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An Altered Peptide Ligand Specifically Inhibits Th2 Cytokine Synthesis by Abrogating TCR Signaling[1]

Alexander Faith,[2] Cezmi A. Akdis, Mübeccel Akdis, Andrea Joss, Daniel Wymann, and Kurt Blaser

Altered peptide ligands (APL) can modify T cell effector function by their diversity in binding to the TCR or MHC class II-presenting molecules. The capacity to inhibit Th2 cytokine production by allergen-specific T cells would contribute to combating allergic inflammation. The presence of APL generated by Ala-substitutions in a synthetic dodeca-peptide spanning an immunodominant epitope of bee venom phospholipase A_2 (PLA) was investigated in human T cells. Four of five substituted peptides reduced proliferation, IL-4, and IFN-γ production by cloned PLA-specific Th0 cells proportionately. However, one APL, PLA-F82A, inhibited IL-4 but had no effect on IFN-γ production. This uncoupling of IL-4 from IFN-γ production was also observed on immunogenic restimulation of the cloned T cells pre-exposed to the APL/APCs. It appeared to result from lower affinity of binding to MHC class II by the APL compared with the native peptide. The APL also inhibited IL-4 production by polyclonal T cells. In consequence of the change in cytokine secretion, the production of IgG4 in vitro increased by PLA-F82A stimulation, compared with the native peptide. Exposure of the cloned T cells to either the APL or the native peptide, in the absence of professional APC, induced anergy such that proliferation and production of IL-4, IL-5, and IL-13 was abrogated on immunogenic rechallenge. Defective T cell activation appeared to result from alterations in transmembrane signaling through the TCR, specifically to lack of tyrosine phosphorylation of the tyrosine kinase, ZAP-70. ***The Journal of Immunology*****, 1999, 162: 1836–1842.**

The secretion of the Th2 cytokines IL-4, IL-5, and IL-13 in affected sites such as skin and lung induces an allergic response (reviewed in Ref. 1). An isotype switch to IgE is dependent on IL-4 or IL-13 (reviewed in Ref. 2), whereas IL-5 promotes the growth and survival of eosinophils (3). Both Th0 and Th2 cells secrete these cytokines, and it is probable that both subsets are implicated as contributing to allergic reactivity (reviewed in Ref. 4).

There has been considerable interest in immunotherapeutic intervention designed to inhibit Th2 cytokine production by allergen-specific T cells (5–7). Recently, immunotherapy using synthetic peptides, spanning dominant epitopes of the major cat and bee-sting allergenic proteins, Fel d 1 and bee venom phospholipase A_2 (PLA),[3] has proved successful in ameliorating allergic symptoms (8, 9). Peptides derived from PLA abrogated specific cytokine production in peripheral T cells from the majority of patients (9). We and others have previously shown that pre-exposure of cloned allergen-specific Th2 cells to native peptide, in the absence of professional APC, results in nonresponsiveness to subsequent immunogenic challenge, including abrogation of IL-4 and IL-13 production (10, 11). Reduced IL-4 and IL-13 production was correlated with altered transmembrane signaling through the TCR, specifically, ablation of $p56^{lck}$ and ZAP-70 tyrosine phosphorylation (10).

The interaction of $CD4^+$ T cells with optimally presented peptide/MHC complex leads to T cell proliferation and cytokine production (reviewed in Ref. 12). However, varying the affinity of ligand for the TCR can alter the profile of cytokine production (13, 14). Certain amino acid substitutions in the native peptide at TCR contact residues, termed altered peptide ligands (APL) (reviewed in Ref. 15), can modify cytokine patterns of both human (16, 17) and murine T cells (18, 19). It has been shown that APL may function as partial agonists, delivering an altered signal to cells, often resulting in anergy (20, 21). Furthermore, peptides substituted at MHC class II-contact residues can divert both human (22) and murine (23, 24) Th2 to Th1 responses. An APL of myelin basic protein, modified at a MHC class II binding residue, reversed the induction of autoimmune disease by the native peptide by altering cytokine patterns of encephalitogenic T cells (25).

Therefore, we examined whether APL could alter Th2 cytokine patterns and/or induce anergy in cloned PLA-specific Th0 cells. Furthermore, the immunological effect of changes in specific T cell cytokine secretion on the isotype production by B cells and the possible mechanism of anergy induction were studied. Compared with native peptide, the ratio of IL-4:IFN-γ was significantly reduced and the IgG4 production increased when cloned PLA-specific Th0 cells were challenged immunogenically with an APL that had a lower affinity for MHC class II. Pre-exposure of Th0 cells to either native peptide or APL-induced anergy, resulting in abrogation of IL-4, IL-5, and IL-13 secretion on immunogenic restimulation. The anergic response was correlated with altered signaling through the TCR, namely lack of phosphorylation of ZAP-70.

Materials and Methods

Ags and reagents

rPLA was prepared as described (26). The synthetic peptides corresponding to $PLA_{81–92}$ (YFVGKMYFNLID) and Ala-substituted $PLA_{81–92}$ peptides were synthesised by Genosys Biotechnologies (Cambridge, U.K.). rIL-2 was kindly provided by Sandoz (Basel, Switzerland). mAbs to ZAP-70 (IgG2a) and phosphotyrosine (ptyr) (RC20) were purchased from

Swiss Institute of Allergy and Asthma Research, Davos, Switzerland

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[2] Address correspondence and reprint requests to Dr. Alexander Faith, Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland. E-mail address: siaf@siaf.unizh.ch

[3] Abbreviations used in this paper: PLA, bee venom phospholipase A_2; APL, altered peptide ligand(s); ptyr, phosphotyrosine.

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Upstate Biotechnology (Lake Placid, New York) and Transduction Laboratories (Lexington, KY), respectively. Anti-CD3 mAb was produced by clone CRL 8001 obtained from American Type Culture Collection (Manassas, VA) and purified by affinity chromatography. mAb to CD3 (OKT3) was kindly donated by Dr. C. Heusser (Novartis, Basel, Switzerland). Anti-CD28 (mAb 15E8) and anti-CD2 (mAbs 4B2 and 6G4) were obtained from the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Purified rabbit anti-mouse Ig was obtained from Dako (Zug, Switzerland). Biotin-N-hydroxysuccinimide-ester (nhs-d-Biotin) and FITC-avidin were obtained from Calbiochem (La Jolla, Ca) and Sigma (Buchs, Switzerland), respectively.

Cells

The T cell clone, BuT1.2A2, derived from a hyperimmunized healthy individual and specific for PLA, has been reported (27). BuT1.2A2 recognizes an epitope spanned by the amino acid sequence, $PLA_{81–92}$ (27), and its cytokine profile, secreting both IL-4 and IFN-γ (28), indicates a Th0 functional phenotype. The clone is restricted by HLA-DP (27). The cloned T cells were resuspended in complete medium, consisting of RPMI 1640, 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 (all from Life Technologies, Basel, Switzerland) and 10% heat-inactivated FCS (Sera-Lab, Crawley Down, Sussex, U.K.). PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed and resuspended in complete medium as above.

Induction of anergy in Th0 cells

Cloned T cells ($1–2 \times 10^6$/ml) were incubated for 16 h in the presence of $PLA_{81–92}$ or PLA-F82A (70 μM) in 24- or 48-well culture plates (Costar, Cambridge, MA). The cells were then washed and incubated for a further 48 h before restimulation with $PLA_{81–91}$ in the presence of autologous, irradiated (5000 rad), EBV-transformed B cells (BuB1; APC) (27). Cloned T cells were also incubated with $PLA_{81–92}$ or PLA-F82A in the presence of APC ($1–2 \times 10^6$/ml) for 5 days and then restimulated with $PLA_{81–92}$/APC, and proliferation and cytokine production measured.

Proliferation assays

For Ag-specific proliferation, cloned T cells (1×10^5/ml) were stimulated with PLA, antigenic peptide, or APL, in the presence of APC (1×10^5/ml), in 200 μl medium in 96-well round-bottom tissue culture plates (Becton Dickinson, Lincoln Park, NJ) in triplicate wells. Proliferation was determined after 48 h by pulsing for a further 16 h with [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (DuPont/NEN, Boston, MA) (1 μCi/well, 37 kBq).

Production of IgG4 in vitro

T cell-depleted PBMC (0.5×10^5) were reconstituted with 0.5×10^5 T cell clone BuT 1.2A2, in 96-well flat-bottom plates (Costar). Cells were stimulated with increasing doses of native peptide or APL in the presence of 5 ng/ml of IL-4 (Novartis) at 37°C in a 5% CO_2 atmosphere (7, 9). IgG4 was determined in supernatants after 12 days of incubation. All experiments were performed in triplicates.

Cytokine and IgG4 measurements

BuT1.2A2 (2×10^5/ml) was stimulated with varying concentrations of PLA or peptides in the presence of autologous EBV-transformed B cells (2×10^5/ml), and supernatants were harvested after 24 h. Cloned T cells (1×10^6/ml) were incubated with $PLA_{81–92}$ or PLA-F82A (70 μM), also in the absence of APC and supernatants-harvested. PBMC were incubated with peptides or a combination of mAbs to CD3 (CRL8001, 0.5 μg/ml), CD2 (0.5 μg/ml), and CD28 (1 μg/ml), and supernatants were harvested after 5 days. The solid phase ELISAs for IL-4, IL-5, IL-13, and IFN-γ have been described before (8, 28, 29). IL-10 was measured using a combination of mAb JES3-9D7 and biotinylated JES3-12G8 (mAbs and IL-10 standard were from PharMingen, San Diego, CA). The sensitivity of the IL-10 ELISA was = 50 pg/ml. IL-2 activity was detected by [^{3}H]TdR uptake by the CTLL-2 line (30).

IgG4 was measured in duplicates by sandwich ELISA as described (7, 9). The assays reached a sensitivity of 0.6 ng/ml of IgG4 World Health Organization 67-97 reference standard.

Affinity measurements by competition assay

The binding affinity of native peptide and APL were determined as previously described (31). $PLA_{81–92}$ was biotinylated and then purified by HPLC using a Nucleosol C18 column (pore size 250 A°, 250 × 2 mm)

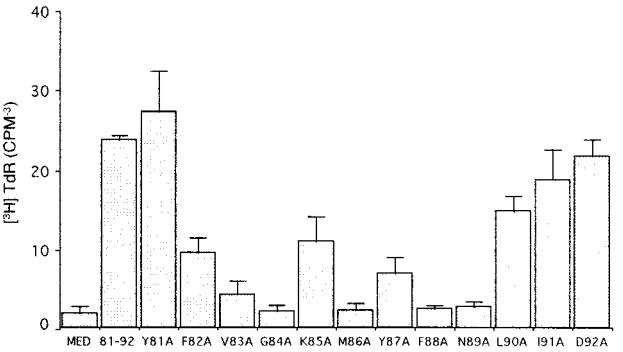

FIGURE 1. Proliferative responses of BuT1.2A2 to $PLA_{81–92}$ and Ala-substituted peptides. BuT1.2A2 (1×10^5/ml) was stimulated with peptides (3 μM) in the presence of autologous EBV (1×10^5/ml).

(Mochereq-Nagel, Oensingen, Switzerland). Fractions were eluted with an acetonitrile gradient at a flow rate of 0.2 ml/min and lyophilized. Binding of biotinylated $PLA_{81–92}$ (25 μg/ml) to BuB1 cells in the presence or absence of varying concentrations of competitor peptides for 5 h at 37°C was detected by addition of avidin-FITC (10 μg/ml). Single color fluorescence was performed as described (29).

Immunoprecipitations

The method was as described (10), with some modifications. BuT1.2A2 was rested in complete medium without growth factors for 4 days and in 2% FCS containing medium for 24 h before experiments. T cells were exposed to $PLA_{81–92}$ or PLA-F82A (70 μM) for 16 h and then washed and incubated for a further 48 h. Cloned T cells (3×10^6) were then stimulated with anti-CD3 (OKT3, 10 μg/ml), cross-linked to plastic (Falcon 2051, Becton Dickinson), and mAb to CD28 (1 μg/ml) for 15 min. T cells were lysed in 0.5% Triton X-100 containing leupeptin and aprotinin (each 10 μg/ml), sodium orthovanadate (1 mM), EDTA (5 mM), and iodoacetamide (5 mM) (all from Sigma). Postnuclear lysates were precleared for 2 h each with Pansorbin (Calbiochem-Novabiochem, San Diego, CA; 10% (v/v) solution of fixed *Staphylococcus aureus* Cowan I, prewashed three times with PBS) and BSA-bound Sepharose-Protein A (Pharmacia, Uppsala, Sweden). Precipitations were performed overnight at 4°C with mAb to ZAP-70, and complexes captured with rabbit anti-mouse Ig bound to Protein G-Sepharose 4B (Pharmacia). Precipitates were washed with cold lysis buffer and boiled in Laemmli sample buffer before electrophoresis.

Immunoblotting

Immunoprecipitates were electrophoresed on 8–16% SDS-PAGE gels (Novex\, San Diego, CA). Proteins were transferred to nitrocellulose blots and blocked with 2% BSA or 3% nonfat milk powder in 0.01 M Tris-buffered saline/0.1 M sodium chloride/0.1% Tween 20. Blots were then probed with mAb to ptyr linked to horse radish peroxidase, stripped and then probed with mAb to ZAP-70, followed by horse radish peroxidase-linked anti-mouse Ig (Transduction Laboratories). Blots were detected by the enhanced chemiluminescence system (Amersham, Little Chalfont, U.K.). Densitometry was performed using a table top scanner (UVP, OmniLab, Switzerland).

Results

The APL, PLAF82A, decreases IL-4:IFN-γ ratio by inhibiting IL-4 production

Peptide analogues with Ala-substitutions at each position of $PLA_{81–92}$ were tested for their effects on proliferation by the T cell clone, BuT1.2A2, in the presence of professional APC. The reduction in the proliferative response of the clone to several substitutions, at positions 82, 83 85, 87, and 90 indicated that these peptides functioned either as weak or partial agonists (Fig. 1). Substitutions at positions 84, 86, 88, and 89 rendered the clone nonresponsive in the proliferation assay, whereas substitutions at the N- and C-terminal residues had no effect. Partial agonists have been shown to modify cytokine production by allergen-specific T cells (15) and therefore, cytokine production in response to the

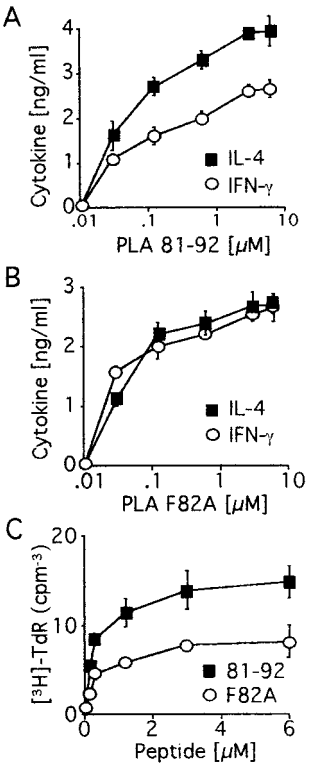

FIGURE 2. Proliferative and cytokine responses of BuT1.2A2 to APL. BuT1.2A2 (2×10^5/ml) was stimulated with increasing concentrations of $PLA_{81–92}$ (*A*) or PLA-F82A (*B*), in the presence of autologous EBV cells (2×10^5/ml), and cytokine production measured. *C*, Proliferative response of BuT1.2A2 (1×10^5/ml) to $PLA_{81–92}$ and PLA-F82A in the presence of equivalent numbers of autologous EBV cells.

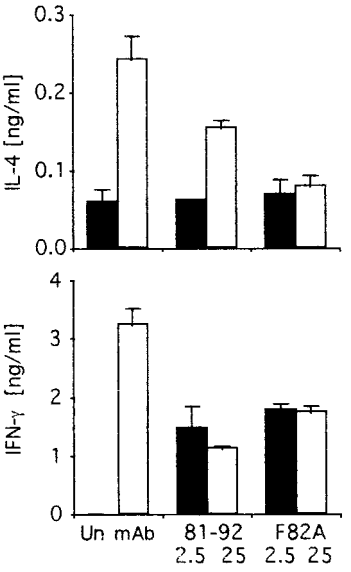

FIGURE 3. Cytokine production by PBMC from an allergic donor in response to $PLA_{81–92}$ and PLA-F82A. PBMC (1×10^6/ml) were stimulated with peptides (2.5 and 25 μg/ml) and mAbs to CD3/CD2/CD28 (mAb) and cytokine production measured after 5 days. "Un" represents unstimulated PBMC.

altered peptides was investigated. Peptides that induced no proliferative response were not included as these appear to induce either no cytokines or in some cases IL-4 alone (17, 32). The pattern of IL-4 and IFN-γ production in response to APL, in general, corresponded to the proliferation data (data not shown). However, for one substitution, F82A, the proportional reduction in IL-4 was greater than that in IFN-γ secretion, and therefore, the IL-4:IFN-γ ratios were decreased compared with $PLA_{81–92}$. Dose-response curves revealed that the IL-4:IFN-γ ratio increased over the concentration range for both $PLA_{81–92}$ and PLA-F82A (Fig. 2, *A* and *B*). At the lowest concentration (0.03 μM), the IL-4:IFN-γ ratios were 1.3 and 0.7 for the native peptide and APL, respectively. At a higher concentration [3 μM], the IL-4:IFN-γ ratios were 1.8 and 1.1, respectively. The reduction in IL-4:IFN-γ ratio by the APL compared with $PLA_{81–92}$ was however, observed over the whole concentration range. Proliferative responses to PLA-F82A also showed significant reductions compared with $PLA_{81–92}$ (Fig. 2*C*), indicating that the APL was a partial rather than a weak agonist.

Cytokine production by PBMC from a bee-sting allergic subject was also investigated. Stimulation of PBMC with mAb to CD3/CD2/CD28 induced production of both IL-4 and IFN-γ (Fig. 3). $PLA_{81–92}$ but not PLA-F82A induced IL-4 secretion. In contrast, the two peptides stimulated secretion of equivalent levels of IFN-γ by polyclonal T cells. Secretion of IL-4 by PBMC from three other allergic donors in response to either peptide was not detectable. Both the native peptide and PLA-F82A induced cytokine production by BuT1.2A2 in the absence as well as the presence of APC (Table I). High levels of IL-10, IL-13, and IL-5 were detected, although it is important to note that the concentration of T cells was 5-fold greater than in the presence of APC. Although both peptides induced similar levels of IFN-γ secretion, production of IL-4 in response to the APL was only 23% of the response to $PLA_{81–92}$. BuT1.2A2 did not produce any IL-2 in response to peptides.

APL stimulation by PLA-F82A increases IgG4 production in vitro

The immunological relevance of the changes in cytokine profile of peptide-specific T cells by the APL, PLA-F82A, was investigated on isotype production in T cell-depleted autologous PBMC substituted with the $PLA_{81–92}$-specific T cell clone BuT1.2A2. It is known that changes in IL-4:IFN-γ ratio directly correlate with production of IgE:IgG4 ratio (7, 28, 33). Indeed, the Th2 cytokine-suppressing APL substantially increased the IgG4 production by B cells, compared with the original $PLA_{81–92}$ peptide and an irrelevant control peptide from a major PLA epitope in vitro. This is demonstrated in Fig. 4.

The APL, PLA-F82A, has a lower binding affinity for HLA-DP than the native peptide

The reduction in IL-4 production by BuT1.2A2 in response to the APL, PLA-F82A, indicated an altered form of TCR triggering, compared with $PLA_{81–92}$. This alteration could result from variation in the binding affinity to either the MHC class II molecule or to the TCR. Therefore, we conducted binding experiments on the autologous EBV-transformed B cell line to investigate this. The T cell clone, BuT1.2A2, is restricted by HLA-DP (27), and >90% of EBV cells expressed HLA-DP (data not shown). The biotinylated $PLA_{81–92}$ bound to 40% of the B cells and the same peptide, used as a competitor at a ratio of 8:1, reduced the number of cells bound by 45% (Fig. 5) and decreased mean fluorescence values from 0.9 to 0.5. In contrast, F82A at the same competitor ratio was unable to reduce cell binding by >8% and mean fluorescence values were decreased from 0.9 to 0.8.

Table I. *Cytokine production by TH0 cells in response to the native peptide $PLA_{81–92}$ and the APL, PLA-F82A, in the absence of APC*

Stimulus	IL-2	IL-4	IL-5	IL-10	IL-13	IFN-γ
None	0.075 (0.01)[b]	UD[c]	1.29 (0.19)	1.82 (0.95)	1.95 (0.02)	0.07 (0.02)
$PLA_{81–92}$[a]	0.055 (0)	2.77 (1.1)	24.02 (0.71)	22.35 (6.2)	22.80 (0.05)	1.87 (0.09)
PLA-F82A	UD	0.63 (0.02)	16.31 (0.92)	19.93 (3.1)	13.12 (1.07)	1.77 (0.06)

[a] BuT1.2A2 (1×10^6/ml) were stimulated with $PLA_{81–92}$ or PLA-F82A (70 μM) in the absence of APC and supernatants were harvested after 24 h.
[b] Mean cytokine ng/ml (1 SD)
[c] UD, undetectable

Induction of anergy by the APL, PLA-F82A, in Th0 cells

We have previously noted that exposure to native peptide in the absence of professional APC induced anergy in cloned PLA-specific Th2 cells (10). The production of IL-4 and IL-13, but not IL-5, was abrogated in response to immunogenic challenge. When BuT1.2A2 was exposed to either $PLA_{81–92}$ or PLA-F82A and restimulated in the presence of native peptide and APC, levels of all cytokines measured, IL-4, IL-5, IL-10, IL-13, and IFN-γ, were reduced compared with untreated T cells (Fig. 6). The APL and native peptide were almost equally effective in inducing anergy in the clone. There were considerable differences in the degree of inhibition for each cytokine. Whereas the production of IL-4 was decreased by 85% and 72% by preincubation with the native peptide or APL, respectively, IFN-γ secretion was only reduced by 25% and 26%, respectively. As PLA-F82A had specifically inhibited IL-4 production in the presence of APC (Fig. 2), BuT1.2A2 was immunogenically restimulated to determine whether anergy could be established in the presence of APC (Fig. 7). Pre-exposure to $PLA_{81–92}$ in the presence of APC had no effect on cytokine production and only a marginal effect on proliferation. In contrast, pre-exposure to PLA-F82A inhibited IL-4 production by 55% on restimulation. This effect was specific as production of IFN-γ and proliferative response were unaffected.

Abrogated tyrosine phosphorylation of ZAP-70 in anergic Th0 cells

We have previously noted abrogated *lck* and ZAP-70 tyrosine phosphorylation in anergic Th2 cells (10), suggesting an alteration in signaling through the TCR. When BuT1.2A2 was exposed to PLA_{81-92} or PLA-F82A in the absence of APC, there was no evidence of ZAP-70 tyrosine phosphorylation up to 60 h after exposure (Fig. 8 and data not shown), a result similar to that previously observed with Th2 cells. To provide a full activating signal, control and anergic Th0 cells were restimulated with a combination of mAbs to both CD3 and CD28. Cells were left for 2 days after exposure to peptides before restimulation to relate the time-course for signal transduction directly to cytokine release. Cloned normal Th0 cells responded to anti-CD3/CD28 stimulation with a strong increase in tyrosine phosphorylation of ZAP-70. Tyrosine-phosphorylated bands, in the 55- to 60-kDa range, were also detected (10). In contrast, in Th0 cells pretreated with either $PLA_{81–92}$ or PLA-F82A, there was no increase in ZAP-70 tyrosine phosphorylation and no associated bands observed.

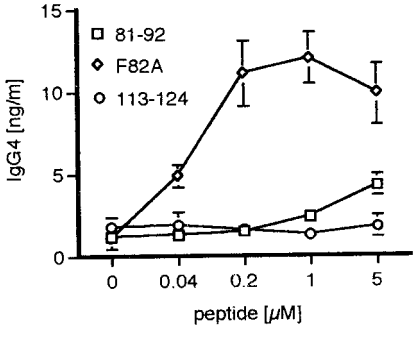

FIGURE 4. Increased production of IgG4 by the APL, PLA-F82A. T cell-depleted PBMC, substituted with the T cell clone BuT1.2A2 from the same donor, were stimulated with the APL, the original T cell peptide $PLA_{81–92}$ or a peptide containing an irrelevant T cell epitope of PLA. The cultures were stimulated with different peptide concentrations for 12 days in the presence of IL-4 (7, 28). Compared with $PLA_{81–92}$, the APL increased the IgG4 synthesis in this system.

Discussion

We have examined the capacity of an APL, derived from $PLA_{81–92}$, to alter cytokine patterns in cloned allergen-specific Th0 cells. The APL, PLA-F82A, reduced IL-4 production but induced similar levels of IFN-γ production in comparison with the native peptide. A decreased IL-4:IFN-γ ratio in human allergen-specific Th0 cells, responding to APL, has been described previously (17, 22), but as a result of increased IFN-γ rather than decreased IL-4 production. Nevertheless, the functional phenotype of polarized human Th2 cells can be skewed, leading to reduction in IL-4 production (34). This indicates plasticity in the regulation of IL-4 secretion that may allow manipulation by a variety of immunotherapeutic means.

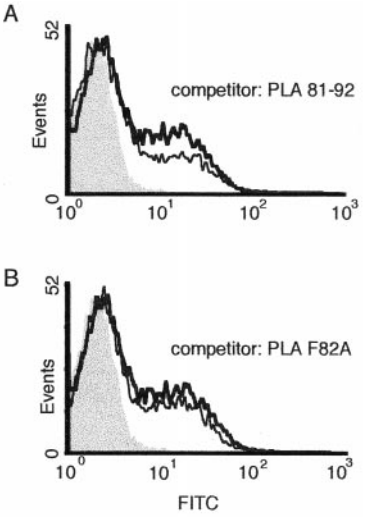

FIGURE 5. Competitive binding of $PLA_{81–92}$ and PLA-F82A to BuB1 cells. Binding of biotinylated $PLA_{81–92}$ (25 μg/ml) to BuB1 (3×10^5): (*A*) in the presence and absence of $PLA_{81–92}$ (200 μg/ml) and (B) in the presence and absence of PLA-F82A (200 μg/ml). The top line represents binding of biotinylated peptide in the absence of competitor. The shaded area represents binding of FITC-avidin control.

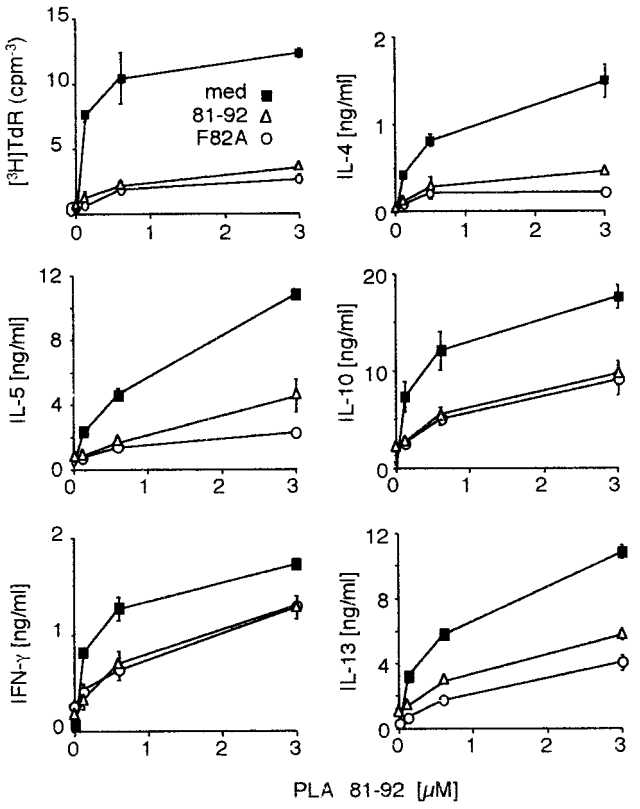

FIGURE 6. Induction of T cell anergy by $PLA_{81–92}$ and PLA-F82A. BuT1.2A2 (1×10^6/ml) was untreated (MED) or exposed to $PLA_{81–92}$ or PLA-F82A (60 μM) for 16 h, washed, and incubated for a further 48 h. Cells were then restimulated with $PLA_{81–92}$ in the presence of autologous EBV. Cytokine production was measured after 24 h.

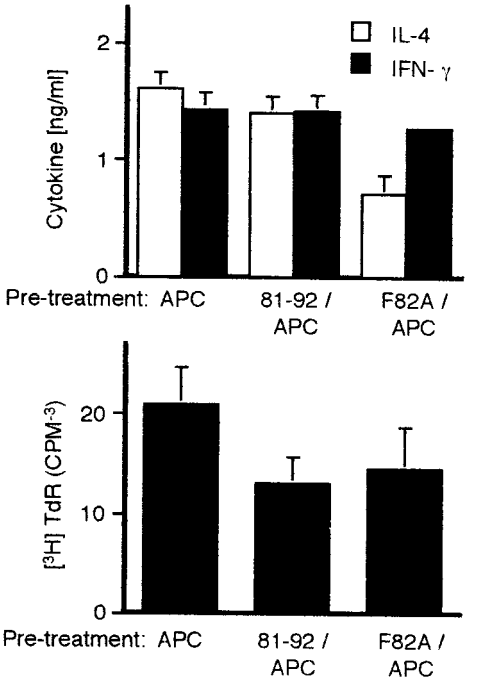

FIGURE 7. Induction of T cell anergy in the presence of APC. BuT1.2A2 (1×10^6/ml) was pretreated by exposure to APC alone, or exposed to $PLA_{81–92}$ or PLA-F82A (60 μM) for 5 days in the presence of APC (1×10^6/ml). T cells were then restimulated with $PLA_{81–92}$ [3 μM] in the presence of APC. Supernatants were harvested after 24 h, and proliferation was measured after 48 h culture.

Diminution of IL-4 production by the APL was observed in the absence as well as presence of APC, indicating that the result was not due to an inhibitory factor produced by APC, but derived from altered binding of the APL to the TCR or the MHC class II molecule. Binding studies indicated that the Phe to Ala substitution at residue 82 reduced the affinity of the peptide for the MHC class II molecule. The clone, BuT1.2A2, is restricted by HLA-DP (27), and no motifs specific for DP have as yet been described. However, the known crystal structures for DR1 and I-E^k binding peptides indicate the presence of a critical anchor residue at the N-terminal position P1 (35, 36). The Phe at position 82 may represent this residue in $PLA_{81–92}$, and by analogy with DR1, this large aromatic residue may be required to fit the corresponding pocket contributed by the HLA-DP molecule.

Previous studies have indicated that APL binding with lower affinity to I-A molecules may skew both naive and cloned murine T cells toward higher levels of IL-4 production (23, 24). However, this has not been found in studies of peptides binding to HLA-DP or -DQ molecules (37, 38). The evidence that IL-4 but not IFN-γ production by polyclonal T cells could also be reduced by PLA-F82A indicated that this was not a property unique to one T cell repertoire.

We have previously reported that increasing the concentration of allergen, and thus increasing the signal strength, increased the IL-4:IFN-γ ratio for the responses of several PLA-specific T cell clones (28). This could also be inferred by the observation that low concentrations of an APL preferentially induced IFN-γ, whereas 10-fold higher concentrations induced both IFN-γ and IL-4 production (19). The specific signals generated by different ligand/TCR interactions and their associated costimulatory signals, in particular through CD28 (39, 40), may primarily determine T cell cytokine responses. These signals are modulated by changes in affinity but not necessarily with only one outcome.

Exposure of BuT1.2A2 to $PLA_{81–92}$ or PLA-F82A, in the absence of APC, rendered the clone functionally inactive on immunogenic restimulation. Production of all Th2 cytokines measured

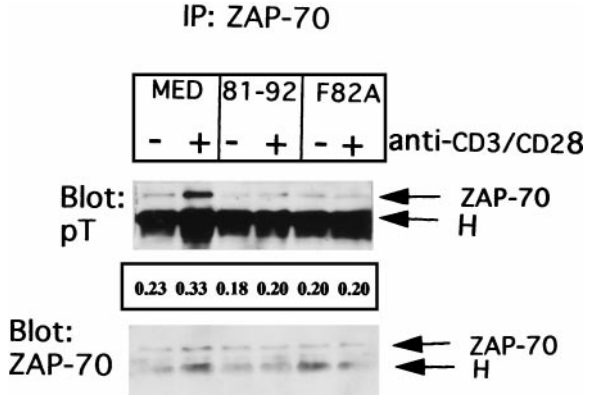

FIGURE 8. Abrogation of ZAP-70 tyrosine phosphorylation in anergic Th0 cells. BuT1.2A2 (2×10^6/ml) was untreated (MED) or exposed to $PLA_{81–92}$ or F82A (60 μM) for 16 h, washed, and incubated for a further 48 h. Cells were then restimulated with a combination of cross-linked mAb to CD3 (10 μg/ml) and soluble mAb to CD28 (1 μg/ml) for 15 min and lysed. Lysates were precleared, then immunoprecipitated with mAb to ZAP-70, and immune complexes were recovered, then resolved on 8–16% SDS gradient gels, immunoblotted with mAb to ptyr, and the densitometry was performed. Blots were then stripped and reblotted with mAb to ZAP-70.

was abrogated and that of IL-4 ablated. We have previously reported that IL-4 production by cloned PLA-specific Th2 cells could also be ablated (10). This indicates that absence of costimulatory signals may alter patterns of Th2 cytokine production by polarised as well as differentiating T cells (reviewed in Ref. 41). Specific inhibition of IL-4 production was observed after exposure to the partial agonist, PLA-F82A, in the presence of APC. Loss of proliferative capacity after exposure to a partial agonist in the presence of APC has been reported (21), but this is the first evidence in human T cells that an APL can partially uncouple IL-4 from IFN-γ production on immunogenic restimulation.

The effect of exposure of BuT1.2A2 to $PLA_{81–92}$ or PLA-F82A in the absence of professional APC was to induce both IFN-γ and Th2 cytokine production. However, secretion of IL-2 was absent. Visually, the cells appeared highly activated, forming large clusters 6–8 h after stimulation (data not shown), indicating presentation of the peptide between the T cells. The induction of anergy may be due to lack of IL-2 that has also been observed in murine models of anergy (41, 42). High levels of IL-10 were secreted on exposure to the peptides that in the absence of IL-2 may also have contributed to the anergic phenotype (43, 44).

We have previously observed that abrogation of IL-4 and IL-13 production by anergic PLA-specific Th2 cells correlated with alterations in transmembrane signaling through the TCR (11). The induction of anergy in cloned PLA-specific Th0 cells was associated with similar changes. Cloned T cells, exposed to either $PLA_{81–92}$ or the APL, PLA-F82A, did not phosphorylate ZAP-70 on restimulation with mAb to CD3/CD28. Activation of ZAP-70 is dependent on the CD4-associated tyrosine kinase, $p56^{lck}$ (45) that has also been shown to regulate the costimulatory receptor, CD28 (46). CD28 was expressed at similar levels on both normal and anergic Th0 cells (data not shown). Therefore, altered signaling through both the TCR and CD28 may contribute to anergic responses of cloned PLA-specific T cells. Although the capacity of PLA-F82A and $PLA_{81–92}$ to induce anergy in Th0 cells was equivalent, it was significant that IL-4 production could be reduced by the APL in comparison with the native peptide. This may be of advantage when considering peptide immunotherapy in allergic patients. Some patients developed postinjection symptoms after treatment with Fel d 1 peptides (8), and this may be due to mediator release after Th2 cytokine production. Recently, we have used a mixture of three major T cell epitope-containing peptides for specific immunotherapy of bee-sting allergic patients (9). Accordingly, it was interesting to see that the APL-induced change in cytokine composition with suppressed IL-4, substantially increased IgG4 in stimulated cell cultures containing the specific T cell clone. IgE could not be generated in measurable amounts in this system because the respective individual was not allergic. This is in accordance with the previous finding that the production of specific IgG4 by memory B cells depends on IFN-γ, the cytokine that suppresses IgE, and that the ratio of specific IgE:IgG4 in vitro directly correlates with IL-4:IFN-γ ratio (33, 47).

In conclusion, an APL of a dominant T cell epitope of the major bee venom allergen has been shown to reduce IL-4 production by cloned allergen-specific Th0 cells and to induce anergy in the cells such, that IL-4, IL-5, and IL-13 production were abrogated on immunogenic restimulation. The responses of anergic T cells were correlated with alterations in transmembrane signaling through the TCR, as demonstrated by ablated ZAP-70 tyrosine phosphorylation. Uncoupling of IL-4 from IFN-γ production changed the isotype production in favour of IgG4. These results indicate that the application of APL, mutated at sensitive sites, altering the secreted-T cell cytokine pattern, may improve the efficacy in specific immunotherapy, either alone, in combination with native peptide or as a component of a recombinant protein.

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