

Prominent IgE-binding and cytokine-inducing capacities of a newly cloned N-terminal region of Der f 14, an apolipoprotein-like house dust mite allergen

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We previously characterized a 177-kDa allergen, M-177, from *Dermatophagoides farinae*. Thereafter, a counterpart to M-177 for *Euroglyphus maynei* was cloned as Eur m 14, and its sequence revealed that two environmental allergens, Mag 1 and Mag 3, are digested fragments of M-177. The aims of this study were to clone the cDNA of Der f 14 corresponding to M-177 and to elucidate the allergenic capacities of the N-terminal fragment of Der f 14 (Der f 14-N). Recombinant allergens were produced as trigger-factor-fused proteins in *Escherichia coli*. Der f 14-N showed the highest IgE-binding frequency among Der f 14-derived fragments in patients allergic to house dust mite by enzyme-linked immunosorbent assay. Der f 14-N showed the highest capacity to induce cell proliferation in murine lymphocyte and human peripheral mononuclear cells among Der f 14-derived fragments. Der f 14-N induced IL-13, IFN- γ and IL-17 production more than Der f 1 and Der f 2 in mouse, and induced IL-5 and IFN- γ production at levels comparable to those of Der f 1 and Der f 2 in some patients. The high prevalence of IgE binding to the Der f 14-N indicates that it could be an important mite allergen.

Keywords: apolipoprotein; Der f 14; *Dermatophagoides farinae*; environmental allergen; house dust mite.

Abbreviations: β Gal, beta-Galactosidase; Dfb, *Dermatophagoides farinae* body extract; ELISA, enzyme-linked immunosorbent assay; FCS, Freund's complete adjuvant; GST, glutathione-S-transferase; HDM, house dust mite; LPS, lipopolysaccharide; Mag, mite allergen gene; TF, trigger factor.

House dust mite (HDM) is a major source of environmental allergens that induce perennial rhinitis and chronic asthma. American HDM (*Dermatophagoides farinae*) and European HDM (*D. pteronyssinus*) are the most common HDM species in humid regions worldwide (1, 2). Another HDM, *Euroglyphus maynei*, is less common than *D. farinae* and *D. pteronyssinus* in homes (1, 3).

Group 1 and 2 allergens are major allergens in HDM; Group 1 allergens are cysteine proteases and group 2 allergens are MD-2-like lipid-binding proteins (4). Groups 1 and 2 allergens of *D. farinae* are named as Der f 1 and Der f 2, respectively. Until now, 31 Der f allergens (Der f 1 to Der f 36) have been formally registered on the database for systematic allergen nomenclature approved by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) Nomenclature Subcommittee.

We previously characterized a 177-kDa HDM allergen named M-177 from *D. farinae* extract (5). M-177 is a 177-kDa allergen that showed 65.8% (25/38) IgE-binding frequency in HDM-allergic patients, and its digested fragments possessed higher IgE-binding frequency (84.2%, 32/38) than that of intact M-177 in the population. M-177 exists as its digested fragments in stored *D. farinae* extract and in environmental conditions, because M-177 is susceptible to proteolytic digestion by proteases such as Der f 1 (6).

One of the digested fragments of M-177 was identified as mite allergen gene (Mag) 3 by immunoblotting experiments using a recombinant Mag 3-specific polyclonal antibody (6). Thereafter, counterpart allergens to *D. farinae* M-177 were cloned from *D. pteronyssinus* and *Euroglyphus maynei* as Der p 14 and Eur m 14, respectively, and their primary sequences revealed that Eur m 14 contains corresponding sequences of Mag 1 and Mag 3, which were previously cloned as *D. farinae*

allergens by our group (7–9). These studies revealed that M-177 of *D. farinae* exists as digested fragments Mag 1, Mag 3 and other fragments in environmental conditions after proteolytic digestion. However, the full length of cDNA for M177 of *D. farinae* has not been cloned and the allergenic capacity of its unidentified region remains unclear.

Der p 14 and Eur m 14 are putative lipid transfer apolipophorins found in insects (7). Lipophorins are multifunctional proteins that have lipid-binding capacity as well as pattern-recognition capacity to induce anti-microbial peptide production as an insect's innate immunity sensor (10, 11). Insect apolipophorin III has the capacity to bind microbe-derived cell wall components such as lipopolysaccharide (LPS), lipoteichoic acid and β -1.3 glucan, and is involved in the immune defence of insects by mediating humoral and cellular immunity (12). Consequently, group 14 allergens are considered to be involved in the immune defence of mites from infection of microbes such as fungi. Insect apolipophorin is homologous to human apolipophorin E, and thus mite's apolipophorin also may act as a stimulator in human innate immune systems (13, 14).

In this study, we aimed to clone the N-terminal unidentified sequence for Der f 14 corresponding to the M-177 allergen from *D. farinae* and to elucidate the allergenic properties of its digested fragments. Towards these ends, we produced Der f 14-derived fragments, Der f 1 and Der f 2 as trigger factor (TF)-fused His-tagged recombinant proteins by *Escherichia coli* (Rosetta-gami DE3) and purified each allergen and TF by Ni-affinity column chromatography. We analysed the IgE-binding frequencies of the Der f 14-derived fragments, Der f 1 and Der f 2 using serum from 34 patients allergic to HDM. We also determined the capacities of the Der f 14-derived fragments, Der f 1, Der f 2 and extract from *D. farinae* body (Dfb) to induce cell proliferation and cytokine production in murine lymphocytes isolated from C3H/HeJ mice sensitized with Dfb emulsified in Freund's complete adjuvant (FCA), as well as cell proliferation and cytokine production in peripheral blood mononuclear cells (PBMCs) from five patients allergic to HDM.

Materials and Methods

Molecular cloning of Der f 14

Primers were designed from the internal primary sequences of M-177 from *E. maynei* and Mag 3 (TTCACGTTCCATAAAAYTNGT and CTGTGCTTTAGCTTGTTTGTT, respectively). The full length of cDNA for Der f 14 was obtained using the primers and *D. farinae* mRNA as templates by the Gene Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT, USA) using the SMART RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions.

Preparation of recombinant proteins

Corresponding regions of the N-terminal sequence of Der f 14 (Der f 14-N), Mag 1 or Mag 3 in Der f 14 (Fig. 1), or full length of cDNA for major HDM allergens Der f 1 or Der f 2 were cloned into pCold TF DNA (Takara, Tokyo) and expressed as recombinant TF-fused proteins with His-tag in *E. coli* (Rosetta-gami DE3)-competent cells (Merck Millipore, Billerica, MA, USA). The cDNAs for Der f 1 and Der f 2 were cloned from *D. farinae* mRNA using primers for each allergen: GTTGGTACCCGTCACGCTTCAATCAAAAC and TT

CCTCGAGTCACATGATTACAACATATGGATA for Der f 1, and TCAGATGCATCATATGATTTCCAAAATCTTGTG and ATGGATCCTTAATCACGGATTTTACCATG for Der f 2. TF-fused allergens Der f 14-N, Mag 1, Mag 3, Der f 1 and Der f 2 were purified using His-trap HP (GE Healthcare Japan, Tokyo), as was TF as a negative control, following the manufacturer's instructions (15).

Soluble and insoluble proteins in lysate of Rosetta-gami (DE3) *E. coli*, purified Der f 14-derived fragments, purified Der f 1, purified Der f 2 and purified TF were loaded onto 10% sodium lauryl sulphate (SDS)-polyacrylamide gel (500 ng each) and electrophoresed under reducing conditions. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a calculation standard. The fractionated proteins were visualized with Coomassie Brilliant Blue (CBB) staining. The fractionated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane and immunostained with 1,000-fold-diluted horseradish peroxidase conjugated anti-His tag antibody (BioLegend, San Diego, CA, USA). The area of the PVDF membrane corresponding to the molecular marker was cut after protein transfer and then stained with CBB to estimate the molecular weights of positive bindings on X-ray film after immunostaining.

Procedures for mouse experiments

C3H/HeJ mice ($n = 3$) were injected with 1 mg Dfb emulsified in FCA into footpads and back skin twice, with 2 weeks between injections. Lymphocytes from inguinal and axilla lymph nodes were collected and pooled, then 8×10^5 lymphocytes were cultured with 160 μ M Der f 14-derived fragments, 160 μ M TF, 100 μ g/ml Dfb, or 50 μ g/ml concanavalin A (Con A) for 3 or 4 days. The optimal dose of Der f 14-derived fragments was determined by a preliminary dose–response study on murine lymphocyte from Dfb/FCA-immunized C3H/HeJ mice (data not shown). For the cytokine assay, plates that had been cultured for 4 days were centrifuged at 1,500 rpm for 10 min at room temperature, and the supernatant was stored at -20°C until assay. The concentrations of IL-5, IL-13 and IFN- γ in the culture supernatant were then quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using antibodies specific to each cytokine (eBioscience, Thermo Fisher Scientific, Yokohama, Japan) following the manufacturer's instructions. For the proliferation assay, 10 nM BrdU was added to the culture after 2 days, and the plate was incubated for an additional 18 hr. BrdU incorporation was analysed using the colorimetric Cell Proliferation ELISA, BrdU kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. All animal experiments were carried out using protocols approved by the Committee on Animal Experimentation of Hiroshima University, Japan.

Human samples

HDM-allergic plasma samples were obtained from 39 subjects (34 for ELISA and 5 for cell culture analysis) selected on the basis of clinical histories and positive ImmunoCAP (Phadia, Thermo Fisher Scientific) scores for HDM (score of 2 or higher). Non-allergic plasma samples from 4 healthy volunteers (3 for ELISA and 1 for cell culture analysis) with negative ImmunoCAP scores for HDM were used as negative controls. The study protocol conformed to the standards set by the Declaration of Helsinki. All experiments were approved by the institutional review board of Takanobashi Central Hospital and were described in detail to all participants before they provided their informed consent and were admitted into the study.

Procedure for ELISA

ELISA was performed according to a method previously reported (16, 17). Briefly, 1 μ g purified protein in 100 μ l bicarbonate buffer (pH 9.4) was coated onto a 96-well microtiter plate (Nunc, Thermo Scientific) at 4°C overnight, which was then blocked with phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS), 0.5% BSA, 0.02% sodium azide and 0.05% Tween 20. Then, 50-fold-diluted serum from 22 allergic patients and 3 healthy volunteers, in PBS containing 10% FBS, 0.02% sodium azide and 0.05% Tween 20, was added to the plate, followed by incubation with 1,000-fold-diluted biotin-conjugated anti-human IgE (epsilon chain-specific; Biosource International, Camarillo, CA, USA) in PBS containing 1% BSA, 0.02% sodium azide and 0.05% Tween 20 for 2 hr. After incubation with 5,000-fold-diluted streptavidin-beta galactosidase (β Gal) conjugate (Roche Diagnostics) in PBS

[illegible]

Fig. 1 Deduced amino acid sequence for Der f 14 and its multiple sequence alignments in mite group 14 allergens. Amino acid sequences of Der f 14 and mite group 14 allergens from *E. maynei* (GenBank accession no. AF149827), *D. pteronyssinus* (AF373221) and *S. scabiei* (JXLN01016333) were aligned using ClustalW on the GenomeNet Bioinformatics computational service (Kyoto University Bioinformatics Center, Kyoto, Japan). The putative signal sequence and conserved region found in lipid transport protein (Vitellogenin domain, Prosite entry: IPR001747) in Der f 14 are indicated by italics and underlining, respectively. Cysteine residues conserved for lipophorin and a potential N-glycosylation site in Der f 14 are indicated in blue and green, respectively. Corresponding sequences for Der f 14-N, Mag 1 and Mag 3 are shown in black, purple and red, respectively, in Der f 14. Gap sequence between Mag 1 and Mag 3 in Der f 14 is shown in orange.

containing 1% BSA, 0.02% sodium azide and 0.05% Tween 20 for 1 hr, 0.2 mM of 4-methylumbelliferyl- β -D-galactopyranoside (Sigma-Aldrich Japan, Tokyo) substrate solution was added to the plate and incubated at 37°C for 2 hr. After the enzymatic reaction was stopped by 0.1 M glycine-NaOH (pH 10.2), fluorescence intensity was measured using a Wallac 1420 ARVOsx Multilabel Counter (Perkin-Elmer, Downers Grove, IL, USA). An arbitrary unit was determined for the fluorescent intensity of binding between 1,000-fold-diluted pooled HDM-allergic serum and 10 ng Dfb as 25 kAU/l determined by a calculation curve between 1,000-fold-diluted pooled serum and serial dilution of Dfb.

Cell culture of human peripheral PBMCs

PBMCs were isolated from 5 HDM-allergic patients and a non-allergic healthy volunteer according to a method previously reported (18, 19). Then, 5×10^5 PBMCs were cultured with 360 μ M Der f 14-N, Mag 1, Mag 3, Der f 1, Der f 2 or TF in AIM-V medium (Invitrogen, Thermo Scientific) with 50 μ g/ml polymyxin B sulfate (Sigma-Aldrich) for 3 days. Proliferation of the human leukocytes and cytokine production were determined by the same method of used in the mouse experiments.

Statistical analysis. Non-repeated ANOVA was applied to compare data among the groups, and when the difference was significant ($P < 0.05$), post hoc analysis using a Tukey test was performed.

Results

Molecular cloning and characterization of Der f 14 in *D. farinae*

cDNA corresponding to *D. farinae* M-177 allergen (Der f 14) was cloned from *D. farinae* mRNA by rapid amplification of cDNA ends (RACE) polymerase chain reaction using primers constructed from the nucleotide sequences of *E. maynei* M-177 (Eur m 14) and Mag 3. The Der f 14 coded a 5,140-bp nucleotide containing a 5,001-bp open reading frame encoding a 1,666-amino acid polypeptide (deposited in GenBank under accession no. LC257981). The deduced amino acid sequence of Der f 14 contains the corresponding amino acid sequences of Mag 1 and Mag 3 (shown in purple and red in Fig. 1). Der f 14 contains a presumed signal sequence in its N-terminus (1–18, indicated in italics) (7) and one putative N-glycosylation site (618–621, indicated in green) (Fig. 1). Der f 14 also contains the Vitellogenin domain profile (33–639, Prosite entry PS51211, indicated by underlining) that is conserved in large lipoproteins (20) and 4 cysteine residues that are important for intracellular disulphide bonds in lipophorins (C25, C73, C115 and C155, shown in blue) (Fig. 1). These features indicate that Der f 14 is an apolipophorin-like allergen similar to Der p 14 and Eur m 14.

Subsequently, the amino acid sequence similarity of Der f 14 was analysed among group 14 apolipophorin-like allergens. Der f 14 has the highest sequence similarity to, and identity with, Eur m 14 (GenBank accession no. AF149827): 98.9% similar and 90.2% identical (Fig. 1). Der f 14 also shows 98.0% similarity to and 87.8% identity with Der p 14 (AF373221), and 88.2% similarity to and 60.1% identity with Sar s 14 from a tick mite, *Sarcoptes scabiei* (JXLN01016333) (Fig. 1). These data suggest that primary sequences of group 14 allergens are highly conserved in mites, including HDM and tick mites.

Preparation of recombinant proteins for Der f 14 and its fragments

Der f 14 is considered to consist of digested fragments in environmental conditions, because it is susceptible to proteolytic digestion by proteases like Der f 1, which is a cysteine protease of *D. farinae* (6). For that reason, Der f 14-derived fragments were produced as an N-terminal fragment of Der f 14 (Der f 14-N), Mag 3 and Mag 1 as TF-fused His-tagged proteins in *E. coli*. The cDNA of each fragment of Der f 14 or HDM major allergen, Der f 1 or Der f 2 was cloned into pCold TF DNA and transfected into competent cells of *E. coli* (Rosetta-gami DE3). Polypeptides for Der f 14-N, Mag 1 and Mag 3 consist of 906, 370 and 341 amino acids and cover 54%, 22% and 20% of the Der f 14 sequence, respectively (Fig. 2A). Calculated molecular weight from primary sequence of each TF-fused protein are 157, 94, 97, 90, 70 and 54 kDa for Der f 14-N, Mag 1, Mag 3, Der f 1, Der f 2 and TF, respectively. The resultant TF-fused proteins were fractionated as soluble proteins after lysis of *E. coli* and showed positive binding to anti-His tag antibody in immunoblotting analysis (Fig. 2B and data not shown). The TF-fused allergens and TF were purified by Ni-immobilized affinity column chromatography. Finally, we succeeded in purifying each Der f 14-derived fragment, Der f 1, Der f 2 and TF, as a single protein on SDS-PAGE after CBB staining (Fig. 2C). We confirmed that the purified proteins were the intended one by immunoblotting analysis with anti-His tag antibody (Fig. 2D). A 150-kDa protein of Der f 2 was considered to be a dimer of Der f 2 (Fig. 2C and D).

Immunological properties of Der f 14-derived fragments in Dfb-sensitized mice

Although it was revealed that Mag 1 possesses a dominant T-cell epitope for murine T cells (51–65 in Mag 1 polypeptide) (21), the capacities to induce murine lymphocyte proliferation remain unclear for Der f 14-N and Mag 3. We stimulated murine lymphocytes isolated from Dfb/FCA-sensitized C3H/HeJ mice with the recombinant Der f 14-derived fragments (Der f 14-N, Mag 1 and Mag 3), Der f 1, Der f 2, TF and Dfb. C3H/HeJ mice have defective LPS signalling due to mutations on *Toll-like receptor 4* (22), allowing us to avoid the undesired effects of probable LPS contamination. The proliferations of murine lymphocytes stimulated with Der f 14-N, Mag 1 and Dfb were statistically significantly different than those with PBS as a negative proliferative control and TF (Fig. 3A). Among the Der f 14-derived fragments and HDM major allergens, Der f 14-N induced higher levels of proliferation than Mag 3, Der f 1 and Der f 2 in murine lymphocyte, which was comparable to that induced by Dfb (Fig. 3A). Mag 1 also induced comparable levels of proliferation with that of Der f 14-N, but the level was not significantly higher than those of Der f 1 and Der f 2 (Fig. 3A).

Interestingly, Der f 14-N induced significantly higher levels of production of Th2-type (IL-5, IL-13), Th1-type (IFN- γ) and Th17-type (IL-17) cytokines compared with PBS and TF, although Dfb induced only Th2-type

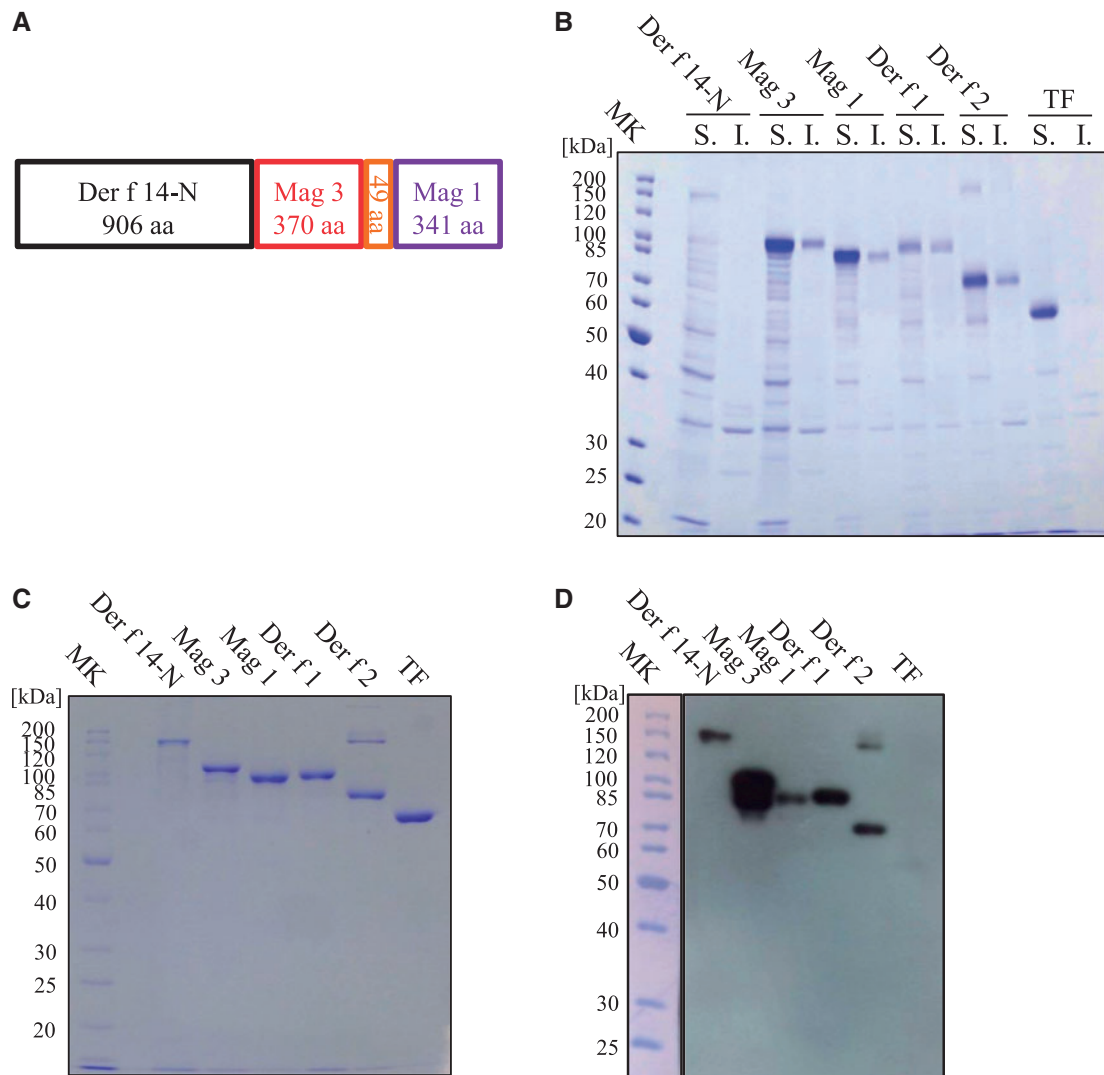


Fig. 2 Purification of recombinant proteins. (A) Diagram of Der f 14-derived fragments. Corresponding regions of Der f 14-N, Mag 1, Mag 3 and gap-sequence between Mag 1 and Mag 3 are shown in Der f 14. (B) Soluble (S.) and insoluble (I.) fractions from *E. coli* lysate transfected with pCold TF DNA cloned with Der f 14-N, Mag 3, Mag 1, Der f 1 or Der f 2 were loaded onto 10% SDS-polyacrylamide gel under reducing conditions and visualized with CBB staining. TF shows *E. coli* lysate transfected with empty pCold TF DNA. (C) Purified TF-fused recombinant Der f 14-N, Mag 3, Mag 1, Der f 1, Der f 2 and TF were loaded onto 10% SDS-polyacrylamide gel under reducing conditions and visualized with CBB staining. (D) Proteins shown in (C) were immunoblotted with anti-His tag antibody. MK, protein weight marker.

cytokine production (Fig. 3B–E). Mag 1 also induced IFN- γ production at higher levels compared with PBS, which was comparable level to that of Der f 14-N (Fig. 3C and D). Der f 1 and Der f 2 did not induce any statistically significantly greater levels of cytokine production than PBS and TF in Dfb/CFA-sensitized C3H/HeJ mice (Fig. 3B–E). These results indicate that Der f 14-N possess a capacity to induce the proliferation of murine lymphocytes comparable to the capacity of Mag 1, and suggest that Der f 14-N is the most potent fragment to induce lymphocyte proliferation and cytokine production in Dfb-sensitized mice.

IgE-binding frequencies and allergenic capacities of Der f 14-derived fragments in human

We next examined the allergenic capacities of Der f 14-derived fragments in human. The IgE-binding frequencies of Der f 14-derived fragments, Der f 1, Der f 2 and TF

were determined by ELISA using plasma from 34 patients allergic to HDM. We defined the IgE binding of each protein as positive if a patient's specific IgE titer to each protein exceeded a threshold (indicated by a red bar for each allergen in Fig. 4A; mean + (3 $\times$ SD) for 3 healthy volunteers: 8.6, 28.9, 3.6, 10.2 and 23.9 kAU/l for Der f 14-N, Mag 3, Mag 1, Der f 1 and Der f 2, respectively). The IgE-binding frequency of IgE binding for each allergen are 61.8%, 14.7%, 38.2%, 70.6% and 85.3% for Der f 14-N, Mag 3, Mag 1, Der f 1 and Der f 2, respectively (Fig. 4A). The IgE-binding frequency of Der f 14-N in the HDM patients was comparable to that of Der f 1, although the titers of IgE specific to Der f 14-N were lower than those of IgEs specific to Der f 1 and Der f 2 (Fig. 4A).

We previously reported that Mag 1 and Mag 3 have the capacity to induce antigen-specific leucocyte proliferation in human comparable to the capacities of Dfb

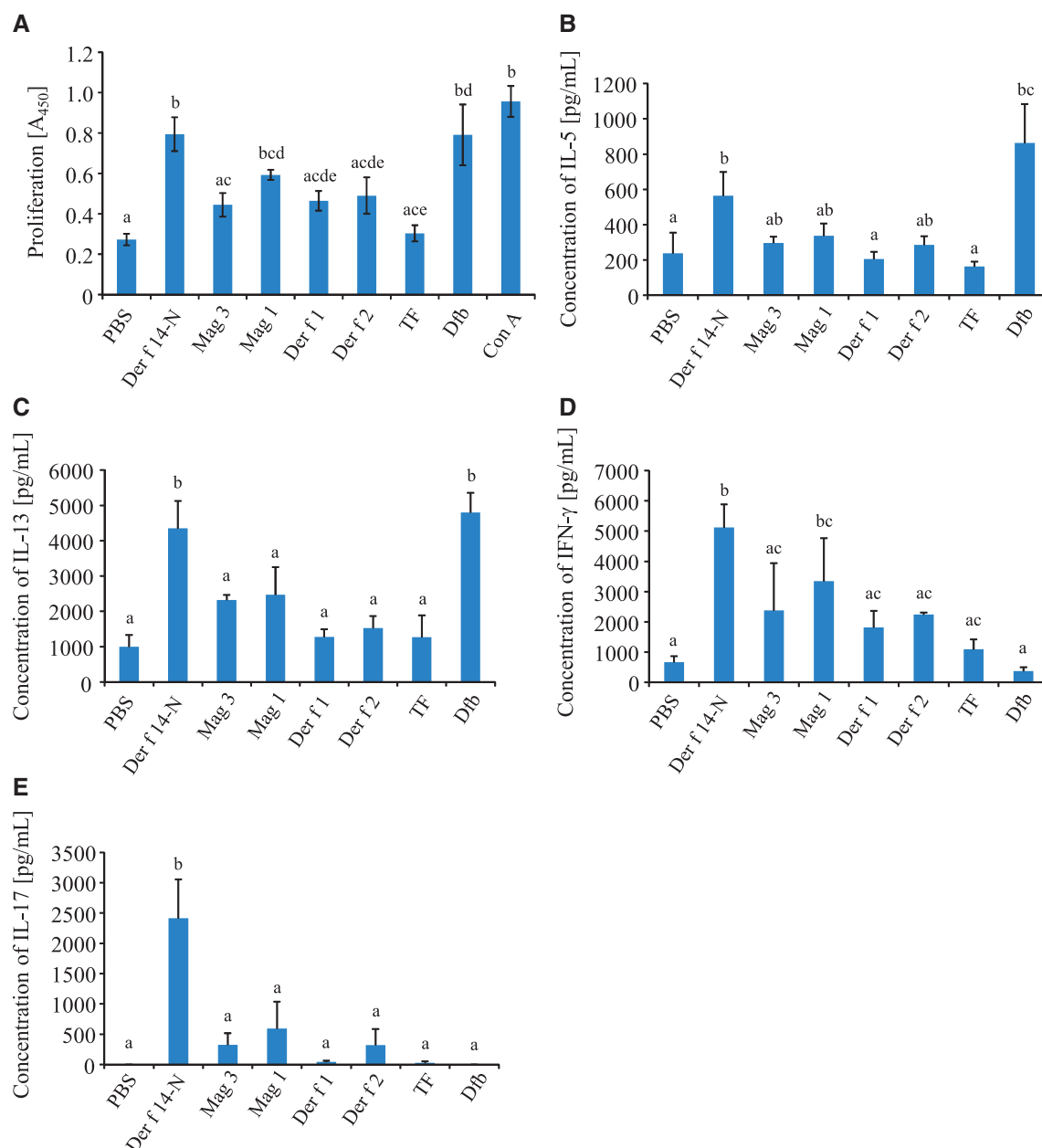


Fig. 3 Proliferation of and cytokine production from murine lymphocytes. Lymphocytes from C3H/HeJ mice sensitized with Dfb emulsified with FCA were cultured with 160 μ M Der f 14-N, Mag 3, Mag 1, Der f 1, Der f 2, TF, 100 μ g/ml Dfb or 50 μ g/ml Con A for 4 or 3 days. (A) Proliferation of lymphocytes was estimated by BrdU incorporation in the last 18 hr of a 3-day cultivation. Amount of incorporated BrdU determined by ELISA is plotted on the Y-axis. (B–E) Cytokine production in culture supernatant after 4 days' culture of murine lymphocyte stimulated with Der f 14-derived fragments, Der f 1, Der f 2, TF or Dfb were analysed by sandwich ELISA for each cytokine. The concentration of each cytokine is plotted on the Y-axis. Statistical analysis was performed using an ANOVA with post hoc Tukey test to determine the significant differences. The bars with different superscripts are significantly different ($P < 0.05$).

and Der f 2 (21, 23). We analysed the capacities of Der f 14-derived fragments, Der f 1 and Der f 2 to induce cell proliferation and cytokine production in 5 HDM-allergic patients' PBMCs. Two of the patients (Patients A and D) showed positive proliferation to Der f 14-N compared with that of PBS as a negative control and of TF (Fig. 4B). Mag 1 induced weak proliferation of PBMCs in 2 of the patients (B and C), but Mag 3 did not induce significant proliferation of PBMCs (Fig. 4B). Der f 1 and Der f 2 induced positive proliferation

in two patients (C and D) and five patients (A, B, C, D and E), respectively (Fig. 4B).

We then analysed cytokine production from PBMCs stimulated with Der f 14-derived fragments, Der f 1 and Der f 2. Similar to the results of the mouse experiment (Fig. 3), in this experiment, Der f 14-N showed the most potent capacity to induce Th2-type (IL-5) and Th1-type (IFN- γ) cytokine production (Patients A and D in Fig. 4C and D) among the Der f 14-derived fragments. Although Mag 1 induced only IL-5 production (Patients B and C), Mag 3 hardly induced

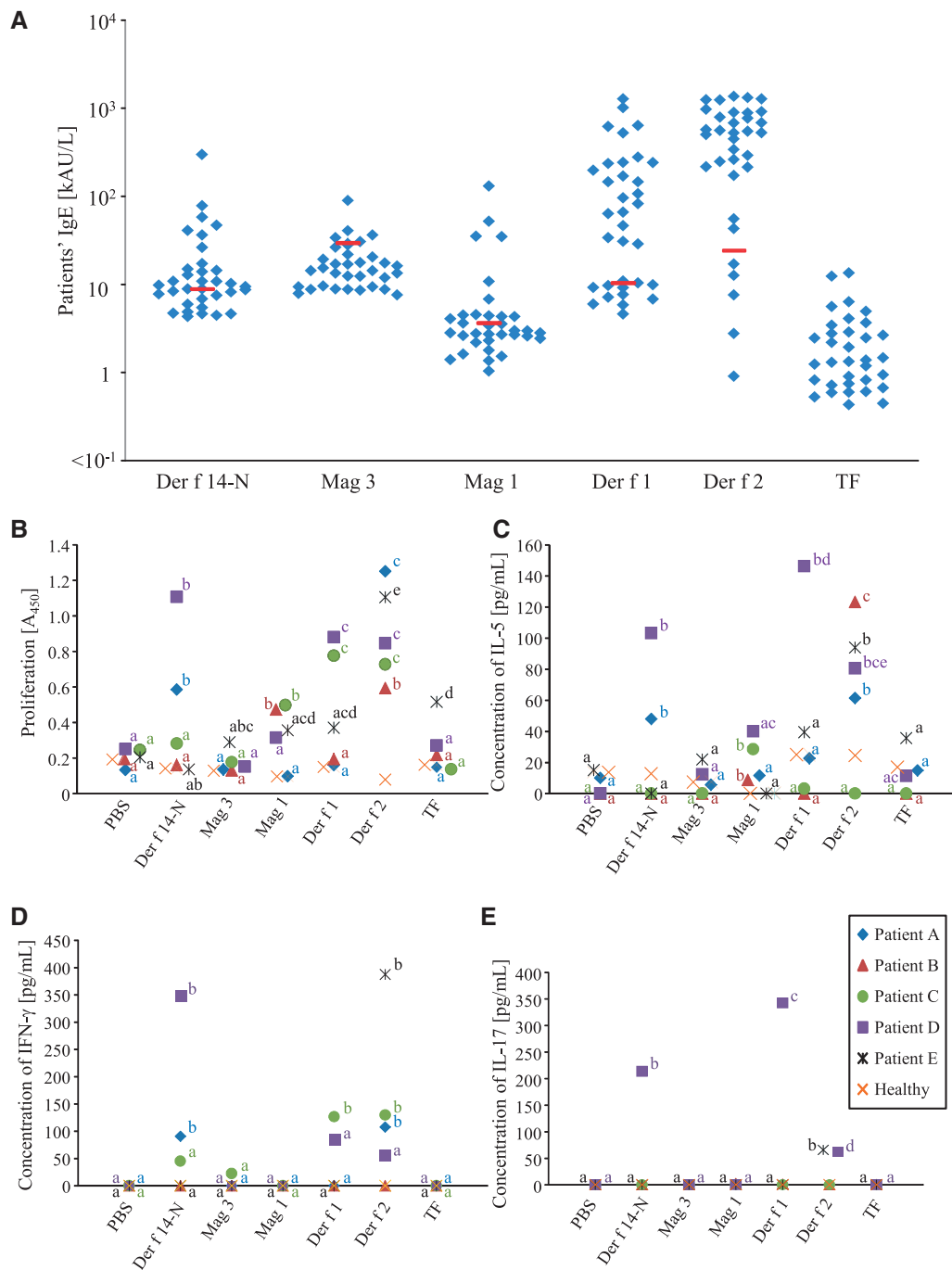


Fig. 4 Allergic capacities of Der f 14-derived fragments in human. (A) Immunoglobulin E titers specific to Der f 14-N, Mag 3, Mag 1, Der f 1, Der f 2 or TF were analysed by ELISA in 34 patients allergic to HDM and 3 non-allergic healthy volunteers. Allergen-specific IgE titers for HDM-allergic patients are plotted on the Y-axis for each allergen. We defined IgE binding of each protein as positive if a patient's specific IgE titer to each protein exceeded a threshold. The threshold of positive binding (indicated by a red bar) was defined as the mean + $(3 \times \text{SD})$ for three healthy volunteers: 8.6 kAU/l for Der f 14-N, 28.9 kAU/l for Mag 3, 3.6 kAU/l for Mag 1, 10.2 kAU/l for Der f 1 and 23.9 kAU/l for Der f 2. (B) Human PBMCs isolated from 5 HDM-allergic patients and 1 non-allergic healthy volunteer were cultured with 360 μM Der f 14-derived fragments, Der f 1, Der f 2 or TF for 3 days with 50 $\mu\text{g}/\text{ml}$ polymyxin B. Cell proliferation was estimated by BrdU incorporation in the last 18 hr of culture. Amount of incorporated BrdU determined by ELISA is plotted on the Y-axis. (C–E) Cytokine production in culture supernatant after 3 days culture of PBMCs stimulated with Der f 14-derived fragments, Der f 1, Der f 2 or TF were analysed by sandwich ELISA for each cytokine. Cytokine concentration is plotted on the Y-axis. (B–E) Statistical analysis was performed using an ANOVA with post hoc Tukey test to determine the significant differences in each allergic patient. The symbols with different superscripts are significantly different ($P < 0.05$).

production of any cytokine tested (Fig. 4C and D). Der f 1 and Der f 2 induced both IL-5 and IFN- γ production in the patients (D in Fig. 4C, and C in Fig. 4D for Der f 1; and Patients A, B, D and E in Fig. 4C and A, C and E in Fig. 4D for Der f 2). IL-17 production was

seen in patient D after stimulation with Der f 14-N, Der f 1 and Der f 2, and was seen weakly in Patient E after stimulation with Der f 2 (Fig. 4E). These results implicate Der f 14-N may be the dominant fragment to induce Th1-type and Th2-type

cytokine production among Der f 14-derived fragments in human.

Discussion

We revealed internal sequences of the N-terminal region of Der f 14 and a 49 amino acid gap sequence between Mag 1 and Mag 3 in Der f 14 (shown in black and orange in Fig. 1). Because Der f 14, originally characterized as M-177, is thought to exist as digested fragments in environmental conditions due to its susceptibility to protease digestion (6), we analysed the allergenic capacity of Der f 14 as Der f 14-derived fragments prepared as recombinant proteins: Der f 14-N, Mag 1 and Mag 3. The results showed that Der f 14's N-terminal region possesses the most potent allergenic capacity for IgE binding and to induce cell proliferation and cytokine production in mouse and human.

Der f 14 showed significant similarity to and identity with other group 14 allergens, namely Der p 14, Eur m 14 and Sar s 14 (Fig. 1). Their high primary sequence identities suggest cross-reactivity among the group 14 allergens, although the cross-reactivity among the allergens from HDM, storage mite and other mite species is controversial (24–27). In a future study, it may be interesting to analyse cross-reactivity among the group 14 allergens by using their corresponding native allergens to estimate the clinical significance of the group 14 allergens in cross-sensitization in HDM and tick mite.

In our previous papers, the IgE-binding frequencies of native Der f 14 (M-177) and glutathione-S-transferase (GST)-fused Mag 3 were 65.8% and 39% by ELISA, respectively, compared with 57% for native Der f 2 (6, 8), and the IgE-binding frequency of β Gal-fused Mag1 was 30% by immunoblotting analysis (9). In our present results, the IgE-binding frequencies of newly identified Der f 14-N (61.8%) and TF-fused Mag 1 (38.2%) were comparable to those of the reported IgE-binding frequencies: native Der f 14 (65.8%) and β Gal-fused Mag 1 (30%) (Fig. 4A and (6, 9)). However, the IgE-binding frequency of TF-fused Mag 3 (14.7%) was significantly lower than that of the reported one: 39% for GST-fused Mag 3 (Fig. 4A and (8)). This difference in the IgE-binding frequency of Mag 3 may be due to the difference in molecular structures of recombinant proteins attributed to the difference in the expression systems used to prepare the recombinant proteins. It is noteworthy that Der f 14-N showed the comparable IgE-binding frequency with native Der f 14 (M-177). This suggests that the N-terminal region (Der f 14-N) is the most important fragment for IgE binding also in undigested native Der f 14. Further studies using a full length of and deletion mutant of Der f 14 may be needed to elucidate the importance of the N-terminal region on the IgE-binding capacity of undigested Der f 14.

Apolipoporphins are involved in murine and human innate immunity, and they induce inflammatory cytokine production after recognizing invading microbes and their components (10–14). Der f 14 has a lipid-binding Vitellogenin domain profile (33–639, Prosite

entry PS51211) in the Der f 14-N region (Fig. 1), so it was hypothesized that Der f 14-N had the most potent capacity to induce cytokine production in Dfb-sensitized mouse and human. In the present results, Der f 14-N showed the highest capacity to induce Th1-type (IFN- γ), Th2-type (IL-5) and Th17-type (IL-17) cytokine production in mouse, and Th1-type (IFN- γ) and Th2-type (IL-5) cytokine production in human (Figs 3 and 4). The size of lipid vehicles co-invading with allergens is reported to influence the balance of Th1- and Th2-type immune responses in mouse (28). The size of lipid bound with the lipid-binding domain of Der f 14-N may determine whether a Th1-type or a Th2-type response will be dominant for Der f 14-specific T cells during the Der f 14 sensitization process in HDM-sensitized patients.

Der f 14-N statistically significantly induced lymphocyte proliferation in Patients A and D compared with that induced by TF (Fig. 4B–D). These data suggest that Der f 14-N possesses human T cell epitope(s) in some patient, because dominant cells proliferated in PBMC under the experimental setting is considered as T cells (29). Further analysis is needed to determine Der f 14-N's T cell epitope(s) using overlapping peptides and PBMC from HDM-allergic patients. The capacities of Der f 14 and its fragments to induce leukocyte proliferation should be further confirmed by future studies including more large numbers of HDM allergic patients.

Th2-type cytokines produced by Th2 cells or follicular T cells are needed to induce class switch recombination in naive B cells into IgE-producing B cells and are needed to promote IgE production from B cells (30, 31). Not only Th2-type cytokines but also proinflammatory cytokines, including IFN- γ and IL-17, are produced at significantly greater levels in the plasma of asthmatic patients than in that of non-allergic healthy subjects (32). In 5 HDM-allergic patients, Der f 14-N induced IL-5, IFN- γ and IL-17 production in Patient D, and IL-5 and IFN- γ production in Patient A (Fig. 4B–D). Der f 14-N's potential to induce both Th2-type and inflammatory (IFN- γ and IL-17) cytokines may contribute to its dominant IgE-binding frequency in some patients (Fig. 4A). Major HDM allergens Der f 1 and Der f 2 also induced IL-5 and IL-17 production in Patient D (Fig. 4B–D). These results suggest that Der f 14-N may contribute to progress in elucidating an asthmatic phenotype in HDM patients to a degree similar to that of Der f 1 and Der f 2.

Der f 14-N induced statistically significant amount of Th1-, Th2- and Th17-type cytokine production from Dfb/CFA-sensitized murine lymphocytes and also induced significant amount of these cytokines in some patients (IL-5 and IFN- γ from Patients A and D, and IL-17 from Patients D) (Figs 3B–E and 4C–E). Although profile of cytokines induced by an allergen is different in individuals and influenced by their clinical history, severity of clinical symptoms and pathophysiological difference (32, 33), these data suggest that Der f 14-N possessed the substantial potential to induce Th1-, Th2- and Th17-type cytokine production. Although the significant amount of cytokines is considered to be produced by antigen-specific T cells in murine lymphocytes

and human PBMC, it is possible that these cytokines may also be produced by antigen-non-specific immune cells, *e.g.* innate lymphoid cells, B cells, basophils or dendritic cells independently of antigen-specificity. Further analysis is needed to demonstrate these cytokines are produced by antigen-specific T cells as antigen-specific manner by immunohistochemical analysis or flow cytometric analysis.

In this study, we cloned the full cDNA sequence of Der f 14 and clarified the dominance of its N-terminal region as an allergen. Der f 14-N possessed the highest IgE-binding frequency among Der f 14-derived fragments and was suggested to possess the potent stimulatory capacity to induce cell proliferation and cytokine production from lymphocytes in HDM-sensitized mice and humans. Further studies are needed to demonstrate the importance of Der f 14-N as an environmental HDM allergen in more large numbers of HDM-allergic patients. We speculate that the involvement of Der f 14-N and other Der f 14-derived fragments as components of component-resolved diagnosis or towards the standardization of vaccines will enable us to more accurately diagnose HDM or to develop more effective allergen-specific immunotherapies. Further studies are also needed to elucidate the physiological contributions of Der f 14-N in the pathology of HDM-allergic patients, and to clarify the amounts of Der f 14-N and other Der f 14-derived fragments in environmental conditions.

In conclusion, the high prevalence of IgE binding to and the potential capacity to induce lymphocyte proliferation and cytokine production of Der f 14-N suggest that Der f 14-N may an important environmental allergen and that further investigations on recombinant Der f 14-derivatives and characterization of the native fragments are warranted.

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Conflict of Interest

None declared.

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