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Bee venom phospholipase A2-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to the antigen concentration*

Protein antigens with both allergenic and immunoprotective properties represent appropriate molecules to study IgE and IgG regulation. We have established a panel of T cell clones specific to bee venom phospholipase A2 (PLA) from human individuals allergic, hyposensitized or immune (protected) to bee sting. All clones obtained were CD3⁺, CD4⁺ and expressed α , β T cell receptor. Depending on the T cell clone, maximal stimulation required 1 to 100 μ g/ml of PLA, and the addition of interleukin (IL)-2 and/or IL-4 increased their antigen-dependent proliferation. Following antigen stimulation, the clones produced IL-4, interferon- γ (IFN- γ) and granulocyte-macrophage colony-stimulating factor. Most clones also produced tumor necrosis factor α (TNF- α) and tumor necrosis factor β (TNF- β), and some produced IL-5 and/or IL-2. Both absolute and relative amounts of secreted cytokines depended on the antigen concentration. At low antigen doses, IL-4 was produced but little or not IFN- γ , whereas at higher PLA concentrations significant amounts of both IL-4 and IFN- γ were obtained. Thus, these PLA-specific T cell clones could be classified according to the changes in the ratio of IL-4/IFN- γ production in response to increasing antigen concentrations. Clones derived from allergic and hyposensitized individuals required higher critical amounts of antigen for IFN- γ induction, and expressed increasing IL-4/IFN- γ ratios with increasing concentrations of PLA. Modulation of cytokine patterns by the dose of the antigen may be a driving force for IgE or IgG formation resulting in allergy or immunoprotection.

1 Introduction

Specific antibodies to protein antigens are elicited after clonal activation, proliferation and differentiation of B lymphocytes [1, 2]. These steps are controlled by T cells and depend on soluble factors. Upon recognition of processed peptides bound to MHC class II molecules on antigen-presenting cells, T lymphocytes become activated and secrete a variety of cytokines [3]. IL-4 [4–6], IL-5 [7–9], IFN- γ [10, 11] and transforming growth factor β (TGF- β) [12] have been shown to be involved as class switching or enhancing factors for different antibody isotypes [13]. In particular IL-4 is an inducer, while IFN- γ is an inhibitor of IgE synthesis [5, 14–18].

Two distinct subsets of mouse CD4⁺ T cells (*i.e.* T_h1 and T_h2) based on cytokine production have been reported [19].

Many subsequent reports have confirmed the existence of such subsets in both mouse and human T cell populations [20–23]. T cell clones producing a mixture of cytokines characteristic for T_h1 and T_h2 have also been described [17, 22–26]. In many cases, responses to bacterial antigens preferentially result in T_h1 lymphocyte stimulation [27, 28], whereas helminth parasites or allergens appear to stimulate T_h2 subsets [23, 27–29]. In some parasitic infections, the type of T_h response observed was reported to be MHC dependent (*e.g.* murine leishmaniasis [30]) or to depend on the stage of infection (*e.g.* in murine schistosomiasis [31] and murine malaria [32]). Bee venom phospholipase A2 (PLA) is a glycoprotein of 134 aminoacids with a molecular weight of 14–16 kDa [33, 34]. It represents the major immunogenic and allergenic component of bee venom [35–37]. Following the initial response to bee sting, individuals mostly develop PLA-specific antibodies of the IgG1 subclass. With repeated exposure, as in beekeepers, subjects may mount protective responses involving mainly the generation of PLA-specific IgG4 antibodies [38, 39]. Alternatively, some individuals may become allergic to bee venom, upon generation of IgE antibodies to PLA [37, 39].

Here we report the establishment of human T cell clones specific to bee venom PLA, the capacity of this allergen to induce different cytokine profiles, the observation that the profile appears to be dependent on the allergic status of the clone donor, and the modulation of these profiles by the dose of the antigen employed. These observations may contribute to a better understanding of the mechanisms of allergy and immunotherapy.

[I 10191]

* This project was supported by the Swiss National Foundation for Scientific Research, Grant nr. 31-26357.89 and CIBA-GEIGY, Ltd., Basel.

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Abbreviation: PLA: Bee venom phospholipase A2

2 Materials and methods

2.1 Antigen

PLA (Sigma, P-9279, Saint Louis, MO) was reduced and carboxymethylated to remove enzymatic activity and associated cell toxic effects [40]. Cell toxicity of PLA was tested by methyl- ^3H thymidine (^3H dThd; Amersham Int., Amersham, GB) incorporation in T cell clones stimulated with phytohemagglutinin (PHA) plus feeders and in EBV-transformed B cells (EBV-B), cultured in the presence of PLA. No toxic effects were observed with chemically altered PLA up to 1 mg/ml, whereas native PLA inhibited ^3H dThd incorporation at 1 $\mu\text{g}/\text{ml}$. The treated PLA showed the same antibody binding capacity in ELISA as the native form.

2.2 Subjects and PBMC isolation

Peripheral blood was obtained by venipuncture from (a) allergic individuals (Re and Wi), recently stung and presenting high levels of serum IgE anti-PLA, (b) a successfully hyposensitized individual (Me) and (c) non-allergic beekeepers (Bu and We). PBMC were isolated by Ficoll-Paque (Pharmacia, Uppsala) density gradient centrifugation, washed three times with Earle's balanced salt solution, counted and resuspended in fresh medium. PBMC of all donors were HLA typed by Dr. M. Fopp, Swiss Red Cross Center, Kantonsspital, St. Gallen, Switzerland, resulting in Re: DR4, DRw8, DQw3, DRw53; Wi: DR3, DR7, DQw2, DRw52/53; Me: DR1, DR12(5), DQw1, DQw3, DRw52; Bu: DR4, DR6, DQw1, DQw3, DQw52/53; We: DR4, DQw3, DRw53. The sera levels of anti-PLA IgE and IgG4 were determined by sandwich ELISA. Mouse anti-human IgE, mAb TN-142 (a gift of Dr. G. Delespesse, University of Montreal, Canada) and alkaline phosphatase-conjugated goat anti-mouse antibody (SBA 1030-04, Birmingham, AL) were used to detect IgE anti-PLA. The PLA-specific IgE content in a standard serum of a bee venom allergic donor (RAST 4⁺), was taken as 100 relative units (RU)/ml. Anti-PLA IgG4 was quantified as described previously [41]. Anti-PLA IgE and IgG4 concentrations in the sera of the different donors are shown in Table 1.

Table 1. Serum concentration of PLA-specific IgE and IgG4 antibodies

	Donor	Anti-PLA IgE RU/ml ^{a)}	Anti-PLA IgG4 $\mu\text{g}/\text{ml}$
Protected	Bu	1.50 $\pm$ 0.39	29.52 $\pm$ 0.63
	We	0.61 $\pm$ 0.08	4.22 $\pm$ 0.09
Hyposensitized	Me	1.43 $\pm$ 0.40	1.57 $\pm$ 0.01
Allergics	Re	394.53 $\pm$ 5.08	3.71 $\pm$ 0.60
	Wi	73.19 $\pm$ 4.71	5.40 $\pm$ 0.47

a) Relative units/ml, compared to an IgE anti-PLA standard serum taken as 100 RU/ml.

2.3 Culture medium

In all experiments RPMI 1640 culture medium was supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids, 1% MEM vitamins, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ kanamycin, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.05 mM 2-ME (all from Gibco, Basel). To this medium was added either 10% autologous serum (RPMI/AS) or 10% fetal calf serum (Sera-lab, Crawley Down, GB; RPMI/FCS). RPMI/AS was used during the *in vitro* priming of PBMC to diminish nonspecific activation. After cloning, T cells were maintained and assayed in RPMI/FCS. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.4 T cell cloning

T cell clones were generated as originally described by Lanzavecchia [42]. Briefly, PBMC were cultured in 200- μl aliquots of 10⁶ cells/ml in RPMI/AS with 10 $\mu\text{g}/\text{ml}$ of PLA in 96-well Costar (Cambridge, MA) flat-bottom plates. After 7 days, human recombinant IL-2 (rIL-2) (Preclinical Research, Sandoz, Ltd., Basel), at 5 ng/ml (50 U/ml) was added. Four days later, aliquots of 25 μl from each primary culture were transferred to 96-well U-bottom plates, washed twice with RPMI/AS and recultured for 48 h in the presence of 10 $\mu\text{g}/\text{ml}$ PLA. After an overnight pulse with 1 $\mu\text{Ci}/\text{well}$ ^3H dThd, PLA-responding cultures were selected and corresponding primary lines were cloned in Terasaki plates (Nunc, Roskilde, Denmark), at 0.3 cell/well in the presence of allogeneic irradiated PBMC and PHA (Wellcome Diagnostics, Dartford, GB). PLA-specific T cell clones that responded to autologous APC plus antigen (stimulation index > 10) were isolated and maintained in culture by periodic restimulation with allogeneic irradiated PBMC and PHA, followed by expansion in IL-2.

2.5 EBV transformation of B cells

PBMC (2.5 $\times$ 10⁶) were resuspended in 5 ml of RPMI/20% FCS containing 30% supernatant (0.45- μm filtered) of the EBV-producing marmoset line B95.8 [43] and 800 ng/ml cyclosporin A (Sandoz, Basel). After 18 h of incubation at 37°C in a 50-ml tissue culture flask (Costar), the cells were washed and seeded at 10⁶ cells/ml, in 24-well Costar plates, in RPMI/FCS containing 800 ng/ml cyclosporin A. After 2 weeks, cultures were expanded in 50- and 250-ml tissue culture flasks.

2.6 T cell proliferation assay

Cultures were set up in 200 μl RPMI/FCS in 96-well flat-bottom microtiter plates. Samples in triplicate, containing 3 $\times$ 10⁴ PLA-specific T cell clones and an equal number of autologous irradiated (6000 rad) EBV-B cells, were incubated for 48 h at 37°C, 5% CO₂, in the presence of different amounts of PLA. Proliferation was measured by liquid scintillation in an LKB β -plate counter after a 12-h pulse with 1 $\mu\text{Ci}/\text{well}$ of ^3H dThd.

2.7 HLA-D restriction

HLA restriction was determined by inhibition of T cell proliferation using mAb directed to monomorphic epitopes on DR, DQ (Clonab DR, Clonab DQ, Biotest, Dreieich, FRG) and DP (B7/21 Becton Dickinson, Basel) molecules. Anti-DRB4 (DRw53) and control anti-class I mAb, both kindly provided by Dr. Z. Nagy, Sandoz, Basel, were also included. Cultures were set up as described in Sect. 2.6 in the presence of PLA and different antibody dilutions. The particular HLA-DRB1 phenotype was determined in some cases by antigen stimulation of the T cell clones presented by allogeneic HLA homozygous EBV-B cells.

2.8 Cytokine requirement and production

One-milliliter cultures of 10^6 cloned T cells and 10^6 irradiated (6000 rad) autologous EBV-B cells were incubated in duplicate at 37°C for 3 to 24 h in the presence of different amounts of PLA. After incubation, supernatants were harvested and assayed for cytokine content. The remaining cells were carefully washed and replated in triplicate at 10^4 cells/well in 96-well microtiter plates with different combinations of exogenously added cytokines. Twenty four hours later, cultures were pulsed with [^{3} H]dThd and radioactivity was evaluated.

2.9 Cytokine detection assays

The IL-2 activity was measured by [^{3} H]dThd uptake of IL-2-dependent CTLL cells [44]. Serial dilutions of rIL-2 (1 U biological activity = 0.1 ng/ml) or IL-2 containing supernatants were incubated with 3×10^3 CTLL cells in 200 μ l for 24 h, followed by a 6-h pulse of 1 μ Ci/well of [^{3} H]dThd. This bioassay detects 50 pg/ml of human rIL-2. IL-5 was estimated by its capacity of supporting growth of the IL-5-sensitive LyH7 B₁₃ cell line (a gift of Dr. R.

Palacios, Basel Institute for Immunology, Basel) as described elsewhere [45].

IL-4 and IFN- γ were measured by sandwich ELISA. The mouse mAb 8F12 [46] and biotinylated 3H4 (prepared by C. H. Heusser) were used for IL-4, and 42-103 (kindly provided by Dr. G. Delespesse, University of Montreal, Canada) and biotinylated KM-48 (Kyowa Medex, Tokyo, Japan) were used for IFN- γ . Sensitivities of the IL-4 and the IFN- γ ELISA were 30 and 50 pg/ml, respectively. Granulocyte-macrophage (GM)-CSF was measured by ELISA using the mouse mAb F1-1-113-4-6 and a biotinylated, polyclonal sheep anti-human GM-CSF, kindly provided by Dr. E. Liehl, Sandoz Research Institute, Vienna, Austria. The sensitivity of this assay was 100 pg/ml. In all cases, the binding to the second antibody was detected by stepwise incubation with alkaline phosphatase-conjugated streptavidin (Tago 6567, Burlingame, CA) and 4-nitrophenyl phosphate disodium salt (Merck, Darmstadt, FRG). TNF- β was measured with a commercial ELISA (Quantikine TNF- β immunoassays, Minneapolis, MN). Since TNF- β can also be secreted by EBV-B cells, values obtained from the T cell clones, were subtracted with the data from APC alone ($1.1 \text{ ng/ml} \pm 0.4$). TNF- α was also estimated by a commercial kit (Endogen Inc., Boston, MA). Both TNF- α and TNF- β ELISA detected 100 pg/ml of the corresponding standard.

3 Results

3.1 Proliferation of PLA-specific T cell clones

Bulk cultures of PLA-responding T cells were produced from PBMC of the beekeepers Bu and We, the hyposensitized donor Me and the allergic individuals Re and Wi. From each donor, four to twelve selected PLA-responding T cell lines were established separately and subsequently cloned and expanded. To avoid a selection of clones

Table 2. Cytokine secretion of PLA-specific T cell clones stimulated with the antigen^{a)}

	HLA restriction	IL-2 [ng/ml]	IFN- γ [ng/ml]	TNF- β [ng/ml]	TNF- α [ng/ml]	GM-CSF [ng/ml]	IL-4 [ng/ml]	IL-5 [U/ml]
Protected								
BuT 1.2A2	DP	20.8 ± 0.4	20.83 ± 0.46	2.45 ± 0.26	4.65 ± 0.05	7.30 ± 0.29	9.48 ± 0.48	36 ± 4
BuT 5.2F10	DQ	3.46 ± 0.6	1.58 ± 0.12	2.75 ± 0.19	0.49 ± 0.05	3.06 ± 0.10	3.66 ± 0.02	$<0.3 \pm 0$
BuT 6.53		nd	0.47 ± 0.06	1.49 ± 0.09	0.37 ± 0.02	4.52 ± 0.02	11.07 ± 0.10	$<0.3 \pm 0$
WeT 2	DRB1* 4.4	3.10 ± 0.2	1.33 ± 0.09	nd	2.47 ± 0.05	4.45 ± 0.40	13.11 ± 0.12	3.67 ± 1
WeT 28	DRB1* 4.4	0.15 ± 0.1	1.67 ± 0.13	nd	nd	4.60 ± 0.32	14.85 ± 0.21	$<0.3 \pm 0$
Hyposensitized								
MeT 1.1A9	DR	$<0.05 \pm 0.0$	0.17 ± 0.01	0.18 ± 0.05	$<0.10 \pm 0.00$	0.96 ± 0.08	1.27 ± 0.00	$<0.3 \pm 0$
MeT 3.2E5		$<0.05 \pm 0.0$	0.17 ± 0.02	0.25 ± 0.08	$<0.10 \pm 0.00$	0.54 ± 0.03	1.51 ± 0.10	$<0.3 \pm 0$
MeT 5.3A7	DQ	$<0.05 \pm 0.0$	0.16 ± 0.01	0.21 ± 0.05	0.60 ± 0.03	2.21 ± 0.05	3.60 ± 0.04	1.4 ± 1
Allergic								
ReT 69	DR	0.31 ± 0.1	0.18 ± 0.01	3.10 ± 0.06	0.13 ± 0.06	2.46 ± 0.02	0.68 ± 0.01	0.6 ± 0
ReT 83		2.56 ± 0.6	0.30 ± 0.01	4.71 ± 0.08	0.15 ± 0.01	2.20 ± 0.02	3.67 ± 0.17	3.7 ± 1
WiT 10.3B2		nd	0.18 ± 0.01	2.11 ± 0.10	$<0.10 \pm 0.00$	2.38 ± 0.04	0.21 ± 0.04	$<0.3 \pm 0$
Non PLA-specific								
BuT 6.21		$<0.05 \pm 0.0$	$<0.05 \pm 0.00$	nd	nd	$<0.10 \pm 0.00$	0.08 ± 0.01	$<0.3 \pm 0$

a) PLA concentration was 50 μ g/ml in all cases except for clon BuT 1.2A2 (0.5 μ g/ml). Supernatants were harvested at 18 h and assayed for the cytokines indicated.

originating from the same precursor, only one specific clone from each microculture was developed. Cytofluorometric analysis showed that all clones belong to the CD3⁺, CD4⁺, CD8⁻ phenotype and express α , β TcR. Inhibition experiments with mAb anti HLA-D, summarized in Table 2, showed that PLA-specific T cell clones can be HLA-DR, -DQ or -DP restricted. As demonstrated in Fig. 1, all PLA-specific T cell clones responded in a dose-dependent manner to the antigen presented by autologous EBV-B cells. Non-PLA-specific control T cell clones, derived from the same individuals showed no significant responses to PLA. Depending on the clone, 1 to 100 μ g/ml of PLA was required for maximal stimulation. Under identical conditions, 50% [³H]dThd incorporation was obtained with clone BuT 1.2A2 at 0.1 μ g/ml of PLA (7×10^{-9} M), whereas 5–40 μ g/ml (3×10^{-7} – 2×10^{-6} M) was required for the other clones. To exclude the possibility that the isolated clones were directed to other bee venom constituents than PLA, they were stimulated with rPLA obtained from *E. coli* cultures (a gift of Dr. M. Suter and T. Dudler, Swiss Institute of Allergy and Asthma Research, Davos). Although slightly more rPLA was required, the clones responded in the same dose-dependent manner to the recombinant product (data not shown).

3.2 Cytokine requirements of PLA-specific T cell clones

PLA stimulation in the presence of autologous APC rendered specific T cell clones sensitive to IL-2 and IL-4. As an example, Fig. 2 shows T cell proliferation of clone WiT 10.3B2 after 18 h of stimulation with different antigen doses followed by repeated washes and reculture in the

presence of exogenous IL-2 and IL-4. The level of the response, measured as [³H]dThd incorporation, correlated with both the dose of interleukins added to the culture and with the concentration of antigen used for stimulation. Higher responses were observed with IL-2. The combination of suboptimal amounts of both cytokines induced an additive effect similar to the optimal proliferation obtained with higher doses of IL-2 alone. IL-1 β , in the range of 0.6 to 5 ng/ml, showed no effect on T cell proliferation at any antigen concentration (data not shown). Uniform cytokine requirements were observed with all clones used in the study.

3.3 Cytokine profile of PLA-specific T cell clones change with the antigen dose

Maximal IL-2 and IL-4 production was reached in culture supernatants after 12 h of stimulation with PLA and autologous APC (data not shown). While IL-4 amounts remained nearly constant during the following 12 h, IL-2 levels decreased significantly, probably due to the shut off in IL-2 mRNA formation [47] and to the consumption by activated T cells. IFN- γ appeared later, reaching plateau levels after 24 h of stimulation (data not shown).

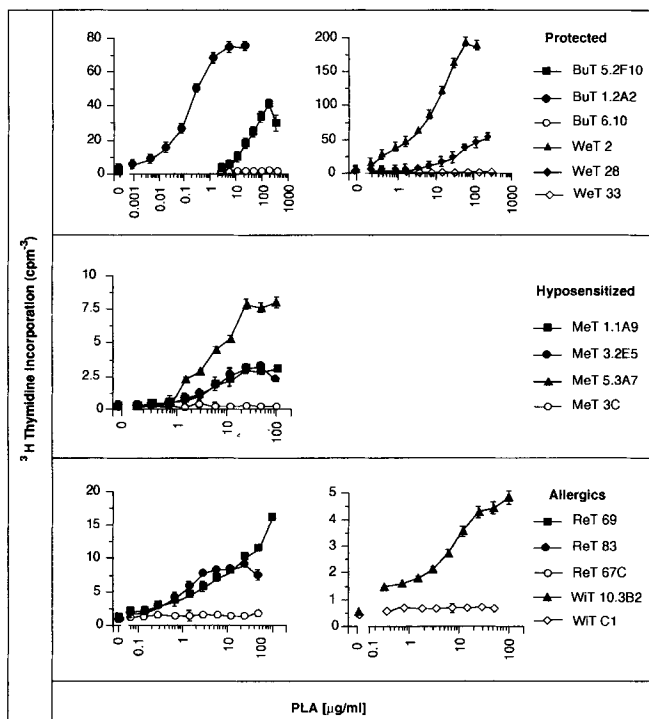


Figure 1. Antigen dose-dependent response of PLA-specific T cells. Filled symbols represent PLA-specific clones, empty symbols PLA nonspecific control T cell clones, derived from the same individuals.

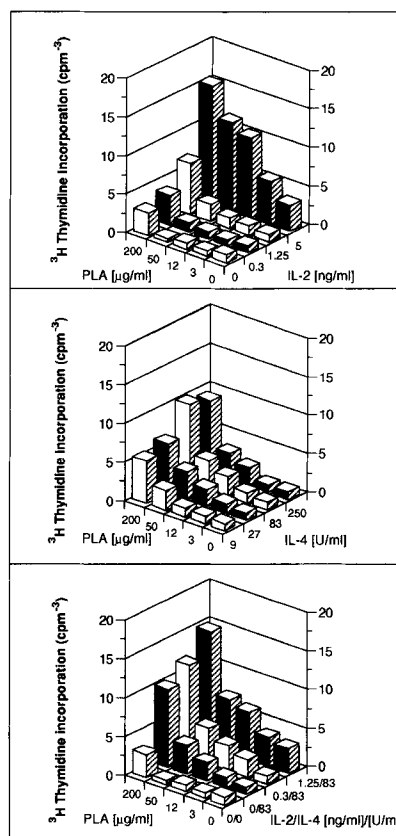


Figure 2. IL-2 and IL-4 requirements of the PLA-specific T cell clone WiT 10.3B2. The bars show [³H]dThd incorporation of triplicate cultures of 2×10^4 T cells after 20 h of incubation with APC and different doses of antigen, followed by an additional 24 h culture with exogenous, IL-2 (upper panel), IL-4 (middle panel) or combinations of IL-2 plus IL-4 (bottom panel). Standard deviation was < 8%.

T cell clones were also assayed for the production of IL-2, IL-4, IL-5, IFN- γ , TNF- α , TNF- β , and GM-CSF after 18–20 h of stimulation with different concentrations of PLA. As shown in Table 2, at plateau levels the stimulation of PLA-specific clones resulted in a mixed cytokine pattern. All clones produced IL-4, IFN- γ and GM-CSF. IL-2 and TNF- β were produced by all clones except those from the hyposensitized individual Me. MeT clones, produced cytokine patterns that, with the exception of the marginal IFN- γ production, are characteristic of T_H2 cells. TNF- α and IL-5 were produced by some but not all clones regardless of whether they were derived from a protected, hyposensitized or allergic individual. These cytokines could not be detected in the supernatant of non-PLA-specific clone BuT 6.21 following the addition of PLA.

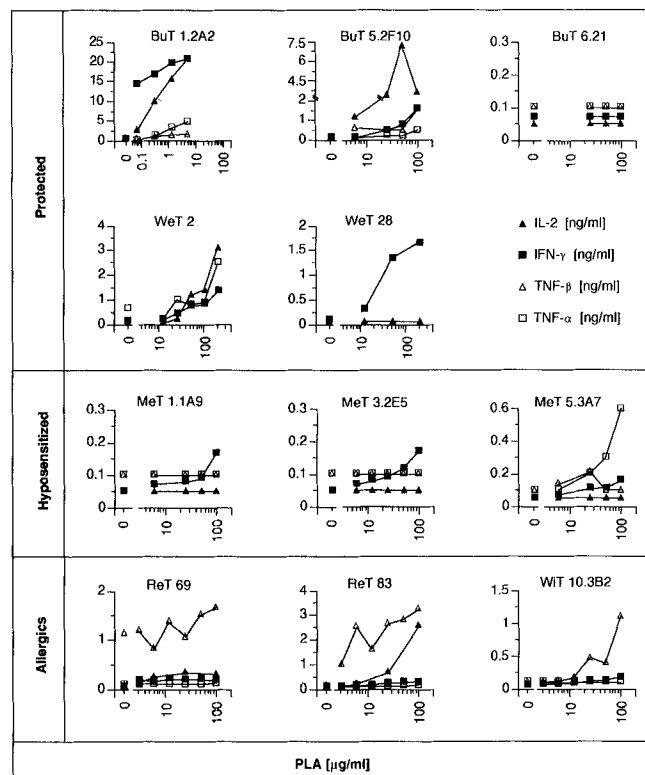
The amounts of each cytokine produced by the various clones differed. Generally, the quantities of cytokines produced by the clones from allergic and hyposensitized individuals were lower than those produced by clones from protected subjects.

A potentially more meaningful comparison is based on the relative amounts of cytokines produced. In clones derived from allergic donors, TNF- β was the cytokine produced in the highest amounts, while among the other clones (*i.e.* clones derived from protected and hyposensitized donors), it was with few exceptions the cytokine produced in the lowest quantity.

Fig. 3 A and B demonstrates the effect of varying the antigen concentration on the amounts of cytokines produced. In general, an increase in the antigen dose resulted in an increase in the cytokine production. The relationship between the amount of cytokine produced and the dose of antigen required was characteristic for the cytokine being measured and the clone being tested. Whereas IL-4 was detectable in culture supernatants of all clones stimulated at low antigen doses, IFN- γ production had a threshold of stimulation comparatively higher in clones derived from allergic and hyposensitized than in the clones from protected individuals. Most of the clones produced little or no IL-2 upon antigen stimulation, however, IL-2 production could be induced with stronger activation signals (*i.e.* cross-linked anti-CD3, not shown).

Since both IL-4 and IFN- γ were secreted by all PLA-specific T cell clones, a comparison based on the ratio of the amounts of these cytokines produced was made and proved to be insightful. In Fig. 4, the change of the IL-4/IFN- γ quotient is presented against the antigen concentration used during stimulation. The results revealed that T cell clones have a characteristic antigen dose-response for IL-4/IFN- γ production. The three MeT clones and ReT 83 showed an increase in the ratio as the antigen dose was increased (correlation coefficients of antigen dose/cytokine ratio ranging from 0.797 to 0.959 and slopes from 0.058 to 0.094). The reverse effect was obtained with clone WeT 2; the quotient decreased as the antigen concentration increased (correlation coefficient of -0.763 and slope of -0.129). The IL-4/IFN- γ ratios in BuT 1.2A2, BuT 5.2F10, WeT 28, ReT 69 and WiT 10.3B2, were unaffected by the different amounts of PLA used ($|\text{slopes}| < 0.050$).

(A)



(B)

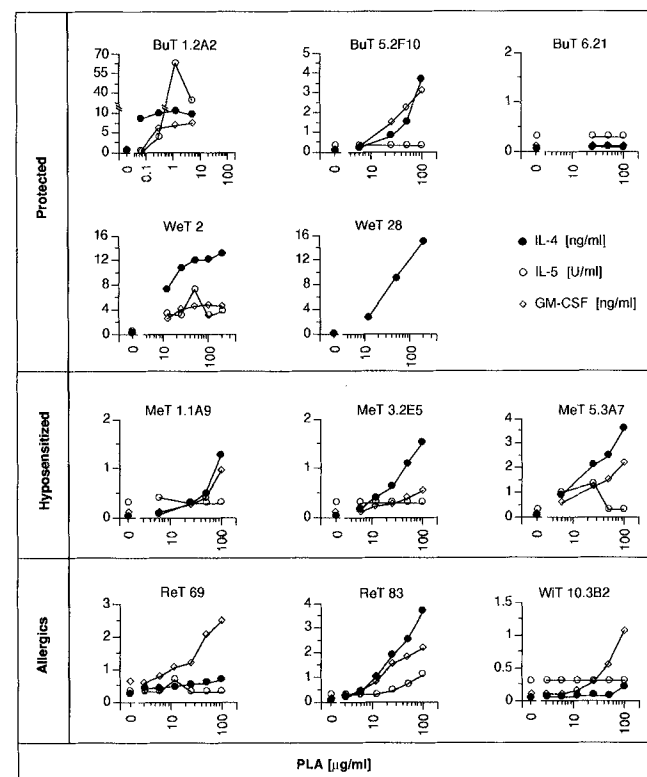


Figure 3. Cytokine production in supernatants of PLA-specific clones after 20 h of stimulation with different antigen doses. BuT 6.21 is a non PLA-specific T cell clone. (A) Cytokines commonly secreted by T_H1 cells. (B) Cytokines of the IL-4 family.

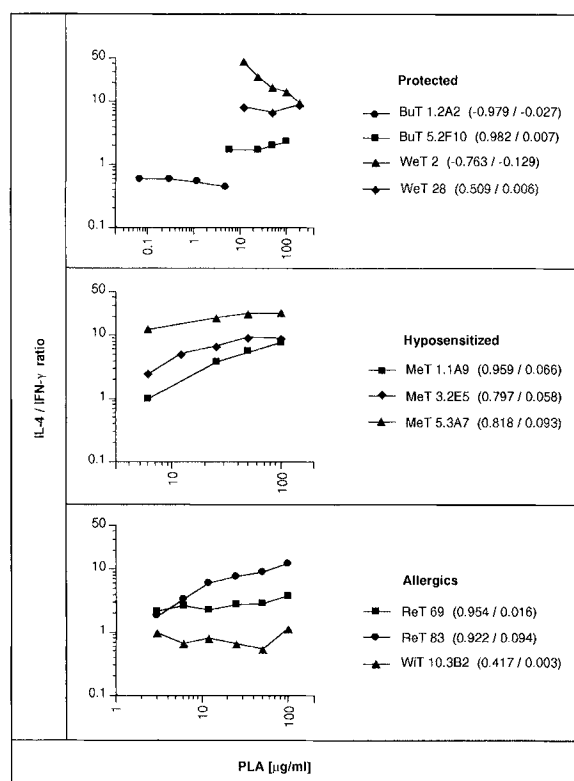


Figure 4. Ratios of IL-4/IFN- γ production in supernatants of PLA-specific T cell clones in response to different doses of antigen. Correlation coefficient and slope of regression line (r/s) are indicated for each curve.

4 Discussion

Accumulating evidence supports a central role for IL-4 and IFN- γ as regulators of immune responsiveness to allergens. IL-4 has been implicated as an inducer of IgE synthesis whereas IFN- γ was described as an antagonist. Great effort has been made in the last years to demonstrate a compartmentalization of these two cytokines into different human T cell subsets. Whereas such separation seems to occur in some antigen responses, in other cases the distinction remains less clear.

All PLA-specific T cell clones described in this article showed similar proliferative responses to the antigen and to exogenous growth factors, even though these clones derived from different individuals with respect to the PLA-specific isotype production and immune status. The T cells grew better in IL-2 than in IL-4, probably because they were maintained in IL-2 and may have up-regulated IL-2 receptors [48]. With the exception of BuT 1.2A2, these clones responded to the antigen preferentially by IL-4 secretion. Moreover, T cell clones can assist autologous B cells to synthesize PLA-specific antibodies (J. Carballido et al., in preparation). Allergen specificity, IL-4-dependent growth, preferential IL-4 secretion and B cell help are characteristics of T_H2 cells. However, the presented clones can not be strictly classified in accordance with the mouse T_H1 or T_H2 subsets, since IL-4 could be expressed together with IFN- γ , TNF- β and in some cases IL-2, provided antigen was present in sufficient amounts.

The influence of the antigen dose on cytokine production was particularly evident in clones WeT 2 and WeT 28. At antigen concentrations of 10 μ g/ml these clones produced IL-4, and little or no detectable IL-2 and IFN- γ (Fig. 3 A and B). These cytokine patterns are characteristic for T_H2 cells. However, a 10-fold increase in the antigen concentration correlated not only with an increase in IL-4 secretion (from 7 to 12 ng/ml for clone WeT 2 and from 3 to 10 ng/ml for clone WeT 28), but also with a significant increase in T_H1-associated cytokines such as IFN- γ (from <0.05 to 1 ng/ml for clone WeT 2 and from 0.28 to 1.5 ng/ml for WeT 28). The same behavior, although less evident with respect to the magnitude of the response, was also observed in the other clones.

The relationship between antigen concentration and the *in vitro* pattern of cytokine production may correlate with *in vivo* responses during immunotherapy. Hyposensitization of individuals allergic to bee sting is a standard clinical procedure [49, 50]. The PLA-specific IgE/IgG4 ratio in serum decreases with prolonged administration of bee venom to allergic patients [38, 49, 51–53]. Since antibody production is regulated by T cell-derived factors, it is reasonable to speculate that elevated antigen levels, artificially maintained in allergic patients during hypsensitization, can modify the cytokine pattern of specific T cells and as a consequence the IgE/IgG4 ratio.

It was demonstrated that certain PLA peptides preferentially bind to particular, isolated HLA-D molecules [54] and that individuals of the HLA-DR4 phenotype develop immunity rather than allergy [55]. Therefore, differences in T cell recognition and activation might exist between allergic and non-allergic individuals. Isotype preference may result from production of different cytokine patterns as a consequence of T cell activation induced by different peptide-HLA complexes [56]. Allergic individuals may not have the capacity to form peptide-HLA complexes with the appropriate affinity to optimally activate potentially protective clones. By increasing the amount of antigen, a larger panel of T cell clones is stimulated, for which the threshold for IFN- γ production is reached. Enhanced IFN- γ production can result in a decrease of the IgE/IgG ratio [13].

From the present study it is not possible to correlate the immune status of different individuals to PLA with restricted cytokine patterns such as those observed in mouse T_H1 and T_H2 cells or in human responses to other allergens. However, it can be noted that in clones from allergic donors the IL-4/IFN- γ ratio tended to tilt in favor of IL-4 production with increasing antigen concentrations, whereas in non-allergic individuals the IL-4/IFN- γ ratio generally remained constant or declined as antigen concentrations were raised. Furthermore, TNF- β production was significantly elevated in clones derived from allergic donors. TNF- β has been already implicated in other diseases such as multiple sclerosis [57], AIDS dementia [58] and experimental allergic encephalomyelitis [59]. The influence of TNF- β production in the allergic state to PLA has to be further elucidated.

We gratefully thank Dr. H. Hengartner, Institute of Pathology, University Hospital of Zurich, Dr. Z. Nagy, Hoffmann-La Roche Inc., Nutley, NJ, Dr. R. Palacios, Basel Institute for Immunology,

Dr. R. Zubler Hôpital Cantonal Universitaire de Genève for their enlightening discussions and Dr. S. Alkan, CIBA-GEIGY Ltd., for providing the protocol for the IFN- γ assay.

Received December 8, 1991; in revised form February 17, 1992.

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