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IgE Binding Capacity of Synthetic and Recombinant Peptides of the Major Storage Mite (*Lepidoglyphus destructor*) Allergen, Lep d 2

Abstract

Background: *Lepidoglyphus destructor* is an important non-pyroglyphid mite species in Europe and a dominant allergen in farming environments. The major allergen of *L. destructor*, Lep d 2, is a protein of 13.2 kD that is recognised by about 90% of sera RAST positive to this mite species. **Methods:** The cDNA of two isoallergens of the Lep d 2 has previously been sequenced and the protein expressed in different protein expression systems. In order to map the B-cell epitopes, the full length protein and the truncated forms of the protein have been expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins. Recombinant Lep d 2 fragments and synthetic overlapping 15 mer peptides spanning Lep d 2 were probed with sera from patients allergic to storage mite. **Results:** The full-length (125 amino acids) GST fusion protein reacted strongly with patient IgE in Western blots and dot blots. Synthetic peptides failed to react with IgE antibodies from mite-allergic patients and the truncated fusion proteins displayed weak IgE-binding capacity. **Conclusion:** We conclude that there are no dominant linear IgE-binding epitopes in Lep d 2. Recombinant or synthetic Lep d 2 fragments may, however, be further evaluated as hypoallergenic candidate molecules for specific immunotherapy.

Key Words

Allergen
 B-cell epitope
 IgE
Lepidoglyphus destructor
 Mite

Introduction

Mites are considered to be one of the major causes of allergy in many parts of the world. Dust mites can be divided into two categories: those primarily found in homes are referred to as house dust mites and those primarily found in farming environments as storage mites. House dust mites belonging to the genus *Dermatophagoides* have been extensively studied [1–4]. Two major allergens, a 25-kD group 1 and a 14-kD group 2, have been identified, cloned and sequenced [5–7]. The storage mite *Lepidoglyphus destructor* is one of the most important non-pyroglyphid mite species

in Europe and a dominant allergen in farming environments [8]. Mite allergens exceed those of pollen and animal danders as a cause of IgE-mediated sensitisation in rural populations [9, 10]. Storage mites may also give rise to IgE-mediated sensitisation in indoor environments of urban populations [11, 12].

The major allergen of *L. destructor*, Lep d 2, is a protein of 13.2 kD (determined from the sequence) and is recognised by about 90% of sera RAST positive to this mite species. Lep d 2 has been cloned, the cDNA of two isoallergens have been sequenced and the protein has been expressed in different heterologous protein expression sys-

tems [13]. Recombinant Lep d 2 shows comparable immunoreactivity to natural Lep d 2 and is able to release histamine from sensitised basophils [14].

Epitope mapping is a way to elicit the primary structure of linear antigenic determinants. A number of epitope mapping studies have been carried out on the *Dermatophagoides* group 2 allergens [5, 7, 15–17]. However, no epitope-mapping studies have previously been carried out on storage mite allergens.

Even though Lep d 2 has only 52% identity to Der p 2, it appears that these allergens are cross-reacting [18], suggesting that there may be common IgE-binding epitopes in these proteins. The aim of this study was to map the B-cell epitopes of the principal allergen of *L. destructor*, Lep d 2 and to compare the results with other group 2 allergens.

Materials and Methods

Production of Recombinant Fusion Proteins

cDNA encoding for mature full-length Lep d 2 [13] and fragments (amino acids 1–46, 1–73, 1–108, 54–125, 73–125 and 40–73) were amplified by PCR. *Bam*HI and *Eco*RI sites were attached to the 5' ends of the 5' primers and 3' primers, respectively. The PCR reactions were performed for 30 cycles in 50- μ l reaction volumes containing 1 $\times$ PFU buffer, 2.5 units of PFU DNA polymerase (Stratagene, La Jolla, Calif., USA), 0.2 mM dNTPs, and 1 mM of each primer. Each cycle consisted of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min 30 s polymerisation at 72°C. The PCR products were purified on Glass Max columns (Gibco BRL, Life Technologies, Paisley, UK) according to the manufacturer's instructions and digested with *Bam*HI and *Eco*RI. After digestion, amplified cDNA was ligated into the *Bam*HI-*Eco*RI site of fusion protein expression vector pGEX-3X (Pharmacia Biotech., Uppsala, Sweden) and transformed into *Escherichia coli* strain XL2-Blue. Each construct was confirmed to be correct by DNA sequencing.

Induction of the GST fusion proteins was performed by inducing the transformed *E. coli* cell cultures (A 600 = 0.5) with 0.1 mM isopropylthiogalactoside (Sigma Chemicals Co., St. Louis, Mo., USA) for 2 h. The cells were harvested, resuspended in PBS and broken by sonication. The fusion proteins were affinity-purified with Glutathione-Sepharose 4B (Pharmacia Biotech.) according to the manufacturer's protocol. Protein concentration was determined by measuring the absorbance at 280 nm.

SDS-PAGE Electrophoresis and Immunoblotting

The Mini-Protean system (Bio-Rad, Richmond, Calif., USA) was used for both the electrophoresis and the electroblotting onto polyvinylidene difluoride (PVDF) membrane (Immobilon Transfer Membrane; pore size 0.45 μ m; Millipore, Bedford, Mass., USA). The purified fusion protein was mixed 1:1 with reducing sample buffer containing 2-mercaptoethanol and boiled for 5 min. The sample was loaded onto a homogeneous 12% polyacrylamide gel. A reference well was used for the low-molecular-weight standard proteins (LMW, Electrophoresis Calibration kit, Pharmacia & Upjohn, Uppsala, Swe-

den). After blotting, the PVDF membrane was blocked with a phosphate buffer (pH 7.4) containing 0.5% Tween.

The membrane was incubated with either a pool of 4 sera from *L. destructor* RAST-positive patients or the four sera individually. The sera were diluted 1:4 and incubated with the membrane overnight (approximately 16 h) at room temperature with gentle shaking. After washing three times in 0.9 % NaCl + 0.5% Tween 20, the membrane strips were first incubated for 2 h with 500 μ l of affinity-purified rabbit anti-human IgE (Miab, Uppsala, Sweden) diluted 1:2,000 and then after washing as previously, incubated with 500 μ l of affinity-purified ALP-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:2,000. After washing the strips in 0.9% NaCl, the allergens were finally visualised by incubating the strips, with Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).

Dot Blot

A Bio-Rad Bio-Dot™ Apparatus was used for the dot blot analysis. A total of 0.5 μ g/dot of purified fusion protein was applied to a supported nitrocellulose membrane (Trans-Blot® Transfer Medium, Bio-Rad). After blocking with buffer containing 0.5% Tween, the membrane was cut into strips with 8 dots (the 7 fusion proteins and glutathione S transferase, GST, alone) on each strip. The strips were incubated overnight at room temperature with 24 individual sera (diluted 1:4) which were RAST positive to varying degrees to *L. destructor* (0.5–90 kU/l). Development of the blot was performed as described for the immunoblot. A positive dot was taken to be a dot that had a greater intensity than GST alone for each individual serum.

Spot Test

Synthetic peptides (table 1), 15 amino acids long, overlapping by 5 amino acids, covering the two isoforms of the Lep d 2 protein (Lep d 2.01 and Lep d 2.02) were synthesised onto a cellulose SPOT's membrane (Custom SPOT's, Genosys Biotechnologies, Pampisford, UK). This SPOT's membrane was analysed with patient sera RAST positive to *L. destructor* and a polyclonal rabbit serum raised against the recombinant Lep d 2 protein. When testing with patient sera, the SPOT's membrane was first incubated with a 1:4 diluted pool of 5 sera or individual sera from *L. destructor* RAST-positive (>20 kU/l) patients overnight at room temperature with mild shaking. After washing according to the supplier's method, the membrane was incubated with 1:2,000 diluted affinity-purified rabbit anti-human IgE (Miab) for 2 h and then after new washings, with 1:200 diluted sheep anti-rabbit β -galactosidase conjugate (Genosys Biotechnologies) for 2 h. Development of the membrane was performed according to the supplier's method. The polyclonal rabbit serum against Lep d 2 was diluted 1:20,000, and developed with the β -galactosidase-conjugated sheep anti-rabbit antibodies as above.

Results

Overlapping peptides 15 amino acid in length and covering the complete sequence of both of the Lep d 2 isoforms [13] were synthesised on a cellulose support (table 1). None of the peptides were recognised by serum IgE from patients RAST positive to *L. destructor* (data not shown), and positive to the full-length recombinant Lep d 2.

Table 1. Peptides of Lep d 2 synthesised on SPOT's membrane

Spot No.	Amino acids	Sequence	Isoform
1	1–15	GKMTFKDCGHGEVTE	Lep d 2.01
2	10–25	GEVTELDITGCSGDT	Lep d 2.01
3	20–35	CSGDTCVIHRGEKMT	Lep d 2.01
4	30–45	GEKMTLEAKFAANQD	Lep d 2.01
5	40–55	AANQDTAKVTIKVLA	Lep d 2.01
6	50–65	IKVLAKVAGTTIQVP	Lep d 2.01
7	60–75	TIQVPGLETGCKFI	Lep d 2.01
8	70–85	GCKFIKCPVKKGEAL	Lep d 2.01
9	80–95	KGEALDFIYSGTIPA	Lep d 2.01
10	90–105	GTIPAITPKVKADVT	Lep d 2.01
11	100–115	KADVTAELIGDHGVM	Lep d 2.01
12	110–125	DHGVMACGTVHGQVE	Lep d 2.01
13	10–25	GEVTELDISGCSGDT	Lep d 2.02
14	20–35	CSGDTCVIHRGQKMT	Lep d 2.02
15	30–45	GQKMTLDAKFAANQD	Lep d 2.02
16	40–55	AANQDTNKVTIKVLA	Lep d 2.02
17	60–75	TIQVPGLETGCKVL	Lep d 2.02
18	70–85	GCKVLKCPVKKGEAL	Lep d 2.02
19	80–95	KGEALDFNYGMTIPA	Lep d 2.02
20	90–105	MTIPAITPKVKADVT	Lep d 2.02
21	100–115	KADVTAELVGDHGVM	Lep d 2.02
22	110–125	DHGVMACGTHGQVE	Lep d 2.02

As a control to see whether the Lep d 2 peptides could be recognised by antibodies, we used a polyclonal rabbit antiserum that had been raised against recombinant Lep d 2. Three peptides reacted with IgG in the polyclonal rabbit antiserum (fig. 1). The three peptides corresponded to amino acids 1–15 (common to both isoforms), 10–25 of Lep d 2.01 and amino acids 10–25 of Lep d 2.02.

Longer overlapping fragments of the Lep d 2.01 isoform were expressed as GST fusion proteins in *E. coli*. The different fragments (amino acids 1–125, 1–108, 1–73, 1–46, 54–125, 73–125 and 40–73) of Lep d 2 were purified from *E. coli* crude extract by affinity chromatography (fig. 2).

Immunoblotting of the different constructs using 4 sera from *L. destructor*-allergic patients (fig. 3) revealed that all of the sera contained specific IgE that strongly detected the fusion with the full-length (amino acids 1–125) Lep d 2. Three of the 4 sera contained IgE which strongly reacted with the fusion protein containing amino acids 1–108 of Lep d 2 but none of the sera recognised any of the other fusion proteins or GST alone.

In order to test a larger number of sera, the GST fusion proteins were applied to a nitrocellulose membrane using a dot blot apparatus. Twenty-four sera were tested (fig. 4) to see whether they contained IgE against the fusion proteins.

The full-length Lep d 2 molecule (fusion protein 1–125) was the most strongly reacting construct among all the sera tested. There was however some detectable IgE binding to other constructs, but this was generally weak (fig. 4).

Discussion

The fact that none of the synthetic peptides were recognised by serum IgE from patients RAST positive to *L. destructor* and positive to the full-length recombinant Lep d 2 indicate that there are no major short linear IgE epitopes in Lep d 2. However, due to the limited number of sera tested (five sera) it cannot entirely be ruled out that some patient sera contain IgE recognising short linear epitopes. The spot-test results with the polyclonal rabbit antiserum are consistent with a linear IgG binding epitope being present in amino acids 10–15 (GEVTE) of Lep d 2. A peptide corresponding to amino acids 65–78 of Der p 2 has been reported to be recognised by 60% of the most positive sera to *D. pteronyssius* [16]. Interestingly, this peptide contained two cysteines (73 and 78) involved in forming the disulphide bridge in Der p 2 that contributes most to stabilising the antigenic structure of this allergen [19]. The peptides from Lep d 2 (8 and 18) which contain the corresponding cysteine residues of Lep d 2 (72–77) are not recognised by IgE in any of the sera tested.

In dot blot analysis, the full-length Lep d 2 molecule (fusion protein 1–125) was the most strongly reacting construct among all of the sera tested. There was however some detectable IgE binding to other constructs, but this was generally weak. Similar results have been obtained in epitope-mapping studies of both the Der p 2 [15, 17] and *D. farinae* (Der f 2) allergens [20].

It is clear from these results that linear sequences of amino acids do not contribute significantly to the IgE-binding epitopes of Lep d 2. It therefore seems likely that the major epitopes are due to the conformation of Lep d 2. Mutagenesis studies on the *Dermatophagoides* group 2 allergens Der p 2 and Der f 2 indicate that the disulphide bridges in these molecules play an important role in stabilising conformational IgE-binding epitopes [19, 21]. IgE binding to many other allergens also seems mainly to depend on conformational rather than linear epitopes [22–24]. In fact, to our knowledge, only one dominant linear peptide epitope that can significantly inhibit patient IgE binding to the whole protein allergen (Par o 1 from *Parieteris officinalis*) has been described [25].

Mapping of conformational epitopes requires more information on the three-dimensional structure of allergens.

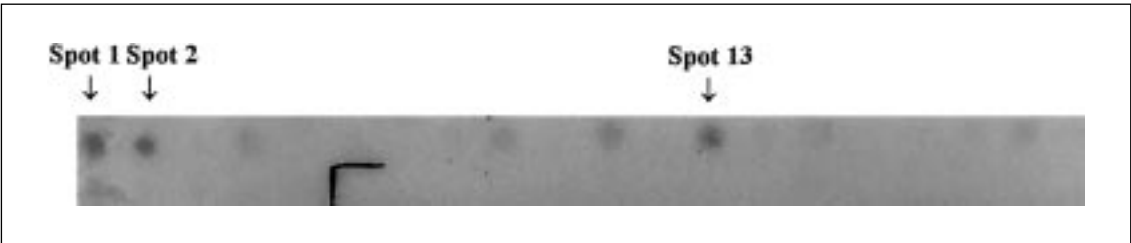


Fig. 1. Reactivity of polyclonal rabbit anti Lep d 2 sera to synthetic peptides of Lep d 2. SPOT's 1, 2 and 13 which were strongly recognised by the sera are marked.

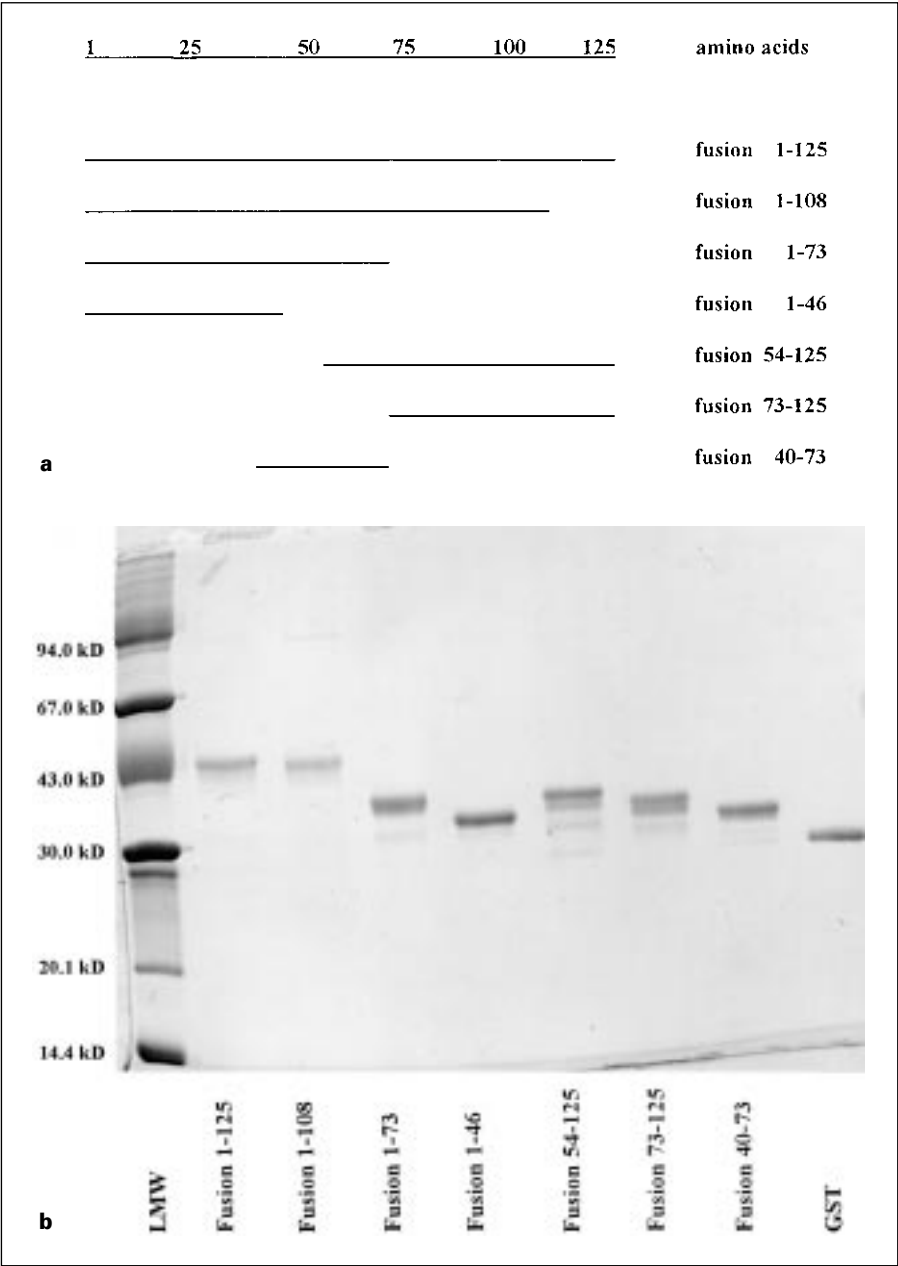


Fig. 2. a Schematic map of the Lep d 2 fragments expressed as GST fusion proteins. **b** Coomassie-Brilliant-Blue-stained SDS polyacrylamide gel of the different purified GST-Lep d 2 fusion proteins.

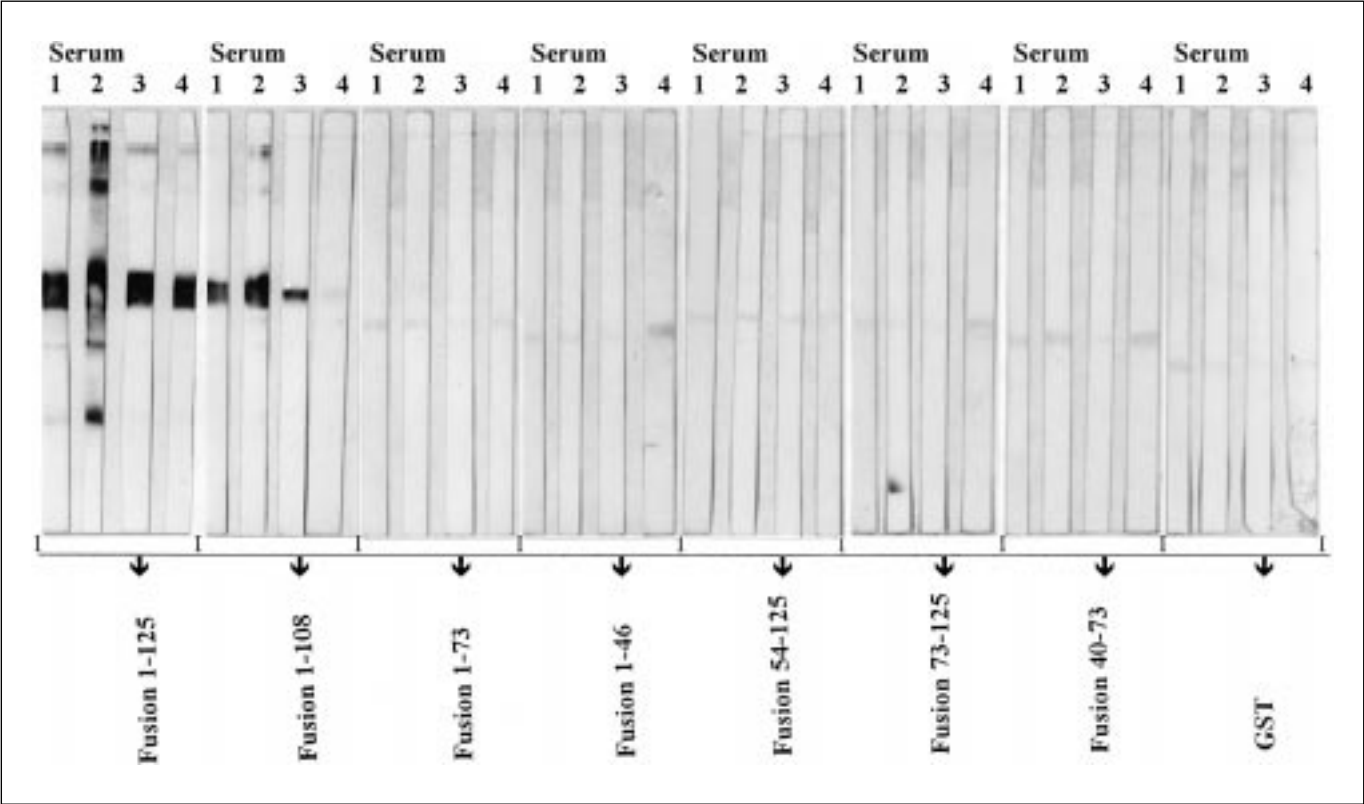


Fig. 3. Reactivity of IgE in individual human patient serum to the different purified GST Lep d 2 fusion proteins and GST alone in Western blotting.

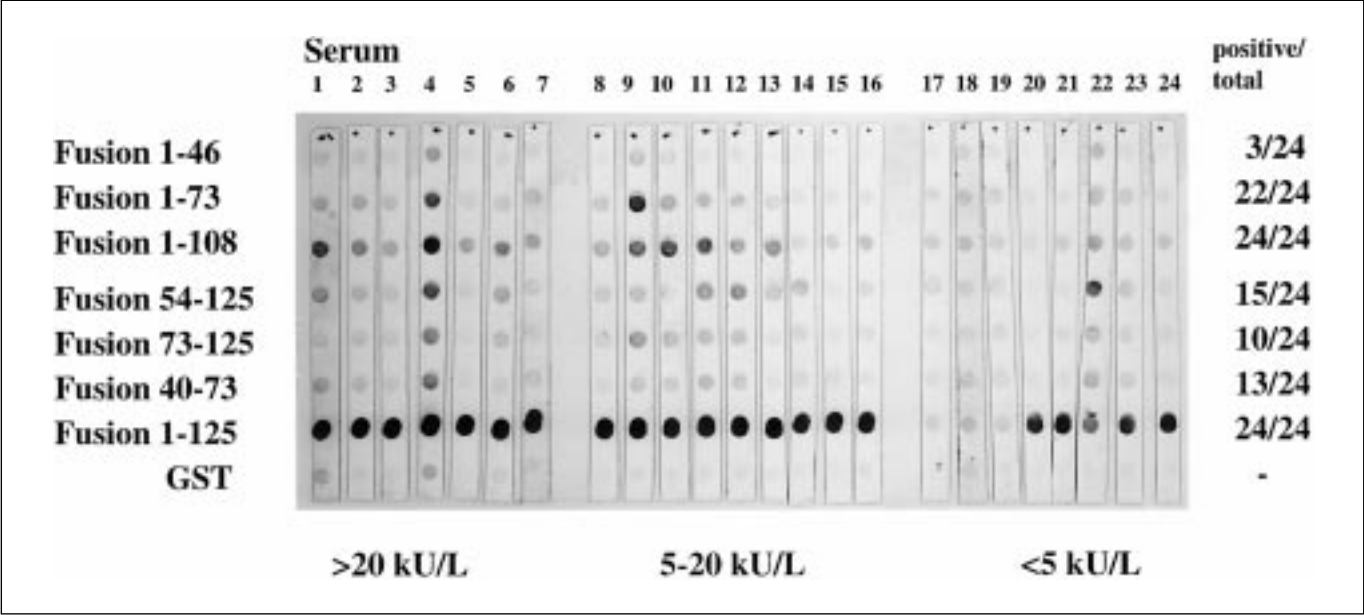


Fig. 4. Reactivity of IgE in individual human patient serum to the different purified GST Lep d 2 fusion proteins and GST alone in dot blot. The sera are divided into three groups according to their RAST value to *L. destructor* whole extract. A positive dot is a dot that has greater intensity than GST alone for each individual serum.

The structure of Bet v 1 has been solved using X-ray crystallography and nuclear magnetic resonance (NMR) techniques [26], and the secondary structure of Der p 2 determined by NMR techniques has recently been reported [27]. We believe that the basis of cross-reactivity of the mite group 2 allergens lies in their three-dimensional structure rather than in linear epitopes. It has been suggested that isoforms of allergens [28], mutated allergens [19] and fragments of allergens with reduced IgE-binding capacity [29] may be useful in immunotherapy as it should be possible to use them in higher concentrations with reduced risk of anaphylaxis. According to current knowledge, T-cell epitopes are represented by small peptides. The fusion proteins 1–46, 40–73 and 73–125 cover the whole length of the Lep d 2 molecule and although no data on T cell epitopes of Lep d 2 are known, they should contain the majority of the T cell epitopes. These fusion proteins, however, have very little

IgE-binding capacity and could therefore be of potential use in immunotherapy. Preliminary results (data not shown) reveal that the fusion protein 1–125 is capable of releasing histamine from whole blood of a mite-allergic patient whereas the fusion proteins 1–108, 1–46, 40–73 and 73–125 do not.

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