

T-cell epitope analysis of Mag 3, an important allergen from the house dust mite, *Dermatophagoides farinae*

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Abstract

Here we describe the detection of T-cell epitope region on the house dust mite allergen Mag 3, which has been shown to trigger T-cell proliferation in mite-allergic asthmatic patients. We first examined murine T-cell epitope using T-cell fraction prepared from recombinant Mag 3 (r-Mag 3)-primed H-2^k mice. Initial proliferation assay with truncated r-Mag 3 indicated that N-terminal 113 amino acid region was required for triggering T-cell activation. Subsequent epitope scanning with synthetic overlapping peptides revealed that T-cell reactive region was assigned within amino acid range 56–75. We also explored human T-cell determinant using specific T-cells from mite-allergic patients. Intriguingly, we found that amino acid range 56–85, a portion partially overlapping with that identified in r-Mag 3-primed mice, was exclusively recognized by T-cells from different patients. Further investigation of unique T-cell epitope region found in this study would provide insight into the development of animal therapeutic model and/or peptide vaccine for asthma. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Allergen; Asthma; House dust mite; Mag 3; T-cell epitope

1. Introduction

Allergens from the house dust mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major causes of allergic disorders such as bronchial asthma, rhinitis, or atopic dermatitis [1,2]. Important mite allergens have been explored via immunochemical, biochemical, and molecular biological techniques, and are now classified as major mite allergen groups. The group 1 (cysteine protease) and 2 families are recognized as primarily important allergens [3–6]. The group 3 (trypsin), 6 (chymotrypsin), and 9 (collagenase) family members, all of which belong to serine proteases, also exhibit strong allergenicity [7–12]. In addition, the groups 4 (amylase), 5, 7, 8 (glutathione *S*-transferase), and 10 (tropomyosin) members have been identified as additional major allergens [13–16].

Elucidation of T-cell activation mechanism against important mite allergen is pivotal for the development of specific immunotherapy, and T-cell epitope analysis is a primary issue of this line of study. T-cell epitope scanning of major mite allergen has been carried out exclusively on the group 1 and 2 members. However, their epitope regions are different among individuals, and/or exist on whole region of the polypeptides [17–23]. These results implicate that designing of peptide vaccine might be complicated when utilizing these family members. To achieve effective peptide-based immunotherapy against a wide variety of patients, it would be preferable to explore dominant mite allergens involved in pathogenic T-cell response, and accumulate information of their T-cell determinants. Unfortunately, however, further intensive effort to search for those important T-cell responsive mite allergens remains to be carried out.

Our group has previously cloned several cDNAs encoding additional important mite allergens [24–27], including the group 10 (tropomyosin), Mag 29 (heat

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shock protein 70 like molecule), Mag 1 (39 kDa allergen), and Mag 3. In these allergens, Mag 1 and Mag 3 have been shown to provoke specific T-cell proliferation in asthmatic patients [28]. Fujii et al. [29] have indicated that dominant T-cell epitope region for nine asthmatic patients exists on the Mag 1 polypeptide, implicating usefulness of this allergen for immunotherapy. Another T-cell responsive antigen Mag 3 shows strong T-cell stimulatory activity as comparable to major allergen Der f 2 [28], suggesting that Mag 3 also is a possible candidate for peptide vaccine. Mag 3 is the IgE-binding peptide fragment that has originally been identified via immunoscreening of *D. farinae* cDNA library with rabbit anti-mite body extract IgG and pooled IgE from allergic patients. *Escherichia coli*-produced r-Mag 3 exhibits IgE binding ability at a frequency of 39% in asthmatic patients [27]. Antiserum raised against r-Mag 3 exclusively cross-reacts a 177 kDa protein in mite body extract, suggesting that natural counterpart is a high-molecular weight allergen. We have shown that this 177 kDa natural allergen, termed M-177, has strong allergenicity as comparable to major allergen Der f 2 [27,30], and that its allergenicity significantly increases *via* naturally occurring proteolysis [31]. More importantly, a recent proteome analysis of *D. farinae* allergens via two-dimensional immunoblotting has demonstrated that Mag 3-like molecule is one of major IgE-binding component [32]. All these evidences suggest that Mag 3 is one of mite allergens having pathological importance, and that elucidation of T-cell determinant on Mag 3 molecule would provide valuable information to develop peptide-based immunotherapy. In the present study, we report T-cell epitope scanning on Mag 3 polypeptide using specific T-cells from r-Mag 3-primed mice and mite-allergic patients. We found that one amino acid region on Mag 3 polypeptide exclusively activated T-cells derived from two distinct allergic individuals, and that this epitope region was also recognized by r-Mag 3-primed murine T-cells.

2. Materials and methods

2.1. Recombinant Mag 3 and its truncated mutants

Expression vector for glutathione-S-transferase (GST)-tagged r-Mag 3 has been previously constructed as described [27]. In this plasmid, *mag 3* cDNA (1049 bp) is inserted into *Sma* I site of pGEX-2T vector (Amersham Pharmacia Biotech, Uppsala, Sweden) via blunt-ended ligation. To prepare expression vectors carrying truncated *mag 3* cDNA (scheme for plasmid construction is shown in Fig. 1A), parental GST-Mag 3 expression vector was cleaved at *Cla* I site within *mag 3* cDNA (nucleotide no. 782 of *mag 3* cDNA, [27]).

Resulting leaner plasmid was then digested by Bal 31 exonuclease (TAKARA, Kyoto, Japan) to delete 3'-*mag 3* coding sequence upstream of the *Cla* I site. In this reaction, deletion also occurs from the *Cla* I site in a sense direction; *mag 3* coding sequence (no. 782–1049) and downstream pGEX-2T vector sequence would be deleted. Bal 31 digest was further cleaved at *Pst* I site on the vector (nucleotide no. 1901) to remove pGEX-2T vector sequence downstream of the *Eco* RI cloning site (no. 940), which has been partially digested via Bal 31 nuclease. Then DNA fragment was linked with 961 bp *Eco* RI/*Pst* I pGEX-2T vector fragment at the *Pst* I site, followed by Klenow fragment-mediated end-blunting and self-ligation to generate deletion constructs.

E. coli BL-21 strain was transformed with resulting expression plasmids, and individual transformant was cultured in 2 × YT medium supplemented with 0.1% isopropyl β-D thiogalactoside (IPTG) to express GST-fusion protein. Purification of fusion protein was carried out using a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech) as described [14]. Prior to final purification of recombinant allergens for T-cell stimulation assay, purified GST-fusion proteins were digested by thrombin (10 U/mg protein) to remove GST tag. Then the thrombin digest was passed through the Glutathione Sepharose affinity column to obtain purified recombinant allergens.

2.2. SDS-PAGE and immunoblotting

Protein samples were resolved by SDS-PAGE according to the discontinuous buffer system by Laemmli [33] on 12.5% acrylamide slab gel. Fractionated proteins were then visualized by 0.25% Coomassie Brilliant Blue (CBB) staining. For immunoblotting, fractionated proteins in the gel were electronically transferred onto a polyvinylidene difluoride (PVDF) filter (Immobilon P, Millipore, MA), and stained with primary rabbit anti-Mag 3 serum IgG previously prepared [27]. Then the blot was stained with secondary peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA). Protein bands were visualized using Immunostaining Kit (Konica, Tokyo, Japan).

2.3. Mice and immunization

Eight to twelve weeks old female C3H/HEJ mice (H-2^k) were purchased from Charles River Japan (Yokohama, Japan). These mice were immunized via foot pats (5 µg/pat) and intraperitoneally (10 µg) with total 20 µg of purified r-Mag 3, which was pre-adsorbed with Freund's complete adjuvant (Difco laboratories, Detroit, MI, USA). After 1 week, mice were sacrificed and T-cell fraction was purified from lymph node

mononuclear cells using a nylon wool column as described [34].

3. T-cell stimulation assay

Prior to performing T-cell stimulation assay, we prepared polyclonal r-Mag 3-specific T-cell lines. To establish murine T-cell line, 2×10^5 lymph node T-cells from r-Mag 3-immunized mice were mixed with r-Mag 3 (5 $\mu\text{g/ml}$) and 2×10^5 mitomycin C (MMC)-treated splenocytes from non-immunized mice as antigen presenting cells (APC). Then the cells were cultured for 5 days in AIM-V serum-free medium (GIBCO/BRL, Grand Island, NY, USA) at 37°C , 5% CO_2 /95% atmosphere. After this stimulation, expanded antigen-specific T-cells were maintained by standard procedure as described [34]. To prepare human T-cell lines, 2×10^5 peripheral blood mononuclear cells (PBMC) from newly diagnosed mite-allergic asthmatic donors (judged by skin test, diameter of erythema > 20 mm) were stimulated with r-Mag 3 (5 $\mu\text{g/ml}$) for 7 days in AIM-V medium. Then proliferating cells (2×10^5) were co-cul-

tured for additional 7 days with antigens, r-human IL-2 (10 U/ml, Genzyme, Cambridge, MA, USA), and freshly prepared 2×10^5 MMC-treated PBMC as APC. To analyze murine or human T-cell response, 2×10^5 T-cell line was cultured with 2×10^5 fresh APC and antigens (5 $\mu\text{g/ml}$ recombinant antigens, or 1 $\mu\text{g/ml}$ synthetic peptides) for 5 days. During the last 18 h, cells were pulsed with 18.5 kBq of tritiated thymidine ($[^3\text{H}]$ -TdR, specific activity of 222 GBq/mmol, Amersham Pharmacia Biotech). After cells were collected using a semiautomatic cell harvester (Abe Kagaku, Chiba, Japan), incorporated $[^3\text{H}]$ -TdR in the cells was measured by liquid scintillation counter [35].

4. Peptide synthesis

Peptide synthesis was performed using materials and instruction from Chiron Technologies (Clayton South, Australia). 15-mer synthetic peptides, which were 10-mer overlapping at five amino acid intervals (total 21 peptides; from p1–15 to p101–115), were synthesized to encompass entire N-terminal 113 amino acid region of Mag 3 polypeptide.

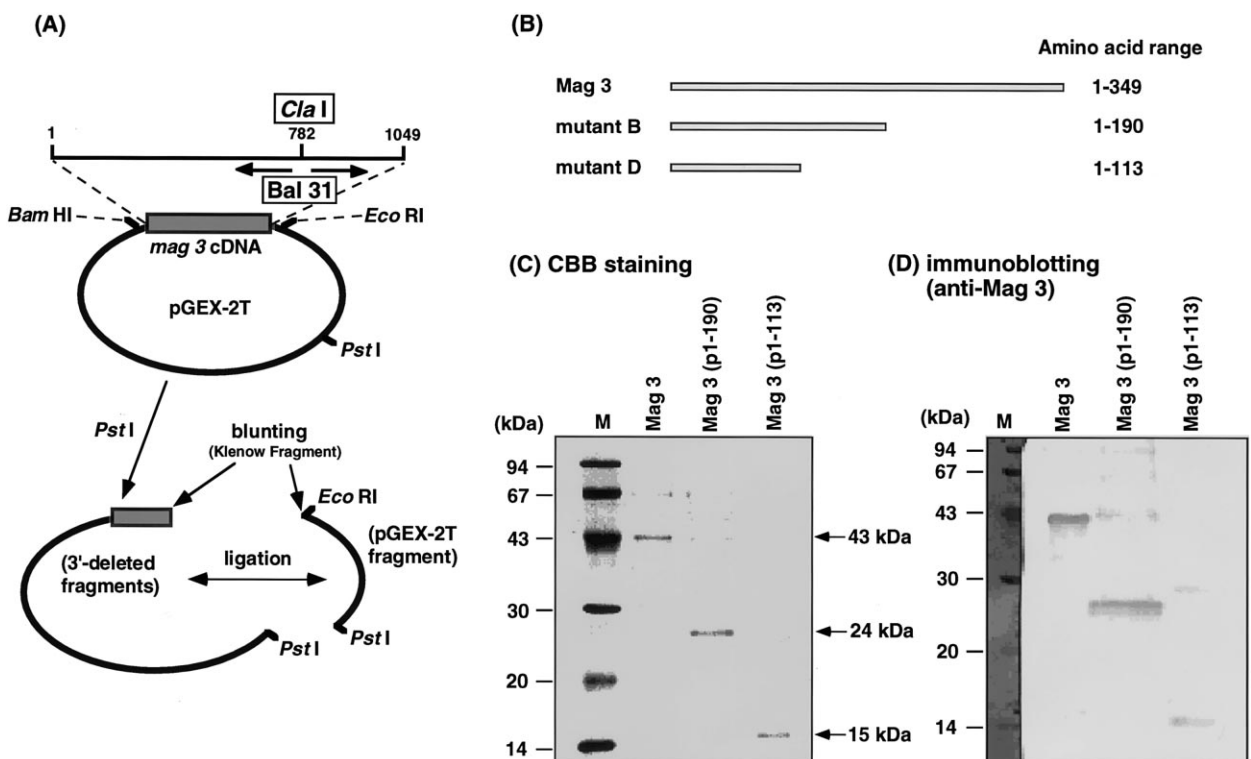


Fig. 1. (A) Scheme for construction of truncated r-Mag 3 expression vector. GST-fusion vector pGEX-2T carrying *mag 3* cDNA was cleaved at internal *Cla* I site, and then digested by *Bal* 31 nuclease for 3'-deletion. *Pst* I-cleaved *Bal* 31 digest was then ligated with *Eco* RI/*Pst* I vector fragment. (B) Schematic representation of Mag 3 polypeptide (349 amino acids) and its C-terminal truncated mutants B and D (190 and 113 amino acids, respectively). DNA sequencing analysis (data not shown) estimated amino acid range of the truncated mutants. (C) SDS-PAGE analysis of purified r-Mag 3 and C-terminal truncated mutants. Purified recombinant proteins (0.5 μg each) were run on a 12.5% polyacrylamide gel, and then visualized by CBB staining. M; molecular weight marker. (D) Immunoblot analysis of purified recombinant allergens by rabbit anti-Mag 3 IgG. After SDS-PAGE shown in (C) was finished, fractionated protein on the gel was transferred onto a PVDF filter, and then stained with anti-Mag 3 antibody. M; CBB staining of molecular weight marker simultaneously transferred onto the filter.

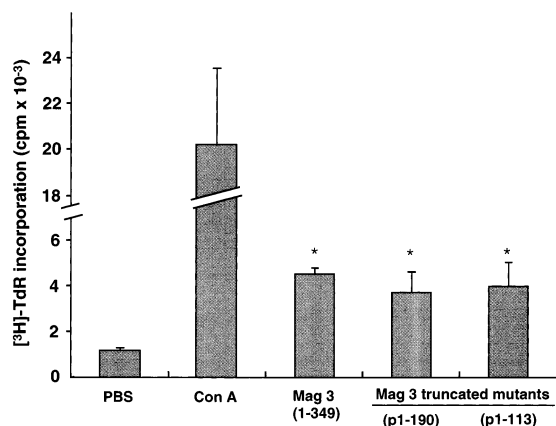


Fig. 2. Proliferative response of r-Mag 3-primed murine T-cells against truncated r-Mag 3. 2×10^5 polyclonal T-cell line was stimulated for 5 days by Con A (10 μ g/ml) or purified recombinant allergens (5 μ g/ml each) together with 2×10^5 MMC-treated syngenic splenocytes as APC. Each sample was tested in triplicate, and data was indicated as mean $\pm$ S.E.M. of [³H]-TdR incorporation. *Significantly proliferated as compared with control phosphate buffered saline (PBS)-supplemented culture ($P < 0.05$).

5. Statistical analysis

Statistical analysis was performed using Student's *t*-test [36]. A confidence level of $P < 0.05$ was evaluated as significant. All experiments were repeated at least three times to confirm their reproducibility, and one typical result was shown as a figure.

6. Results

6.1. Expression and purification of r-Mag 3 and its truncated mutants

To prepare C-terminal truncated r-Mag 3, we deleted 3'-coding sequence of *mag 3* cDNA by Bal 31 exonuclease digestion (as shown in Fig. 1A). Resulting GST-fused deleted constructs were then introduced into *E. coli* BL-21 strain to express recombinant GST-fusion protein. We confirmed successful expression of GST-Mag 3 and truncated mutants by SDS-PAGE and anti-Mag 3 immunoblot assay (data not shown). Among several truncated mutants obtained, we chose two mutants (mutant B and D, schematic map is shown in Fig. 1B) for further analysis, since their molecular weights (24 and 15 kDa; preliminary assessment by SDS-PAGE) were appropriate for initial epitope scanning. DNA sequencing analysis of these two mutants (data not shown) indicated that their amino acid ranges were 1–190 and 1–113, respectively. After primary purification of fusion protein via Glutathione Sepharose affinity chromatography, GST-Mag 3 and two GST-fused deletion mutants were digested with thrombin to remove the GST-tag. Then the thrombin

digest was applied through a Glutathione Sepharose column to purify recombinant proteins. SDS-PAGE analysis of purified 43 kDa r-Mag 3 and its C-terminal truncated 24 and 15 kDa mutants are indicated in Fig. 1C. Anti-Mag 3 immunoblot assay (Fig. 1D) and N-terminal amino acid sequencing analysis (data not shown) demonstrated that these recombinant proteins are Mag 3 and its derivatives.

6.2. N-terminal 113 amino acid portion of r-Mag 3 is required for triggering T-cell activation in r-Mag 3-immunized H-2^k mice

To assess T-cell reactive region on the Mag 3 polypeptide, we first tested proliferative response of T-cell line prepared from r-Mag 3-immunized C3H/HeJ mice (H-2^k haplotype). The reason why we chose endotoxin-hyporesponsive C3H/HeJ strain was to minimize the possibility of undesired effect of lipopolysaccharide, which might be contaminated in our recombinant allergen preparation from *E. coli* expression system. As shown in Fig. 2, murine r-Mag 3-primed T-cells significantly proliferated (indicated by asterisks; $P < 0.05$, as compared with control PBS-supplemented culture) in response to both of two truncated mutants, p1–190 and p1–113. Their proliferative response was comparable to that observed in parental r-Mag 3-stimulated culture. From these results, murine T-cell reactive region was found to reside within N-terminal 113 amino acids of Mag 3 polypeptide.

6.3. Assignment of murine T-cell epitope region using synthetic overlapping peptides

To further assign murine T-cell determinant region, we next performed stimulation assay using 21 synthetic overlapping peptides (p1–15 to p101–115; 15-mer, 10-mer overlapping at five amino acids intervals) which cover entire N-terminal 113 residues of the Mag 3 molecule. As shown in Fig. 3, significant T-cell response (shown by asterisks; $P < 0.05$, as compared with control PBS-supplemented culture) was observed in culture wells stimulated with peptides p56–70 and p61–75. In contrast, no significant proliferative response was observed in wells stimulated with other peptide samples. These results suggest that murine (C3H background) T-cell determinant was assigned within amino acid range 56–75 of the Mag 3 molecule.

6.4. T-cell reactive region for mite-allergic patients is partially overlapping with those identified in r-Mag 3-primed H-2^k mice

We next explored human T-cell determinant using two mite-sensitized asthmatic donors responsive to r-Mag 3. Proliferation assay with r-Mag 3 and two

truncated mutants (p1–190 and p1–113) revealed that both of the two mutants were able to trigger significant T-cell proliferation (indicated by asterisks; $P < 0.05$, as compared with PBS-added culture) as comparable to parental r-Mag 3 (Fig. 4). This proliferative response was evident in both patient cases A and B. These results suggest that Mag 3 T-cell determinant region for these mite-allergic individuals, as also seen in r-Mag 3-immunized H-2^k mice, resides within N terminal 113 amino acids of the Mag 3 polypeptide.

To scan T-cell responsive region on the N-terminal 113 amino acids, we subsequently performed stimulation assay with the same overlapping peptides as used in murine epitope analysis. In the patient case A, significant T-cell proliferation ($P < 0.05$, as compared with PBS-supplemented culture) was observed when stimulated with peptides p56–70, p61–75, and p71–85 (Fig. 5). In another patient case B, we also found a similar stimulatory profile to that seen in patient A; significant T-cell activation was triggered in cultures stimulated with p56–70, p61–75, and p71–85 (Fig. 5). In contrast, no significant proliferative response was detected against other peptides including internal p66–80 in both of the patients. These results suggest that, at least in these two Mag 3 responsive allergic donors we tested, Mag 3 T-cell reactive region is assigned within amino acid range 56–85, a portion partially overlapping with that identified in r-Mag 3-primed H-2^k mice (56–75).

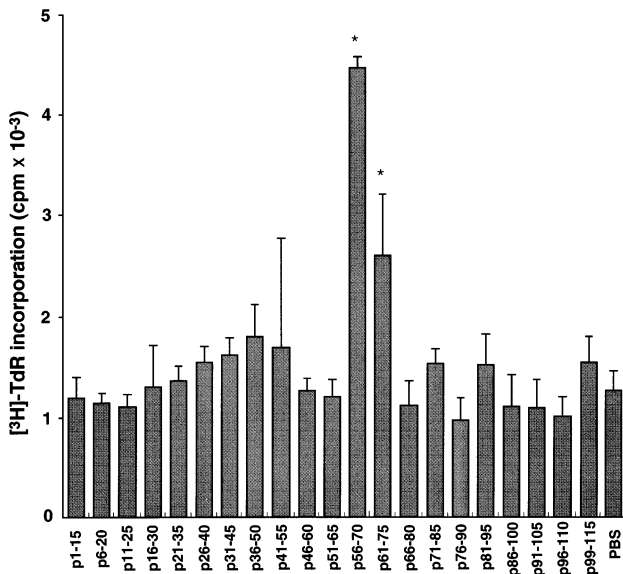


Fig. 3. Murine T-cell epitope scanning via synthetic overlapping peptides. 2×10^5 polyclonal T-cell line was cultured for 5 days with 2×10^5 APC and 21 overlapping peptides (1 μ g/ml each) which cover N-terminal 113 amino acids of Mag 3 polypeptide. *Significantly responded as compared with control PBS-added culture ($P < 0.05$).

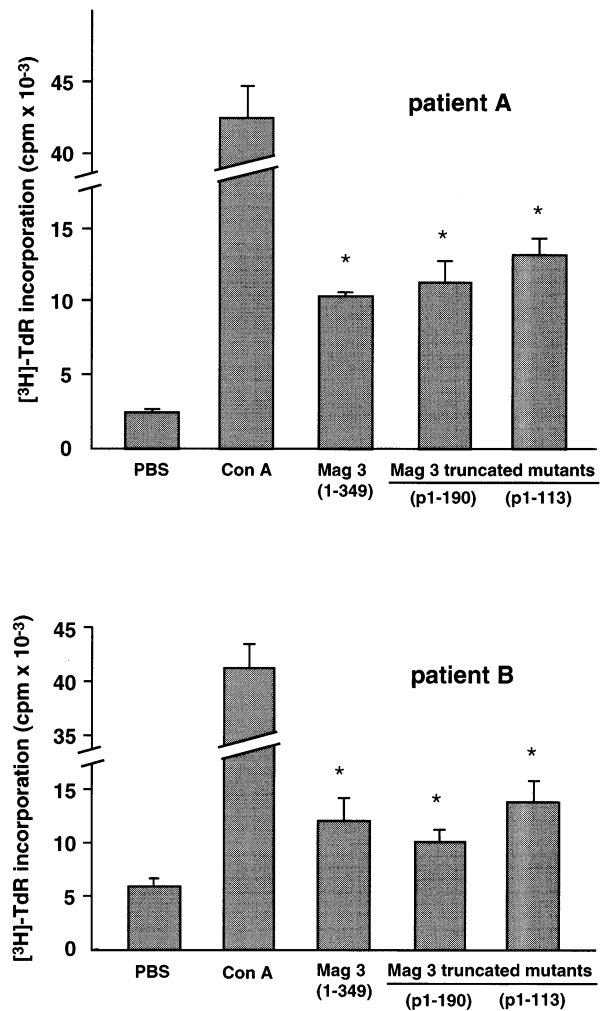


Fig. 4. T-cell determinant region for mite-allergic patients also resides within N-terminal 113 amino acids of Mag 3 polypeptide. 2×10^5 patients' T-cell line and 2×10^5 MMC-treated PBMC from the same donor (as APC) were stimulated with Con A (10 μ g/ml) or purified recombinant allergens (5 μ g/ml each) for 5 days. Two r-Mag 3-responsive patient cases (A and B) were examined. *Significantly proliferated as compared with control PBS-supplemented culture ($P < 0.05$).

7. Discussion

In previous studies, our group has surveyed human T-cell response against several recombinant mite antigens [28], and found two T-cell-responsive allergens, Mag 1 [26] and Mag 3 [27], both of whose primary structures are distinct from classified major mite allergens. Especially, Mag 3 has been shown to provoke strong T-cell proliferation as comparable to major allergen Der f 2 [28], suggesting that Mag 3 is a valuable antigen not only to elucidate T-cell activation mechanism in mite-allergic patients, but also to design peptide vaccine for treatment of asthma. As the first step to address these issues, here we analyzed human and murine T-cell reactive region on the Mag 3 polypeptide.

Polyclonal T-cell line from r-Mag 3-primed H-2^k mice responded against amino acid range 56–75 (Fig. 3), suggesting that T-cell determinant for C3H background exists on this region. In contrast to our results on Mag 3, murine T-cell epitope on the group 1 and 2 allergens has been reported to be heterogeneous. Epitopes on Der p 1 have been assigned in amino acid range 21–49, 78–100, 110–131, and 197–202 for H-2^b mice [37], whereas H-2^d-restricted Der p 1 epitope has been found within 111–139 [38]. Hoyne et al. [39] have reported that Der p 2 responsive H-2^b murine T-cells recognize 11–35, 78–104 and 105–129, and that epitopes for H-2^k mice are within 36–50 and 78–104. Our result that Mag 3-primed T-cells recognize relatively restricted range on the polypeptide suggests that Mag 3 is useful mite allergen for construction of simplified T-cell-based murine therapeutic model when utilizing the H-2^k background.

Epitope scanning with two mite-allergic patients indicated that amino acid range 56–85 serves as T-cell responsive region (Fig. 5), implicating that Mag 3 also contains T-cell determinants for allergic patients. Identified human T-cell determinants residing on the group 1 and 2 allergens seem to be more heterogeneous than those found in allergen primed mice; Der p 1 epitope has been identified within multiple regions or whole

region of the polypeptide [17–20]. Der p 2 also has been reported to contain multiple human T-cell determinants throughout the polypeptide [21–23]. To develop effective peptide vaccine, identification of dominant T-cell epitope for a variety of patients is preferable. In this regard, Mag 3 could be relatively applicable mite antigen to design peptide vaccine, although further inspection against increased patient cases should be required.

Our data that N-terminal 113 amino acid region of Mag 3 is still capable of murine and human T-cell activation indicate that p114–349 is dispensable for provoking T-cell proliferation. However, a possibility remains that this C-terminal portion might include T-cell determinant other than our results. To exclude the case that T-cell response against truncated mutants (p1–113 and p1–190) might impair or abolish when tested in an equal epitope number-condition, we also performed stimulation assay at equivalent antigen molar (200 nM). But we observed no obvious difference in T-cell response as compared with that performed in an equal protein weight (5 µg) shown in Figs. 2 and 4 (data not shown). This clearly rules out the case raised above, although we cannot completely exclude the possibility that p114–349 has other T-cell determinants. Epitope scanning in p114–349 should therefore be tested to assess this point, together with study on fine epitope identification of Mag 3 polypeptide.

T-cell lines from allergic donors responded to amino acid range 56–85 (shown in Fig. 5), which is slightly broader than that observed in r-Mag 3-immunized mice (amino acids 56–75, in Fig. 3). In these donors, multiple T-cell repertoire recognizing distinct T-cell determinants might participate in the proliferative response. We found significant cell proliferation when stimulated with the p56–70, p61–75, or p71–85, whereas no T-cell response was observed against p66–80, which is 10-mer overlapping with the T-cell responsive p61–75 or p71–85 peptides. Although the reason why this internal p66–80 was negative for T-cell activation is currently unknown, several explanations could be possible. One case is that anti-p61–75 T-cell repertoire recognizing 66–75 cannot respond to p66–80 owing to the existence of non-overlapping extra five amino acids (i.e. amino acid range 76–80) which exert inhibitory effect on T-cell activation. Alternatively, p66–80 might contain only partial T-cell determinant for anti-p71–85 repertoire, and therefore is insufficient for triggering cell proliferation of p71–85-responsive T-cells. It is also possible that antigen-specific unresponsiveness, growth arrest, or cell death might be induced by p66–80 stimulation. Elucidation of T-cell responses via Mag 3-specific T-cell clones would clarify these questions.

It is intriguing that human T-cell lines from distinct patients respond exclusively to amino acid range 56–75, which is also T-cell reactive region for Mag 3-im-

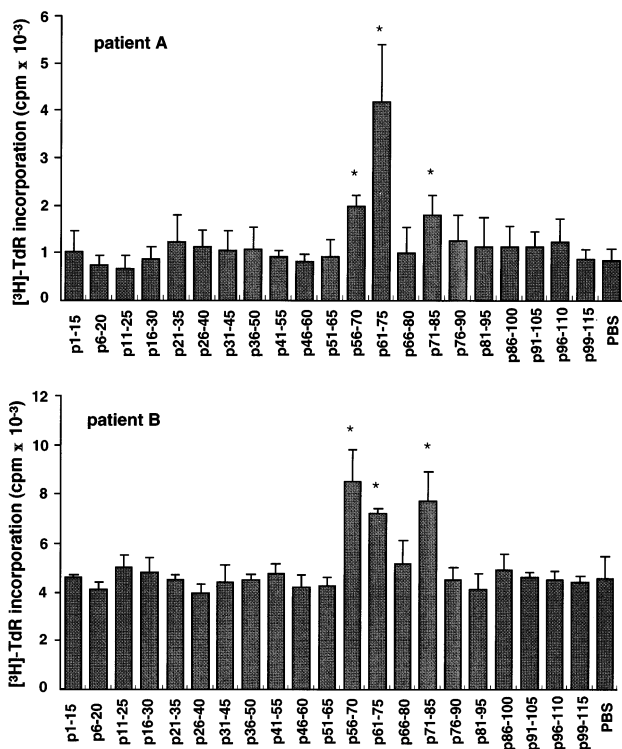


Fig. 5. Detection of human T-cell epitope via synthetic overlapping peptides. 2×10^5 patients' T-cell line and 2×10^5 APC were stimulated with 1 µg/ml overlapping peptides (also used in murine T-cell epitope scanning, Fig. 3) for 5 days. *Significantly proliferated as compared with control PBS-added culture ($P < 0.05$).

munized H-2^k mice. This raises a possibility that T-cell determinant in 56–75 is shared within human and mice, despite their difference in major histocompatibility complex (MHC) structure. Evidence for such cross-recognition of murine H-2-peptide complex by human leukocyte antigen (HLA)-restricted T-cells has been reported [40]. Given that Mag 3 T-cell determinant structure or peptide-MHC-T cell receptor interaction profile is similar within these species, it would be possible to set a practical murine in vivo therapeutic model that mimics human T-cell recognition and/or activation. Overlapping p56–75 region could be applicable for performing pilot animal therapeutic trial via peptide modification. We also observed that T-cell-responsive profile against overlapping peptides was quite similar within allergic donors we tested; both of the patients' T-cells were activated in response to p56–70, p61–75, and p71–85, whereas they showed no cell proliferation by p66–80 stimulation (Fig. 5). This leads us to anticipate the existence of shared T-cell determinants for distinct HLA haplotypes. In fact, our group has previously demonstrated that T-cell responsive mite allergen Mag 1 also has immunodominant T-cell epitope region shared within nine allergic patients [29]. To assess above possibilities, fine T-cell determinant mapping for allergic patients or r-Mag 3-primed mice is now in progress using deleted or altered analog peptides. We are also trying to establish a panel of Mag 3-specific T-cell clones. These analyses would facilitate to test whether there are any analogies and/or correlation in T-cell recognition within distinct species or HLA haplotypes, and also provide valuable insight into designing of peptide vaccine for immunotherapy. Our preliminary observation indicated that T-cell response to r-Mag 3 in one mite-allergic donor (patient A in the present study) was accompanied by interferon- γ production whereas no interleukin-4 secretion (data not shown). This implies that Mag 3 is a potent mite antigen that predisposes patients' helper T (Th) cell balance to polarize anti-allergic Th1 phenotype.

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