177 by degradation. Furthermore, the *in vitro* allergenic activity of sM-177 and M-177 was examined using the histamine release assay in samples from eight individuals. The histamine release profiles of four patients are shown in Fig. 6. The profiles of the other two patients and the two healthy donors are not shown because there was no histamine release. The four

profiles showed different patterns of histamine release: in one, histamine release was only caused by stimulation with sM-177 (Fig. 6a); in two, the release curve with sM-177 was higher than that with M-177 (Fig. 6b,d); and in the fourth, the peak release occurred at a lower concentration of sM-177 than that brought about by M-177 (Fig. 6c). Every pattern implied that sM-177 would more strongly stimulate a patient's basophils than intact M-177.

Discussion

Immunoblotting analysis using the specific antibody to the recombinant Mag 3 showed that the electrophoretic behaviour of specific antigens was different in every preparation. M-177 was present in large amounts in the fresh extract obtained from purified mite bodies, whereas in stored extracts the intact M-177 molecule was replaced by a large number of anti-Mag 3 cross-reactive antigens smaller than 177 kDa (Fig. 1b). To explain this observation, it was shown that in stored extracts M-177 was degraded into varied fragments that keep its antigenicity (Fig. 2a and 2b).

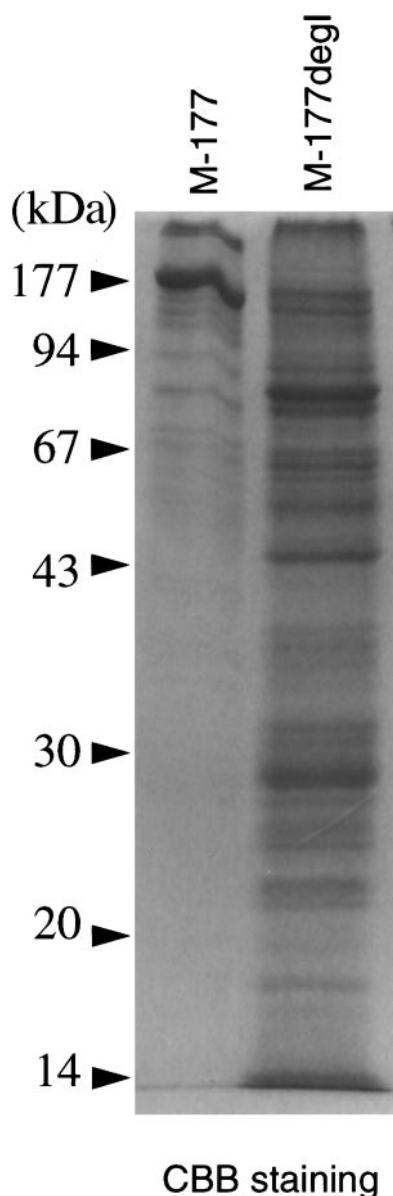


Fig. 3. Degradation of M-177 by incubation overnight at 37°C in the presence (left lane) or absence (right lane) of Der f 1. Samples (20 µg) were separated on 12.5% SDS-polyacrylamide gels, followed by CBB staining.

Furthermore, it was shown that the degradation of M-177 occurred in dead mites in the same manner (data not shown). Under a microscope, mite skeletons are seen to be the major impurity in the mite fraction of Df-I centrifuged with NaCl-saturated solution (data not shown). In the authors' experience the skeletons are recovered with the mite bodies in the floating fraction and cannot be separated. In fact, a number of the cross-reactive antigens were detected in the conventional extract, Dfb-I (Fig. 1b). The authors' confirmed that their improved purification method was effective in

removing the skeletons. Df-II contained only intact mite bodies (data not shown), and almost no cross-reactive antigens were seen in Dfb-II, the extract of this purified preparation. These findings suggest that the length of storage (comparing Dfb-I with sDfb-I) and the number of impurities in the mite fraction (comparing Dfb-I with Dfb-II) are the major source of the cross-reactive antigens. Mite proteases are suggested as the most likely cause of the breakdown of M-177, because the degradation was suppressed by the addition of protease inhibitors and brought about by the addition of Der f 1 (Fig. 2c and Fig. 3). In addition, chymotrypsin, trypsin, human and bovine thrombin are all capable of degrading M-177 into various similar fragments, while Der f 2 cannot (data not shown). In fact, a variety of enzymatic activities have been detected in crude mite antigens [12] and several mite allergens have been identified as enzymes. Der 1, 3, 4, 6, 8 and 9 are cysteine protease, serine protease, amylase, chymotrypsin, glutathione transferase and collagenolytic serine protease, respectively [13–18]. In other ELISA inhibition experiments, the authors also confirmed that M-177 constitutes as much as 0.9 and 0.4% of the total protein in Dfb-II and Dfb-I, respectively (submitted for publication). These findings strongly suggest that this high molecular weight allergen produces the heterogeneity seen in the antigenic composition of crude mite antigens.

The heterogeneity of allergens has been a major problem in the identification of mite allergens. The so-called 'major' allergen molecules are invariably identified in all crude antigen preparations, but a large number of 'minor' antigens are poorly characterized and the number of such antigens has not yet been accurately determined. This heterogeneity is thought to be the result of differences between mite species, culture conditions, extraction procedures, and the length and conditions of storage. However, until now the molecular mechanism producing this heterogeneity remained unresolved [4–6]. This phenomenon can be explained by the fragmentation of M-177, which produces a large variety of fragments over a wide range of molecular sizes that retain antigenic activity in extracts (Fig. 2b).

Interestingly, the IgE reactivity of the fragmented antigen sM-177 was 1.8 times higher than that of the intact form (Fig. 5a). The results of the ELISA inhibition assay helped us to explain the increased IgE reactivity (Fig. 5b,c). The profiles of the inhibition curves suggest that specific IgE for M-177 in patients' sera consists of two populations of IgE: one that recognizes the epitopes present on the surface of the intact molecule and another that recognizes the epitope structures produced on the fragments by degradation. It was possible that the capacity of M-177 as an allergen might be reduced by degradation. However, the histamine-release test showed that sM-177 stimulates patients' basophils more strongly than M-177, indicating that the increased IgE reactivity directly reflects its allergenic activity (Fig. 6a–d).

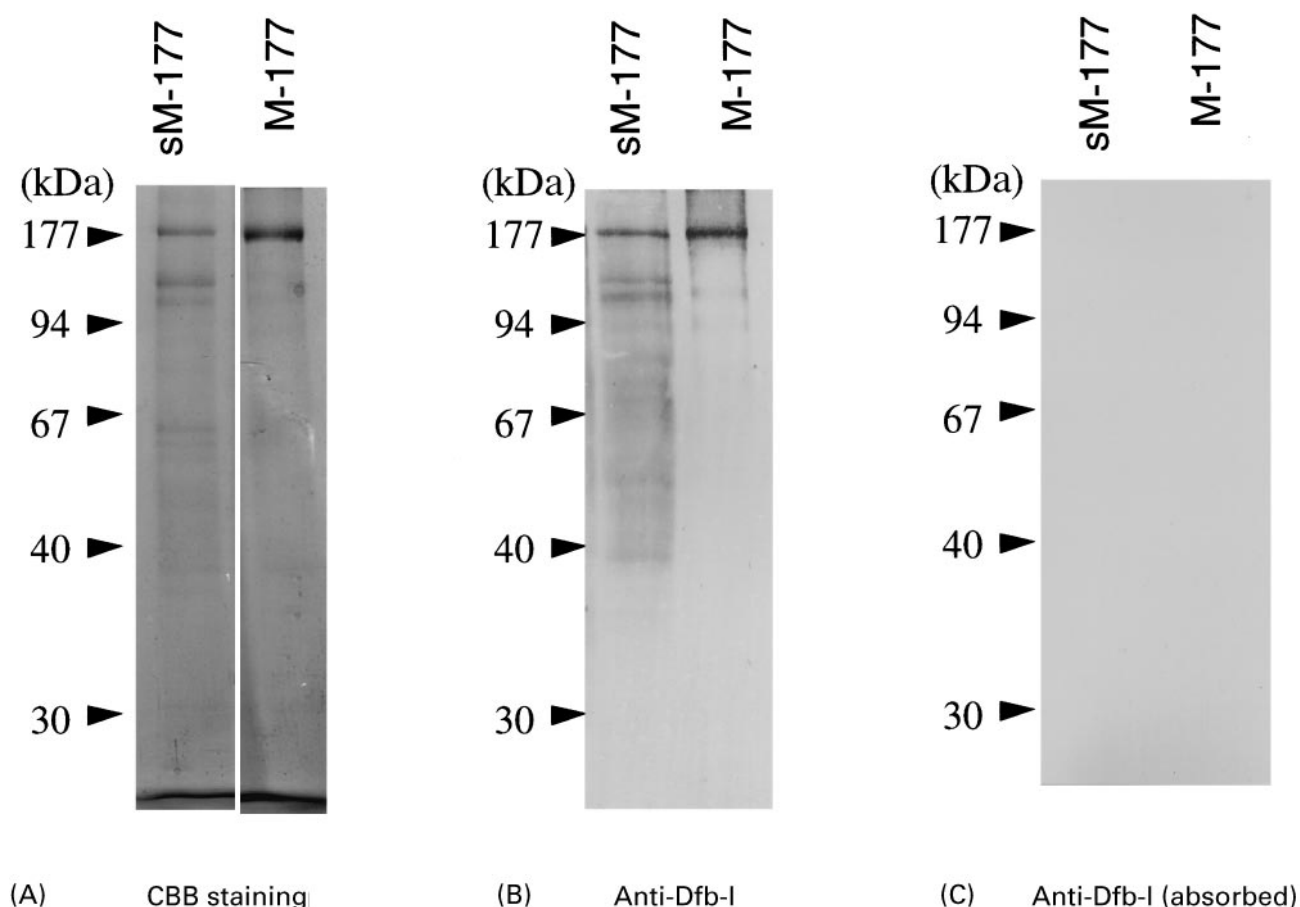


Fig. 4. SDS-PAGE and immunoblotting analysis of M-177 and sM-177. Samples were separated on 10% SDS-polyacrylamide gels, followed by CBB staining (2 μ g, panel a), or immunoblotting with anti-Dfb-I antiserum (0.5 μ g, panel b) and absorbed with a mixture of 20 μ g/mL M-177 and 20 μ g/mL M-177 deg II (0.5 μ g, panel c).

The other *D. farinae* allergens, Der f 1 and Der f 2, are well-known, major mite allergens. Takai *et al.* reported on the allergenic activity of cysteine variants of Der f 2. They showed that the IgE reactivity of a cysteine double mutant, C8/119S, was markedly reduced compared with that of the wild-type [19]. Yasueda *et al.* reported that the IgE reactivity of Der f 1 was heat-sensitive [20]. Unlike the results of this study, both these findings suggest that patient's IgE recognized the antigenic structures of the native allergens more readily than those of the denatured antigens. The electrophoretic mobility and characteristics of Der f 1 on the SDS gels and stained membranes were maintained, in contrast with the results with M-177 (Fig. 1c and Fig. 2d).

Der f 1 behaved in the same manner as Der f 2 (data not shown). These results suggest that Der f 1 and Der f 2 molecules are much more stable than M-177. The authors believe that the IgE recognition reflects the immune response to the structure of allergens when allergic patients have been sensitized.

In this paper, the electrophoretic behaviour and the allergenic activity of the high-molecular-weight mite allergen M-177 in stored samples was analysed. The authors demonstrated that various properties of M-177 were produced by the degradation of crude antigens, and that the allergenic activity of fragmented M-177 was higher than that of the intact antigen.

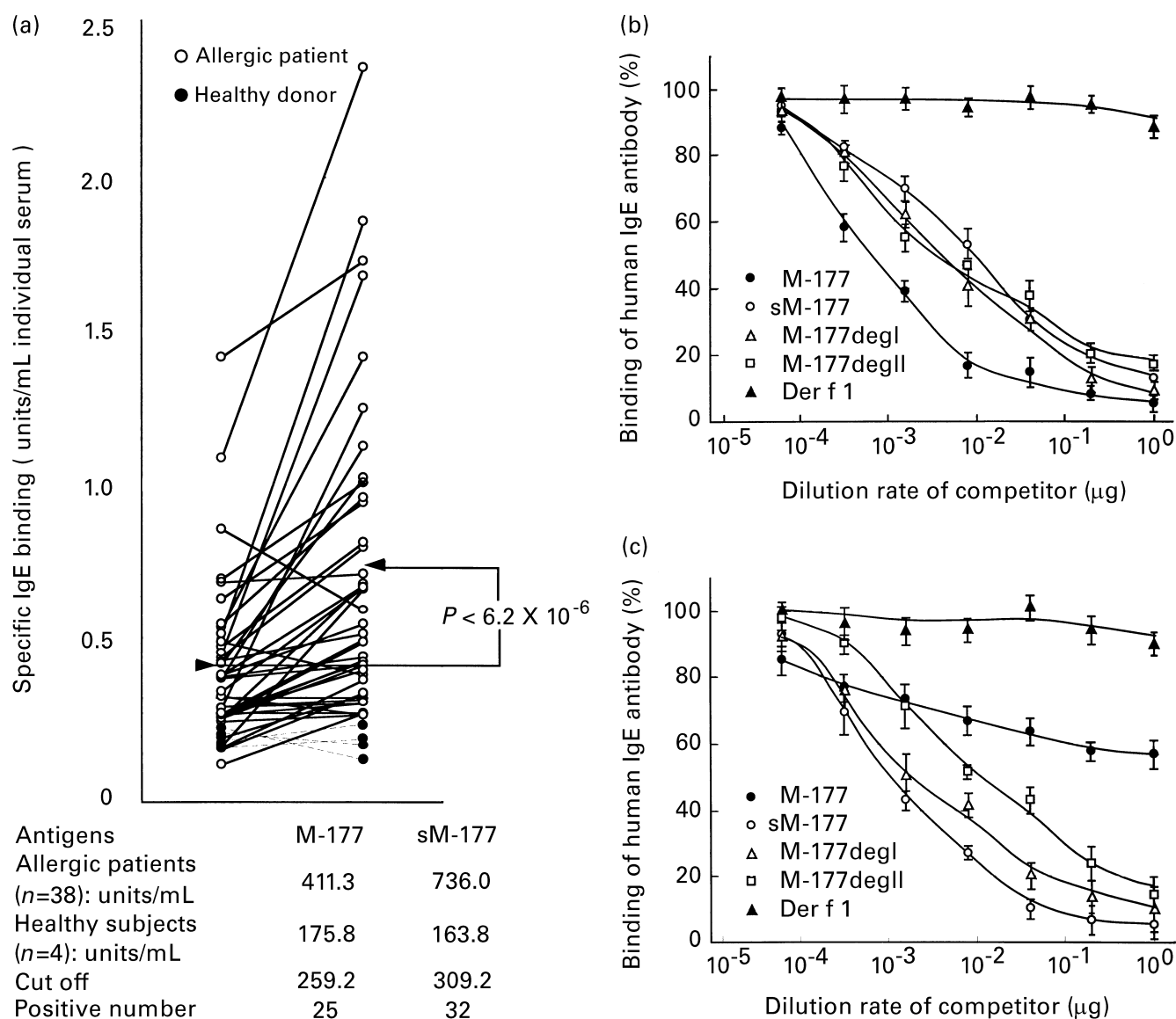


Fig. 5. IgE reactivity of M-177 and sM-177. IgE reactivity to M-177 and sM-177 (a) was analysed by ELISA. Cut off values were determined by adding the mean + 3 SD of the difference between the measured value for individual serum samples and the average patient value, to the mean IgE level in healthy donors. Significant differences were determined using a one-sample *t*-test. Values represent the mean of two experiments. Competitive inhibition of human IgE binding to (b) M-177 and (c) sM-177 was analysed by ELISA inhibition using the pooled sera from double-positive to both M-177 and sM-177 ($n = 10$, in order of reactivity). Values represent mean plus SEM of the three experiments.

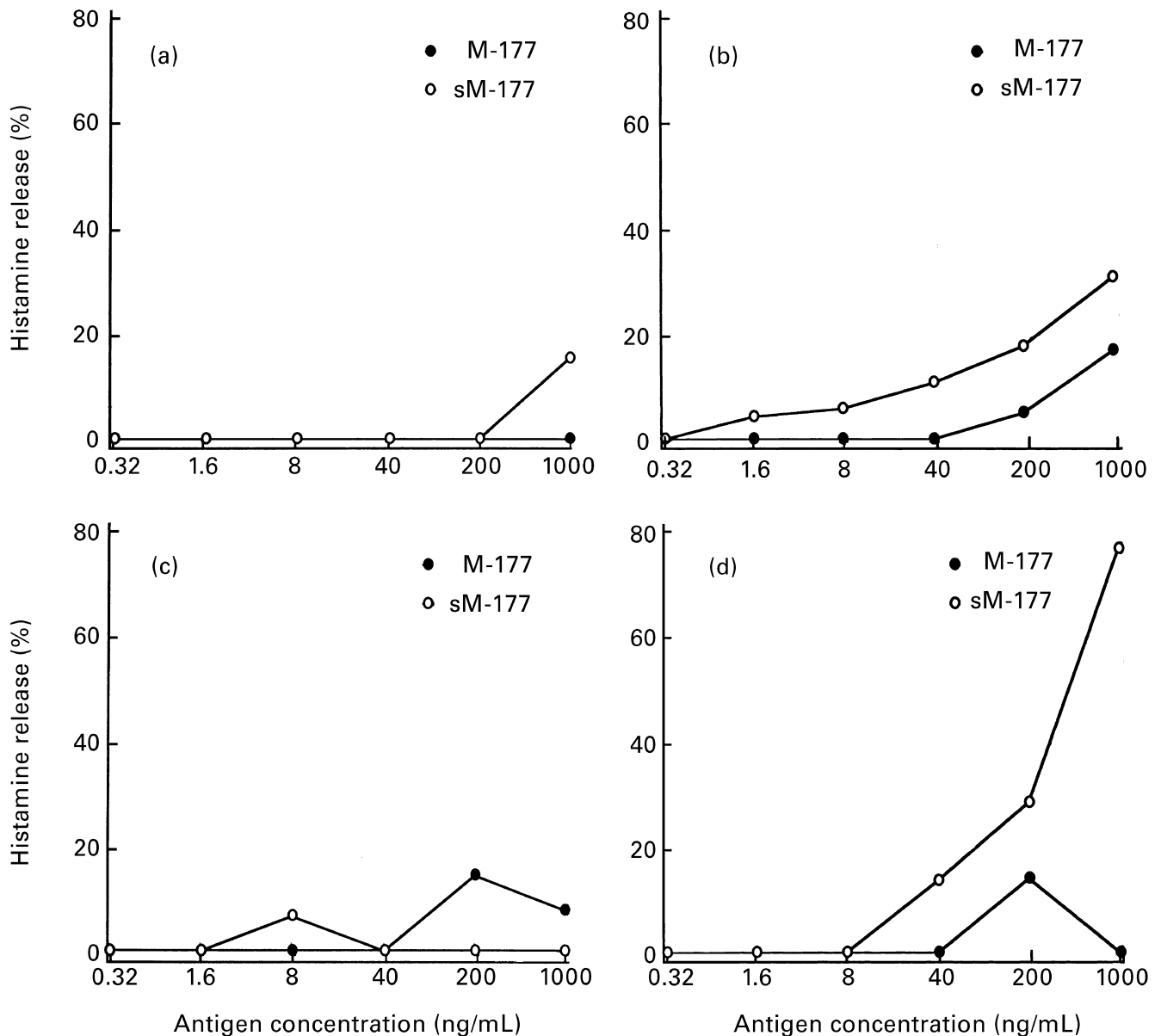


Fig. 6. Histamine release from basophils stimulated with M-177 and sM-177. Two hundred microliters of the washed cell suspension were added to the same volume of Hanks' buffer containing a series of appropriately diluted M-177 or sM-177. The concentration of histamine in the supernatant of the incubation mixture was measured by HPLC.

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