

T Cell Epitope Specificity in Human Allergic and Nonallergic Subjects to Bee Venom Phospholipase A2¹

José M. Carballido,^{2*} Nicole Carballido-Perrig,^{*} Martin K. Kägi,[†] Rob H. Meloen,[‡] Brunello Wüthrich,[†] Christoph H. Heusser,[§] and Kurt Blaser^{*}

^{*}Swiss Institute of Allergy and Asthma Research, Davos, Switzerland; [†]Allergy Unit, Department of Dermatology, University Hospital, Zürich, Switzerland; [‡]Central Veterinary Institute, Lelystad, The Netherlands; and [§]Pharmaceuticals Research, CIBA, Ltd., Basel, Switzerland

ABSTRACT. Phospholipase A2 (PLA) is a biochemically fully defined glycoprotein, representing the main allergen of bee venom. We have established CD4⁺ T cell clones specific to PLA from subjects allergic and nonallergic to bee sting. By screening the epitope specificity of these clones with 62 synthetic overlapping dodecapeptides representing the PLA molecule, two immunogenic epitopes, PLA 81–92 and PLA 113–124, were identified. Additional screening, using longer peptides of up to 18 residues, revealed a third epitope at position PLA 45–62. These epitopes are recognized by specific T cell clones in association with HLA-DP and -DQ molecules, although HLA-DR-associated responses to PLA exist. Primary in vitro proliferation of unfractionated PBMC from bee sting allergic, hyposensitized, or hyperimmune subjects to PLA-derived peptides revealed the same immunogenic sites as found in the T cell clones. Among the different groups of individuals, proliferative responses to the PLA molecule and fragments thereof were generally higher in allergic patients than in nonallergic subjects. Thus, at least three linear epitopes are involved in T cell recognition of PLA, independently whether or not subjects are allergic. *Journal of Immunology*, 1993, 150: 3582.

The Ag specificity of an immune response is determined by the activation of specific lymphocyte clones through their surface Ag receptors. Whereas surface Ig on B lymphocytes recognize native protein structures of Ag in solution, the TCR generally recognize a complex of processed antigenic peptide bound to self MHC molecules on APC (1, 2). CD4⁺ T cells become activated upon presentation of exogenous proteins associated with HLA class II Ag (3, 4).

The generation of T-dependent B cell responses results

from elaborated synergistic and antagonistic regulatory signals that control B cell activation, proliferation, and subsequent differentiation into plasma cells. The cross-linking of surface Ig by the Ag originates the first signal and initiates processing and presentation of the Ag to CD4⁺ T cells (5). The T lymphocytes become stimulated and deliver signals to B cells, via both physical contact and T cell-derived soluble factors (6). The qualitative and quantitative composition of the cytokines released from activated T cells regulates the isotype produced by B cells (7). Since the pattern of cytokines generated in vivo can be modulated by the antigenic peptide structure or the MHC haplotype recognized by T cells (8), a better knowledge of the elements involved in the ternary peptide/MHC/TCR complexes is crucial to understand the development of antibody-mediated allergic diseases and their therapy.

With the recent availability of the molecular structure of defined allergens, some T cell epitopes have been localized in *Dermatophagoides pteronyssinus* allergen I (*Der p* I) (9, 10), *Ambrosia artemisiifolia* allergen I (*Amb a* I) (11), rat

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² Address correspondence and reprint requests to José M. Carballido, Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH-7270 Davos, Switzerland.

α -2 urinary protein (RA2UG) (12), and bee venom apamin (13).

PLA³ is the main allergenic component of bee venom (14, 15). It is a glycoprotein of 14 to 16 kDa consisting of 134 amino acids (16) with known three-dimensional crystal structure (17). After initial response to bee sting, generally low affinity anti-PLA IgG1 antibodies are elicited (18). With repeated exposure, most subjects elicit high affinity IgG4 antibodies to PLA (18, 19). However, some individuals generate PLA-specific IgE antibodies and develop bee sting allergy (15, 20). It is known that IgE antibody formation requires T cell contact and IL-4 (21), however, the mechanisms of T cell activation leading to a preferential IL-4 production remain to be identified.

Little is known about molecular interactions involved in the immune response to PLA. In the mouse, the existence of conformational PLA epitopes that stimulate T cell hybridomas has been suggested (22). In humans, the capacity of PLA-derived peptides to bind HLA-DR1, -2, -5, and DRw52a molecules (23) and the association of the HLA-DR4 haplotype with nonallergic responses in bee-stung subjects have been reported (24).

This work was aimed to identify epitope structures within PLA and to correlate human T cell responses elicited by defined peptides with states of bee sting allergy. The dissection and correct manipulation of the elements involved in T cell activation could contribute to the development of new approaches in allergen-specific immunotherapy.

Materials and Methods

Ag

PLA (P-9279; Sigma) was reduced and carboxymethylated as described (25) to remove enzymatic activity and associated cell toxic effects. Dodecapeptides, overlapping 10 sequential amino acids from PLA, were synthesized by pin technology using a modification of the Pepscan method as described elsewhere (26). PLA 81–92 and 113–124 fragments were synthesized by Dr. Schneider and Dr. Rolli (Institute for Clinical Immunology, Inselspital, University of Bern, Switzerland), as described for other peptides (27). The purity was higher than 95%. PLA peptides 1–15, 16–31, 32–44, 66–82, and 120–134 were produced and kindly provided by Dr. Kamber, Dr. Flörsheimer, and Dr. Hartmann, CIBA Ltd., Basel, Switzerland. The purity of these fragments was higher than 95%. The octadecapeptides comprising the PLA sequences 8–25, 24–41, 36–53, 45–62, 54–71, 74–91, 76–93, 81–98, 94–111, 107–124, 111–128, and 114–131 were purchased from Cambridge Research Biochemicals, Ltd., Northwich, United Kingdom. Purity

ranged from 80 to 95%, depending on the sequence. None of the above peptides was recognized by serum IgG or IgE from bee sting-sensitized donors by using different ELISA techniques. The chemically treated PLA showed antibody-binding capacity in ELISA similar to that of the native form.

Human subjects

Three groups of bee venom-sensitized donors representing allergic ($n = 20$), hyposensitized ($n = 8$), and nonallergic hyperimmune ($n = 12$) subjects, summarized in Table I, were studied. Allergic donors presented immediate type reactions after bee sting, classified according to the method of Müller (28). The amount of bee venom-specific IgE, measured by Phadebas radioallergosorbent or CAP test (Pharmacia, Uppsala, Sweden), ranged from 2.8 to 483 PRU, with an average 187 PRU. Allergic donors responded positively to intracutaneous bee venom skin test (Pharmalgen; Pharmacia), and presented elevated serum levels of anti-PLA IgE compared to specific IgG4 antibodies. These subjects did not receive bee venom immunotherapy at the time of blood collection. The subjects of the hyposensitized group originally showed general reactions to bee sting and were exposed to conventional hyposensitization for at least 1 yr with the exception of donor Ak, who was rush-desensitized. The group of hyperimmune individuals is represented by beekeepers without clinical history of bee sting allergy. They were CAP class ≤ 1 (≤ 0.5 PRU) and expressed serum anti-PLA antibodies mainly of the IgG4 subclass.

Cells and culture conditions

Cloning and maintenance of PLA-specific T cell clones and EBV transformation of autologous B cells were previously described in detail (29). Peripheral blood was obtained by venipuncture from bee venom-sensitized subjects after informed consent. PBMC were isolated by Ficoll-Paque (Pharmacia) density gradient centrifugation, counted, and resuspended in fresh medium. RPMI 1640 culture medium supplemented with 1 mM sodium pyruvate, 1% MEM non-essential amino acids, 1% MEM vitamins, 2 mM L-glutamine, 100 μ g/ml kanamycin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.05 mM 2-ME (all from Gibco, Basel, Switzerland) was used in all cases as either RPMI/AS or RPMI/FCS (Sera-lab, Crawley Down, United Kingdom). Experiments with T cell clones were set up in RPMI/FCS. Experiments involving PBMC were performed in RPMI/AS to avoid unspecific stimulation.

Proliferation and HLA-D restriction of PLA-specific T cell clones

Cultures were performed during 48 h at 37°C, 5% CO₂ in 200 μ l RPMI/FCS using 96-well flat bottomed microtiter

³ Abbreviations used in this paper: PLA, bee venom phospholipase A2; EBV-B, EBV-transformed B cells; SI, stimulation index; PRU, Pharmacia relative units; RPMI/AS, RPMI supplemented with 10% autologous serum; RPMI/FCS, RPMI supplemented with 10% FCS.

Table 1
Clinical profile of bee venom-sensitized donors

Donor	Reaction Type ^a	Radioallergosorbent/ CAP Tests (PRU)	Skin Test ^b (µg/ml)	Anti-PLA IgE (RU/ml) ^c	Anti-PLA IgG4 (µg/ml)	Specific Therapy
Allergic						
Al	SLR ^d	187.0	≥0.1	165.38 ± 6.75	20.71 ± 5.29	
Ax	IV	3.9	≥1.0	7.41 ± 0.43	0.04 ± 1.27	
Dd	III	17.9	≥0.001	41.46 ± 3.70	1.67 ± 0.25	
Eu	III	36.0	≥0.001	39.49 ± 2.11	3.16 ± 0.69	
Hu	II	5.5	≥0.01	15.68 ± 1.17	0.76 ± 0.05	
Gh	III	66.5	≥0.01	47.14 ± 1.71	1.25 ± 0.12	
Jg	SLR	8.3	≥0.1	37.90 ± 2.05	0.66 ± 0.02	
Ka	SLR	297.0	≥0.01	67.10 ± 3.40	0.01 ± 0.01	
Kd	IV	14.3	≥0.0001	43.29 ± 1.27	0.25 ± 0.01	
Mb	SLR	21.0	≥0.01	26.35 ± 2.08	2.62 ± 0.80	
Mm	II	4.8	≥0.001	39.11 ± 1.23	2.75 ± 0.37	
Re	II	42.1	ND	401.83 ± 6.71	3.30 ± 0.47	
Rh	III	45.0	ND	50.12 ± 4.70	3.91 ± 0.72	
Rr	SLR	483.0	≥0.01	120.01 ± 2.80	2.94 ± 0.51	
Rs	II	3.5	≥0.01	27.92 ± 0.56	3.17 ± 1.12	
St	IV	12.2	ND	71.11 ± 2.04	4.17 ± 0.23	
Ur	III	33.0	≥0.0001	270.33 ± 9.39	21.39 ± 1.14	
Wh	II	2.8	≥0.01	40.62 ± 3.74	5.06 ± 0.62	
Wi	IV	7.4	ND	65.39 ± 7.63	6.86 ± 0.80	
Ws	III	49.9	≥0.001	59.41 ± 4.01	5.90 ± 1.11	
Hyposensitized						
Ak	IV	25.7	≥0.01	25.16 ± 1.17	8.80 ± 0.73	1992–
Bs	III	3.5	≥0.1	7.92 ± 1.09	2.27 ± 0.56	1990–
Fi	II	4.2	ND	5.92 ± 1.09	5.59 ± 0.55	1986–
Hb	IV	19.0	≥0.01	15.16 ± 1.16	7.18 ± 1.01	1983–
Jo	IV	3.0	ND	6.72 ± 1.69	0.80 ± 0.00	1970–1976
Ke	III	3.5	≥0.01	8.44 ± 0.48	1.03 ± 0.12	1989–
Me	IV	14.1	ND	10.39 ± 1.23	0.51 ± 0.02	1989–
Mj	III	2.6	≥0.1	12.16 ± 0.07	0.92 ± 0.13	1991–
Hyperimmune						
Ag	NCH	0.1	ND	0.02 ± 0.50	33.85 ± 0.89	
Bu	NCH	0.4	ND	1.49 ± 0.44	29.55 ± 0.56	
Ch	NCH	0.5	ND	2.72 ± 1.72	24.09 ± 1.88	
Fu	NCH	Ø	ND	0.95 ± 0.20	7.37 ± 0.92	
Gb	NCH	0.1	ND	1.31 ± 0.12	26.28 ± 0.47	
Hh	NCH	Ø	ND	0.47 ± 0.04	1.33 ± 0.14	
Hs	NCH	Ø	ND	0.01 ± 0.08	48.56 ± 1.65	
Mi	NCH	Ø	ND	1.22 ± 0.44	46.15 ± 2.89	
Mr	NCH	Ø	ND	0.03 ± 0.07	71.43 ± 1.24	
Rb	NCH	0.3	ND	2.27 ± 0.12	17.72 ± 1.37	
We	NCH	0.3	ND	1.58 ± 0.41	8.42 ± 0.06	
Wx	NCH	Ø	ND	1.22 ± 0.30	12.13 ± 0.16	

^a Reactions according to the classification of Mueller (28).

^b Numbers represent µg/ml of bee venom presentation able to induce a positive intracutaneous reaction.

^c Relative units ± SD compared to a standard serum (100 relative units/ml) collected from an allergic radioallergosorbent test class 4 patient.

^d SLR, severe local reaction; I, light general reaction; II, moderate general reaction; III, severe general reaction; IV, shock reaction; NCH, beekeepers with no clinical history of bee sting allergy; Ø, not detectable.

plates. Samples in triplicate, containing 3×10^4 PLA-specific T cell clones and an equal number of autologous irradiated (5000 rad) EBV-B cells, were incubated in the presence of different amounts of PLA. For epitope mapping, all PLA-specific T cell clones were stimulated with the full panel of 62 sequentially overlapping dodecapeptides, synthesized by pin technology, and with the battery of 19 longer fragments, mostly octadecapeptides, described in Figure 3. Each of the 62 dodecapeptides was resuspended in PBS and administered at a final concentration of 0.7 µM. This corresponds to concentrations from 0.91 to 1.23 µg/ml, depending on the molecular mass of the peptides. All 19

longer peptides were diluted in PBS and titrated from 0.07 to 6 µM in 1:3 step dilutions. Proliferation was measured by liquid scintillation in a Pharmacia-Wallac β-plate counter after a 12-h pulse with 1 µCi/well of [³H]TdR. HLA restriction was determined by inhibition of T cell proliferation, using suboptimal amounts of PLA and different mAb directed to monomorphic epitopes on HLA-DR, DQ (Clonab DR, Clonab DQ; Biotest, Dreieich, Germany), or -DP (B/721; Becton-Dickinson, Basel, Switzerland) molecules. Anti-DRB4 (DRw53) and control anti-class I mAb, both kindly provided by Dr. Z. Nagy, Sandoz, Basel, Switzerland, were included.

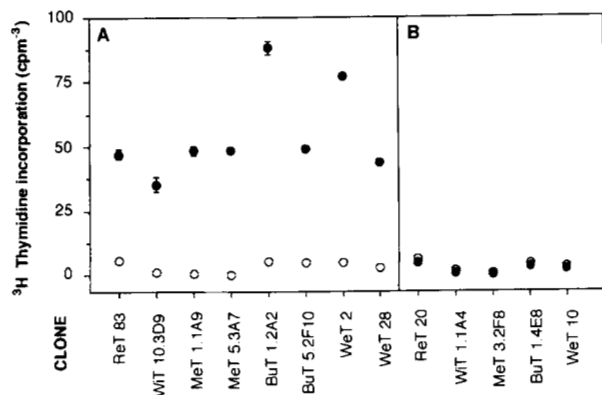


FIGURE 1. T cell responses of (A) PLA-specific and (B) PLA-nonspecific T cell clones in the presence of autologous irradiated EBV-B cells. ●, With 0.3 μ M PLA; ○, without Ag.

PBMC proliferation assay

Responses of unfractionated PBMC to immunogenic peptides were determined by [^{3} H]TdR incorporation of 3×10^5 PBMC incubated with equimolar amounts (from 6 to 0.07 μ M in 1:3 steps) of PLA or PLA-derived peptides. Cultures were set up in 200 μ l of RPMI/AS in triplicate wells of 96-well flat bottomed Costar plates, incubated for 5 days, and followed with an additional overnight pulse of 1 μ Ci/well of [^{3} H]TdR. The SI was calculated as the ratio of mean cpm obtained in the presence of Ag to mean cpm in the absence of Ag.

Results

Ag specificity of T cell clones

To study the immune response to bee venom PLA, we have generated a panel of CD4⁺ T cell clones from individuals allergic (Re, Wi), hyposensitized (Me), and nonallergic hyperimmune (Bu, We) to bee sting (29). As shown in Figure 1A, all PLA-specific T cell clones proliferated in response to the Ag presented by autologous irradiated EBV-B cells. The T cell proliferation was not due to nonspecific mitogenic effects of PLA, since non-PLA-specific clones derived from the same subjects did not show any significant response under the same conditions (Fig. 1B). The PLA-specific T cell clones were generated against the entire PLA molecule (29). They were isolated from different in vitro priming conditions and different subjects to avoid the selection of cells originating from the same clone.

T cell epitope mapping of bee venom PLA

Based on the published sequence (16), 62 sequentially overlapping dodecapeptides representing the full length of the PLA molecule were synthesized. The capability of equimolar concentrations of these dodecapeptides to induce proliferation in all PLA-specific T cell clones shown in Figure 1A was assayed using autologous irradiated EBV-B cells. As demonstrated in Figure 2, two T cell epitopes could be

located in the C-terminal half of PLA. The clone BuT 1.2A2 clearly responded to the peptide PLA 81–92. A reduced response was also observed with the overlapping fragment PLA 79–90, and no significant signals were detected with any of the other dodecapeptides. The clone BuT 5.2F10 recognized an epitope located at position 113–124 within the PLA molecule. This clone also showed an overlapping response to the fragment PLA 115–126. The remaining PLA-specific clones did not proliferate in response to any of the 62 PLA-derived dodecapeptides assayed under the same conditions.

Since the amount of protein that can be obtained by synthesis with pin technology is limited, and since small peptides can easily be degraded in culture, all PLA-specific T cell clones were further evaluated against a series of 19 longer fragments available in higher amounts. This battery of mainly octadecapeptides, as presented in Figure 3, which includes the immunogenic epitopes PLA 81–92 and PLA 113–124, was titrated over a range of five concentrations. As shown in Figure 4A, clone BuT 1.2A2, which proliferated in response to PLA 81–92, also recognized to the same or slightly higher degree the longer fragments including this epitope. Clone BuT 5.2F10 could be stimulated in a dose-dependent manner by the PLA-derived peptides comprising residues 107–124, 111–128, and 114–131 as well as the dodecapeptide PLA 113–124 (data not shown). On the other hand, clone MeT 5.3A7, which did not respond to 0.7 μ M of any of the 62 dodecapeptides, proliferated in a dose-dependent manner to 0.07 to 6 μ M PLA 45–62 or to equimolar doses of the entire PLA molecule (Fig. 4B).

T cell epitopes of PLA are recognized by specific T cell clones in association with different HLA-D molecules

As shown in Figure 5A, specific proliferation of clone BuT 1.2A2 can be blocked using mAb directed to monomorphic determinants in the HLA-DP molecules. The mAb against HLA-DR, DRw53, DQ or against HLA class I molecules failed to inhibit the T cell proliferation. Similar experiments performed with clone BuT 5.2F10, represented in Figure 3B, demonstrated that this clone is HLA-DQ restricted. The clone MeT 5.3A7, which recognizes PLA 45–62, was also shown to be restricted to HLA-DQ (29).

Stimulatory capacity of PLA 45–62, PLA 81–92, and PLA 113–124 among PBMC of allergic and nonallergic subjects

To investigate the relevance of the defined epitopes, T cell proliferation of mononuclear cells, freshly isolated from bee sting allergic and nonallergic subjects, was tested against PLA and the PLA-derived peptides presented in Figure 3. Ag were added at the same concentrations as in the experiments shown in Figure 4. In each case, maximal proliferative response required different concentrations of

	BuT 1.2A2		BuT 5.2F10	
	cpm ⁻¹		cpm ⁻¹	
	MEAN	STDEV	MEAN	STDEV
80 ↓				
90 ↓				
100 ↓				
110 ↓				
120 ↓				
130 ↓				
CLKNSADTISYFVGKMYFNLIIDTKCYKLEHPVTGCGERTEGRLHYTVDKSKPKVYQWFDLRKY				
LKNSADTISYFD	1217	40	1441	67
NSADTISYFVGD	1061	59	1149	6
ADTISYFVGKMD	1210	9	1303	54
TISSYFVGKMYFD	1161	159	1344	80
SSYFVGKMYFNLD	7132	325	1295	113
YFVGKMYFNLIID	32913	248	1105	27
VGKMYFNLIIDTKD	1176	190	1305	257
KMYFNLIIDTKCYD	1136	82	1045	36
YFNLIIDTKCYKLD	1099	146	1400	226
NLIIDTKCYKLEHD	1056	96	1143	37
IDTKCYKLEHPVD	956	137	886	353
TKCYKLEHPVTGD	1195	245	1152	46
CYKLEHPVTGCGD	1134	61	1037	20
KLEHPVTGCGERD	1428	110	1109	61
EHPVTGCCERTED	1418	18	1136	175
PVTGCCERTEGRD	1223	100	1113	66
TGCCERTEGRLD	1416	130	1366	150
CCERTEGRLHYD	1299	4	1304	7
ERTEGRLHYTV	1224	65	1162	33
TEGRLHYTVDKD	1321	96	1300	111
GRCLHYTVDKSKD	1264	217	1767	99
CLHYTVDKSKPKD	1290	215	43360	5288
HYTVDKSKPKVYD	1481	99	6825	341
TVDKSKPKVYQWD	1385	6	2346	356
DKSKPKVYQWFD	1360	2	1062	28
SKPKVYQWFDLRD	1280	79	1013	1
PKVYQWFDLRKYD	1455	3	1200	11
No antigen	1302	98	1332	110

FIGURE 2. Epitope mapping of PLA using overlapping dodecapeptides. Only the C-terminal end of the molecule is shown. No significant responses were detected with the peptides representing the other half of PLA. Immunogenic epitopes: PLA-81–92 and PLA 113–124 are shown in **bold**. The aspartic acid residue at the C-terminal side of all peptides results from the D-P-C spacer to the solid phase in peptide synthesis. All peptides were assayed at 0.7 μ M.

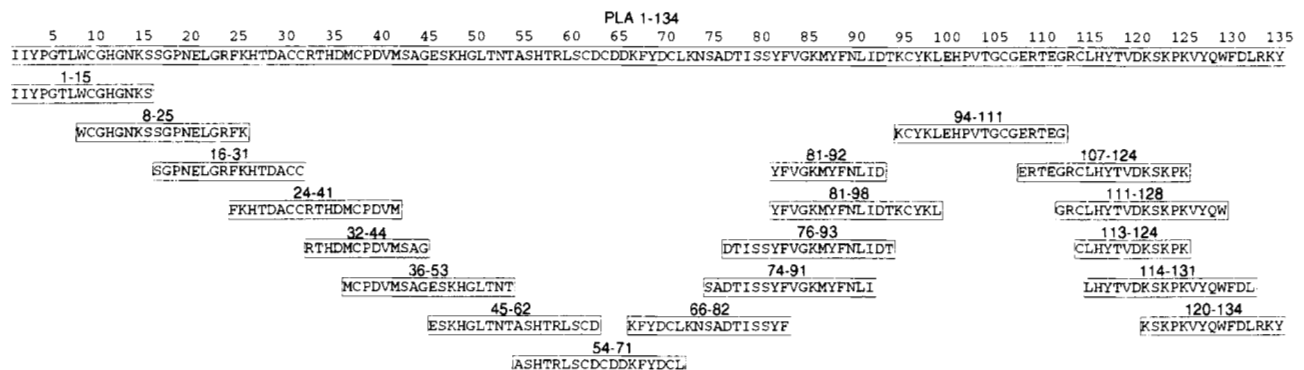


FIGURE 3. Amino acid sequence of PLA and the 19 PLA-derived peptides used to induce T cell activation of cloned T cells and unfractionated PBMC.

Ag and depended on the subject used in the experiment. The geometrical mean of the highest SI to PLA or PLA-derived peptides in each group of donors is presented in Figure 6.

The SI obtained with unfractionated PBMC were considerably lower than those observed with cloned cells. This is mainly due to the low frequency of T cells specific for a particular Ag and to the high background obtained (8,000 to 30,000 cpm) resulting from the increased cell number necessary to perform the experiments. Highest responses to both PLA and PLA-derived peptides were obtained in the group of allergic patients ($n = 20$). The mean responses obtained with PBMC from hyposensitized ($n = 8$) and hy-

perimmune ($n = 12$) donors were generally lower. Although the extent of the responses was higher in allergic than in nonallergic subjects, in all cases significant responses corresponded to the three immunogenic sites mapped with T cell clones. No additional epitopes were found (Fig. 6).

Figure 6 also presents the mean of the responses with SI > 3 . This reflects values higher than the background response plus twofold its SE. Among the allergic subjects, 65% showed SI > 3 in response to the entire PLA molecule, whereas 14% of the hyposensitized and 33% of the hyperimmune donors were in this range. The extent of T cell

FIGURE 4. T cell proliferation of (A) BuT 1.2A2 and (B) MeT 5.3A7 in response to PLA and its derived peptides shown in Figure 3. The amino acid residues of responding fragments are indicated. Nonresponding peptides are represented by open circles.

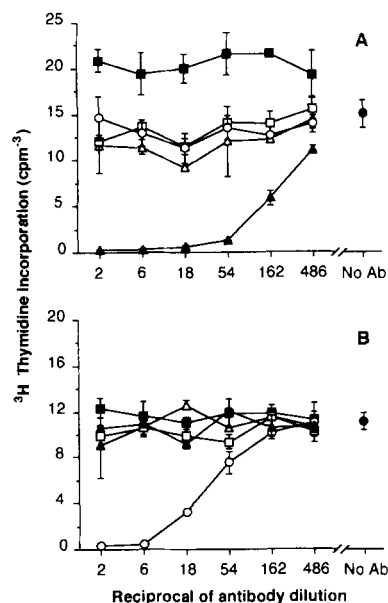
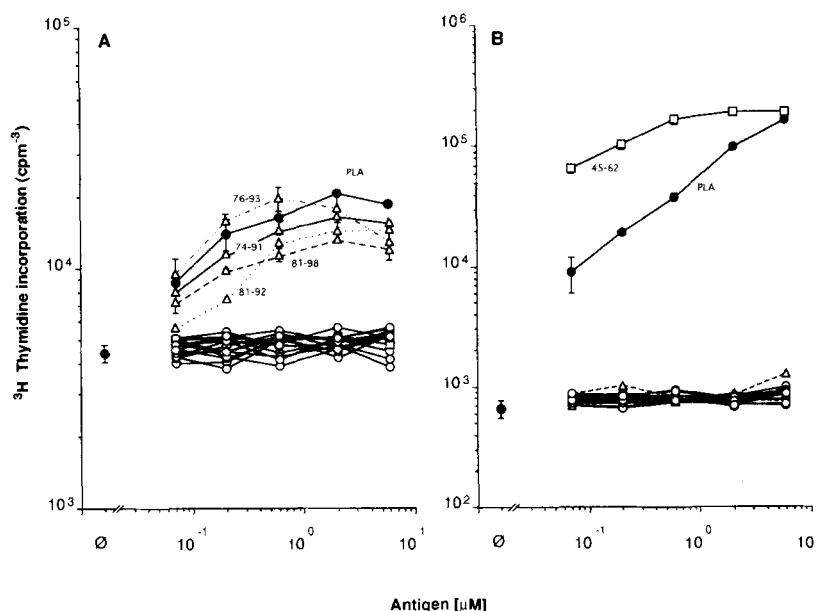


FIGURE 5. Inhibition of Ag stimulation of PLA-specific T cell clones by neutralizing mAb to HLA. PLA at 0.3 μ M was presented by autologous irradiated EBV-B cells. A, BuT 1.2A2; B, BuT 5.2F10; Δ , anti-class I; $\blacksquare$, anti-DR; $\square$, anti-DRw53; $\circ$, anti-DQ; $\blacktriangle$, anti-DP; $\bullet$, without antibody.

responses in hyposensitized subjects was lower compared to those of the other two groups.

Whereas similar percentages of individuals from the different groups responded to the immunogenic site including PLA residues 45–62, the mean proliferative response was highest in the allergic group.

In all groups of donors, PLA 76–93 induced the highest proliferative response among the four peptides that define the immunogenic site PLA 74–98. The fragments PLA 76–93 and PLA 81–98 were recognized by the same proportion of subjects in the allergic and hyperimmune group,

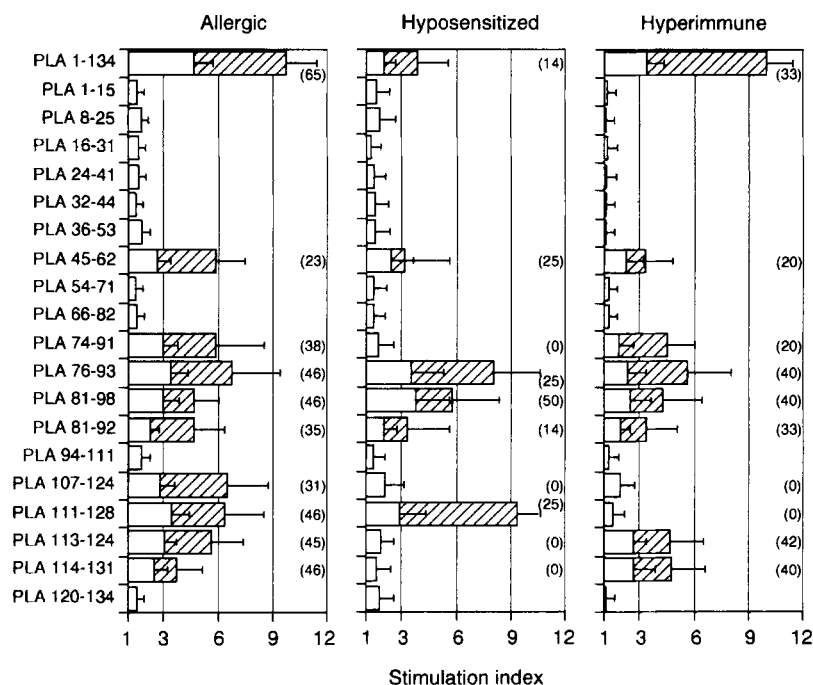
whereas most of the hyposensitized donors preferentially recognized PLA 81–98.

In the third immunogenic site, 45% of allergic subjects recognized the dodecapeptide PLA 113–124. The octadecapeptide PLA 111–128 induced similar reactivity in a similar number of donors. Elongation at the N-terminal end of the dodecapeptide slightly increased the reactivity but decreased the percentage of responders. The addition of residues at the C-terminal end resulted in lower reactivity with same number of responders. The percentage of hyposensitized donors displaying SI > 3 for this immunogenic site was 25%. Their responses were exclusively directed to PLA 111–128. The T cell responses in hyperimmune subjects were equally directed to PLA-113–124 and 114–131.

Discussion

By using human T cell clones, it was possible to determine three linear epitopes in the PLA molecule. As shown in Figure 7, these epitopes are separated in both primary and tertiary protein structures. PLA 45–62 is located in the middle loop connecting the first and second α -helices, PLA 81–92 in the front helix, and the epitope PLA 113–124 in the C-terminal loop of PLA (Fig. 7). All epitopes contain an amino acid motif predicted by Rothbard and Taylor (30). The epitopes PLA 45–62 and PLA 81–92 were predictable by the amphipathic α -helix model of DeLisi and Berzofsky (31). The highest homology in both primary and tertiary protein structures between PLA and other phospholipases, including human phospholipase A2, is expressed in the Ca^{2+} -binding loop (residues 9–13) and in the two anti-parallel helices including the active site of the enzyme (residues 25–37 and 61–74). This might explain why sequences such as PLA 66–82, which display two Rothbard motifs and the highest amphipathic score (Fig. 7), failed to stimulate

FIGURE 6. T cell proliferation of unfractionated PBMC from bee sting-sensitized donors. PBMC from allergic ($n = 20$), hyposensitized ($n = 8$), and hyperimmune ($n = 12$) subjects were stimulated with 6, 2, 0.6, 0.2, and $0.07 \mu\text{M}$ PLA and PLA-derived peptides. *White bars*, geometrical mean of the SI induced by optimal Ag concentrations. *Hatched bars*, mean of the responses with SI > 3 (background with unstimulated cells plus twofold its SE). *Numbers in brackets*, percentage of donors displaying SI > 3.



T cell clones and PBMC from bee sting-sensitized donors.

PLA-specific clones other than BuT 1.2A2 and BuT 5.2F10 failed to proliferate in response to the synthetic overlapping dodecapeptides produced by the pin technology. This could be due to the insufficient length of the peptides. Most of the MHC class I-associated peptides consist of eight to nine amino acids (32–34), with the central five to seven residues contributing to the majority of the specific contacts (35, 36). On the other hand, sequence analysis of peptides bound to murine MHC class II molecules revealed that naturally processed peptides are of 13 to 18 residues long (37, 38), although they can be shortened to some extent without loss of binding activity (38). Indeed, additional screening of the T cell clones by using octadecapeptides instead of dodecapeptides permitted the identification of the epitope PLA 45–62. It might be that activation of the other PLA-specific T cell clones requires even longer peptides. Flanking regions outside the epitope, exerting positive influence on T cell recognition and activation (39, 40), or targeting the peptides to the adequate intracellular compartments (41, 42) have been reported. Degradation of certain peptides in the culture before internalization by the APC could also play a role. Hence, although MeT 5.3A7 proliferated with $0.07 \mu\text{M}$ PLA 45–62, it did not respond to $0.7 \mu\text{M}$ of any of the overlapping dodecapeptides contained in this fragment. As suggested by Kuo et al. (22) for the murine response to PLA or by O'Hehir and Lamb (10) for the human response to house dust mite allergens, some T cell responses may require conformational epitopes. Such determinants could be represented by discontinuous peptide sequences linked by cystine bridges. The reason why not all PLA-specific T cell clones

could be mapped may be due to factors such as insufficient peptide length, peptide degradation, and/or conformational requirements. Therefore, we cannot exclude the existence of other T cell epitopes in PLA.

That MeT 5.3A7, BuT 1.2A2, and BuT 5.2F10 clones responded to PLA 45–62, PLA 81–92, and PLA 113–124, respectively, without cross-reactivity, demonstrates that T cell proliferation was not due to nonspecific mitogenic effects of the peptide sequences. Clones MeT 5.3A7 and BuT 5.2F10 are HLA-DQ-restricted, whereas BuT 1.2A2 is restricted to HLA-DP. On the other hand, other PLA-specific clones such as WeT 2 are HLA-DR restricted (43), and epitope- as well as PLA-induced proliferation of PBMC can be suppressed by antibodies to HLA-DR determinants (data not shown). This indicates that PLA responses are not restricted to a particular HLA-D class.

As other allergens, PLA has the capacity to elicit antibody responses of different isotypes (18, 20). Since isotype selection is controlled by T cell-derived cytokines (7), one could speculate that allergic and nonallergic responses result from the activation of different T cell populations differing in epitope specificity. Indeed, Soloway et al. (8) recently demonstrated that the substitution of only one single amino acid in a T cell epitope was sufficient to abolish IgG1- and IgE-associated hypersensitivity in different mouse strains. Peptide mapping of unfractionated PBMC from bee sting-sensitized subjects could not associate allergic or nonallergic states with preferential recognition of particular epitopes. This suggests that other factors beside T cell epitope specificity may be crucial for the induction of differential cytokine patterns and, in consequence, different antibody isotypes. Thus, T cells from both allergic

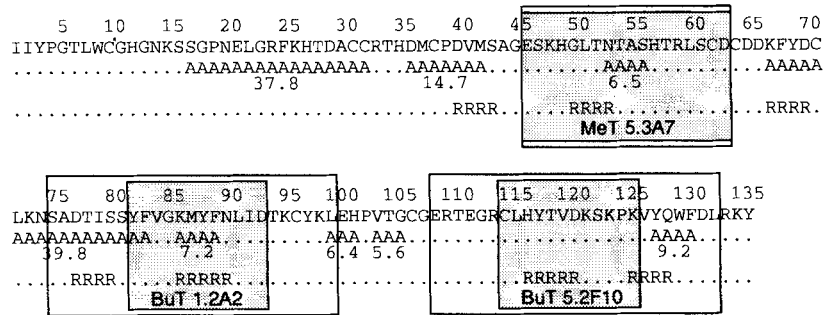
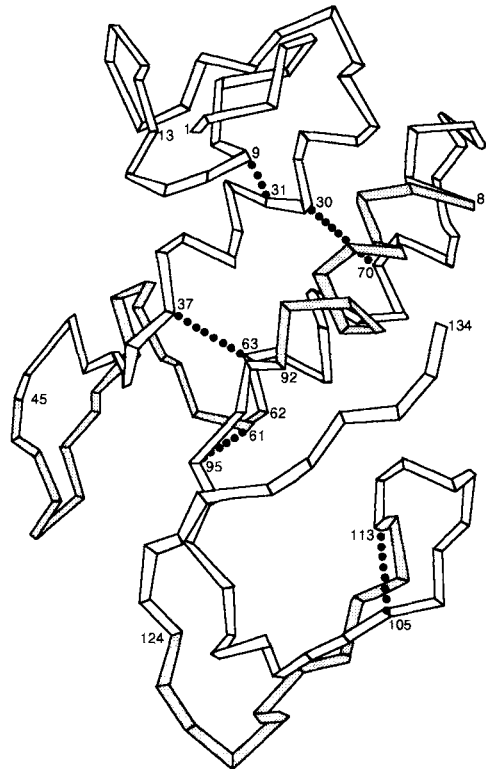


FIGURE 7. *Top*, Primary structure of PLA. A, Position of predicted T cell epitopes with corresponding amphipathic score using the AMPHI program (31); R, T cell epitopes predicted by the amino acid motif of Rothbard and Taylor (30); *empty boxes*, three immunogenic areas experimentally found; *shaded boxes*, minimum sequences able to stimulate the PLA-specific T cell clones MeT 5.3A7, BuT 1.2A2, and BuT 5.2F10. *Bottom*, Schematic representation of the backbone of bee venom PLA indicating the localization of the three minimal T cell epitopes.



and nonallergic subjects might be able to recognize the same epitopes on the PLA molecule, but the association of these peptides with different class II molecules might result in qualitative and quantitative different cytokine production (44).

The highest proportion of individuals responding to the entire PLA molecule was observed in the allergic group. Moreover, a considerable number of allergic patients displayed significant proliferative responses to each of the immunogenic peptides. In contrast, proliferation to the same PLA fragments was achieved by a lower percentage of subjects from the hyposensitized and the hyperimmune groups. This depressed proliferation cannot necessarily be attributed to particular epitope preferences and might rather be due to T cell tolerance in consequence of repeated contact with the allergen. Potent immunogenic epitopes are also strong tolerogens (35, 45). Accordingly, immunization of allergic patients with such peptides or amino acid-substituted analogues (46) could be used to induce unresponsiveness to bee sting without risk of undesired

anaphylactic reactions that accompany classical immunotherapy.

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