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IgE and T-cell responses to high-molecular weight allergens from bee venom

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Summary

Background Bee venom contains multiple allergens with a wide distribution of molecular weight. In contrast with conventional bee venom desensitization, peptide or recombinant allergen immunotherapy may have to take into account patients' individual patterns of humoral or cellular response.

Objective To study immunoglobulin (Ig)E and T-cell responses to high-molecular weight bee venom allergens ≥ 50 kDa.

Methods Bee venom proteins were separated by size exclusion chromatography and fractions were characterized by one and two-dimensional gel electrophoresis. IgE antibody binding to bee venom fractions was analysed by immunoblotting and T-cell responses by proliferation assay.

Results Among 38 bee venom-hypersensitive patients, IgE recognition pattern of bee venom allergens varied greatly. IgE bound mainly to phospholipase A_2 and furthermore to several proteins ≥ 50 kDa (50, 54, 69, 84 and 94 kDa). N-terminal sequences of these proteins showed no homology with known proteins. In addition, peripheral mononuclear cells from patients as well as from nonatopic donors strongly proliferated in response to those proteins.

Conclusions Although present in low amounts, high-molecular weight allergens from bee venom elicit strong IgE and T-cell responses, and may need to be considered as clinically relevant. Therefore, the development of peptide or recombinant protein-based immunotherapy for bee venom allergy may require careful characterization of such allergens.

Keywords: IgE, bee venom, T-cells, immunotherapy

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Introduction

Bee venom (BV) (*Apis mellifera*) is a complex mixture of peptides and glycoproteins, most of which are enzymes [1]. Major allergens include phospholipase A_2 (PLA_2), hyaluronidase, allergen C and acid phosphatase as previously shown using various techniques including radioallergosorbent test (RAST) and crossed immunoelectrophoresis [2–6]. Among the peptides, only melittin was recognized by immunoglobulin (Ig)E in a minority of patients [7]. Best characterized BV allergens are PLA_2 [8,9], hyaluronidase [10,11] and acid phosphatase [12] with molecular weights (MW) of 16–20, 41 and 45 kDa, respectively. Currently, complete protein and cDNA sequence data for PLA_2 and hyaluronidase are available [9,11,13]. The N-terminal sequence and the sequence of some proteolytic peptides derived from acid phosphatase have also been reported [14] and PLA_2 crystal structure has recently been resolved [15]. Apart from PLA_2, which accounts for approximately 12% of BV dry weight, hyaluronidase, acid phosphatase and allergens of higher MW each represent less then 2% of dry weight [2,3,16].

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Venom immunotherapy (VIT) provides efficient protection in about 80% of patients [17]. Although detailed mechanisms underlying VIT are not yet understood, T-cells seem to play a crucial role [18–20]. We have recently described a novel immunotherapy strategy based on long (50–60 mer) overlapping peptides mapping the whole PLA_2 molecule [21,22]. In order to consider the development of a BV allergy vaccine based on synthetic peptides or recombinant allergens, it appears essential to identify and characterize the most allergenic proteins. In this report, we focus on proteins ≥ 50 kDa and have identified five with a respective MW of 50, 54, 69, 84 and 94 kDa. These proteins represented only a minor proportion of total BV proteins. However, they strongly bound IgE antibodies from >60% hypersensitive patients and were able to induce a vigorous T cell response, suggesting that they may represent clinically relevant allergens.

Materials and methods

Patients

Sera were collected from 38 BV hypersensitive patients (grades II to IV according to Mueller's classification) [23] and peripheral blood mononuclear cells (PBMC) from six patients. All hypersensitive patients had BV specific IgE (≥ 0.35 kU/L) as tested by CAP® system (Pharmacia, Uppsala, Sweden) and positive intradermal skin tests. Furthermore, PBMC and sera were obtained from four BV nonhypersensitive controls which included two individuals with a history of hay fever (positive specific IgE for grass pollen, positive skin-prick test to grass pollen); all were negative for specific IgE to whole BV (≥ 0.35 kU/L). The research protocol was accepted by the Hospital Review Board. All patients gave their written informed consent to participate in the study.

Size exclusion gel chromatography and protein analysis

Some 250 mg lyophilized whole BV (*Apis mellifera*) (Latoxan, Rosans, France) was dissolved in 50% formic acid. Particles were removed by centrifugation and filtration prior to sample application to a Sephadex G-100 column (2.5×100 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 50% formic acid. Fractions of 4.3 mL were collected at a flow rate of 6.5 mL/h. Each fraction was lyophilized, dissolved in 0.02 N acetic acid and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (gradient gel, 8–20%) [24]. Apparent MW was calculated as described [25]. Alternatively, proteins were separated by two-dimensional gel electrophoresis and stained as described [26]. Briefly, samples were prepared for isoelectric focusing in a reducing buffer (8 M urea, 1,4-dithioerythritol) and separated on Immobiline DryStrip (Pharmacia, Uppsala, Sweden). Proteins were resolved in a 9–16% gradient gel in the second dimension. Protein concentration was determined by BCA Protein Assay (Pierce, Socochim SA, Lausanne, Switzerland)

N-terminal sequence analysis

Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) (Millipore, Volketswil, Switzerland) and stained with amido black (Merck, Geneva, Switzerland). *N*-terminal sequence analysis was carried out with a pulse-liquid phase microsequencer, model 473 A (Applied Biosystems, Foster City, CA, USA) using standard programs. EMBL and SWISS-PROT data bases were searched for protein sequence homology applying the Wisconsin Package Version 9.1 software (Genetics Computer Group, Madison, WI, USA).

Immunoblot assay

Proteins were separated by SDS-PAGE (8–20% gradient) under nonreducing conditions and blotted to PVDF membranes in CAPS/methanol buffer (10 mM CAPS, 10% methanol, pH 11). Transferred proteins were visualized by staining with Protogold® (Chemie Brunschwig, Basel, Switzerland). Membranes were blocked with nonfat milk (5%) in Tris-buffered salt solution containing 0.05% Tween 20 (TTBS), and subsequently incubated with patients sera (1/10 in TTBS) for 24–72 h at 4 °C. Specific IgE binding was detected using a biotinylated monoclonal mouse antihuman IgE antibody (Pharmingen, Hamburg, Germany) followed by incubation with streptavidin conjugated horseradish peroxidase (HRP) (UBI, Lucerna Chem AG, Luzern, Switzerland). Peroxidase reactivity was visualized by enhanced chemiluminescence (ECL, Amersham, UK). For IgG antibody detection sera were diluted 1:100 in TTBS 1% milk and incubated for 2 h at RT. Specific antibody binding was detected with a HRP conjugated mouse antihuman IgG antibody (Pharmingen, Hamburg, Germany). Peroxidase reactivity was visualized by enhanced chemiluminescence.

Proliferation assay

PBMC were isolated from heparinized blood by gradient density centrifugation (Ficoll®, Pharmacia, Sweden). PBMC (2×10^5 cells/well) were cultured in quadruplicates in 96-well plates in RPMI 1640 (Gibco, Basel, Switzerland) medium supplemented with 10% AB^+ serum (Swiss Red Cross, Bern, Switzerland) alone or in the presence of 10 μg/mL antigen. After 5 days of culture in 5% CO_2 at 37 °C, cells were pulsed with 1 μCi/well [^{3}H]-thymidine (Du Pont NEN products, Boston MA, USA) and harvested 18 h later.

Results were expressed as cpm of [^{3}H]-thymidine incorporated into DNA. All patients' PBMC responded to tetanus toxoid (10 μg/mL) (Swiss Serum Institute, Bern, Switzerland) which was used as positive control for proliferation.

Detection of enzymatic activity in BV

Pools #1 to #8 were tested for enzymatic activity of hyaluronidase, PLA_2 and acid phosphatase as described elsewhere [12,27,28].

Results

Analysis of BV proteins separated by gel filtration

Proteins from BV were separated by size exclusion gel chromatography (Fig. 1). From 250 mg BV applied to the G-100 column, 0.5% (1.3 mg) was recovered in peak I and 0.85% (2.1 mg) in peak II. Each fraction was analysed by SDS-PAGE. PLA_2 could be identified as the main protein eluting in peak III. Fractions 28–53, which contained proteins > 20 kDa eluting ahead of peak III, were grouped in eight pools for further analysis. Pools were tested for PLA_2, hyaluronidase and acid phosphatase enzymatic activity (Fig. 1). PLA_2 activity could be detected in increasing strength in pools #4 to #8. Hyaluronidase activity was found in pools #3 through #6, with a maximum in peak II (pool #3 and #4). Acid phosphatase coeluted with hyaluronidase, since weak acid phosphatase activity was found in peak II. No activity for the three enzymes was detected in peak I (pool #1 and #2). SDS-PAGE analysis of pool #1 and #2 revealed six bands eluting in the first peak with an apparent MW of 50, 54, 69, 84, 94 and 170 kDa, respectively (Fig. 2). In pool #3 and #4 (peak II), a major broad band migrated at about 43 kDa. Furthermore weak bands $\geq$ 50 kDa, also present in peak I, as well as proteins migrating as a doublet at 38 kDa were observed. Pools #5 to #8 contained proteins ranging from 19 to 45 kDa with bands migrating at 19, 21, 25, 36 and 43 kDa, respectively.

Short N-terminal sequences could be determined for 43, 50, 54, 69 and 94 kDa proteins (Table 1). The sequence determined for the 43 kDa band was homologous to hyaluronidase [11,29]. None of the other sequences showed any homology to either acid phosphatase [14], other BV proteins or proteins from data bases. Furthermore, although incomplete, distinct sequences were disclosed for each band.

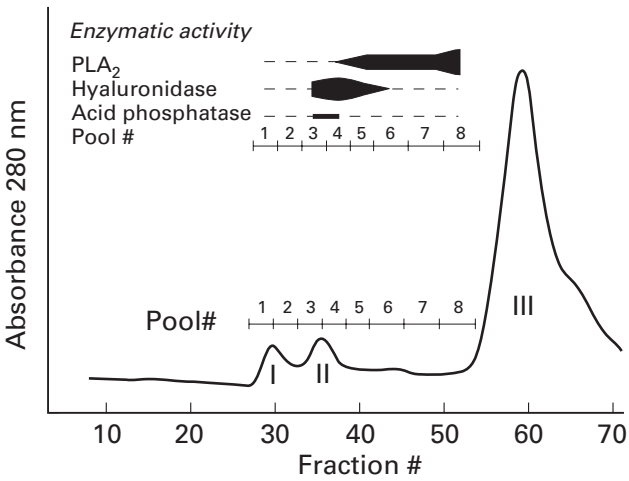

Fig. 1. Elution profile of whole bee venom applied to a Sephadex G-100 column. The eight pools derived from fractions 28–53 are indicated. Major peaks were numbered in Roman numerals. Relative intensity of phospholipase A_2 (PLA_2), hyaluronidase and acid phosphatase specific enzymatic activity in pools #1 to #8 is shown graphically (insert); dashed line: no activity detected.

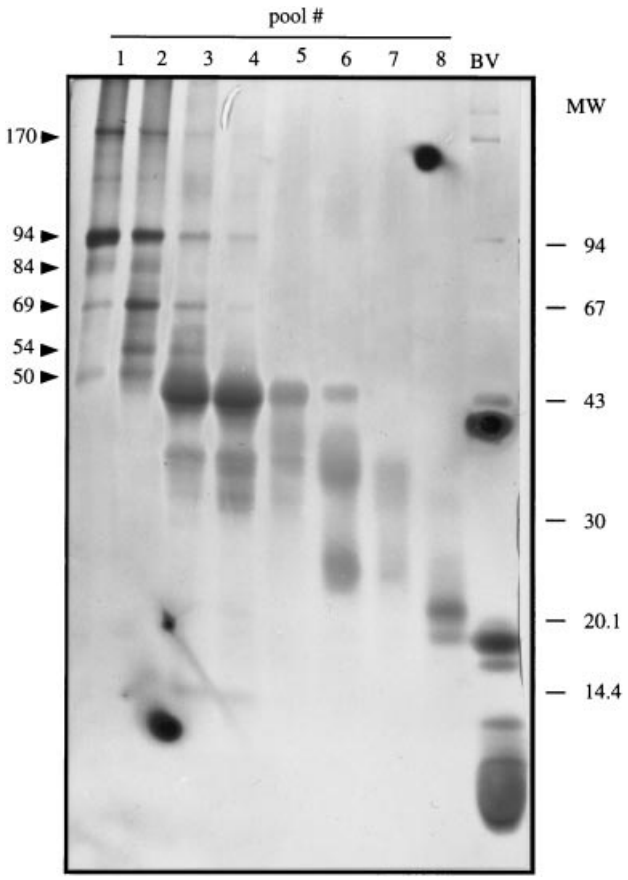

Fig. 2. SDS-PAGE analysis of bee venom (BV) pools #1 to #8 (lanes 1–8). Molecular weight standards are indicated on the right margin. Proteins were separated under reducing conditions and revealed by silver staining.

Table 1. N-terminal sequences from high molecular weight allergens.

Protein M_w	N-terminal sequence
94 kDa	(D/Q)E(P/I)LEXLX
84 kDa	ND
69 kDa	DXQVPKV
54 kDa	XXAERPNQAS
50 kDa	XYSLRDFKANIF
43 kDa	PDNXKXVREFNV

X, no assignment; ND, not determined.

IgE binding to unfractionated BV

IgE antibodies were shown to recognize preferentially conformational epitopes [30,31]. Therefore nonreducing conditions were used for SDS-PAGE and transfer to PVDF membrane in order to preserve protein conformation to a maximum. Sera from BV hypersensitive patients ($n = 38$) were analysed by immunoblot for specific IgE binding to unfractionated BV. Representative immunoblots from 12 out of 38 sera are shown in Fig. 3. Globally IgE binding patterns to BV proteins varied considerably among patients. Proteins most frequently recognized were PLA_2 (doublet at 17 kDa) and the band at 43 kDa. Weaker but readily detectable binding to proteins of 50, 69, 84 and 94 kDa was noted. Large variations in IgE recognition pattern were observed for those proteins; 24 of the 38 sera (63%) contained IgE specific to BV allergens ≥ 50 kDa.

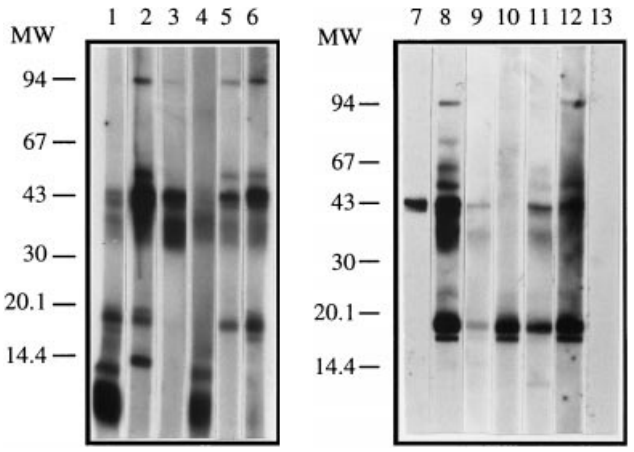

Fig. 3. Variability of immunoglobulin (Ig)E recognition patterns of whole bee vemom (BV) proteins in BV-hypersensitive patients. BV proteins separated by SDS-PAGE (preparative gel; approximately 20 μg/lane) and transferred to polyvinylidene difluoride membrane were incubated with 12 sera from BV-hypersensitive patients (lanes 1–12) and specific IgE binding was detected by chemiluminescence. Lane 13: control serum from a grass pollen allergic but BV-nonhypersensitive donor.

IgE binding to fractionated BV proteins

To more precisely characterize IgE binding to less abundant BV proteins, six sera from BV hypersensitive patients were incubated individually to enriched pools #1 to #8 and analysed by immunoblotting (Fig. 4). In pool #1 and #2, substantial IgE binding to 50, 69 and 94 kDa proteins was detected in five patients, although quantitatively lower in comparison with IgE binding to hyaluronidase and PLA_2. Weak binding to 54 kDa and 84 kDa proteins was observed in two (panel II, IV) and three patients, respectively (panel II, IV, V). IgE antibodies to proteins ≥ 50 kDa, present in small quantities in pools #3 and #4, were detected in four sera. In these pools, the 43 kDa band was visible in all patients, and even exclusively in one patient (panel VII).

High MW proteins were separated by 2D-PAGE (Fig. 5a), transferred to PVDF membrane and incubated for IgE detection with pooled sera from four hypersensitive patients (Fig. 5b). IgE disclosed spots migrating at 50 kDa (pI $\approx$ 6.6), 69 kDa (pI $\approx$ 6.4) and 94 kDa (pI $\approx$ 5.9). Hyaluronidase (pI > 7.5) and other spots (pI $\approx$ 5.7) at 43 kDa also bound IgE. The position of these latter spots (43 kDa, pI $\approx$ 5.7) was compatible with published results for acid phosphatase [32]. Taken together, these results indicated that apart from major allergens (PLA_2, hyaluronidase and acid phosphatase) most BV hypersensitive patients developed IgE against proteins ≥ 50 kDa.

T-cell response to fractionated BV proteins

To examine the T cell response to high MW proteins, PBMC from six BV hypersensitive patients and four BV nonhypersensitive controls were stimulated with pools #1 to #8 (10 μg/mL). PBMC isolated from BV hypersensitive patients proliferated vigorously in response to pools #1 to #4, as did cells from BV nonhypersensitive controls (Fig. 6a). However, in contrast to hypersensitive patients, all sera from BV nonhypersensitive controls were negative for IgE, but were positive for IgG antibodies which recognized mainly PLA_2 and in one case proteins of 43 and 54 kDa (Fig. 6b). BV hypersensitive patients' IgG binding pattern was very similar to their IgE pattern with less variation in proteins > 40 kDa, revealing only two bands at 43 and 54 kDa.

Discussion

In this study, we have shown that IgE recognition of BV proteins ≥ 50 kDa was highly prevalent (> 60% patients).

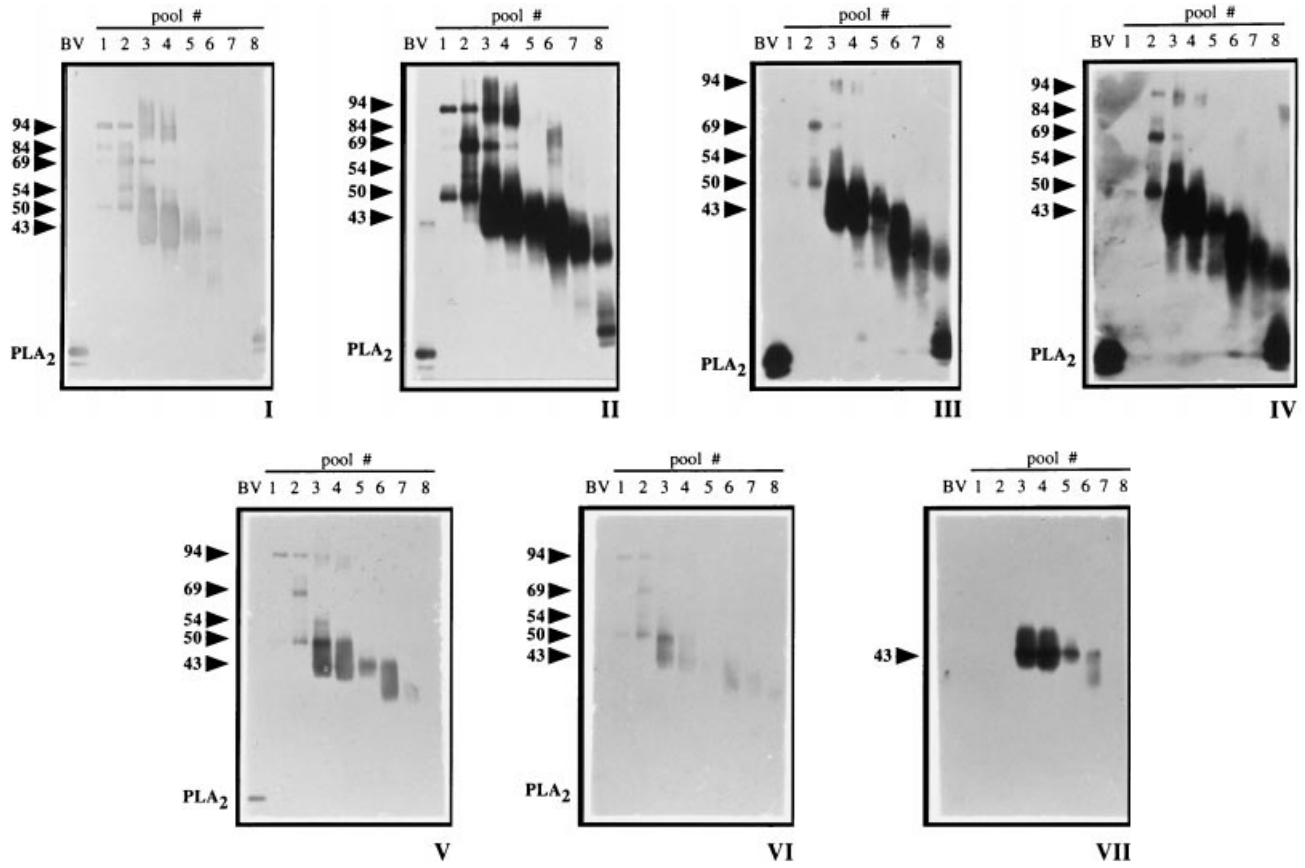

Fig. 4. Detailed analysis of immunoglobulin (Ig)E binding patterns of six bee venom (BV) hypersensitive patients to proteins of pools #1 to #8. Proteins were separated under nonreducing conditions and IgE immunoblot was performed as described in Fig. 3. Pools #1 to #8 (2 μg/lane): lanes 1–8; BV (4.5 μg): whole bee venom. Major high molecular weight bands are indicated (arrows). Panel I: total protein staining with Protogold®; panels II to VII: polyvinylidene difluoride membranes were incubated with serum from hypersensitive patients.

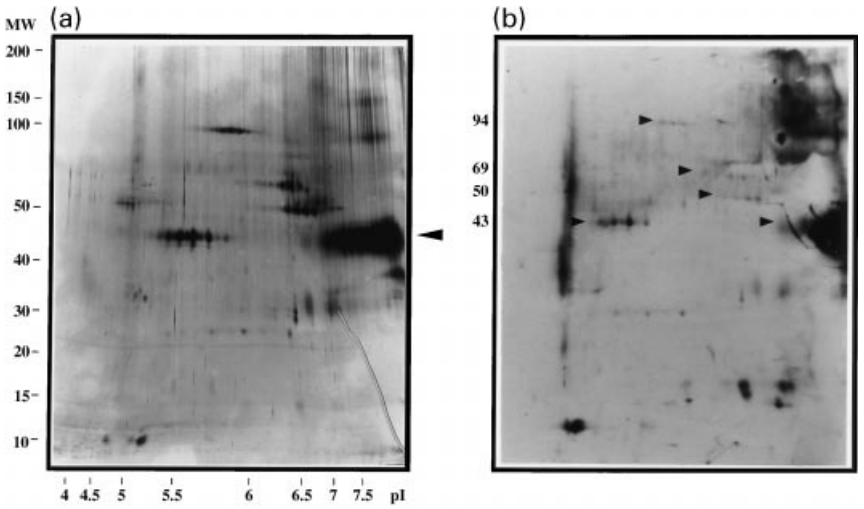

Fig. 5. 2D-PAGE analysis of bee venom (BV) fraction-containing proteins >40 kDa. (a) Proteins were revealed by silver staining. Arrow indicates position of hyaluronidase and acid phosphatase. Approximate molecular and pI are indicated. (b) Immunoglobulin (Ig)E immunoblot analysis of proteins shown in (a) (as described in Fig. 3). Pooled sera of four BV hypersensitive donors were examined for IgE binding. Arrows and labels indicate spots corresponding to bands detected in Fig. 4.

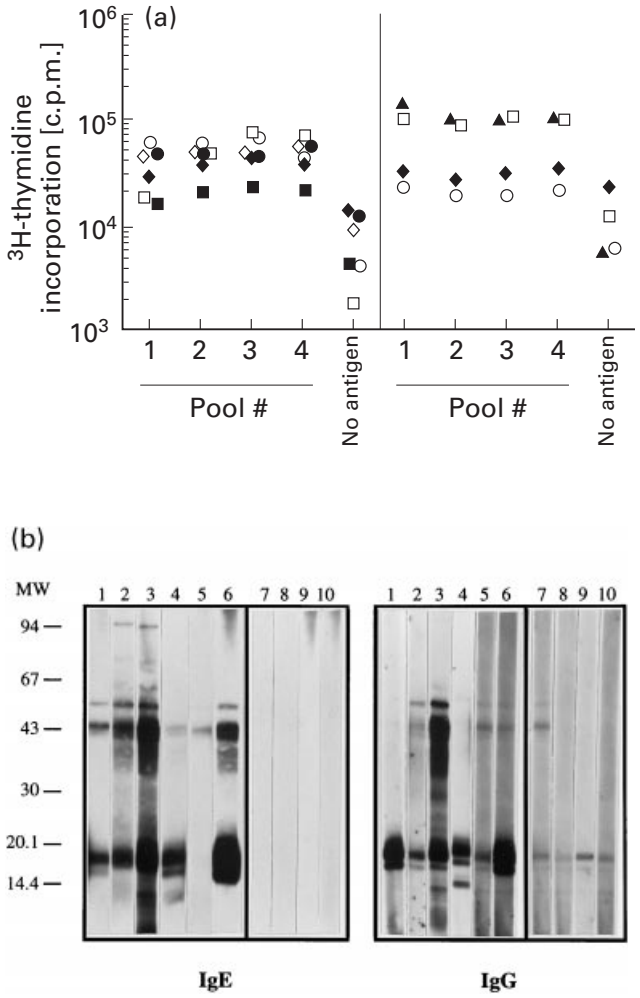

Fig. 6. T-cell and antibody responses to bee venom (BV) antigens. (a) Proliferative response of peripheral blood mononuclear cells from six BV-hypersensitive patients (left panel) and four BV-nonhypersensitive controls (right panel) to BV proteins enriched in pool #1 to #4. Results are expressed as means of quadruplicates (SD < 15%), indicating [^{3}H]-thymidine incorporation into DNA (c.p.m.). (b) Immunoglobulin (Ig)E and IgG immunoblot analysis of donors shown in (a) (as described in Fig. 3); BV-hypersensitive: lanes 1–6; BV-nonhypersensitive controls: lanes 7–10.

These high MW proteins represented only a very small amount of poorly characterized BV proteins which had not previously been described as relevant allergens. Furthermore, they were able to vigorously stimulate T-cells isolated from BV hypersensitive patients.

Five out of six proteins of 50, 54, 69, 84 and 94 kDa, present in pool #1 and #2, were recognized by IgE antibodies from BV-hypersensitive patients. This considerably increases the number of potential BV allergens reported to date. N-terminal sequences derived from these proteins showed that they were distinct from each other and that they had no homology to known proteins. Only two IgE binding proteins were reported so far in this MW range: a weak IgE binding 52 kDa protein in a single patient [33] and allergen C. Allergen C has been described as an IgE binding protein of 102–105 kDa [32,34]. We however, did not detect a protein with a comparable MW in our enriched fractions. The 94 kDa protein might correspond to allergen C admitting a difference of about 10 kDa resulting from the use of different MW standard proteins. However, proteins such as hyaluronidase and PLA_2 were found at their expected MW, which might contradict this hypothesis. Furthermore, two groups reported faint non IgE binding bands at 71 and 87 kDa [34] as well as 63 and 80 kDa [16], respectively. These bands may correspond to proteins at 69 kDa and 84 kDa in our analysis. However, in contrast to those reports, IgE binding to these proteins was readily detectable. It was suggested in previous studies that some BV proteins might form aggregates [16,34]; therefore we used denaturing conditions during gel filtration to reduce noncovalent aggregation. Detection of IgE binding to aggregates was unlikely in our analysis, since proteins were dissociated in 2D-PAGE analysis and N-terminal sequence analysis disclosed distinct sequences for each band (see Table 1). Although substantial, IgE recognition of BV high MW allergens was quantitatively lower than IgE recognition of the major allergens PLA_2 and hyaluronidase. The question of the clinical relevance of this quantitative difference is more difficult to address. Since there is usually no strict correlation between IgE serum titers and allergic reactions [35–37], only provocation tests with purified allergens would allow a definite answer to this question.

A vigorous T-cell proliferative response to pools #1 to #4 was observed in the BV hypersensitive group and in BV nonhypersensitive controls (Fig. 6a). Remarkably, although proteins from #1 and #2 are much less abundant in BV than proteins from #3 and #4 (hyaluronidase and acid phosphatase), they are comparable in terms of T-cell proliferation. Cytokine profiles of T-cells from hypersensitive patients should differ from that detected for BV nonhypersensitive controls' T-cells [38,39], since only IgG anti BV proteins were detected in the latter group (Fig. 6b). In a recent study [21], we observed a progressive increase in T-cell proliferation to PLA_2 during the incremental dose period of specific BV immunotherapy, contrasting with T-cell hyporesponsiveness in patients treated for more than 3 years. The early increase in T-cell proliferation coupled to a large variability in IgE response to BV allergens may expose patients during IT to the risk of sensitization to proteins to which they were not sensitized previously [40]. Taken together, these data support a better characterization of individual T-cell and IgE responses to allergens and may lead to a need for customization of BV extract for VIT. Further standardization of IT preparations, using either peptides or recombinant

allergens, supports the need for further studies of T-cell response during IT and a better characterization of allergen extracts. Our data strongly suggest that high MW allergens from BV should be added to the list of relevant candidates for recombinant or peptide-based IT.

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