

T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*

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Summary

Background The dust mite *Lepidoglyphus destructor* is an important cause of allergic reactions to dust, especially in farming environments. Two isoforms, recombinant (r)Lep d 2.01 and rLep d 2.02, of the major allergen Lep d 2, have previously been expressed as recombinant proteins. These isoforms differ 10.4% at the amino acid level. Furthermore, a mutant form of Lep d 2.01 (rLep d 2.6Cys) with a highly reduced IgE reactivity, has also been produced.

Objective To investigate the T cell responses to the recombinant isoforms of Lep d 2, the Lep d 2.6Cys mutant and peptides of Lep d 2, in allergic and non-allergic individuals.

Methods Peripheral blood mononuclear cells from 18 allergic and 16 non-allergic individuals were stimulated with the different antigens and the proliferative responses were measured. The cytokine production (interleukin (IL)-4, IL-5 and interferon (IFN)- γ) were measured by ELISA.

Results Higher T cell proliferation was measured to isoform 01 than to 02 in 28/34 subjects. The responses to rLep d 2.6Cys were lower than to isoform 01 in most subjects, but higher than to Lep d 2.02. Two immuno-dominant peptides, corresponding to amino acid residue 11–25 and 61–75 were identified. The atopic subjects produced significantly lower IFN- γ in response to Lep d 2.01 as compared to the non-atopics.

Conclusions There was a significant difference in T cell response between the two isoforms of rLep d 2. The hypoallergenic mutant rLep d 2.6Cys was able to evoke a T cell response with a magnitude which is between the two isoforms. Amino acid residue 11–25 and 61–75 are the most frequently recognized parts of Lep d 2 and are likely to contain the immuno-dominant T cell epitopes.

Keywords: mite, *Lepidoglyphus destructor*, Lep d 2, recombinant allergen, T cells, mutant allergen, isoforms, peptides, cytokines

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Introduction

Allergic reactions to mites is a great problem, affecting people all over the world [1]. The dust mites, including

house dust mites and the formerly designated storage mites, have been extensively studied and characterized and it has been shown that the majority of mite allergic subjects are sensitized to the group 1 and group 2 allergens [2–4]. The dust mite *Lepidoglyphus destructor* is an important cause of allergic reactions to dust in farming environments.

The group 1 mite allergens have been shown to be

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cysteine proteases [5] while the biological function of the group 2 allergens remains unknown. The group 2 allergens have a calculated molecular mass of approximately 14 kDa and have a structure that is stabilized by three disulphide bonds [6]. The group 2 allergens have been shown to be readily expressed as recombinant proteins in *E. coli* with preserved IgE reactivities [7–10].

We have previously cloned [11] and expressed two isoforms of Lep d 2, recombinant (r)Lep d 2.01 and rLep d 2.02, in *E. coli* as well as in a baculovirus system [9]. The deduced amino acid sequence of Lep d 2.02 differs in 13 residues out of 125 (10.4%) as compared to Lep d 2.01. No significant difference in IgE binding between the two isoforms was found when compared *in vitro* by immunoblotting, RAST inhibition, histamine release and ELISA [9] or *in vivo* by skin prick test [12].

Well-characterized recombinant allergens are valuable tools for improved diagnostics and for therapy of allergy, since they provide pure and well-characterized allergens in contrast to the crude allergen extracts in use today. In addition, modified recombinant allergens with reduced IgE reactivity, are believed to be potential tools for improved specific immunotherapy if it can be shown that they evoke T cell responses. The use of hypoallergenic modified allergens would increase the safety of immunotherapy by reducing the risks of anaphylactic reactions and also give the possibility of applying higher allergen doses, which may be of benefit for the clinical outcome of the treatment [13]. Mutants of recombinant Der p 2, Der f 2 and Lep d 2 have been produced where the cysteine residues in the allergens have been changed by site-directed mutagenesis, leading to altered structures due to disruption in the formation of disulphide bonds. This has resulted in various degrees of reduction in IgE binding [14–16]. We have recently produced several different mutants of rLep d 2, which demonstrated different reactivities with IgE antibodies [16]. One mutant form of Lep d 2.01, rLep d 2.6Cys, lacking all the 3 naturally occurring disulphide bonds, has no detectable IgE reactivity in immunoblotting and demonstrated a more than 100-fold reduced IgE binding in RAST-inhibition experiments as compared to the wild-type non-mutated rLep d 2. Furthermore, 15/18 rLep d 2 skin prick test positive subjects showed no skin reaction to the rLep d 2.6Cys mutant (Kronqvist, unpublished data).

T cell epitope mapping has been performed for other group 2 mite allergens and van Neerven *et al.* [17] found that Der p 2 contains multiple T cell epitopes that may differ from individual to individual and that T cell lines from five atopic individuals reacted with at least 10 different epitopes on the molecule. O'Hehir *et al.* [18] found that T cell epitopes are present in all regions of Der p 2 but peptides 61–86 and 78–104 were the most frequently

recognized. In addition, half of the patients responded to T cell epitopes in the region 11–50 of Der p 2.

In this study, we carefully evaluated the T cell response to Lep d 2 using the two recombinant isoforms of Lep d 2, the hypoallergenic rLep d 2.6Cys mutant, non-IgE binding peptides of Lep d 2 and partly purified native Lep d 2.

Materials and methods

Atopic patients and healthy controls

The patients included in the study were 18 farmers (mean age 44 years, range 31–58 years) from the Swedish island of Gotland, all with an occupational exposure to *L. destructor*. They were all skin prick test positive to rLep d 2.01 and rLep d 2.02 as well as *L. destructor* whole extract [12]. As control subjects, 16 non-atopic healthy individuals (mean age 30 years; range 21–46 years) were used.

The Ethics committee of the Karolinska Institutet, Stockholm, Sweden, approved the study.

Stimulating antigens

Partially purified native (n)Lep d 2 was prepared from *L. destructor* extract by precipitation with 3.3 M ammonium sulphate as described earlier [9].

Recombinant Lep d 2.01 and rLep d 2.02, including C-terminal 6Histidine-tags, were produced as previously described in detail [9] using the expression vector pET-17b (Novagen, R & D systems, Abingdon, UK) in the *E. coli* expression strain BL21(DE3)pLysS (Stratagene, La Jolla, CA, USA). The rLep d 2.6Cys mutant was produced by oligonucleotide-directed mutagenesis of the cDNA encoding isoform Lep d 2.01 as described previously [16] in the phage vector system M13mp19, according to the method of Kunkel [19] as modified by Geisseloder [20]. Oligonucleotides were designed to introduce altered bases at the 6 cysteine codons, resulting in serine codons and thereby preventing the three naturally occurring disulphide bonds to form in the mutant protein. Expression of rLep d 2.6Cys was performed as described earlier [16].

Thirteen synthetic peptides were used in this study, each was 15 amino acid residues in length and with 5 amino acid residues overlap in each end, Fig. 1. These peptides, synthesized by MTC peptide Synthesis (Karolinska Institutet, Stockholm, Sweden), have previously been investigated in a separate study and shown to be non IgE-binding [21].

All antigens were diluted in sterile phosphate buffered saline (PBS). Five µg/mL of phytohaemagglutinin (PHA) (Pharmacia Biotech, Uppsala, Sweden) were used as a

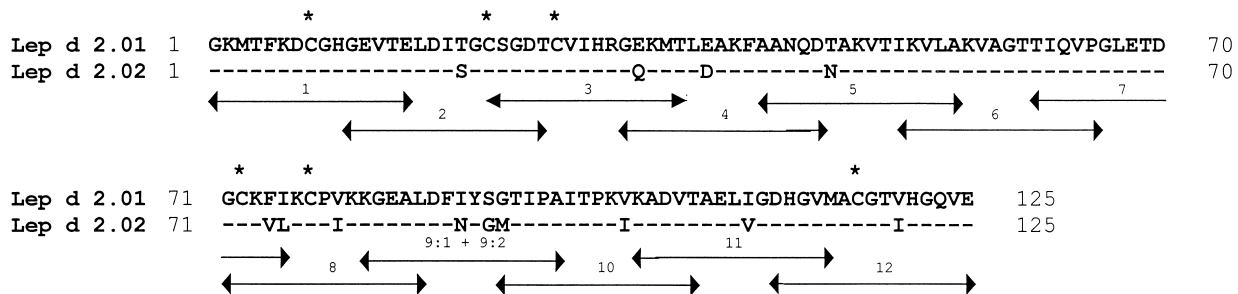


Fig. 1. Synthetic peptides of Lep d 2 used for stimulation of PBMCs. The peptides were 15 amino acids in length and were overlapping by 5 amino acids at each end. The peptides were designed to isoform Lep d 2.01 except for peptide 9, which were synthesized in two variants i. e 9 : 1 corresponding to Lep d 2.01 and 9 : 2 corresponding to Lep d 2.02. Dashes (-) represent amino acids that are conserved between the isoforms. The cysteine residues are indicated with asterisks.

positive control in the proliferation assays and PBS and medium alone were used as negative controls.

Proliferation assay of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on Ficoll Paque (Pharmacia Biotech) and cultured in round-bottomed 96-well culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) at 2×10^5 cells/well in Iscove's modified Dulbecco's medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 5% pooled heat-inactivated human AB⁺ sera, 25 µg/mL gentamicin, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercapto-ethanol. The PBMCs were stimulated with two concentrations of the antigens (1 and 10 µg/mL) and grown for 7 days (37 °C, 6% CO₂). During the last 18 h of culture, 1.0 µCi/well of [³H]thymidine (Amersham, Buckinghamshire, UK) was added to each well and the thymidine incorporation was determined by scintillation counting. Triplicate cultures were set up and the results were expressed as mean cpm values and as stimulation indexes (SI) which were calculated as mean cpm for antigen-stimulated PBMCs divided by mean cpm for PBMC cultures in medium alone. Table 1 shows the highest SI obtained, regardless of the concentration (1 µg/mL or 10 µg/mL) used. In the majority of cases, the highest proliferation was achieved with 10 µg/mL.

Initially, T cell lines were established and stimulated with antigen in the presence of autologous irradiated PBMCs. However, since high background proliferation was obtained, leading to low SI values for the whole allergens and complete masking of the peptide responses, PBMC cultures were used throughout the study. The T cell responses to the peptides were also masked by the presence of human AB sera in the culture media and therefore, the peptide studies were carried out in serum-free medium.

Consequently, PBMCs (2×10^5 cells/well) were cultured in Aim-V serum free medium (Gibco BRL) for 7 days (37 °C, 6% CO₂) in the presence of 100 µg/mL of the respective peptide. The proliferative responses to the peptides were carried out using thawed cells and the proliferation were determined as described above.

Cultures for cytokine production

Long-term PBMC cultures were grown to obtain supernatants for cytokine measurement [22]. PBMCs (2.5×10^6 cells/well) were cultured in Iscove's modified Dulbecco's medium (Gibco BRL) with the supplements described above, in flat-bottomed 12 well culture plates (2.5 mL/well) (Falcon), and were stimulated with rLep d 2.01 (10 µg/mL). At day 5 of culturing, 20 U/mL of interleukin (IL)-2 (Pharmacia Biotech) was added and the cells were cultured for another 3 days when 20 U/mL of IL-2 was added together with 1.5 mL of fresh media. The supernatants were then collected at day 11 and kept in -20 °C until assessment.

Cytokine measurements

IL-4, IL-5 and interferon (IFN)-γ were measured in long-term PBMC culture supernatants by solid phase ELISA using the CytoscreenTM Human IL-4 Ultrasensitive kit (Biosource, Camarillo, CA, USA), CytoscreenTM Human IL-5 kit (Biosource) and the QantikineR Human IFN-γ, Immunoassay kit (RD Systems, Minneapolis, MN, USA), respectively, following the manufacturers protocols. The detection range for the IL-4 assay was 0.39–25 pg/mL, 4.0–750 pg/mL for the IL-5 assay and 3.0–10 000 pg/mL for the IFN-γ assay. The samples were analysed in duplicates. Samples with concentrations above the upper detection range were further diluted and re-analysed.

Table 1. Stimulation indexes to the Lep d 2 variants measured after 7 days of culture, serum levels of *L. destructor* specific IgE and skin prick test to rLep d 2.01

Subject	rLep d 2.01 (SI)	rLep d 2.02 (SI)	rLep d 2.6Cys (SI)	nLep d 2* (SI)	sIgE <i>L. destructor</i> kU/L†	SPT rLep d 2.01
1	2.4	1.6	1.6	nd	1.87	3 +
2	34	11.9	22	18	3.0	3 +
3	3.0	1.3	3.1	nd	3.21	4 +
4	4.0	1.1	2.5	nd	0.37	2 +
5	8.5	3.2	1.8	nd	1.51	3 +
6	4.7	2.5	4.7	5.1	0.42	3 +
7	12	3.6	3.7	nd	< 0.35	2 +
8	2.1	0.8	3.4	nd	1.96	3 +
9	3.0	1.0	1.9	nd	5.04	3 +
10	4.8	2.9	4.0	4.3	8.47	3 +
11	4.7	1.9	2.8	5.0	2.12	3 +
12	8.2	2.5	3.9	nd	1.8	3 +
13	0.2	0.6	0.2	nd	0.66	3 +
14	1.0	0.7	0.9	nd	3.8	3 +
15	3.2	0.7	0.9	nd	< 0.35	3 +
16	2.8	1.0	1.1	12.3	0.58	3 +
17	5.8	3.2	6.8	nd	12.6	4 +
18	5.0	1.5	4.3	6.4	3.92	4 +
na 1	23.7	24.5	30.7	nd	< 0.35	nd
na 2	0.7	0.9	0.7	nd	< 0.35	nd
na 3	2.6	2.2	1.1	nd	< 0.35	nd
na 4	7.4	6.7	3.1	nd	< 0.35	nd
na 5	2.6	7.0	1.1	nd	< 0.35	nd
na 6	2.9	3.0	4.4	nd	< 0.35	nd
na 7	2.7	2.4	1.6	nd	< 0.35	nd
na 8	2.1	2.0	1.1	nd	< 0.35	nd
na 9	1.8	1.6	1.2	nd	< 0.35	nd
na 10	3.9	2.2	2.9	nd	< 0.35	nd
na 11	4.5	1.4	2.5	nd	< 0.35	nd
na 12	2.2	1.4	1.4	nd	< 0.35	nd
na 13	6.1	2.0	4.1	nd	< 0.35	nd
na 14	5.0	4.3	4.0	nd	< 0.35	nd
na 15	9.7	8.3	8.2	nd	< 0.35	nd
na 16	6.3	2.4	3.8	nd	< 0.35	nd

nd = not done; SI = Stimulation index; SPT = skin prick test.

1–18 = atopic subjects, na 1–16 = non-atopic subjects.

*partly purified native Lep d 2.

†Pharmacia & Upjohn CAP System FEIA, Pharmacia AB Diagnostics, Uppsala, Sweden. Reference range < 0.35 kU_A/L.

Statistical analysis

Friedman ANOVA and Wilcoxon matched pairs test were used for comparison of proliferation to the different rLep d 2 variants. Mann–Whitney *U*-test was used for comparison of differences in proliferation and cytokine production between atopic subjects and healthy controls. Correlation was calculated using the Spearman rank correlation analysis. *P* < 0.05 was considered statistically significant.

Results

Proliferative responses to partially purified native Lep d 2

To compare the proliferative response to rLep d 2 with the response to the native allergen, PBMCs from 6 *L. destructor* allergic subjects were stimulated with partially purified native Lep d 2 and rLep d 2.01. There was a correlation between the proliferation to nLep d 2 and rLep d 2.01

($r_s = 0.89$, $P = 0.01$) in these six selected subjects. The results showed nearly equal reactions in three subjects (SI nLep d 2: 4.3–5.1, SI rLep d 2.01: 4.7–4.8), one subject reacted stronger to rLep d 2.01 (SI = 34) than to nLep d 2 (SI = 18) and two subjects reacted stronger to the native allergen (SI = 6.4 and 12.3) than to rLep d 2.01 (SI = 5.0 and 2.8). The stimulation indexes are shown in Table 1.

Proliferative responses to rLep d 2.01 and rLep d 2.02

In the whole study population ($n = 34$), a correlation between the proliferation to rLep d 2.01 and rLep d 2.02 ($r_s = 0.7$, $P < 0.001$) was found. However, the SI values (Table 1) for rLep d 2.01 was significantly higher than for rLep d 2.02 ($P < 0.001$), Fig. 2.

In 17 of the 18 *L. destructor* allergic patients the proliferative response to rLep d 2.01 was higher than to rLep d 2.02. Sixteen patients had SI values higher than 2.0 to isoform 01 (range 2.1–34.8) and 7 to isoform 02 (range 2.1–11.9). The difference between the two isoforms varied

between 1.5 and 5.5 times higher response to rLep d 2.01 (median value = 2.5). No correlation was found between SI values for rLep d 2.01 and RAST values for *L. destructor* whole extract.

In the group of 16 controls, the proliferative response varied as in the allergic group and 12/16 of the subjects had a stronger reaction to rLep d 2.01 than to rLep d 2.02. The SI values for rLep d 2.01 ranged between 0.7 and 23.7 and for rLep d 2.02, between 0.9 and 24.5, Table 1. There was no significant difference in proliferation to rLep d 2.01 ($P = 0.6$) and rLep d 2.02 ($P = 0.08$) between the atopic and the nonatopic subjects.

Proliferative response to rLep d 2.6Cys mutant

The proliferation to mutant rLep d 2.6Cys was significantly lower than to rLep d 2.01 ($P = 0.005$) but significantly higher than to rLep d 2.02 ($P = 0.035$). Two patients reacted stronger to rLep d 2.6Cys than to rLep d 2.01. Five of the 18 patients had equal reactions to the two allergens, 11 reacted stronger to rLep d 2.01 (1.5–5 times more). In the non-allergic group, the SI values ranged between 0.7 and 23.7 to rLep d 2.01 and between 0.7 and 30.7 to rLep d 2.6Cys. Thirteen out of 16 had a higher proliferative response to rLep d 2.01 than to rLep d 2.6Cys. Two controls reacted stronger to the rLep d 2.6Cys mutant than to rLep d 2.01. The stimulation indexes are shown in Table 1. Although the SI values were lower, rLep d 2.6Cys showed a good correlation with rLep d 2.01 ($r_s = 0.8$, $P = 0.001$). No significant difference in proliferation against rLep d 2.6Cys was observed between patients and controls ($P = 0.9$).

Proliferative responses to peptides of Lep d 2

Responses to Lep d 2 derived peptides were investigated in 13 of the 18 *L. destructor* allergic subjects and in all of the non-atopic controls ($n = 16$). SI values higher than 2.0 were regarded as positive and the results are shown in Table 2.

Two out of 13 allergic subjects responded to peptide 1 (amino acid (aa) 1–15), 7 to peptide 2 (aa 11–25), 1 to peptide 4 (aa 31–45), 12 to peptide 7 (aa 61–75), 1 to peptide 8 (aa 71–85), 3 to peptide 9 : 1, corresponding to aa 81–95 in isoform Lep d 2.01, 3 to peptide 9 : 2, corresponding to aa 81–95 in isoform Lep d 2.02, 2 to peptide 11 (aa 101–115) and 4 to peptide 12 (aa 111–125). None of the patients had an SI > 2.0 to peptide 3, 5, 6 or 10. One patient did not respond with SI > 2.0 to any of the peptides. Three of the atopic subjects responded to one peptide, 3 to two peptides, 2 to three peptides, 3 to four peptides and one patient responded with SI > 2.0 to eight of the peptides.

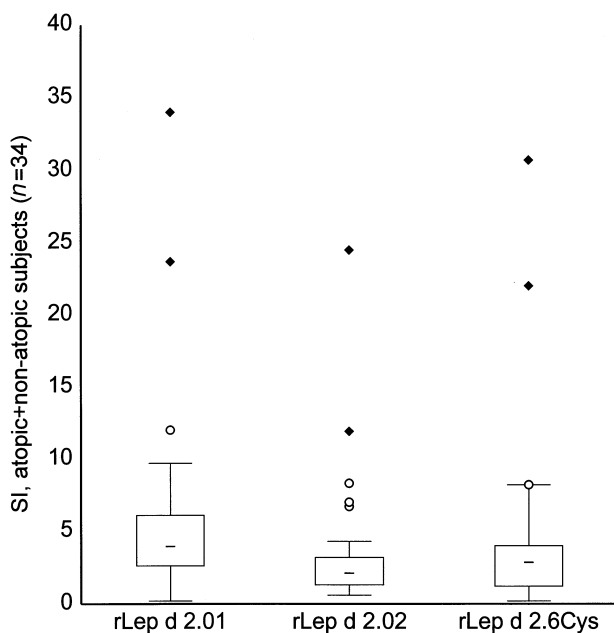


Fig. 2. Stimulation indexes to rLep d 2.01, rLep d 2.02 and rLep d 2.6Cys. Boxes enclose 25th–75th percentile and medians are marked. PBMC from 18 *L. destructor* allergic patients and 16 non-allergic controls were stimulated with rLep d 2.01, rLep d 2.02 and rLep d 2.6Cys for 7 days. Stimulation indexes were calculated as mean cpm for antigen-stimulated PBMC divided by mean cpm for un-stimulated PBMC. Significantly higher proliferation was obtained to rLep d 2.01 compared to rLep d 2.02 ($P = 0.001$). The proliferation to rLep d 2.6Cys was significantly lower than to rLep d 2.01 ($P = 0.005$) but significantly higher than to rLep d 2.02 ($P = 0.035$).

Table 2. Stimulation indexes (SI) to the Lep d 2 peptides after 7 days of culture

Subject	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Peptide 6	Peptide 7	Peptide 8	Peptide 9 : 1	Peptide 9 : 2	Peptide 10	Peptide 11	Peptide 12
2		3.9		4.9			3.9						
4		3.0					4.3						2.7
5							2.8		4.0	5.4		2.4	
6							4.3						2.7
7	4.0	3.3					4.9			2.0			
10							2.7						
12							3.2						
14	2.9	3.2					2.5	3.4	3.6	2.4		3.5	2.6
15		2.5					10						
16							4.5						
17		2.1					5.3						
18		3.1					4.5		3.2				3.9
na 2			2.0										
na 3		2.6					2.3			2.3			3.4
na 5							4.7	2.7		2.1			
na 10	5.5	4.9					3.9						2.4
na 11							3.2	3.5					
na 12		3.4					5.6	2.8					3.1
na 13	6.0	2.4					5.9						
na 15	2.7						2.9						
na 16								2.1					

2–18 = atopic subjects, na 2–16 = non-atopic subjects.

Stimulation indexes ≥ 2.0 are given.

Atopic subject no. 11 and non-atopic subjects 1, 4, 6–9 and 14 gave no specific proliferation to any of the peptides (SI < 2.0).

In the non-atopic group, 7/16 did not respond with SI > 2.0 to any of the peptides. Three out of 16 responded to peptide 1, 4 to peptide 2, 1 to peptide 3, 7 to peptide 7, 4 to peptide 8, 2 to peptide 9 : 2 and 3/16 to peptide 12. None of the non-atopics had SI > 2.0 to peptide 4, 5, 6, 9 : 1, 10 or 11. Two healthy controls responded to one peptide, 2 to two peptides, 2 to three peptides and three to four peptides.

In total, 11/21 of the subjects who responded to the peptides did react with peptide 2 (aa 11–25) and 19/21 of these subjects reacted to peptide 7 (aa 61–75).

Cytokine production

The results of the IFN- γ measurements are shown in Fig. 3. The IFN- γ production was significantly lower in the atopics as compared to the non-atopic subjects. The levels of IFN- γ in the atopic group varied between (113–11 900 pg/mL, median 1139 pg/mL) and in the non-atopic group (306–22 174 pg/mL, median 4387 pg/mL), $P = 0.02$.

The levels of IL-4 were higher in the atopic group (0.5–103 pg/mL, median 12.3 pg/mL) in comparison with the

non-atopic group (0.89–72.2 pg/mL, median 4.9 pg/mL), Fig. 4(a). Likewise, the atopic subjects produced higher IL-5 (170–1743 pg/mL, median 569 pg/mL) than the non-atopic subjects (26.5–1922 pg/mL, median 250 pg/mL), Fig. 4(b). Although the production of IL-4 and IL-5 was higher in the atopic group it did not reach statistical significance. However, significantly higher IL-4/IFN- γ ratio ($P = 0.02$) and IL-5/IFN- γ ratio ($P = 0.003$) was found in the atopic group. When cytokine production was compared with the SI values obtained for rLep d 2.01, a correlation ($r_s = 0.7$, $P = 0.003$) between proliferation and production of IFN- γ was found in the group of healthy controls, Fig. 5, but not in the atopic subjects.

Discussion

The use of recombinant allergens in diagnosis and treatment of allergic disease require a careful evaluation of the immunological properties of these proteins. The individual reaction pattern of patients with respect to both humoral and cellular responses has to be studied to ensure an optimal clinical outcome. Cloning of allergen genes

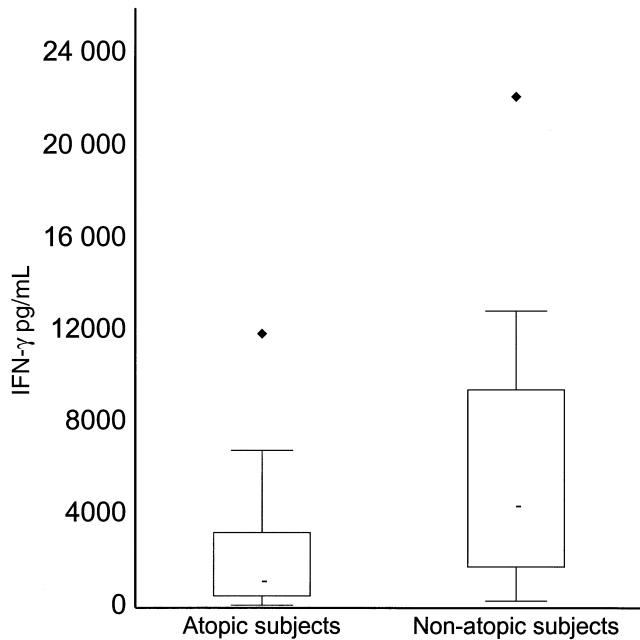


Fig. 3. IFN- γ production in long-term PBMC cultures. 2.5×10^6 cells were cultured in the presence of 10 $\mu\text{g/mL}$ rLep d 2.01 for 11 days. Significantly lower ($P = 0.02$) IFN- γ production was measured in long-term PBMC cultures from atopic individuals ($n = 18$) compared to non-atopic controls ($n = 16$).

have shown that several major allergens exist as multiple isoforms due to polymorphism in the genes [4]. It has also been shown that different sources of mite allergens can contain different spectra of isoforms, i.e. isoforms of allergens isolated from environmental mites and from commercial mite extracts have been shown to differ [23]. This becomes an important aspect when preparing reagents for therapeutic purposes since the isoforms to which the patient is sensitized may not be present in the preparations available for clinical use.

In this study we found that most of the subjects had a higher PBMC response to isoform 01 than to isoform 02 of Lep d 2. Interestingly, *in vitro* studies [9] and skin prick test [12] have previously shown that the isoforms have comparable IgE reactivity. The deduced amino acid sequence of Lep d 2.02 differs in 13 residues out of 125 (10.4%) as compared to Lep d 2.01. When we first cloned these allergens [11] we observed that the cDNA encoding isoform 01 was more frequently found in the *L. destructor* mites we used than that of Lep d 2.02. This may reflect that Lep d 2.01 is a more common isoform than Lep d 2.02, leading to a higher degree of sensitization to this isoform. The peptides used in this study were designed according to the Lep d 2.01 sequence, except for peptide 9 (aa 81–95) which was synthesized in two versions, corresponding to the isoforms Lep d 2.01 and Lep d 2.02. The by far most

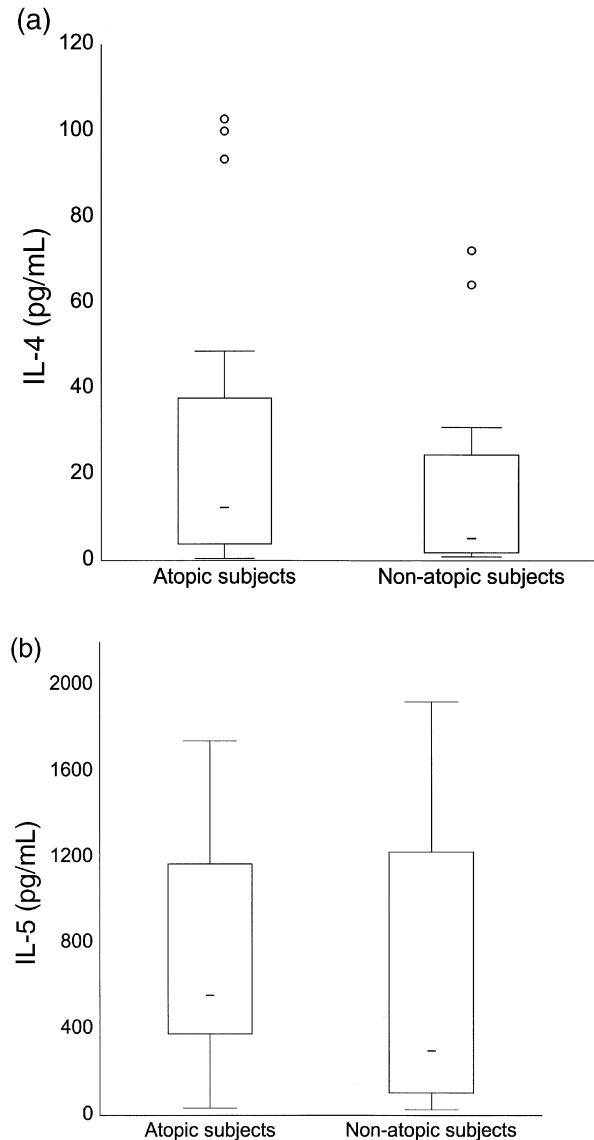


Fig. 4. IL-4 and IL-5 production in long-term PBMC cultures. 2.5×10^6 cells were cultured in the presence of 10 $\mu\text{g/mL}$ rLep d 2.01 for 11 days. There were no significant differences in the levels of (a) IL-4 production and (b) IL-5 production between atopic ($n = 18$) and non-atopic subjects ($n = 16$).

immuno-dominant peptide, peptide 7, differ in two amino acid positions compared to Lep d 2.02 (aa 74 F/V and aa 75 I/L) and this fact might partly explain why we observed a lower proliferative response to Lep d 2.02 than to Lep d 2.01. The importance of these individual amino acids should be further evaluated.

The rLep d 2.6Cys mutant showed a lower degree of T cell reactivity than the non-mutated rLep d 2.01 in a majority of the patients although the reversed situation was found in four subjects with higher responses to the mutant.

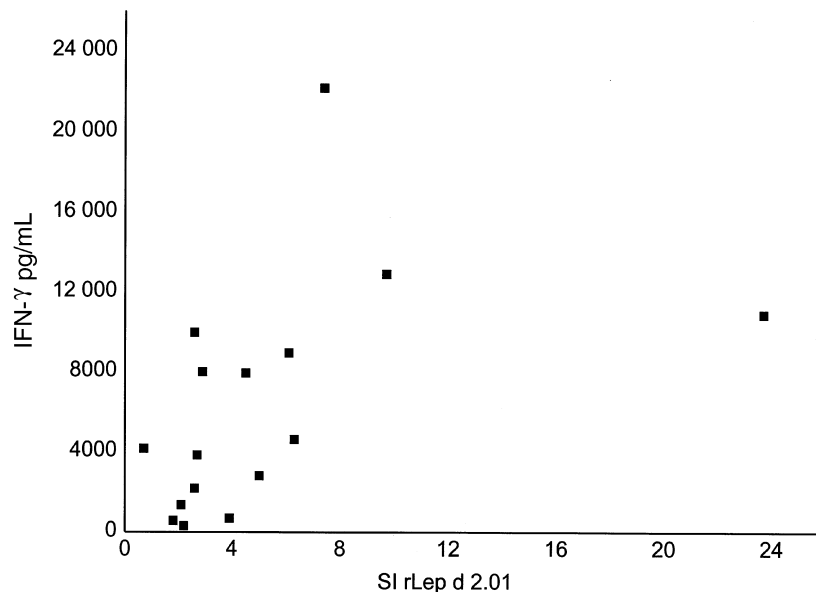


Fig. 5. Correlation between stimulation indexes to rLep d 2.01 and IFN- γ production. A positive correlation ($r_s = 0.7$, $P = 0.003$) was found between proliferation to rLep d 2.01 and IFN- γ production in the group of non-atopic controls ($n = 16$).

However, the proliferation to rLep d 2.6Cys was significantly higher than to rLep d 2.02. The reason why the mutant gave a lower T cell reactivity than rLep d 2.01 in the majority of subjects might be due to the fact that the structure of this mutant is irreversibly denatured, since all the cysteines are substituted for serine residues in order to prevent the disulphide bonds to form. This may lead to a change in the recognition by antigen-presenting cells, in the processing and ultimately in the presentation of peptides to the T cells. The most frequently recognized peptides i.e. peptide 2, 7 and 12 all contain cysteines, namely C21 in peptide 2, C72 in peptide 7 and C117 in peptide 12. It is possible that point mutations at these sites in Lep d 2.6Cys also influence the T cell epitopes. However, it remains to be elucidated if this reduction in T cell reactivity will affect the beneficial aspects of using this mutant in specific immunotherapy in *L. destructor* allergic patients when considering its greatly reduced IgE reactivity. The rLep d 2.6Cys mutant have been used in skin prick test in the same patients investigated in this study. All of the patients showed a positive skin prick test to non-mutated rLep d 2 but only 3 of them had a weak positive Skin prick test to rLep d 2.6Cys while 15 were negative even at the highest concentration applied (100 μ g/mL) (Kronqvist, unpublished data). These results indicate promising possibilities to apply even higher doses of the allergen *in vivo* with reduced risk of anaphylactic reactions.

It has previously been shown that non-allergic individuals have an *in vitro* T cell response to inhaled allergens [18,24–26] and that they react to the same epitopes as allergic individuals [27,28]. We also observed T cell responses to the recombinant *L. destructor* allergens among the non-atopic subjects and there was no significant

difference in the proliferation as compared to the atopic subjects. Furthermore, in agreement with other studies [27,28], we found no differences in epitope recognition between atopic subjects and healthy controls. However, the non-atopic subjects produced significantly higher IFN- γ in response to rLep d 2.01 and had significantly lower IL-4 : IFN- γ and IL-5 : IFN- γ ratios. Furthermore, there was a correlation between the SI values for Lep d 2.01 and IFN- γ production in the non-atopic subjects, indicating that a strong proliferative response is accompanied by a Th1-like response in the non-atopic individuals.

There might be an advantage of using whole modified allergens instead of peptides since the whole T cell epitope repertoire is present in the full length protein and epitope specificity may vary considerably between patients [17,18]. For example, in the study on Der p 2 T cell epitopes performed by O'Hehir *et al.* [18], each of the peptides investigated were shown to be detected by at least three of the 18 subjects tested. However, the responses to the Lep d 2 peptides were more homogenous. Peptide 2 (aa 11–25) and peptide 7 (aa 61–75) were the most frequently recognized peptides and may contain the most relevant T cell epitopes of Lep d 2. These immuno-dominant peptides may be considered for designing peptide vaccine for Lep d 2-immunotherapy. In order to perform a complete mapping of the CD4 T cell epitopes of Lep d 2, peptides with greater overlap, e.g. 11 amino acids overlap, have to be used. Müller *et al.* [29] have in a human system, performed immunotherapy using a mix of three immuno-dominant T cell peptides from the bee venom allergen PLA₂, which resulted in epitope-specific anergy in peripheral T cells. In addition, Hoyne *et al.* [30] have demonstrated linked suppression to Der p 1 in mice by administration of Der p 1

peptides suggesting that immuno-dominant T cell peptides can be used for immunotherapy.

In this study, we have carefully evaluated the capacity of different Lep d 2 variants to evoke T cell responses. It has previously been demonstrated that isoforms of allergens can differ in their capacity to evoke immune responses [31] and we now show for Lep d 2 that the isoforms differ in their ability to stimulate T cells. This aspect becomes important when designing immunotherapy schemes since it must be ensured that the patient in fact reacts to the specific isoform used. We have also demonstrated that the hypoallergenic rLep d 2.6Cys is able to induce a T cell response, although lower than to the non-mutated counterpart. Furthermore, we found that Lep d 2 contains two major immuno-dominant regions. Further studies are needed to evaluate the use of rLep d 2.6Cys for immunotherapy and to investigate if administration of the immuno-dominant peptides would have a protective effect and lead to linked suppression to the whole allergen.

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