

Monoclonal antibodies against recombinant Der f 3 reveal localization of Der f 3 in the gut and faecal pellets of *Dermatophagoides farinae*

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Abstract Home dust mite derived materials are known to be a major source of problematic inhalant allergens. The aim of this study was to determine the localization of the group 3 allergen, Der f 3, within *Dermatophagoides farinae*, in order to assess the relative importance of excreted materials and nonexcreted body components as allergen sources. Recombinant Der f 3 (rDer f 3) was expressed in bacteria and purified as an immunogen for production of monoclonal antibodies (mAb) against it. *Dermatophagoides farinae* mites and their faecal pellets were embedded in paraffin, and serial sections were immunoprobed with mAb clone 3D3 against Der f 3. *D. farinae* midgut mucosa, gut contents and faecal pellets were strongly immunopositive for Der f 3. Der f 3 immunoreactive products were not detected in any other internal organs of the mite. These results suggest that Der f 3 allergen may be synthesized in and secreted from the digestive tract and excreted from the mite's body in the faecal pellets.

Keywords Der f 3 · *Dermatophagoides farinae* · Localization

Introduction

Allergic diseases are increasing in prevalence all over the world and have become a global health problem (Furuta et al. 2005). However, the way in which susceptible individuals become sensitized to allergens remains unclear (Meza and Gershwin 1997). Various kinds of allergens can cause common allergic diseases such as allergic asthma, allergic rhinitis and atopic dermatitis. Home dust mite derived materials, especially from the species *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f), are a major source of inhalant allergens that induce allergic diseases. There are about 30 reported allergen components produced by *D. farinae* dust mites (Thomas et al. 2002).

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Indeed, over 50% of clinically observed allergic diseases have been attributed to them (Platts-Mills and Chapman 1987).

Der f 3 isolated from *D. farinae* (Heymann et al. 1989) is a group-3 allergenic protein produced by house dust mites. It is a serine protease with trypsin-like activity enabling it to activate the kallikrein-kinin system, which is involved in various allergic reactions in human plasma (Takahashi et al. 1990). Der f 3 encodes a mature protein, 232 amino acids (aa) in length, with a 27-aa-long pre-pro sequence at the N-terminus indicative a secretory protein identity. It shows sequence variation similar to that observed in group 1 and group 2 dust mite allergens (Nishiyama et al. 1995). A search of the BLAST protein database confirmed that the Der f 3 clone is ~84% homologous with Der p 3. Smith et al. obtained natural Der f3 by affinity purification with a monoclonal antibody (mAb), and aa sequence data was generated for both of these proteins and used to construct DNA probes to screen a *D. pteronyssinus* cDNA library by hybridization; the screen resulted in the isolation of a recombinant Der p 3 cDNA clone (Smith et al. 1994).

There is much uncertainty concerning the allergenic properties of Der f 3 given that it has been shown to have widely variable extents (9–100%) of immunoglobulin E (IgE) binding frequency with the sera of mite-allergic patients (Heymann et al. 1989; Stewart et al. 1992). Biochemical experiments in which group 3 allergens were extracted from faecal pellets have suggested that Der f 3 allergens are excreted in fecal pellets, which can become airborne and hence are an important cause of asthma (Ando et al. 1993; Holt et al. 2003). However, Der f 3's secretion pathway and physiological role in mites have yet to be elucidated.

The localization of specific allergens in the mite body could help to elucidate the physiology of sensitizing processes as well as the biological functions of individual allergens. Thus, the synthesis and secretion sites of the Der f 3 allergen within *D. farinae* mite bodies needs to be thoroughly investigated in order for its biological actions to be fully understood. In the current study, we developed Der f 3 specific monoclonal antibodies and used immunohistochemistry to examine the localization of the Der f 3 allergen in *D. farinae* mites.

Materials and methods

Cloning, expression, purification and refolding of rDer f 3

Total RNA was extracted from cultured *D. farinae* mites that originally had been obtained from Southern China domestic environments. Der f 3 cDNA was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and cloned. Its sequence has been published in GenBank (Accession no. EU312162). Amplified Der f 3 cDNA was subcloned into PET-His expression vector (Qinbaosheng Biotech, China), and recombinant Der f 3 (rDer f 3) overexpression was induced by 1 mM of isopropyl- β -thiogalactoside (Sigma, St. Louis, MO, USA) as inclusion bodies in *Escherichia coli* BL21(DE3)plysS (Novagen, USA). The rDer f 3 product was purified to a 95% purity level by affinity column chromatography using Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN, Germany) and then allowed to refold (Cai et al. 2007).

Production of mAbs against rDer f 3

Der f 3 specific mAbs were produced as described previously (Yong et al. 1999). Briefly, Sp2/0 myeloma cells were fused with the spleen cells of BALA/c mice that had been

immunized with 30 µg rDer f 3 mixed with an incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) three times at 3-week intervals. Supernatant solutions were screened for rDer f 3 specific antibody production by ELISA. Antibody positive hybrids were cloned twice by limiting dilution, and frozen cell stocks were stored in liquid nitrogen. Isotyping was carried out by ELISA with an isotyping kit for mAbs according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). The mAbs were produced as ascites and purified by affinity chromatography over recombinant Protein G (Gamma Bind Plus; GE, USA). Ascites titres of these mAbs were assayed by indirect ELISA with 100 ng/well rDer f 3-coated plates.

Immunoblot analysis

The protein samples included extracts of *D. pteronyssinus* (Allergy Laboratories, Inc, USA), *D. farinae* (Allergy Laboratories, Inc, USA), *E9-Df-D. farinae* allergen standard (FDA Rockville, MD, USA), *E. coli* BL21(DE3)plysS lysate without rDer f 3 expression and *Pichia pastoris* strain GS115 lysate without rDer f 3 expression. The samples were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes (Bio-Rad, USA) for western blot analysis. Immunoblot analysis was performed according to the procedures described by Gillespie and Hudspeth (1991). The nitrocellulose membranes were reacted with mAbs and then incubated with 1:1,000 diluted goat anti-human IgG conjugated with horseradish peroxidase (HRP; Kirkegaard & Perry Laboratories, USA). Colour was developed with DAB (3,3'-diaminobenzidine) substrate solution (Amresco, USA).

Primary epitope analysis of mAbs

For primary epitope analysis, the epitopes of rDer f 3 (1–262 aa) were predicted using DNASTar Protean software. We selected three fragments from rDer f 3 comprising 126 aa: A (residues 72–119), B (residues 124–174) and C (residues 178–262). In order to obtain these three dissected polypeptides, the three cDNA fragments were produced by PCR amplification and ligated into pTrc-CKS prokaryotic expression vectors constructed by our laboratory. CKS is an ideal fusion partner since it is expressed as the highest observable expression level of up to 70% soluble protein in *E. coli* (Hayden et al. 1995). Furthermore, its reliability was further confirmed when it was used to express HIV antigens for an FDA-approved diagnostic Abbot second generation ELISA kit (US Patent No. 6511812). Each recombinant fusion peptide was expressed in *E. coli* and purified from bacterial lysates using a HisTrap Kit (Amersham Pharmacia Biotech). Immunoblotting and indirect ELISAs were performed with each CKS-fusion peptide against mAbs, using CKS as a control protein.

Preparation of sections

Dermatophagoides farine mites were cultured at 25°C and 75% relative humidity in a small air-filtrated room. Faecal pellets that were present in the culture medium with ~100 live mites after 24 h in culture were collected with a brush under a stereomicroscope. The mites and their faecal pellets were fixed in 4% paraformaldehyde for 16 h at room temperature, washed in phosphate buffered saline (PBS) three times (10 min each) to remove

the fixative, dehydrated through graded alcohols, and subjected to two xylene washes to remove the alcohol. The specimens were then embedded in paraffin (melting point, 54°C) and allowed to harden overnight at room temperature. Seven-micrometre serial sections were cut in a transverse plane of the mite bodies and the faecal pellets. All sections were collected serially on glass slides coated with poly-L-lysine. Consecutive sections (3–5) were collected on each glass slide and processed for morphology and immunohistology studies (Wu et al. 2007).

Morphological study and Der f 3 immunohistochemistry

Dermatophagoides farinae sections on every third slide of serial sections were dewaxed, rinsed in tap water, and stained with haematoxylin and eosin (H&E) for general morphological study of the mite body cavity. Slides were selected randomly from the serial sections and processed for immunochemistry to identify Der f 3 allergen in mite bodies and faecal pellets. The sections were dewaxed, rinsed in tap water and placed in PBS. The sections were incubated in PBS containing 5% sheep serum for 20 min at room temperature to block unspecific binding and then incubated in 1: 5,000 anti-Der f 3 mAb in PBS at 4°C overnight. After being washed in PBS three times (2 min each), the sections were incubated with 1:100 biotin-labelled sheep anti-mouse IgG (Siam, USA) and in PBS for 30 min at room temperature. The sections were washed in PBS (2 min) and then incubated in PBS with 1:100 fluorescent isothiocyanate (FITC)-streptavidin complex (Sigma–Aldrich, St. Louis, MO, USA) for 30 min. After being washed in PBS, the sections were counterstained with 0.01% Evans blue (Fluka, USA) for 5 min at room temperature. Finally, the sections were mounted in buffered glycerol. On negative control sections, the clone 3D3 mAb was replaced with normal mouse serum during the incubation. All incubations were carried out in a humid chamber. Der f 3 immunoreactivity in mite bodies and faecal pellets was examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Results

Monoclonal antibodies to rDer f 3

One hundred and 24 hybridomas were screened by ELISA. Ten clones (8.1%) were found to be positive. Finally, two clones, 3D3 and 2A8, which produced mAbs with high affinity to rDer f 3, were established through a limiting dilution. Both mAb clones were purified and found to be species-specific. Immunoblot analysis indicated that they reacted specifically with *D. farinae* extracts, and did not cross-react with *D. pteronyssinus* extracts (Fig. 1). Both mAb clones (3D3 and 2A8) were found to be of the IgG1 subtype. The 3D3 mAb had a better ascite titre (819,200) and was thus selected for subsequent Der f 3 localization experiments.

Primary epitope analysis

Recombinant polypeptides were purified using a HisTrap Kit (data not shown). As shown in Fig. 2, both mAbs, 2A8 and 3D3, showed reactivity in ELISAs to A polypeptide (72–119 aa) and the full length rDer f 3 (Fig. 2a). Reactivities were confirmed by immunoblot analysis (Fig. 2b, c).

Fig. 1 Immunoblot analysis of mAb 3D3 specificity. M: marker; 1 mAb 3D3 reacted with *D. farinae* extracts, 2 mAb 3D3 reacted with *D. farinae* extract, 3 mAb 3D3 reacted with *D. pteronyssinus* extract, 4 *E. coli* BL21(DE3)plysS lysate without rDer f 3 expression, 5 *P. pastoris* strain GS115 lysate without rDer f 3 expression. mAb 2A8 had similar reactions (data not shown)

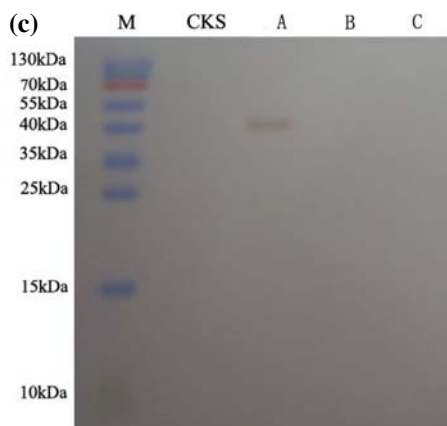
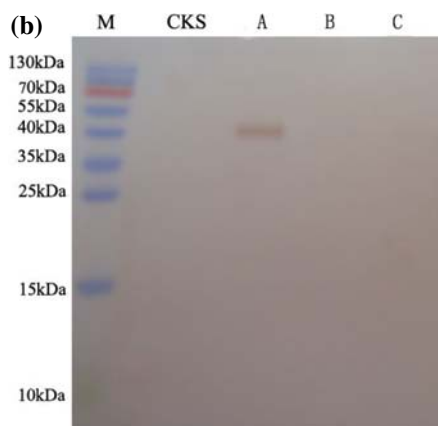
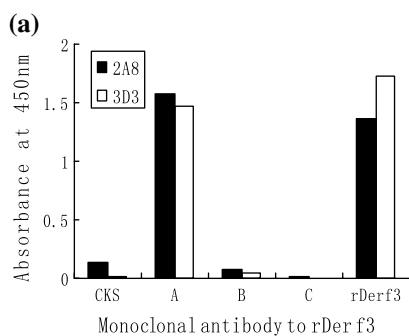
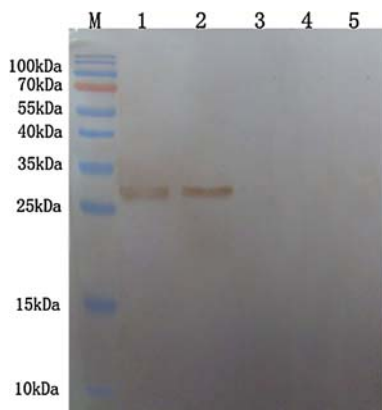


Fig. 2 Primary epitope analysis of mAbs. mAb reactivity to three dissected rDer f 3 fragments (A 72–119 aa, B 124–174 aa, C 178–262 aa) and full-length rDer f 3 was examined by ELISA (a). Each peptide was electrophoresed onto 15% SDS–polyacrylamide gel and immunoblotting was performed with monoclonal antibody 2A8 (b) and 3D3 (c)

General morphological study

Examination of longitudinal H&E-stained sections revealed the general morphological structures of the *D. farinae* body cavity. The external structure, chitin crust, and the internal structures, such as the midgut, hindgut, and reproductive tract, were all clearly demonstrated. The digestive tract occupied a major portion of the body cavity. The gut contents were present in the lumen of both the midgut and the hindgut. The midgut was located close to the central part of the body cavity (data not shown).

Distribution of Der f 3 immunoreactivity

Examination of specimens under a fluorescence microscope revealed Der f 3 presence in paraffin sections of mite bodies (Fig. 3) and defecated faecal pellets in the culture medium (Fig. 4). Examination of longitudinal *D. farinae* sections labelled with mouse 3D3 mAbs revealed Der f 3 immunoreactivity in the digestive tract. No other internal organs were positively labelled by the mouse anti-Der f 3 antibodies. Der f 3 immunoreactive products

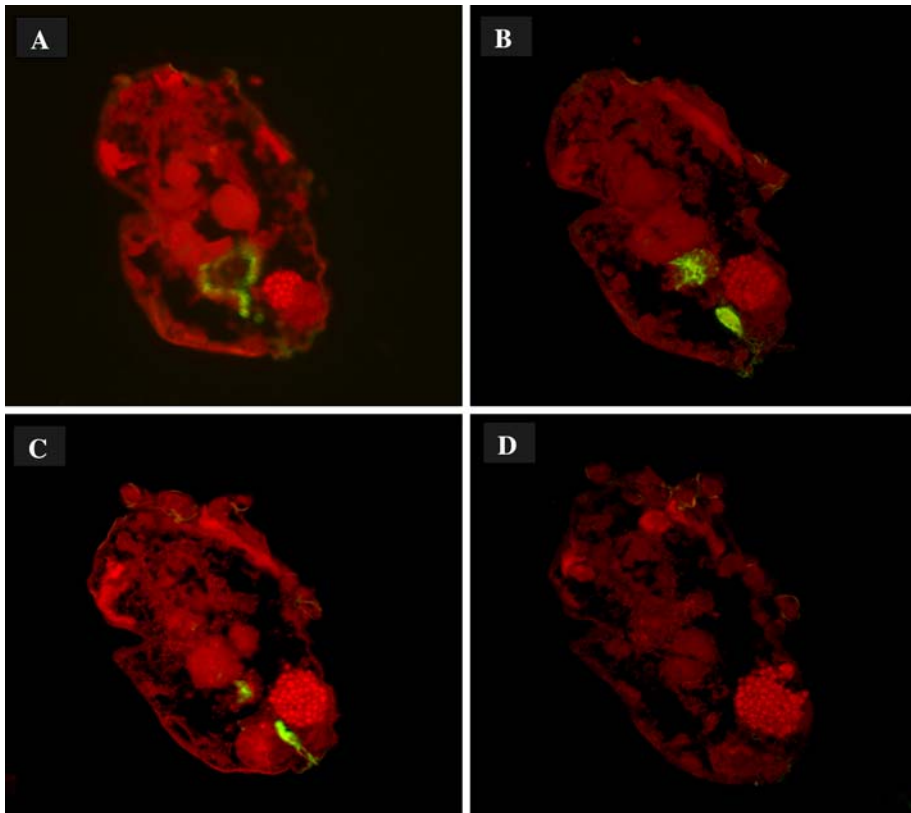


Fig. 3 Immunoprobings of Der f 3 in serial longitudinal sections of paraffin embedded *Dermatophagoides farinae* body. The epithelial lining of the anterior midgut (A), gut contents (B) and posterior midgut (C) reacted strongly reaction. The distinctive gut staining pattern was absent in negative control sections incubated with normal mouse serum (D)

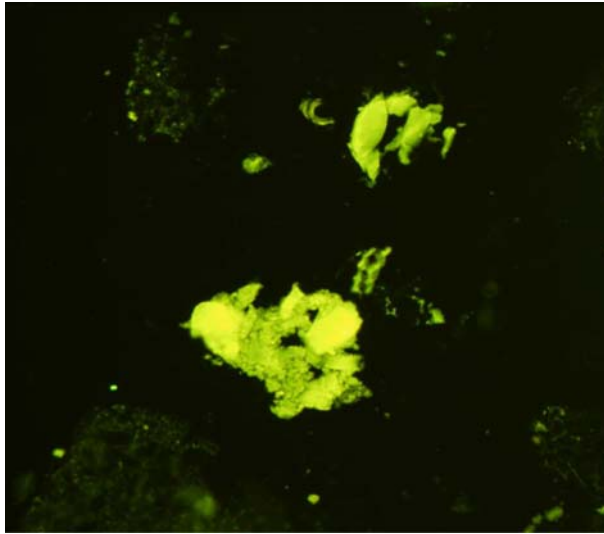


Fig. 4 Der f 3 immunoreactivity of *Dermatophagoides farinae* faecal pellets. Photomicrograph of a section of faecal pellets in culture medium showing robustly labelled Der f 3 immunoreactive products. Culture medium alone (without mite or faecal specimens) was not immunoreactive (image was dark, not shown)

were observed in the anterior midgut in the absence of ingested materials (Fig. 3A), posterior midgut (Fig. 3C) and in the gut contents of the lumen (Fig. 3B). No obvious differences in labelling intensity or pattern were observed between male and female specimens (photomicrographs not shown). *Dermatophagoides farinae* faecal pellets exhibited strong Der f 3 immunoreactivity (Fig. 4). No Der f 3 immunoreactivity was observed in any of the negative control (normal mouse serum) sections of mite bodies (Fig. 3D) or culture medium alone (without mite or faecal specimens; data not shown).

Discussion

The present immunohistochemistry study demonstrated strong labelling of Der f 3 allergen in the midgut of *D. farinae* mites with Der f 3-specific clone 3D3 mAb. Ample Der f 3 was likewise found in Der f faecal pellets. These findings may imply that Der f 3 is synthesized in the mite's intestinal epithelium, secreted in the lumen of the anterior midgut, mixed with digestive materials, and ultimately incorporated into the mite faecal pellets. These results are consistent with the previous prediction based on biochemical experiments that Der f 3 is a secretory protein (Ando et al. 1993).

The present findings in *D. farinae* are consistent with prior work employing human serum containing mite-specific IgE in *D. pteronyssinus* showing a preponderance of allergens in the *D. pteronyssinus* anterior midgut and faeces (Tovey and Baldo 1990). It has also been demonstrated using immunohistochemical methods that Der p 1, Der p 2 and Der f 2 allergens, respectively, are localized to the digestive tract and concentrated in the faecal pellets (Rees et al. 1992; Liu et al. 2005; Park et al. 2000; Jeong et al. 2002). In addition, two-site capture ELISA was developed using two different mAbs for quantitating Der f 3 in our laboratory. We obtained two house dust mite extracts: one from pure mite



Fig. 5 Difference between Derf 3 and Der p3 revealed by aa sequence comparison analysis. The primary epitope recognized by mAb 3D3 and 2A8 is between residue 72 and residue 119. There is a 15 aa difference in this 48-aa stretch

bodies and the other from a mixture of mite bodies and mite faecal pellets. Derf 3 allergen could be detected in both extracts, but a higher content of Der f 3 was present in the mixture than in the bodies alone (data not shown). These data indicated that although Der f 3 existed in both mite body and faecal pellets, it was much more concentrated in the faecal pellets. These results also further confirmed the localization of Der f 3 in *D. farinae*. This information will be helpful in guiding the selection of the material (i.e., bodies or body-faeces mixture) that should be used for vaccine and diagnostic reagent preparation in the future.

mAb-based immunoassays have been applied to monitor and assess dust mite allergen contents in the indoor environment. In current study, mAbs with a high affinity for rDer f 3 were obtained. Immunoblot analysis indicated that they reacted specifically with *D. farinae* extracts and exhibited no cross-reactivity with *D. pteronyssinus* extracts. In order to further confirm the specificity of the anti-Der f 3 mAbs, we conducted experiments in which we analyzed mAb epitopes. The primary Der f 3 epitope analysis results suggested that the epitope recognized by mAb 3D3 and 2A8 is between residue 72 and residue 119; aa sequence comparison analysis showed that the difference between Der f 3 and Der p 3 allergens is mostly concentrated in this fragment (15 of these 48 aa are different, 31%; Fig. 5). In view of the above immunoblot and primary epitope analysis findings, we deduced that our mAbs are specific to Der f.

In conclusion, this study demonstrated that Der f 3 immunoreactivity was localized to the midgut, gut contents and faecal pellets of *D. farinae* mites, suggesting that the Der f 3 allergen is synthesized in and secreted from midgut epithelia and excreted from the mite body in faecal pellets. This localization indicates that, in addition to live mites that deposit substances in human domicile materials and air conditioning filters (Liu et al. 2005), mite faecal pellets and decomposing carcasses, which contain group 1, 2, and 3 allergens, also have the potential to produce airborne inhalant allergenic substances.

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