Epitope-specific T Cell Tolerance to Phospholipase A_2 in Bee Venom Immunotherapy and Recovery by IL-2 and IL-15 In Vitro

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Abstract

Bee venom phospholipase A_2 (PLA) is the major allergen in bee sting allergy. It displays three peptide and a glycopeptide T cell epitopes, which are recognized by both allergic and non-allergic bee venom sensitized subjects. In this study PLA- and PLA epitope-specific T cell and cytokine responses in PBMC of bee sting allergic patients were investigated before and after 2 mo of rush immunotherapy with whole bee venom. After successful immunotherapy, PLA and T cell epitope peptide-specific T cell proliferation was suppressed. In addition the PLA- and peptide-induced secretion of type 2 (IL-4, IL-5, and IL-13), as well as type 1 (IL-2 and IFN-γ) cytokines were abolished, whereas tetanus toxoid-induced cytokine production and proliferation remained unchanged. By culturing PBMC with Ag in the presence of IL-2 or IL-15 the specifically tolerized T cell response could be restored with respect to specific proliferation and secretion of the type 1 T cell cytokines, IL-2 and IFN-γ. In contrast, IL-4, IL-5, and IL-13 remained suppressed. Treatment of tolerized T cells with IL-4 only partially restored proliferation and induced formation of distinct type 2 cytokine pattern. In spite of the allergen-specific tolerance in T cells, in vitro produced anti-PLA IgE and IgG4 Ab and their corresponding serum levels slightly increased during immunotherapy, while the PLA-specific IgE/IgG4 ratio changed in favor of IgG4. These findings indicate that bee venom immunotherapy induces a state of peripheral tolerance in allergen-specific T cells, but not in specific B cells. The state of T cell tolerance and cytokine pattern can be in vitro modulated by the cytokines IL-2, IL-4, and IL-15, suggesting the importance of microenvironmental cytokines leading to success or failure in immunotherapy. (*J. Clin. Invest.* 1996. 98:1676–1683.) Key words: bee venom allergy • T cell anergy • interleukin-4 • anti-PLA IgE • anti-PLA IgG4 • recovery from anergy

Introduction

The precise mechanisms by which allergen immunotherapy achieves clinical improvement in allergic patients is still poorly understood. Induction and rise in IgG antibodies, particularly of the IgG4 class (1–3), or the generation of $CD8^+$ cells which may modulate allergen-specific IgE production, have been claimed to be responsible for successful immunotherapy (4). Other observations demonstrated reduced numbers of mast cells and secretion of mediators (5, 6). Similarly, numbers and activation of eosinophils were reduced in inflamed tissue upon immunotherapy (7, 8). It is well known that in the pathogenesis of allergic diseases the production of IL-4 and IL-13 by $CD4^+$ T cells is critical for the induction of IgE synthesis (9, 10), while IL-5 is important in eosinophil differentiation and survival (11, 12). Thus, it has been demonstrated that allergen-specific $CD4^+$ T cells isolated from patients with allergic diseases produce higher levels of IL-4 and IL-5 compared to those from sensitized nonallergic individuals (13, 14). Moreover, successful allergen immunotherapy of allergic rhinitis and bee venom allergy was shown to be associated with decreased IL-4 and IL-5 production in $CD4^+$ T cells and PBMC (15, 16).

Bee venom phospholipase A_2 (PLA)[1] is a 14–16-kD glycoprotein, consisting of 134 amino acids (17) and displaying a single carbohydrate side chain at the residue Asn^{13}. It represents the major allergenic compound in bee venom (BV)[1] (18, 19) and from 86 bee venom-allergic patients with positive skin test, 85 reacted to PLA (18). PLA can elicit both IgE mediated allergy and normal immunity to bee sting which generally is associated with high affinity IgG4 anti-PLA antibodies (18, 20, 21). Successful immunotherapy is accompanied by an increase in PLA-specific IgE and IgG4 Abs (16, 20, 22–24), but decline of their ratio in favor of IgG4 (3). Recently, in PLA-specific T cell clones and PBMC from bee venom-allergic patients and sensitized nonallergic subjects, three T cell stimulating peptide sequences in the PLA molecule have been identified, corresponding to $PLA^{45–62}$, $PLA^{81–92}$, and $PLA^{113–124}$ (3) and an MHC class II–restricted T cell epitope involving the carbohydrate side chain at the residue Asn^{13} (25). In the present study we investigated the epitope-specific T cell responses to PLA and the capacity of B cells to produce PLA-specific IgE and IgG4 Ab before and after bee venom immunotherapy (BV-IT). We demonstrated that epitope-specific peripheral tolerance to the main BV allergen PLA is induced in T cells but not in B cells during BV-IT and that tolerized T cells can be reacti-

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1. *Abbreviations used in this paper:* BV, bee venom; BV-IT, bee venom immunotherapy; degPLA, deglycosilated PLA; PLA, bee venom phospholipase; sCD40L, soluble CD40 ligand; TT, tetanus toxoid.

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vated by the cytokines IL-2 and IL-15 to secrete distinct Th1 type cytokine patterns.

Methods

Study population. Five bee venom allergic individuals (mean age: 47 yr) with a history of severe systemic allergic reactions of grade III-IV (26) after a bee sting were included in the study. All patients demonstrated positive intracutaneous reactions to honey bee venom (ALK, Horsholm, Denmark) at a concentration of $< 10^{-4}$ g/liter and bee venom specific serum IgE Abs > 1 kU/liter as estimated by Phadezym CAP (Pharmacia Fine Chemicals, Uppsala, Sweden). Blood samples were taken before and 2 mo after starting bee venom immunotherapy.

Bee venom immunotherapy. Under intensive care conditions 0.1, 1, 10, and 20 μg bee venom (ALK) were administered s.c. at 30-min intervals in the upper arms and then 30 and 50 μg at 60-min intervals, reaching a cumulative dose of 111.1 μg. At day 7 two booster injections of 50 μg were administered, followed by 100 μg booster injections at day 21 and day 50. Thereafter, 100 μg boosters were given at intervals of 4 wk (27).

Reagents. PLA was from Boehringer GmbH (Mannheim, Germany) and PLA devoid of carbohydrate (degPLA)[1] was obtained by enzymatic deglycosylation of PLA after incubating 1 mg PLA for 24 h at 37°C with 1 U of endoglycosidase and F/N glycosidase (Boehringer GmbH) in 25 mM Tris-HCl, 10 mM EDTA, at pH 8, in the absence of denaturating agents. Deglycosylation was monitored by SDS-PAGE and Coomasie brilliant blue R-250 (Bio-Rad, Richmond, CA) staining (25). T cell epitope peptides corresponding to PLA^{45-62} (PI), PLA^{81-92} (PII), and $PLA^{113-124}$ (PIII) were synthesized by Drs. C.H. Schneider and H.P. Rolli (Institute for Clinical Immunology and Allergology, Inselspital, University of Bern, Switzerland), as described (28). These three peptides were not recognized by specific IgE or IgG4 directed to PLA. Tetanus toxoid (TT) was from the Swiss Serum and Vaccine Institute (Bern, Switzerland). The following recombinant cytokines were kindly provided as indicated: IL-2 was from Sandoz Ltd. (Basel, Switzerland) and IL-4 from Ciba Geigy Ltd. (Basel, Switzerland). IL-15 was from PeproTech, Inc. (Rocky Hill, NJ). Soluble CD40L (sCD40L) was produced from the transfected cell line 8-40-1, originally generated by Dr. P. Lane (Institute for Immunology, Basel, Switzerland) and cultured for 3 d in CG medium (Vitromex GmbH, Vilshofen, Germany) and standardized according to highest IgE inducing capacity after 12 d culture of PBMC co-stimulated with 25 ng/ml of IL-4 (29). Supernatants obtained from the corresponding untransfected cell line J558L (kindly provided by Dr. M. Reth, University of Freiburg, Freiburg, Germany) were used as control.

T cell cultures. PBMC were isolated by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed three times and resuspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (all from GIBCO BRL, Basel, Switzerland) and 10% heat-inactivated FCS (Sera-Lab Ltd., Sussex, England). Cells (10^6/ml) were then stimulated in a 24-well plate (Costar Corp., Cambridge, MA), with either native PLA, degPLA, or T cell epitope-bearing peptides all in 1 μM concentration. TT was used at 0.01 U/ml. Cultures were expanded in medium supplemented at day 7 with a mixture of IL-2 (25 U/ml) and IL-4 (25 ng/ml). After 12 d, 10^6 cells were washed three times with PBS and restimulated with 1 μM of the same antigen as before, in the presence of 10^6 autologous, 3,000 Rad-irradiated PBMC in 24-well tissue culture plates, in duplicates. Supernatants were harvested at selected time points obtained from preliminary kinetics studies, 16 h later for determination of IL-2 and IL-4 and 48 h later for IL-5, IL-13, and IFN-γ. To determine T cell proliferation, 2×10^5 PBMC were stimulated with 1 μM Ags in 200 μl medium in 96-well flat bottom tissue culture plates, in triplicates for 6 d and pulsed with 1 μCi of [^{3}H]thymidine (DuPont-NEN, Boston, MA). Incorporation of the labeled nucleotide was determined after 20 h in a LKB β plate reader (Wallac; Pharmacia, Turku, Finland).

In vitro recovery of tolerized T cells. Experiments to re-establish T cell responsiveness were performed by culturing 2×10^6 PBMC for 12 d with 50 U/ml of IL-2, or 50 ng/ml of IL-15, or 50 ng/ml of IL-4 in the presence of 1 μM PLA, degPLA, or T cell epitope peptides. Cultures were expanded with medium containing the same cytokines. The cells were then washed three times and 10^6 cells were restimulated with the same Ag in the presence of 10^6 irradiated autologous PBMC in a 24-well culture plate. Cytokines were measured in supernatants taken at the above indicated time points. Recovery of T cell proliferation from tolerance was determined in 10^5 T cells treated with either of the cytokines above and Ag which were cultured together with 10^5 autologous irradiated PBMC and the same Ag (1 μM) in 96-well plates. Proliferation was determined at day 5 after 20 h of [^{3}H]thymidine incorporation.

Measurement of cytokines. The solid phase ELISA for IFN-γ was described before (30). Briefly, 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with mAb 43–11 to human IFN-γ and developed with biotinylated mAb 45–15 and extravidine peroxidase (Sigma Chemical Co.). O-phenylenediamine dihydrochloride (Sigma Chemical Co.) was used in citrate buffer, pH 5, as the chromogenic substrate. The sensitivity of the IFN-γ ELISA was < 20 pg/ml. For the detection of IL-13 the mAb JES8-5A2 (kindly provided by Dr. J. De Vries, DNAX, Palo Alto, CA) was used for coating. Recombinant IL-13 from PeproTech, Inc. was used as a standard. Rabbit anti-IL-13 (PeproTech, Inc.) and alkaline phosphatase-labeled goat anti–rabbit Ab (Zymed Labs, Inc., San Francisco, CA) were used for detection. The chromogenic substrate was 4-nitrophenyl phosphate disodium hexahydrate (E. Merck, Darmstadt, Germany) in diethanolamine buffer, pH 9.8. The detection limit was 300 pg/ml of IL-13. IL-4 and IL-5 were measured by chemiluminescent immuno-assay as described for IFN-γ (30). Briefly, mAbs 3H4 and TRFK5 respectively

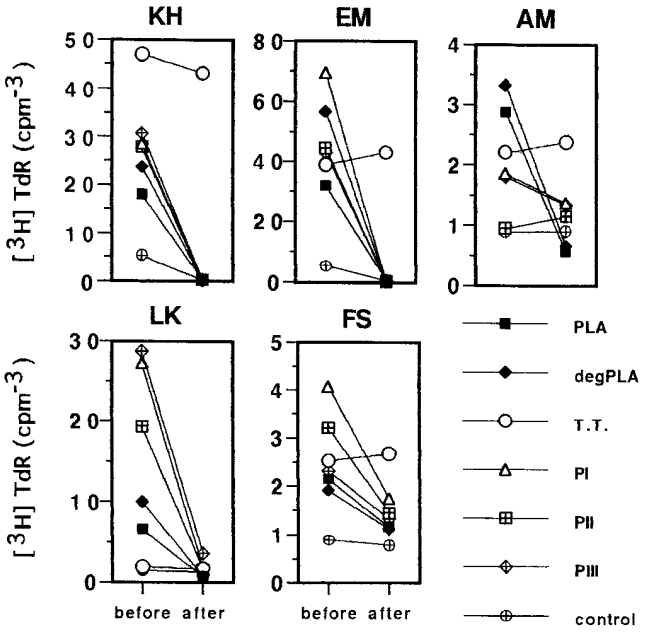

Figure 1. Changes of proliferation of PBMC before and after 2 mo of BV-IT. PBMC of five BV-IT patients were stimulated with PLA, degPLA, three T cell epitope peptides, or control Ag (TT) before and after 2 mo of immunotherapy. [^{3}H]Thymidine incorporation was measured after 7 d. Proliferation to PLA, degPLA, and T cell epitope peptides showed significant decrease after 2 mo ($P < 0.001$) while TT-specific proliferation did not change.

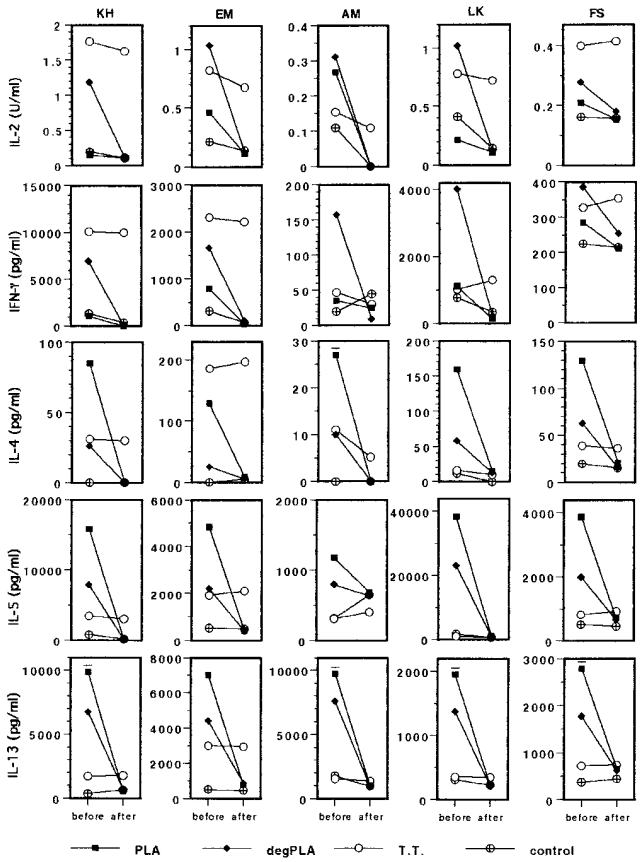

Figure 2. Changes of IL-2, IFN-γ, IL-4, IL-5, and IL-13 secretion in response to specific-allergen (PLA), degPLA, and control antigen (TT). PBMC of five patients were stimulated with either of PLA, degPLA, and TT before and after 2 mo of BV-IT. Cultures were supplemented with IL-2 and IL-4 at day 7 and expanded when necessary. Cells were washed and restimulated with the same antigens in the presence of autologous irradiated PBMC at day 12. Supernatants were harvested 16 h later for IL-2 and IL-4, and 48 h later for IFN-γ, IL-5, and IL-13 determination. All of the determined cytokines showed significant decrease in response to PLA and degPLA ($P < 0.001$) but not to TT.

(Ciba Geigy, Ltd.) were used for coating 96-well white plates (Microlite; Dynatech AG, Chantilly, VA). For detection mAbs 8F12 (Ciba Geigy) and JES15A10 (PharMingen, St Louis, MO) were labeled with 2,6-dimethyl-4-(*N*-succinimidyl-oxyl-carbonyl)-phenyl-10-ethylacridinium-carboxylate-methasulphate (DMAE-HNS) according to Weeks and Woodhead (31) and used at a final concentration of 0.3 μg/ml simultaneously with standards or samples. The plates were incubated 2 h at room temperature and chemiluminescence was detected immediately after the injection of 0.5% H_2O_2 in 0.1 N nitric acid and 0.25 N NaOH solution by a 96-well luminometer (model LB 96; Berthold AG, Wildbad, Germany) after two washings with PBS and once with water. The detection limit was < 5 pg/ml for IL-4 and < 10 pg/ml for IL-5. The IL-2 activity was measured by [^{3}H]thymidine uptake of CTLL cells as described (32).

Induction of PLA-specific Ig production. PBMC (2.5×10^6/well/5 ml) were cultured in 6-well tissue culture plates (Costar Corp.) in the presence of 0.1 ng/ml PLA, 25 ng/ml IL-4, and 25% sCD40L containing cell supernatant in the above medium further supplemented with 40 μg/ml human transferrin, 4 μg/ml bovine insulin (both from Sigma Chemical Co.) (33). Supernatants were harvested at day 12 for determination of PLA-specific IgE and IgG4 Abs.

Measurement of Ig isotypes. The IgE and IgG4 anti-PLA antibody contents in patients serum and culture supernatants were measured in duplicates by ELISA (34–36). PLA-specific antibodies were detected in 0.5 μg/well PLA-coated ELISA plates (Maxisorb) which were incubated with patients' serum and culture supernatants at different dilutions. Biotinylated anti-IgE mAb 6-7 (Ciba Geigy, Ltd.) and peroxidase-labeled extravidine (Sigma Chemical Co.) were used to develop IgE anti-PLA. Anti-IgG4 mAb RJ4 (Oxoid, Ltd., Basingstoke, UK) and peroxidase-labeled anti–mouse Ig antibody (Tago, Inc., Burlingame, CA) were used in IgG4 anti-PLA ELISA. Sera, calibrated according to bee venom RAST (Pharmacia Diagnostics AB, Uppsala, Sweden), from allergic patients were used as IgE anti-PLA standard. Human PLA-specific IgG4/λ mAb BVA2 (37) was used as a standard to determine IgG4 anti-PLA. The sensitivity of these ELISAs were < 0.05 U/ml of IgE anti-PLA and < 0.1 ng/ml of IgG4 anti-PLA.

Statistical analysis. Student's *t* test for paired samples was used for statistical analysis to compare results obtained before and after immunotherapy.

Results

BV-IT results in specific T cell tolerance to PLA and related T cell epitopes. PLA represents the major allergenic and immunogenic compound in bee venom. Accordingly, we investigated possible changes in immunological properties towards this key allergen in consequence of BV-IT. The results of T

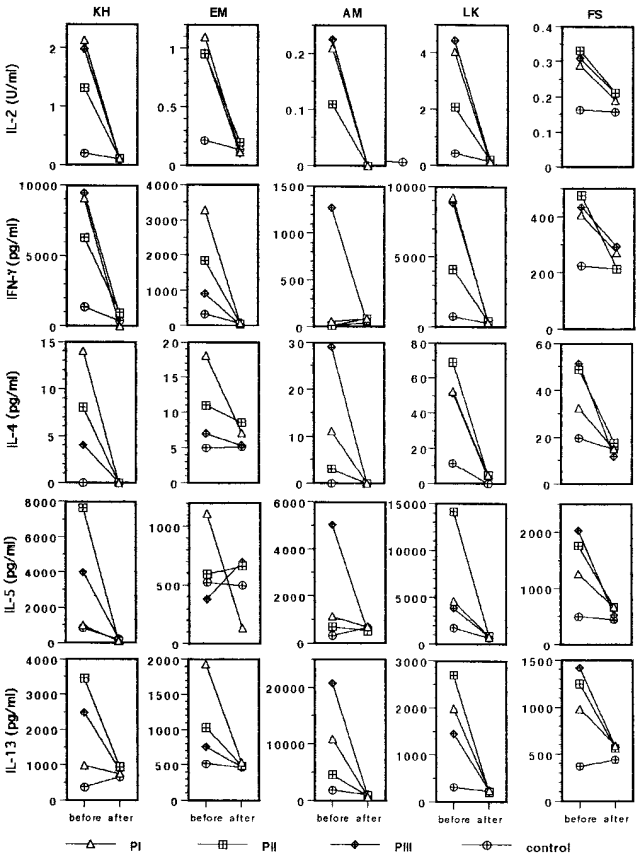

Figure 3. Changes of IL-2, IFN-γ, IL-4, IL-5, and IL-13 secretion in response to T cell epitope peptides of PLA. PBMC were stimulated with three PLA T cell epitope peptides. Cells were expanded, restimulated, and cytokines were measured as in the legend of Fig. 2.

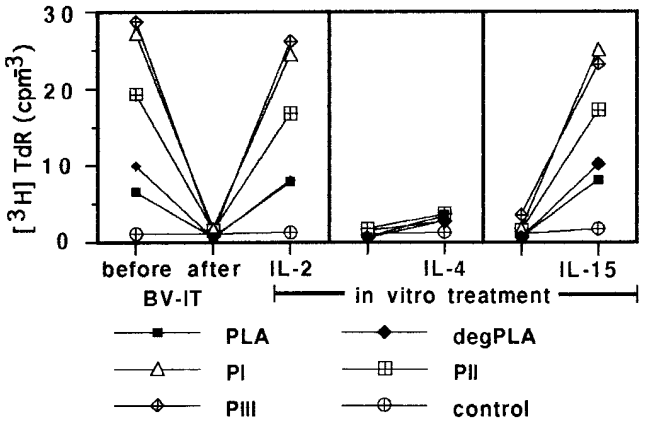

Figure 4. In vitro re-establishment of BV-IT induced whole PLA and epitope specific T cell unresponsiveness by IL-2, IL-4, and IL-15. PBMC were obtained from a patient after 2 mo BV-IT and cultured with either of PLA, degPLA, and three T cell epitope peptides in the presence of either IL-2, or IL-15, or IL-4. At day 12, 10^5 cells were washed and restimulated with the same antigens in the presence of 10^5 autologous irradiated PBMC. Proliferation was determined after 5 d by 20 h incorporation of [^{3}H]thymidine. Data shown are from a representative experiment of patient (LK). Similar results showing re-establishment of T cell response were obtained from three other patients after two months of BV-IT.

cell proliferation from five bee venom allergic patients before and after immunotherapy are presented in Fig. 1. Before BV-IT, all patients responded to both glycosylated and deglycosylated PLA. Four patients recognized all three epitopes to different extents and one patient (AM) reacted against two epitopes. After 2 mo of BV-IT, T cells were found unresponsive specifically against the entire PLA allergen ($P < 0.001$), while the response to the TT control Ag was not affected. Moreover, specific tolerance was also induced to all three T cell epitopes ($P < 0.001$). In Fig. 2, allergen-induced cytokine production in response to glycosylated and deglycosylated PLA is demonstrated. In these experiments, PBMC were antigen specifically stimulated and responding cells were expanded with a mixture of IL-2 and IL-4. Cytokines were measured after washing and restimulation with the same Ags, in the presence of irradiated autologous PBMC. Before BV-IT, in all cases, native PLA elicited increased Th2 type cytokines IL-4, IL-5, IL-13 compared to the deglycosylated variant, whereas IL-2 and IFN-γ secretion in response to native PLA were relatively low. After 2 mo of immunotherapy the PLA-induced production of both Th1 cytokines, IL-2, IFN-γ, and Th2 cytokines IL-4, IL-5 and IL-13 showed a significant decrease to almost control levels ($P < 0.001$). In contrast, cytokine profile to the TT control was not affected by the BV-IT ($P > 0.05$).

As expected from the above PLA results, peptide induced cytokine synthesis including both Th1- and Th2-type cytokines were also abolished in patients subjected to BV-IT ($P < 0.001$), as shown in Fig. 3.

Allergen-specific response of T cells tolerized by BV-IT can be re-established by cytokine treatment in vitro. Because unresponsiveness in specific T cell proliferation and cytokine production was achieved by BV-IT, we investigated conditions by which the anergized T cell responses could be restored. For this purpose 2×10^6 PBMC were cultured with Ag in the presence of IL-2 or IL-4 or IL-15 for 12 d. Cells were then washed and restimulated with the same Ag in the presence of autologous PBMC as APC. As can be seen from the representative experiment with patient LK in Fig. 4, the abrogated Ag-specific T cell proliferation was almost fully recovered. Same results were observed after IL-15 treatment of T cells tolerized in BV-IT. The proliferative response could not be restored by IL-4 treatment.

Differential recovery of Th1 and Th2 cytokine production in tolerized T cells in vitro. Cytokine patterns of antigen-specific T cells in allergic patients are of great importance in the patho-

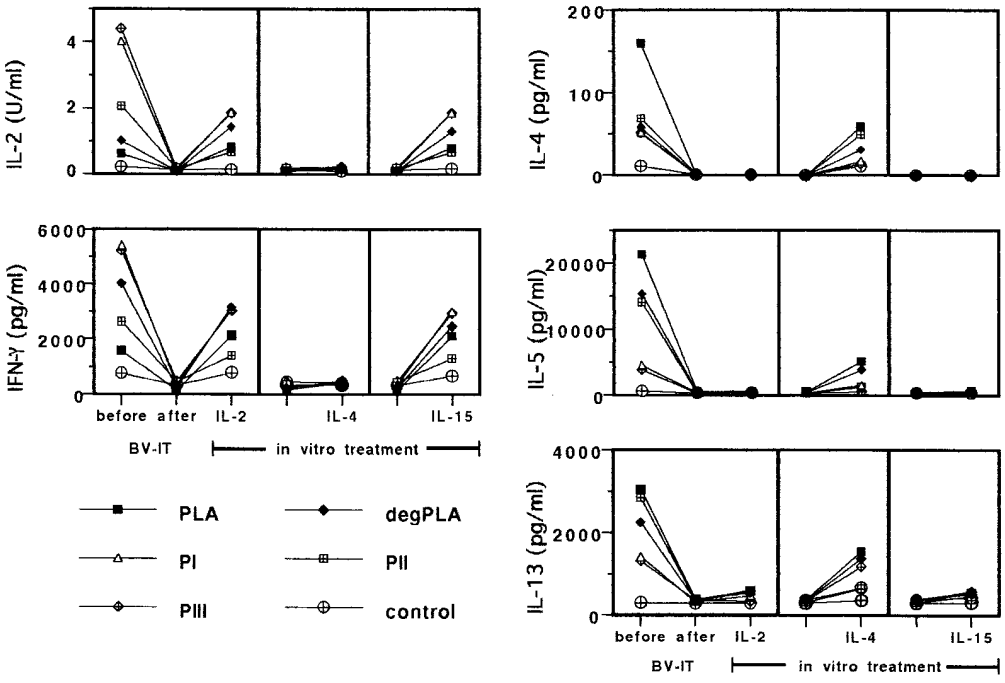

Figure 5. Differential recovery of Th1 and Th2 cytokine production of tolerized T cells in vitro. PBMC were treated as in the legend of Fig. 4. At day 12 10^6 cells were washed and restimulated with the same antigens in the presence of 10^6 autologous irradiated PBMC. Supernatants were harvested 16 h later for IL-2 and IL-4, and 48 h later for IFN-γ, IL-5, and IL-13 determination. Data shown are from a representative experiment of patient (LK). Same results showing re-establishment of T cell cytokine response were obtained from three other patients after 2 mo of BV-IT.

genesis of allergic diseases. Thus, we further analysed the changes in cytokine production of nonresponsive T cells after culturing in cytokine-supplemented medium. PBMC isolated after BV-IT were cultured for 12 d with 1 μM Ag in the presence of each of the three cytokines, IL-2, IL-4, or IL-15. The cells were then washed and restimulated with the same antigens in the presence of irradiated autologous PBMC as described above. The results of a representative experiment with patient LK are shown in Fig. 5. The capacity of Ag-induced IL-2 and IFN-γ secretion was re-established by both IL-2 and IL-15 but not by IL-4 treatment. Interestingly, culturing of cells with Ag plus IL-2 or IL-15 did not restore the production of IL-4, IL-5, and IL-13, and initiated a distinct Th1 type cytokine pattern. In contrast, when cells were cultured with IL-4 and Ag, IL-4, IL-5, and IL-13 production were recovered from the tolerant state. The amounts of Th2 cytokines produced by the restored cells reached 9–67% of the initial levels before BV-IT.

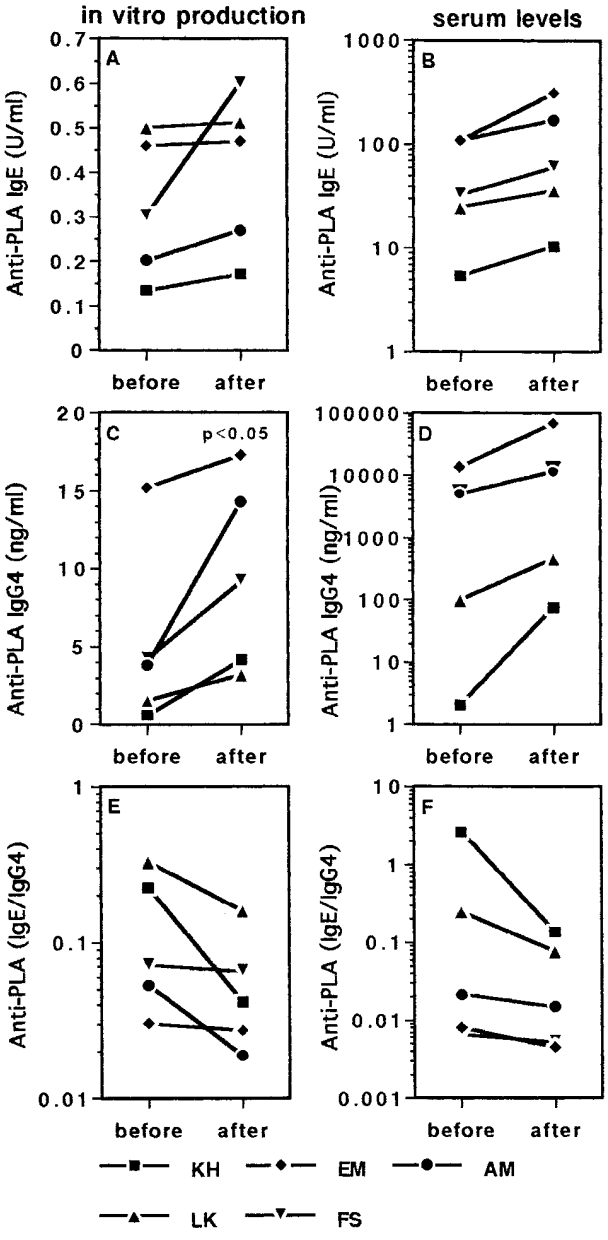

Figure 6. In vitro production and serum levels of PLA-specific IgE and IgG4. Before and after 2 mo of immunotherapy, PBMC (2.5×10^6) were cultured in the presence of PLA, IL-4, and sCD40L. Supernatants were harvested at day 12 for PLA-specific IgE (*A*) and IgG4 (*C*) determination. Corresponding serum levels of anti-PLA IgE (*B*) and anti-PLA IgG4 (*D*) were measured and plotted. The ratios of anti-PLA IgE/IgG4 before BV-IT and after 2 mo were calculated and plotted as *E* (in vitro induced) and *F* (serum levels). Both in vitro induced and serum levels of PLA-specific IgE and IgG4 showed a tendency to increase after 2 mo of BV-IT whereas PLA-specific IgE to IgG4 ratios showed a decrease in all of the patients. The difference was statistically significant for in vitro anti-PLA IgG4 formation ($P < 0.05$).

BV-IT does not reduce the capacity of B cells to produce PLA-specific IgE and IgG4 Ab in vitro. Although peripheral T cell tolerance was demonstrated towards the BV major allergen PLA and related epitopes after 2 mo of BV-IT, the B cell capacity to produce PLA-specific IgE and IgG4 Abs was of essential importance. This was analysed in a culture system which allows the in vitro production of PLA-specific IgE and IgG4 Abs by stimulation of PBMC through CD40 in the presence of IL-4 and optimum amounts of PLA (33). Fig. 6 illustrates the capacity of PBMC to produce PLA-specific IgE and IgG4 after 12 d of culture with PLA/IL-4/sCD40L. Generally, in vitro induced anti-PLA IgE and IgG4 in corresponding sera showed a tendency to increase during BV-IT. The difference was statistically significant for the in vitro anti-PLA IgG4 formation ($P < 0.05$). More importantly, although both isotypes slightly increased during the time of treatment, the ratio of specific IgE/IgG4 decreased in favor of IgG4 in average by a factor of 12.1 in serum and 2.3 in cultures. These results suggest that during BV-IT allergen-specific B cells were not tolerized and express their entire capacity to produce specific Abs.

Discussion

In this study we have demonstrated that BV-IT for 2 mo of individuals suffering from bee sting allergy is associated with a significant reduction in T cell proliferative response and cytokine secretion to the epitopes of the major BV allergen, PLA. The T cell tolerance induced by BV-IT was specific since TT-specific responses were not altered. In previous studies a relationship between decreased allergen-specific T cell proliferation and successful immunotherapy has been demonstrated in various atopic diseases including allergy to ragweed, cat, grass pollen, and bee venom (15, 16, 38–40). In a recent study a decrease in IL-4 and IL-5, but increase in IFN-γ production by BV-IT was reported (16). It is plausible that methodological differences in re-stimulation of T cells by anti-CD3 instead of antigen-specific stimulation may be responsible for differences in cytokine production (16). In this study we demonstrate that BV-IT induces peripheral tolerance in T cells and tolerized T cells did not show a distinction in cytokine profiles; both Th1 and Th2 type cytokine production were suppressed by immunotherapy.

Proposed mechanisms of peripheral T cell unresponsiveness include the induction of antigen-specific anergy (41, 42) and the production of humoral and cellular suppressor factors (43, 44). We know that T cells can specifically be rendered unresponsive if the antigen is administered under conditions which do not deliver co-stimulatory signals to T cells (45). In general, antigens display several T cell epitopes, some of them being immunodominant (46, 47). Immunodominant antigenic

determinants can also be strong inducers of T cell unresponsiveness (48) and administration of soluble antigens to animals can functionally inactivate antigen-specific peripheral T cells (49, 50). The observed T cell unresponsiveness achieved in this study is probably similar to the mechanisms of anergy induction in vitro in T cells as initially described for human Th clones reactive to influenza hemagglutinin (51) or in mice to house dust mite allergen (52). We attempted to analyze changes in surface molecule expression before and after immunotherapy in PBMC and found no difference in surface expression of CD40L, CD30 expression on CD4, or CD8 T cells (data not shown). However, the low frequency of antigen-specific T cells in peripheral blood makes it difficult to measure the changes in specific T cells.

T cell anergy is distinguished from suppression in that antigen-specific T cell responses can be recovered by the addition of IL-2 in the presence of antigen (53). Signaling through the common γ-chain of IL-2, IL-4, and IL-7 in the presence of antigen was shown to prevent the induction of anergy (54). Although Th0 and Th1 type clones are more susceptible to tolerance induction (49, 55) anergy could be induced in human allergen specific Th2 cells in relevant peptide or anti-CD3 treatment in the systems lacking second signals (56, 57). In both cases addition of IL-2 but not IL-4 alone reversed the anergic state (56, 57). In addition, the B cell help for IgE production of anergized human type 2 T cell clones was restored by IL-2. This effect was enhanced by simultaneous addition of IL-4 (56). In our study the specifically anergized T cell responses could be restored by culturing the cells with allergen and IL-2 or IL-15, but only in terms of T cell proliferation and Th1 type cytokine secretion (IL-2 and IFN-γ). IL-15 shares many of the activities on T cells and B cells previously defined for IL-2 (58, 59). This can be explained by the fact that IL-15 uses the IL-2 receptor complex for signaling (58). However, whereas IL-2 expression is restricted to activated T cells, IL-15 mRNA has been detected in most other cell types and tissues. Strongest expression was demonstrated in monocytes, epithelial cell lines, muscle, and placenta (58). Thus IL-15 may act as an important microevironmental cytokine to regulate tolerance and recovery in immunotherapy.

In contrast to IL-2 and IL-15, treatment of the tolerized T cells with IL-4 re-established the allergen induced Th2 cytokine production only at lower levels. Although recovery of Th2 cytokines was observed, IL-4 treatment of tolerized cells did not restore proliferation as previously reported (57). The in vitro modulation of T cell anergy induced by BV-IT suggests that: (*a*) cytokines produced in the microenvironment can govern the secondary induction of distinct Th1 or Th2 cytokine patterns that are associated with either normal immunity against an allergen and successful immunotherapy or with further development of allergy and inflammation; (*b*) specifically tolerized T cells circulate in the peripheral blood and migrate into lymphoid tissues where they can recover by the local cytokine milieu. This recovery may occur more readily in vivo than in vitro which may explain the necessity to prolong immunotherapy up to 3 yr (60). In this state of immune response it seems that high amounts of the respective allergen is decisive for the induction of Th1 cytokines and the synthesis of specific IgG4 Ab (61).

Many studies have demonstrated that during BV-IT, after initial increase, allergen-specific serum IgE decreases over a period of months to years (16, 62). In the present study we observed a non-responsiveness at the T cell level after 2 mo of BV-IT. However, following supplementation of PBMC culture with a T cell cytokine and T cell surface molecule such as IL-4 and sCD40L (63, 64) and PLA, B cells appeared to preserve their capacity to secrete even increased amounts of anti-PLA IgE and anti-PLA IgG4. In parallel, serum levels of both PLA-specific antibodies tended to increase. The question remains why the specific IgE and IgG4 responses increase after BV-IT. The change in ratio of IgE to IgG4 was in the favor of IgG4 and more pronounced in serum antibodies. This may be due to the different half lives of the two isotypes in vivo. Most likely, because of a nonresponsiveness was achieved in antigen-specific CD4+ T cells, the influence of cytotoxic 1 type CD8+ cells, natural killer cells and many other environmental cells on the change in cytokine balance in favor of IFN-γ and less Th2 cytokines such as IL-4 and IL-13, preferring the secretion of IgG4, and exceeding suppressive effects on IgE may be of major relevance (36, 65).

The understanding of tolerance induction by allergen immunotherapy is of great interest for a better knowledge of the pathophysiological mechanisms in allergy. Our present study demonstrates that immunotherapy leads to peripheral T cell tolerance without inhibiting apparent B cell function of specific IgE and IgG4 Ab production. The association of successful immunotherapy with decreased T cell reactivity to the immunodominant T cell epitopes and further modulation of cytokine profile of the tolerized T cells presents a way of possible treatment of certain allergies directly with epitope peptides which specifically tolerize the relevant regulatory T cells.

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