

Delineation of PLA₂ epitopes using short or long overlapping synthetic peptides: interest for specific immunotherapy

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Summary

Background Venom immunotherapy is definitely indicated in severe systemic anaphylactic reactions to bee stings, but is not devoided of risks of anaphylaxis. Safer methods of immunotherapy need to be developed.

Objective To delineate phospholipase A₂ T-cell epitopes using short 15mer vs long 40–60mer overlapping peptides, and to approach the potential interest of a venom immunotherapy based on the use of long peptides (1–60, 51–99, 90–134) mapping the whole phospholipase A₂ molecule vs a restricted number of immunodominant epitopes.

Methods Proliferation of a CD8⁺ T cell depleted peripheral blood mononuclear cell fraction and short-term T-cell lines from unselected bee venom hypersensitive patients in response to phospholipase A₂ synthetic peptides.

Results Whereas T-cell proliferation to 15mer overlapping peptides was weak, T-cell response to long overlapping peptides was in contrast vigorous in all patients, mostly directed to C-terminal peptide 90–134. Our results did not support the concept of rare dominant T-cell epitopes, and disclosed T-cell responses to multiple epitopes in several patients. No significant IgE-binding to long overlapping peptides was detected except in one patient against peptide 90–134.

Conclusion 15mer peptides might not be sensitive enough to fully delineate all potential T-cell epitopes scattered along the allergen. Since they do not bind IgE *in vitro* or only weakly, and taking into account a T-cell response frequently directed to multiple epitopes, long overlapping peptides may represent ideal tools for immunotherapy.

Keywords: immunotherapy, hypersensitivity, T cells, bee venom, peptides, epitopes

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Introduction

Bee venom hypersensitivity is now a well defined indication for immunotherapy in patients with severe systemic anaphylactic reactions (grade III or IV according to Müller) [1–4]. Conventional venom immunotherapy (VIT) based on a standardized preparation of bee venom allergens provides complete protection in at least 80% of patients after a 3–5 year desensitization [5,6]. However, VIT, like other forms

of specific immunotherapy, may expose patients to systemic anaphylactic reactions, sometimes severe, in particular during the incremental dose period [7–9]. Though the mechanisms of conventional immunotherapy are not yet fully understood, VIT effects on T cells probably play a crucial role. After 3–5 years of treatment, VIT appears to modify the profile of cytokines secreted by T cells with a decrease in IL-4 and IL-5, while IFN γ is increased [10,11].

Peptide immunotherapy could mimic conventional VIT with several potential advantages [12]. The use of linear T-cell epitopes avoid IgE crosslinking and thus decrease the potential risk of anaphylaxis of conventional VIT. Peptides

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could, in these conditions, be administered at high dose and might induce a state of short-lived T-cell anergy, as suggested in mouse and rat models with immunogenic peptides containing immunodominant T-cell epitopes for Fel d 1 [13], the hen egg lysozyme [14], myelin basic protein [15,16] and type II collagen [17]. However, the use of T-cell epitopes requires as a prerequisite a precise delineation of the most frequently encountered epitopes of a given allergen. Furthermore, since T-cell recognition of antigen/MHC class II complex on antigen presenting cells is dependent on individual patient's HLA expression [18], the practical approach of peptide immunotherapy with minimal epitopes may become considerably more complicated. To define a peptide immunotherapy strategy for bee venom hypersensitivity, we studied in this paper the T-cell response to one of the major bee venom allergens, phospholipase A₂ (PLA₂), using 15mer overlapping peptides, and compared it *in vitro* with results obtained using three long overlapping peptides (40–60mer peptides) covering the whole PLA₂ molecule (peptides 1–60, 51–99 and 90–134).

Materials and methods

Patients

Ten unselected BV hypersensitive patients from our outpatients clinics were enrolled in the study. Inclusion criteria were systemic allergic reactions (degree III–IV) following a honey bee field sting (according to H. L. Müller's classification [19]), positive immediate intradermal skin tests (presence of a weal-and-flare reaction at BV concentration <0.1 µg/mL) and elevated specific IgE levels for whole bee venom (>0.35 kU/L as titrated by CAP system, Pharmacia, Uppsala, Sweden).

Reagents

Whole BV and PLA₂ were purchased from Latoxan (Rosans, France). PLA₂ was detoxified by reduction and carboxymethylation followed by cleavage with cyanogen bromide as described [20]. Tetanus toxoid (TT) (Swiss Serum Institute, Bern, Switzerland) was used as control antigen. Fifteen amino acid long peptides, overlapping the preceding one by five residues and three 40–50 amino acid long peptides with an overlap of 10 residues mapping the entire PLA₂ were generated using a multipetide synthesizer (Automated multiple peptide synthesizer, ABIMED, Germany). F-moc, t-Bu strategy was used according to Atherton *et al.* [21]. Peptides were purified by gel chromatography and used at a final concentration of 10 µg/mL. Amino acid analysis, analytical HPLC and mass spectrometry were used to assess composition and purity of peptides. For determination of MHC class II restriction,

MoAb BL2 (anti-DR), and MoAb SPVL3 (anti-DQ) were purchased from Immunotech SA, France. MoAb B7.21 is directed against DP and is a gift from Stefan Demotz, Institute of Biochemistry, Epalinges, Switzerland.

HLA class II DNA typing

HLA-DR generic typing (DR1–DR18, DR51, DR52) was performed on genomic DNA by PCR/SSO oligotyping on microtiter plate [22]. The analysis of HLA-DRB1, HLA-DQB1 and -DPB1 subtypes (high resolution typing) was performed by PCR/SSO-oligotyping on dot blot [23].

Cell preparation

PBMC were isolated from heparinized blood by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). For CD8⁺ cell depletion, PBMC were suspended in PBS containing 2% human AB⁺ serum (Swiss Red Cross, Bern, Switzerland) and 5 mM EDTA, then incubated at the concentration of 10⁷ cells/80 µL with anti-CD8 MoAb coated magnetic beads for 15 min. Cells were then separated in a magnetic cell sorter (MACS[®], Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany). CD8⁺ depleted fraction contained <1% CD8⁺ and >70% CD4⁺ T cells, as determined by flow cytometry (Epics Profile II, Coulter, Miami, FL, USA).

PBMC proliferation assays

PBMC (2 × 10⁵/well) from each donor were cultured in octoplicates in 96 well round bottom plates (Nunc, Roskilde, Denmark) in RPMI medium (Gibco, Basel, Switzerland) containing 10% AB⁺ serum (Swiss Red Cross, Bern, Switzerland) with appropriate concentration of antigen, according to Reece *et al.* [24]. After 6 days of culture in 5% CO₂ at 37°C, cells were harvested following the addition of 1 µCi of ³H-thymidine/well (Du Pont NEN Products Boston, MA, USA) during the final 18 h of culture. Results were calculated in cpm of ³H-thymidine incorporated into DNA. Since eight replicates for each peptide were used, T-cell response to a defined PLA₂ epitope was considered positive when mean of octoplicates in cpm >2 SD over background or stimulation index >2.

Short-term T-cell lines

T-cell lines were derived from PBMC of hypersensitive donors stimulated in 24 well plates (Nunc) (10⁶ cells/mL) with a mixture of three 40–60mer peptides (10 µg/mL) in the presence of autologous, irradiated PBMC (2500 rad) and maintained in supplemented 10% AB⁺/RPMI 1640 medium as described above. T-cell lines were restimulated after 7 and

14 days with autologous, irradiated PBMC and adequate antigen (10 µg/mL). Recombinant human IL-2 (rhuIL-2) (10 U/mL, Glaxo, Geneva, Switzerland) was added from day 9, twice a week. At day 14, to test for specificity, T cell lines were stimulated (1×10^5 cells/well in triplicates) with antigens and irradiated feeder cells. Seventy-two hours later, T cells were pulsed with 1 µCi of ^3H -Thymidine for 18 h and harvested. Results of proliferation were expressed as cpm of ^3H -thymidine incorporated into DNA.

T-cell cloning

T cell blasts from short-term PLA₂ specific T-cell lines were isolated from Percoll gradient and cloned at 0.3 cells/well in Terasaki plates supplemented with rhuIL-2 (20 U/mL), in the presence of autologous irradiated PBMC or EBV-transformed B cells, as described above. After expansion in 96 well plates, cloned T cells were tested for specificity against short 15 amino acid long peptides mapping PLA₂.

Immunoblotting

Proteins and peptides were separated by SDS-PAGE and blotted to PVDF membranes (Millipore AG, Volketswil, Switzerland) using a CAPS/methanol buffer (10 mM CAPS, 10% methanol, pH 11). Membranes were blocked with 10% fetal calf serum in Tris-buffered salt solution containing 0.05% Tween 20, then incubated for 72 h at 4°C with patients sera diluted 1/10 in Tris-buffer/0.05% Tween. After washing in Tris-buffer/0.05% Tween, IgE binding was assessed by membrane incubation with a horseradish peroxidase conjugated mouse anti-human IgE antibody (ANAWA, Wangen, Switzerland) and revealed by enhanced chemiluminescence (ECL, Amersham, UK).

Results

CD8⁻ PBMC response to 15mer peptides

Since CD4⁺ T cells play a crucial role in IgE regulation via IL-4 production and appear to be one of the target of specific immunotherapy [25,26], proliferative response to cyanogen bromide treated PLA₂ and PLA₂ peptides was examined on a CD8⁺ depleted fraction of PBMC (CD8⁻ PBMC) from BV hypersensitive patients. CD8⁻ PBMC were incubated either with short 15 amino acid long peptides overlapping the preceding one by five amino acids, or with three 40–60mer peptides covering the whole PLA₂ molecule, 1–60, 51–99 and 90–134. All peptides were used at the concentration of 10 µg/mL and were tested in octuplicates. T-cell response was considered positive when mean proliferation to a given peptide was > 2 SD or two stimulation indexes over background. Primary T-cell response to short 15 amino acid

long overlapping peptides varied largely between patients (Fig. 1). In general, cell proliferation was weak (mean stimulation index 2.1, range 1.4–4.1), and in three out of 10 patients, T cells did not even respond to any of the short peptides, despite the presence in each of those patients of serum anti-PLA₂ specific IgE. In the seven patients with a positive response as defined above, a T-cell proliferation was observed in response to a few peptides only (in general less than 3), except in one patient (patient 3), whose T-cell response was directed to multiple epitopes, and concerned peptides 1–15, 21–35, 31–45, 51–65, 71–85, 81–95, 101–115, 111–125 and 121–134 (Fig. 1). This, however, did not allow definition of a particular cluster of dominant epitopes, except perhaps peptide 81–99 to which three out of 10 patients responded. Taken together, these data show that mapping PLA₂ T-cell epitopes with 15 amino acid long overlapping peptides in an unselected group of patients was essentially inconclusive.

CD8⁻ PBMC response to long overlapping peptides

In contrast, mapping PLA₂ with three 40–60mer peptides (1–60, 51–99, 90–134) individually or as a mixture allowed detection of a T-cell response in all patients. T-cell response was enhanced compared with the response obtained with short peptides, despite an approximately five times lower molarity with 40–60mer peptides (Fig. 1B and Fig. 2). In eight out of 10 patients, T cells proliferated to the C-terminal peptide 90–134, in four to peptide 51–99 and in one patient to the N-terminal peptide 1–60. This distribution of T-cell response was also paralleled by the intensity of T-cell proliferation with the strongest response against peptide 90–134 (Fig. 2). All patients responded to a mixture of the three 40–60mer peptides, even though in two of them (patients 8 and 9), PBMC proliferation was weak and present only in this situation (Fig. 1B and Fig. 2). In two patients, proliferation to the three long peptide mixture was not paralleled by proliferation to PLA₂. Though these patients' T-cell responses to the 40–60mer peptide mixture were the weakest of the group, this may also suggest that detoxification of PLA₂ with cyanogen bromide may have disrupted methionine containing T-cell epitopes. Proliferation to tetanus toxoid used as control, was present in all patients. Taken together, our results on primary CD8⁻ PBMC indicate that short overlapping peptides are only weak inducers of T-cell responses in most patients. T-cell response analysis can be improved using 40–60mer peptides. This probably has to be ascribed to the capacity of 40–60mer peptides to recruit multiple minimal T-cell epitopes. Furthermore, our results, from an unselected group of patients, underline the difficulty in precisely delineating minimal T-cell epitopes from hypersensitive patients, and do not support the notion of a small number of dominant

(a)

Patients \ Peptides	1	2	3	4	5	6	7	8	9	10
1-15										
11-25										
21-35										
31-45										
41-55										
51-65										
61-75										
71-85										
81-99										
91-105										
101-115										
111-125										
121-134										

(b)

1-60										
51-99										
90-134										
3LP										
PLA ₂										
TT										

Fig. 1. Mapping PLA₂ with short (a) or long (b) peptides. CD8⁺ PBMC were stimulated for 6 days with 15mer peptides (10 µg/mL) or 40–60mer peptides (10 µg/mL) from PLA₂, as well as with a mixture of the three long peptides (3LP) (10 µg/mL), cyanogen bromide treated PLA₂ (10 µg/mL) and tetanus toxoid (TT) as a non-allergen control (10 µg/mL). PLA₂ epitopes inducing a ³H-thymidine incorporation into DNA (in cpm) >2 SD over background were marked by a shaded box.

epitopes, an observation which questions the use of short peptides for specific immunotherapy in atopy. In contrast, 40–60mer peptides were effective T-cell stimulators and allowed determination of the region of the allergen in which T-cell epitopes could be located, even in this particular hypersensitivity model, where patients are rarely in contact with the allergen.

Short-term T-cell line response to PLA₂ peptides

Since short overlapping peptides did not allow precise

delineation of PLA₂ T-cell epitopes using CD8⁺ PBMC, short term T-cell lines from seven bee venom hypersensitive patients were established. T-cell line responses to short overlapping peptides and to 40–60mer peptides are examined in Fig. 3. Two patterns of T-cell response to short peptides were observed: a T-cell response to multiple epitopes in three patients (patients 3, 4 and 5), and a response restricted to a few epitopes in three (patients 1, 2, 11). In general, most of the epitopes which induced a primary T-cell response were also able to induce a T-cell line proliferation. However, responses to some epitopes were

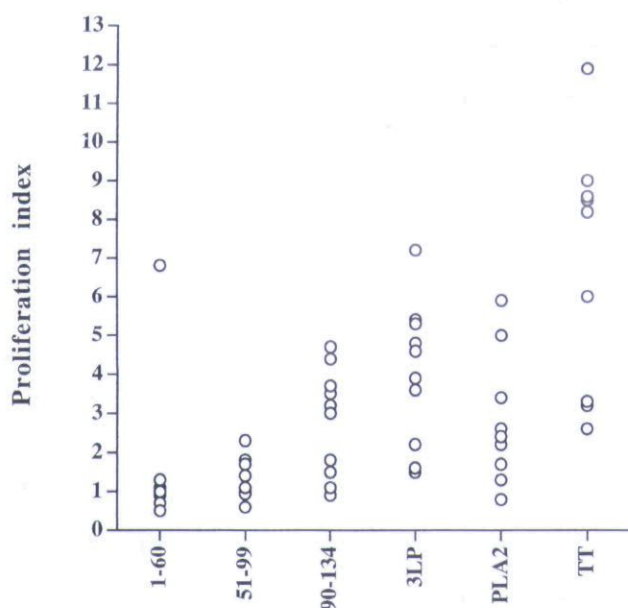


Fig. 2. Mapping PLA₂ with long overlapping peptides: proliferation of CD8⁺ PBMC in response to 40–60mer peptides, PLA₂ or TT as described above, expressed as stimulation index (SI).

only detectable in T-cell lines. Again, stimulation indices obtained with 40–60mer peptides were higher (Fig. 4) and confirmed a predominant response directed against epitopes from the C-terminal peptide 90–134, followed by peptide 51–99, then 1–60, which induced a proliferation in three patients' cell lines.

Identification of a novel PLA₂ T-cell epitope

A T-cell response to the N-terminal peptide 1–60 was present in only one patient in primary cultures, and in three patients in short-term lines. Therefore, various T-cell clones were derived from a patient (MLI) who did not respond to peptide 1–60 in primary culture. One of these clones, clone 8A, strongly proliferated in response to peptide 21–35, even at concentration as low as 5 ng/mL and reached a maximum between 1 and 5 μ g/mL, suggesting a high affinity of clone 8A for this peptide (Fig. 5a). Further mapping of the epitope recognized by clone 8A was performed with peptides covering amino acid sequence 16–35 and 19–38 (Fig. 5b). Interestingly, when clone 8A was incubated with 20mer epitopes 16–35 and 19–38, an enhancement of the proliferative response was observed at the optimal concentration of 1 μ g/mL, indicating that the optimal epitope was located within the sequence 16–35 or possibly 19–35. Subsequently, T-cell lines from patients 3, 4 and 5 were found strongly reactive to epitope 21–35, whereas the PBMC response of the same patients to epitope

21–35 was absent (patient 4) or at the limit of significance (patients 3 and 5).

Since previous reports indicated that T cell clones directed against PLA₂ were mainly DP/DQ-restricted [37], an analysis of the MHC class II restriction for clone 8A was performed (Fig. 6). Proliferation of clone 8A to peptide 21–35 was inhibited in a dose-dependent manner by a monoclonal antibody directed against DR (MoAb BL2). In contrast monoclonal antibodies directed to DP (MoAb B7.21) or DQ (MoAb SPVL3) did not inhibit clone proliferation. EBV-transformed B cells derived from patient MLI were HLA typed by PCR and expressed the DR allele DRB1 0301 and DRB1 *1501. As anti-DR MoAb BL2 recognizes a monomorphic region of DR, the allele expressing the MHC class II molecule responsible for presenting peptide 21–35 to clone 8A could not be determined.

In vitro IgE-binding to PLA₂ and its long peptides

IgE-binding to 40–60mer PLA₂ peptides and to native PLA₂ was examined by western blotting using chemiluminescence (Fig. 7). In 10 patients, a strong IgE binding to PLA₂ was detected, but no binding to peptides 1–60, 51–99 or 90–134 was demonstrated, except in one patient for peptide 90–134.

Discussion

Immunotherapy based on a restricted number of dominant T-cell epitopes using peptides derived from allergens has raised considerable interest, since peptides are efficient tolerogens, and in principle do not bind IgE [12,27]. Induction of T cell non-responsiveness was initially described *in vitro* in T-cell clones incubated with high (supraoptimal) concentration of peptides. These T-cell clones failed to respond to a second challenge in the presence of antigen presenting cells [28,29]. More recently, non-responsiveness to further antigenic stimulation in allergen-specific TH₂ clones was obtained using allergen-derived peptides in the absence of antigen presenting cells [29]. *In vivo*, the induction of T cell non-responsiveness has mainly been reported in animal models of autoimmunity [15,30,31]. In a murine model of allergy, IL-2 production in response to Fel d 1 was abrogated by prior treatment of animals with Fel d 1 peptides [13]. Clinical trials based on specific immunotherapy with two 27 amino acid long peptides derived from Fel d 1 in patients with cat allergy are currently in progress. Only minor systemic anaphylactic-like side effects have been reported, though none of the patients so far has developed anti-peptide IgE. Despite the advantage of the absence of IgE crosslinking, the immediate and long term clinical effect of an immunotherapy based on a restricted number of short peptides (minimal immunodominant T-cell

(a)

Patients Peptides	1	2	3	4	5	11	12
1-15							
11-25							
21-35							
31-45							
41-55							
51-65							
61-75							
71-85							
81-99							
91-105							
101-115							
111-125							
121-134							

(b)

1-60					ND		ND
51-99					ND		ND
90-134					ND		ND
3LP							
PLA ₂					ND		ND

Fig. 3. Mapping PLA₂ with long overlapping peptides using short-term T-cell lines: T cell blasts were stimulated for 3 days with 15mer peptides (10 µg/mL) or 40–60mer peptides (10 µg/mL) from PLA₂, as well as with a mixture of the three long peptides (3LP) (10 µg/mL), or PLA₂ (10 µg/mL). PLA₂ epitopes inducing a ³H-thymidine incorporation into DNA (in cpm) >2 SD over background were marked by a shaded box.

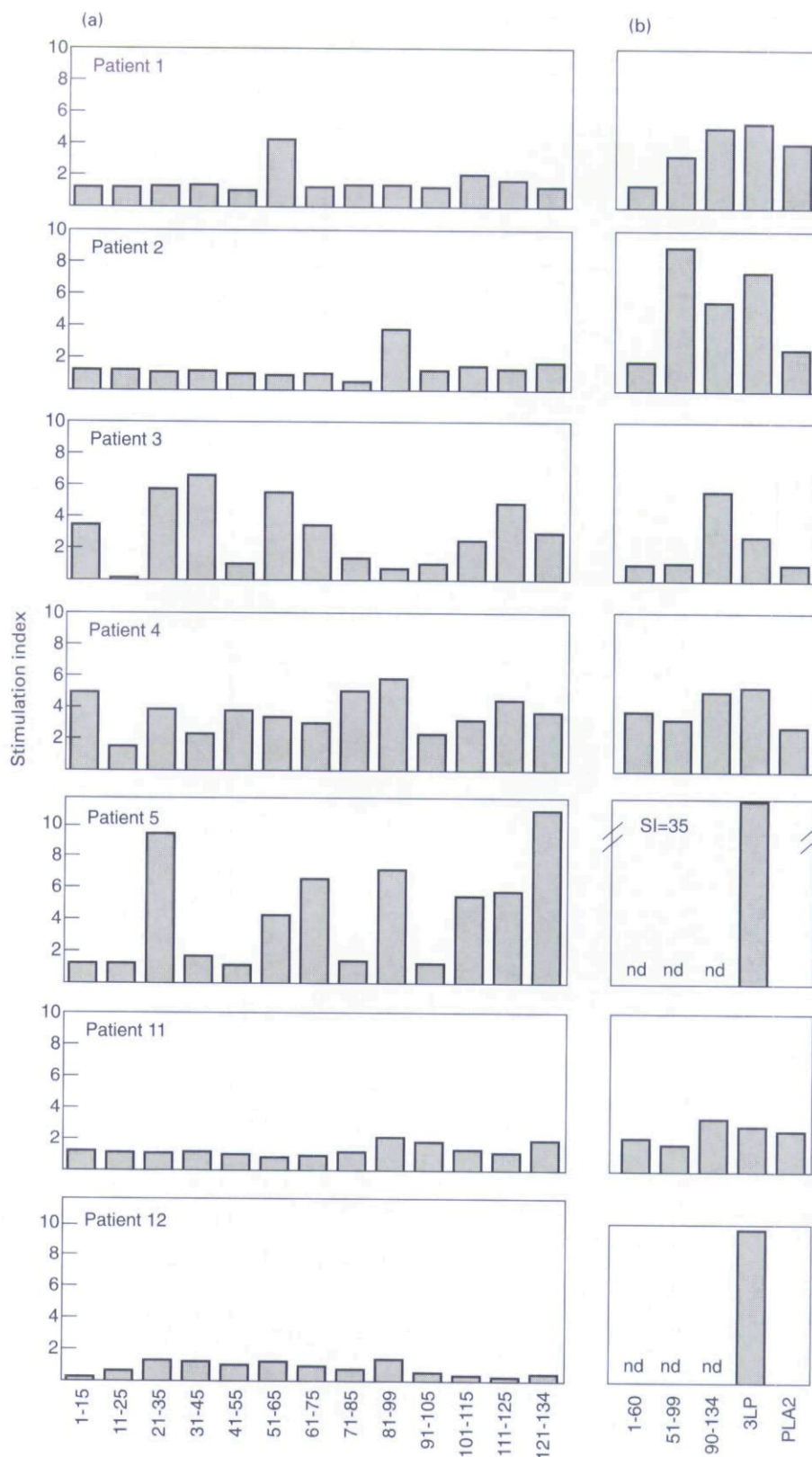


Fig. 4. Mapping PLA₂ with long overlapping peptides using short-term T-cell lines: T-cell blasts response to 15mer peptides (a) or 40–60mer peptides, 40–60mer peptide mixture (3LP) or PLA₂ (b) at the concentrations indicated in Fig. 3, expressed as stimulation index (SI).

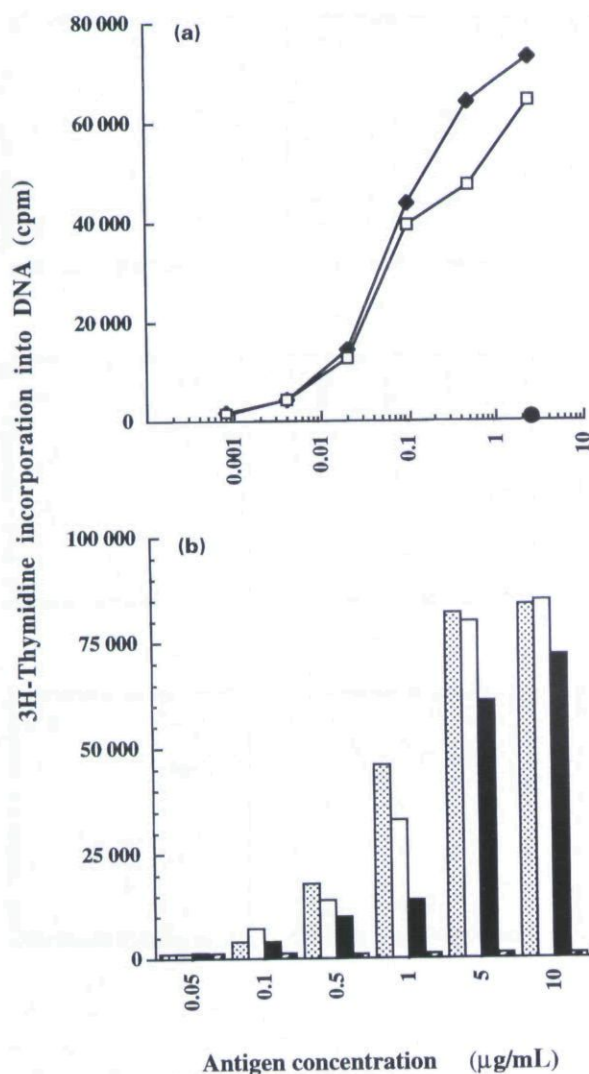


Fig. 5. Precise delineation of a novel PLA₂ epitope recognized by clone 8A. (a) Clone 8A proliferation to peptide 21-35 and to PLA₂, (b) Precise epitope mapping with peptides covering amino acids 16-38. (a) □, PLA₂; ♦, peptide 21-35; ●, unstimulated; (b) ▨, peptide 16-35; □, peptide 19-38; ■, peptide 21-35; ▤, peptide 11-25.

epitopes) are unclear, especially when high molecular weight allergens are concerned. Most allergens contain multiple T cell activation-inducing epitopes [32-35]. Whereas in initial studies on an allergen such as PLA₂, T-cell epitope delineation tended to demonstrate the dominance of a restricted number of epitopes [36,37], our results indicate that, in a significant number of patients, a T-cell response to multiple epitopes of PLA₂ was present. This observation is relevant with regard to the elaboration of an allergy vaccine. Indeed, although mouse studies have indicated that T-cell responses to complex native proteins can be limited to a few immunodominant epitopes [38-40], studies in human using tetanus toxoid or Fel d 1 as antigen

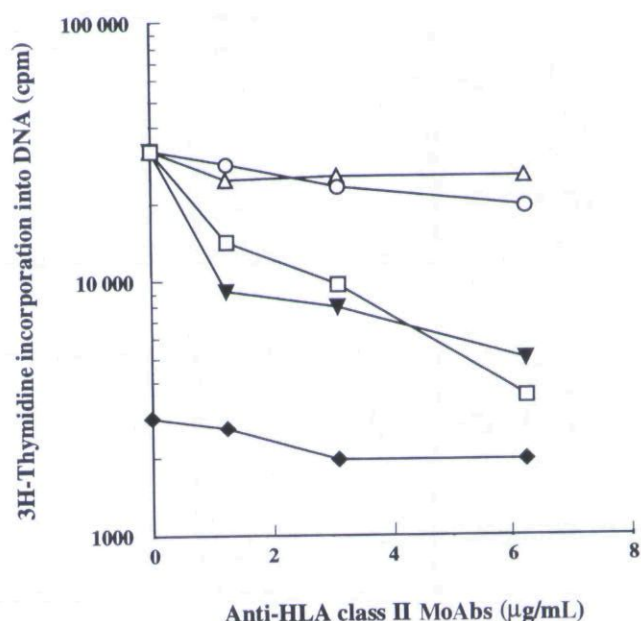


Fig. 6. Clone 8A is DR restricted determination of HLA class II restriction of T cell clone 8A. Prior to stimulation with antigen (1 μg/mL), clone 8A was incubated as indicated for 30' with anti-DR (BL2), -DP (B7.21) or -DQ (SPVL3) MoAb (10 μg/mL), or pan-anti-HLA class II MoAb 3B12, in the presence of autologous EBV-B cells. Control corresponds to T cells incubated with anti-HLA class II MoAbs in the absence of peptide 21-35. □, anti-DR; △, anti-DP; ○, anti-DQ; ▼, anti-DR/DP/DQ; ♦, control.

do not support this notion [24,35]. In consequence, the presence of less frequent (non-immunodominant) clones may render the approach of tolerization with a restricted set of peptides non-operational [41]. Clone 8A which recognizes a previously undescribed 21-35 epitope, might represent such a clinically relevant non-immunodominant epitope, since through IL-4 production, the clone may sustain IgE production by B cells. The use of a limited number of dominant peptide epitopes for immunotherapy may only transiently tolerize patients' T cells, until non-immunodominant epitope specific T cells become activated. In this respect, long overlapping peptides covering the whole allergen would have the advantage of including both dominant and non-dominant T-cell epitopes. Because CD4⁺ T cells are critical in the induction of IgE synthesis [26,42-44], our epitope mapping was here restricted to *in vitro* proliferation assays of a CD4⁺ PBMC fraction. This however, does not exclude a role for the CD8⁺ T cell subpopulation in downregulating the IgE response, via the induction of IFNγ producing CD8⁺ T cells for instance [45]. The possible contribution of CD8⁺ T cells in the allergic response has recently been suggested [46,47].

In our unselected group of BV hypersensitive patients described here, the delineation of T-cell epitopes using 15mer peptides had clear practical limitation, and was

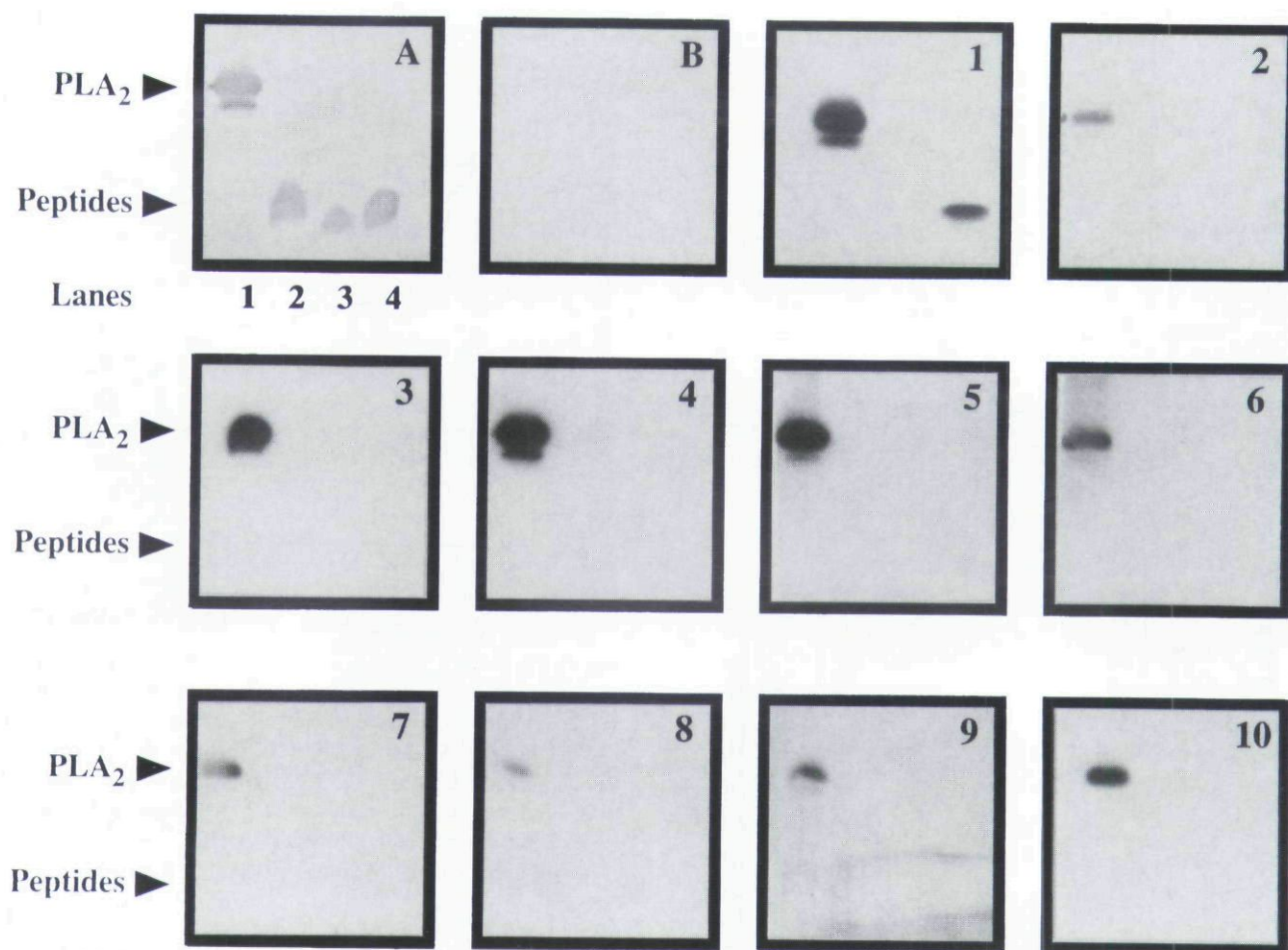


Fig. 7. Long peptides do not bind IgE. BV (lane 1, 10 μ g) and peptides 1–60, 51–99 and 90–134 (lane 2 through 4, 2 μ g) were separated by SDS-PAGE and blotted to PVDF membrane. Specific IgE binding was detected by chemiluminescence. PLA₂ and long peptide position are indicated on the left side. Panel A: Total protein staining with Protogold® (BioCell, England), panel B: membrane was incubated with the serum of an atopic, but non-BV hypersensitive patient, panels 1–10: membranes were incubated with sera from BV hypersensitive patients; IgE binding (to peptide 90–134) was detected in only one patient (panel 1).

of low sensitivity, often unreliable, inducing in CD8⁺ PBMC stimulation indexes at the limit of significance, in most cases. Short 15mer peptides may actually only target a restricted number of specific T-cell clones, some epitopes (immunodominant or non-immunodominant) remaining undetected. The low proliferative response has probably to be ascribed to the low T cell precursor frequency in patients who have been only rarely in contact with the allergen and sometimes long before tests were performed. Although a significant T-cell proliferative response to short peptides was obtained in short term T-cell lines, the pattern of T-cell epitopes may still be questioned, due to bias of *in vitro* selection of T cell clones. In contrast 40–60mer peptides mapping the whole allergen were able to stimulate a significant T-cell response even in CD8⁺ PBMC, which appears to be mainly directed against peptides 51–99 and

90–134. These regions include epitopes most often described previously by other groups [36,37].

Thus, apart from interest in their diagnostic use in mapping the most immunogenic regions of the allergen, long overlapping peptides may also represent a valuable alternative to immunotherapy with native allergens, particularly PLA₂. First, they have the above discussed advantage of possibly including all T-cell epitopes. With respect to MHC class II presentation, which may not be optimal with peptide overlaps of nine or 10 amino acids, the design of longer overlaps may even decrease the risk of incompletely covering some epitopes and may be considered [48,49]. Second, long peptides are able to efficiently stimulate T-cell responses in hypersensitive patients and may even be better tolerogens than short peptides. Third, and most importantly, in spite of their length, the 50–60 amino acid long peptides covering

PLA₂ *in vitro* do not bind IgE. IgE mainly recognizes conformational structures on PLA₂ [50], which even as an entire unfolded allergen has been shown to only minimally bind IgE [51,52]. Although the risk of crosslinking IgE may be higher with long rather than with short peptides, this does not appear to occur in our case, at least *in vitro*, and suggests that long peptides may also be safe tools for immunotherapy. *In vivo* intradermal tests will be necessary to definitively demonstrate this hypothesis. Fourth, another disadvantage of a restricted number of dominant peptides would be the necessity to HLA type patients. Indeed, T cell epitopes are restricted by the different MHC class II antigens [32,33], including HLA-DRβ3 and -DRβ4 gene products [53]. The use of long peptides would avoid the need for HLA-typing patients or defining individual patient T cell epitope profiles, a task which would be laborious and time-consuming in clinical practice. Clinical trials to verify these hypothesis are in progress.

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References

- Valentine MD. Insect venom allergy: diagnosis and treatment. *J Allergy Clin Immunol* 1984; 73:299–304.
- Reisman RE, Livingston A. Venom immunotherapy: 10 years of experience with administration of single venoms and 50 µg maintenance doses. *J Allergy Clin Immunol* 1992; 89:1189–95.
- Reisman RE. Venom hypersensitivity. *J Allergy Clin Immunol* 1994; 94:651–8.
- Müller U, Mosbech H. Position paper: Immunotherapy with hymenoptera venoms. *Allergy* 1993; 48(Suppl. 14):37–46.
- Golden DBK, Kwitrovich KA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Discontinuing venom immunotherapy: Outcome after five years. *J Allergy Clin Immunol* 1996; 97:579–87.
- Müller U, Berchtold E, Helbling A. Honey bee venom allergy: results of a sting challenge 1 year after stopping successful venom immunotherapy in 86 patients. *J Allergy Clin Immunol* 1991; 87:702–9.
- Lockey RF, Benedict LM, Turkeltaub PC, Bukantz SC. Fatalities from immunotherapy (IT) and skin testing (ST). *J Allergy Clin Immunol* 1987; 79:660–77.
- Lockey RF, Turkeltaub PC, Olive ES, Hubbard JM, Baird-Warren IA, Bukantz SC. The Hymenoptera venom study. III: Safety of venom immunotherapy. *J Allergy Clin Immunol* 1990; 86:775–80.
- Lockey RF. Adverse reactions associated with skin testing and immunotherapy. *Allergy Proc* 1995; 16:293–6.
- Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995; 154: 4187–94.
- McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: Comparison of rush and conventional immunotherapy. *Clin Experiment Allergy* 1995; 25:828–38.
- Wallner BP, Geffer ML. Immunotherapy with T cell reactive peptides derived from allergens. *Allergy* 1994; 49:302–8.
- Briner TJ, Kuo MC, Keating KM, Rogers BL, Greenstein JL. Peripheral T cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen, Fel d I. *Proc Natl Acad Sci (USA)* 1993; 90:7608–12.
- Adorini L, Muller S, Cardineaux F, Lehman PV, Falcioni F, Nagy ZA. In vivo competition between self peptides and foreign antigens in T cell activation. *Nature* 1988; 33:623–5.
- Gaur A, Wiers B, Liu A, Rothbard J, Fathman CG. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science* 1992; 258:1491–4.
- Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol* 1990; 8:579–621.
- Ku G, Kronenberg M, Peacock DJ, Tempst P. Prevention of autoimmune experimental arthritis with a peptide fragment of type II collagen. *Eur J Immunol* 1993; 23:591–9.
- Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.)* 1974; 248:701–2.
- Müller HL. Diagnosis and treatment of insect sensitivity. *J Asthma Res* 1966; 3:331–3.
- Demotz S, Lanzavecchia A, Eisel U, Niemann H, Widmann C, Corradin G. Delineation of several DR-restricted tetanus toxin T cell epitopes. *J Immunol* 1989; 142:394–402.
- Atherton E, Logan CJ, Sheppard RC. Peptide synthesis. Part 2. *Bioorg Chem* 1979; 8:351.
- Cros P, Allibert P, Mandrand B, Tiercy J-M, Mach B. Genetic testing on microtiter plates: oligonucleotide genotyping of HLA polymorphism. *Lancet* 1992; 340:870–3.
- Tiercy J-M, Grundschober C, Jeannet M, Mach B. A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes. In: K. M. Hui and J. Bidwell, eds. *Handbook of HLA Typing Techniques*. Boca Raton: CRC Press Inc., 1993:117–47.
- Reece JC, Geysen HM, Rodda SJ. Mapping the major human T helper epitopes of tetanus toxoid: the emerging picture. *J Immunol* 1993; 151:6175–84.
- Pène J, Rousset F, Brière F, Chrétien J, Bonnefoy J-Y, Spits H, Yokota T, Arai N, Banchereau J, de Vries J. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferon γ and α and prostaglandin E2. *Proc Natl Acad Sci (USA)* 1988; 85:6880–4.
- Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med* 1993; 178:2123–2130.

- 27 Yssel H, Fasler S, Lamb J, de Vries JE. Induction of non-responsiveness in human allergen-specific type 2 T helper cells. *Curr Opin Immunol* 1994; 6:847–52.
- 28 Lamb JR, Skidmore BJ, Green N, Chiller JM, Feldmann M. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza haemagglutinin. *J Exp Med* 1983; 157:1434–47.
- 29 Fasler S, Aversa G, Terr A, Thestrupedersen K, de Vries JE, Yssel H. Peptide-induced anergy in allergen-specific human Th2 cells results in lack of cytokine production and B cell help for IgE synthesis — Reversal by IL-2, not by IL-4 or IL-13. *J Immunol* 1995; 155:4199–206.
- 30 Wraith DC, McDevitt HO, Steinman L, Acha-Orbea H. T cell recognition as the target for immune intervention in autoimmune disease. *Cell* 1989; 57:709–15.
- 31 Critchfield JM, Racke MK, Zunika-Pflücker JC, Cannella B, Raine CS, Goverman J, Lenardo MJ. T-cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science* 1994; 263:1139–43.
- 32 Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, de Vries JE. T cell activation inducing epitopes of the house dust mite allergen Der p I. Induction of a restricted cytokine production profile of Der p I-specific T cell clones upon antigen-specific activation. *J Immunol* 1992; 148:738–45.
- 33 Verhoef A, Higgins JA, Thorpe CJ, Marsh SG, Hayball JD, Lamb JR, O'Hehir RE. Clonal analysis of the atopic immune response to the group 2 allergen of dermatophagoides spp.: identification of HLA-DR and -DQ restricted T cell epitopes. *Int Immunol* 1993; 5:1589–97.
- 34 van Neerven JR, vant'Hof W, Ringrose JH, Jansen HM, Aalberse RC, Wierenga EA, Kapsenberg ML. T cell epitopes of house dust mite major allergen Der p II. *J Immunol* 1993; 151:2326–35.
- 35 van Neerven RJ, van Pol MM, van Milligen FJ, Jansen HM, Aalberse RC, Kapsenberg ML. Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol* 1994; 152:4203–10.
- 36 Dhillon M, Roberts C, Nunn T, Kuo M. Mapping human T cell epitopes on phospholipase A2: The major bee-venom allergen. *J Allergy Clin Immunol* 1992; 90:42–51.
- 37 Carballido JM, Carballido-Perrig N, Kägi MK, Meloen RH, Wüthrich B, Heusser CH, Blaser K. T cell epitope specificity in human allergic and non-allergic individuals to bee venom phospholipase A2. *J Immunol* 1993; 150:3582–91.
- 38 Baumhüter S, Wallace CJA, Proudfoot AEI, Bron C, Corradin G. Multiple T cell antigenic determinants identified within a limited region of the horse cytochrome c molecule. *Eur J Immunol* 1987; 17:651–6.
- 39 Gammon G, Shastri N, Cogswell J, Wilbur S, Sadegh-Nasseri S, Krzych U, Miller A, Sercarz E. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol Rev* 1987; 98:53–73.
- 40 Perkins DL, Berriz G, Kamradt T, Smith JA, Geftner ML. Immunodominance: intramolecular competition between T cell epitopes. *J Immunol* 1991; 146:2137–44.
- 41 Gammon G, Sercarz E. How some T cells escape tolerance induction. *Nature* 1989; 342:183–5.
- 42 DeKruyff RH, Turner T, Abrams JS, Palladino MA, Umetsu DT. Induction of human IgE synthesis by CD4+ T cell clones: requirement for interleukin 4 and low molecular weight B cell growth factor. *J Exp Med* 1989; 170:1477–93.
- 43 Walker C, Virchow J-C, Bruijnzeel PLB, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J Immunol* 1991; 146:1829–35.
- 44 Coffman RL, Seymour BW, Hudak S, Jackson J, Rennick D. Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* 1989; 245:308–10.
- 45 McMenamin C, Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J Exp Med* 1993; 178:889–899.
- 46 Holmes BJ, Diaz-Sanchez D, Lawrence RA, Bell EB, Maizels RM, Kemeny DM. The contrasting effects of CD8(+) T cells on primary, established and Nippostrongylus brasiliensis-induced IgE responses. *Immunology* 1996; 88:252–60.
- 47 Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D, Lee TH. The role of CD8+ T cells in immunoglobulin E regulation. *Allergy* 1995; 50:9–14.
- 48 Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991; 353:622–7.
- 49 Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey HM, Sette A. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* 1992; 256:1817–20.
- 50 Schneider T, Lang AB, Carballido JM, Babi LFS, Dudler T, Kägi MK, Blaser K, Suter M. Human monoclonal or polyclonal antibodies recognize predominantly discontinuous epitopes on bee venom phospholipase A2. *J Allergy Clin Immunol* 1994; 94:61–70.
- 51 Forster E, Dudler T, Gmachl M, Aberer W, Urbanek R, Suter M. Natural and recombinant enzymatically active or inactive bee venom phospholipase A(2) has the same potency to release histamine from basophils in patients with Hymenoptera allergy. *J Allergy Clin Immunol* 1995; 95:1229–35.
- 52 Müller UR, Dudler T, Schneider T, Cramer R, Fischer H, Skrbic D, Maibach R, Blaser K, Suter M. Type I skin reactivity to native and recombinant phospholipase A(2) from honeybee venom is similar. *J Allergy Clin Immunol* 1995; 96:395–402.
- 53 O'Hehir RE, Mach B, Berte C, Greelaw R, Tiercy J-M, Bal V, Lechler RI, Trowsdale J, Lamb JR. Direct evidence for a functional role of HLA-DRB1 and -DRB3 gene products in the recognition of Dermatophagoides spp. (house dust mite) by helper T lymphocytes. *Int Immunol* 1990; 2:885–92.

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