

Peptide-Induced Anergy in Allergen-Specific Human Th2 Cells Results in Lack of Cytokine Production and B Cell Help for IgE Synthesis

Reversal by IL-2, not by IL-4 or IL-13

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The induction of anergy in T cells is believed to be the result of triggering of the TCR in the absence of adequate costimulation mediated through the interaction of CD28 and its ligands, CD80 and CD86. Here, we demonstrate that stimulation of human group I allergen in *Dermatophagoides pteronyssinus* extract (Der p 1)-specific CD4⁺ Th2-like T cell clones with Der p 1-derived peptides in the absence of professional APC results in a state of nonresponsiveness. The induction of anergy occurred despite the expression of high levels of CD28, CD80, and CD86 on the surface of the T cell clones and was not prevented by the addition of anti-CD28 mAb. The anergic, Der p 1-specific, Th2 cells failed to mobilize calcium from intracellular stores, to proliferate, and to produce IL-2, IL-4, IL-13, GM-CSF, and TNF- α following optimal stimulation with Der p 1-derived peptide and autologous APC. However, they mobilized intracellular calcium following stimulation with Ca²⁺-ionophore and produced all of the above cytokines, including IFN- γ , when stimulated with phorbol ester and Ca²⁺ ionophore. These results indicate that the anergic T cell clones are capable of responding to signals circumventing the TCR/CD3 complex activation pathway. In contrast to T cell clones optimally activated with peptide and APC, anergic T cells failed to induce IgG4 and IgE synthesis when cocultured with B cells, even in the presence of exogenous IL-4 or IL-13. Anergic T cells expressed normal levels of CD40L, suggesting that their inability to help in Ig production by B cells is due to conditions other than a lack of expression of this molecule. Finally, exogenous IL-2 restored the helper function of anergic Th2 T cells for IgE production by B cells, which was greatly enhanced by the addition of IL-4 or IL-13. These data suggest that induction of anergy in allergen-specific Th2 T cells by allergen-derived peptides may play an important role in the successful desensitization of allergic patients. *The Journal of Immunology*, 1995, 155: 4199–4206.

Most human T cell clones specific for various allergens generated from peripheral blood or skin biopsies of atopic patients have been shown to belong to the Th2-like subset (1–4). Cocultivation of activated Th2 T cell clones with purified B cells results in IgE production, which is partially blocked by neutralizing anti-IL-4 mAb, indicating that IL-4 production by these T cell clones plays a major role in the induction of IgE synthesis in vitro (5–7).

Studies using mouse models have indicated that IL-4 is also responsible for IgE production in vivo. Administration of neutralizing anti-IL-4 mAb to mice blocked the production of IgE in vivo as a result of infection with *Nippostrongylus braziliensis* (8),

whereas mice in which the IL-4 gene was disrupted failed to produce IgE following infection with parasites (8, 9). Also in humans, there is strong, albeit indirect, evidence that allergen-specific IL-4-producing T cells play a critical role in the induction and maintenance of IgE synthesis in vivo (10, 11).

Recently, it was reported that another T cell-derived cytokine, IL-13, also induces naive human B cells to switch to IgE-producing cells, independently of IL-4 (12). Although the relative contributions of IL-4 and IL-13 to the induction of human IgE synthesis in vivo remain to be determined, it is likely that prevention or inhibition of IL-4 and IL-13 production by allergen-specific CD4⁺ T cell clones may provide an efficient way to block allergen-induced IgE synthesis and IgE-mediated allergic diseases.

Early studies using human and mouse T cell clones showed that interaction of the TCR with Ag in the absence of costimulation did not result in activation of the cells, but rather led to a state of nonresponsiveness or anergy during which the cells no longer proliferate or produce IL-2 following stimulation with Ag and APC (13, 14). In murine Th0 and Th1 clones, these costimulatory signals, which are required for optimal activation and IL-2 production, have been shown to be partially mediated via the interaction of CD28 with CD80 (reviewed in Ref. 15) and possibly with CD86 (16, 17). Murine Th2 cells, on the other hand, produce IL-4, which acts as an autocrine growth factor, in the absence of costimulatory signals. However, they require costimulation via APC to induce responsiveness to IL-4 (18). Therefore, it has been suggested that CD28 also acts as a costimulatory signal for Th2 cells, most likely

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by inducing the endogenous production of IL-1 which, in an autocrine manner, induces responsiveness to IL-4. Although all three murine Th subsets can be tolerized *in vitro* (14, 19–21), it seems that Th2 cells are less susceptible to tolerance induction (14, 19). The resistance of mouse Th2 clones to tolerance induction was found in one study to be associated with the presence of IL-4 (22). Recently, however, it has been shown that murine Th2 cells too can be rendered anergic by incubation with altered peptides (21).

In the present study, we determined whether human Der p 1-specific⁴ Th2 T cell clones could be rendered functionally nonresponsive to subsequent antigenic stimulation. It is shown that T cell clones incubated for 16 h with peptides representing two different T cell activation-inducing epitopes on Der p 1 become unresponsive to further stimulation via the TCR/CD3 complex. These anergic Th2 clones failed to produce cytokines and to proliferate in response to stimulation by Der p 1 and APC. In addition, they failed to provide B cell help for the synthesis of IgM, IgG, and IgE, even in the presence of exogenous IL-4 or IL-13, whereas the B cell helper function of these Th2 cells was restored by the addition of IL-2.

Materials and Methods

T cell clones and reagents

The T cell clones NP-12 and NP-44, which are specific for the Tyr⁹⁴-Arg¹⁰⁴ epitope of Der p 1, the major allergen in house dust and restricted HLA-DR1101, have been described previously (3). The Der p 1-specific T cell clone AR-142, which is reactive with the Der p 1-derived peptide Ala¹⁷¹-Gly¹⁸² in the context of HLA-DR1501, was generated from the PBMC of an atopic patient (S. Fasler, H. Yassel, unpublished observations). All Der p 1-specific T cell clones have a Th2-like cytokine production profile, as characterized by the high levels of IL-4 and IL-5 and low levels of IFN- γ production following Ag-specific activation. Optimal T cell proliferation was observed at concentrations of 1 μ g/ml for each peptide and T cell clone at an EBV-LCL/T cell ratio of 2:1. T cell clones (2×10^5 cells/ml) were cultured in 24-well Linbro plates (Flow Laboratories, McLean, VA) by stimulation every 2 wk with a feeder cell mixture consisting of irradiated (4000 rad) allogeneic PBMC (10^6 cells/ml), irradiated (5000 rad) autologous EBV-LCL (10^5 cells/ml), and purified PHA (0.1 μ g/ml; Wellcome Diagnostics, Dartford, UK), as described previously (22). Three or four days after each restimulation with feeder cells, the cultures were further expanded in culture medium containing 20 U/ml rIL-2 (a kind gift from Dr. G. Zurawski, DNAX Research Institute). All cloned T cell lines and the EBV-LCL NPR and ATR, used as autologous APC for the NP and AR T cell clones, respectively, were free of mycoplasma and were cultured in a modification of Iscove's medium, described by Yssel et al. (23), supplemented with 1% human serum (Gemini, Calabasas, CA). All experiments were conducted in this culture medium unless indicated otherwise. HLA-matched monocytes (generously provided by Drs. C. Figdor and R. Huybens, Netherlands Cancer Institute, Amsterdam, The Netherlands) were isolated from the peripheral blood of healthy donors by centrifugal elutriation, as described previously (24). Monocyte preparations were >95% pure, as judged by nonspecific esterase staining and immunofluorometric analysis. Recombinant Der p 1 was produced in *Escherichia coli*, after expression of genomic DNA encoding Der p 1 (kindly provided by Dr. W. Thomas, Princess Margaret Hospital, Perth, Australia), as a glutathione transferase fusion protein in a pGEX-2T expression vector, followed by purification of the *E. coli* homogenate by affinity chromatography on glutathione-Sepharose 4B (Pharmacia, Uppsala, Sweden) and treatment of the beads with thrombin, as previously described (3). Der p 1 peptides, representing minimal T cell activation-inducing epitopes, were synthesized on an Advanced Chemtech 350 Synthesizer (Louisville, KY) using 9-fluorenylmethyloxycarbonyl-chemistry, according to the manufacturer's instructions. The peptides were characterized by laser desorption mass spectrometry and peptide sequencing or quantitative amino acid analysis and had a purity of about 90%.

Induction of anergy

One million T cell clones were incubated with different concentrations of specific synthetic peptides in the absence of APC in 24-well Linbro plates in a final volume of 1 ml. T cell clones, used as a negative control, were incubated for the same period of time with culture medium or the nonrelevant peptide Ser⁴⁵-Ser⁶⁷. In experiments in which optimally activated T cells were used, 10^6 T cell clones were stimulated with 2×10^5 purified HLA-matched monocytes/ml or 10^6 autologous EBV-LCL that had been pulsed for 24 h with 1 μ g/ml of specific Der p 1 or specific peptide. After 16 h of incubation, the cells were washed, and supernatants were collected for cytokine analysis. After two additional washes with culture medium, the cells were used in various assays.

mAb, immunofluorescence, and flow cytometry

Methods of immunofluorescence staining, flow cytometry, and data analysis were described by Lanier and Recktenwald (25). Flow cytometry was performed using a FACScan (Becton Dickinson Co., San Jose, CA). The mAb used in this study were anti-CD2, anti-CD3 ϵ , anti-CD4, and anti-CD25 mAb (Becton Dickinson); anti-CD80 mAb L307, anti-CD86 mAb IT.2 (16), and anti-CD28 mAb L293 (26) (kind gifts from Drs. L. Lanier, DNAX, and M. Azuma, Dental University, Tokyo, Japan); and anti-CD40L mAb LL48 (a kind gift from Dr. J. Banchereau, Schering-Plough Research Laboratories, Dardilly, France).

Proliferation assay

Cellular proliferation in response to Ag-specific stimulation of the T cells was measured using an [³H]TdR assay, as previously described (27). In brief, 10^5 T cells were mixed with 2×10^4 irradiated HLA-matched monocytes or 2×10^5 autologous EBV-LCL, which had been pulsed overnight with native Ag or with synthetic peptide (1 μ g/ml), in a flat bottom 96-well tissue culture plate (Flow) in a total volume of 200 μ l. After 72 h of incubation at 37°C, 1 μ Ci (37 kBq) of [³H]TdR (Amersham International, Arlington Heights, IL) was added to each well. Four hours later, the cells were harvested onto glass fiber strips, and the amount of incorporated [³H]TdR was measured by liquid scintillation counting. The results are expressed as the mean of triplicate cultures $\pm$ the SD.

Cytokine production assays

One million T cell clones per ml were cocultured with 2×10^6 autologous Ag-pulsed EBV-LCL or with the combination of the phorbol ester TPA (1 ng/ml) and the Ca²⁺ ionophore A23187 (500 ng/ml; Calbiochem, La Jolla, CA) in 24-well Linbro tissue culture plates. After 48 h of activation, supernatants were harvested, spun at 250 $\times$ g to remove any remaining cells, aliquoted, and stored at -80°C before testing. Cytokine levels in the supernatants of activated T cells were determined by ELISA, using cytokine-specific Ab for human IL-4 (28), IFN- γ (29), TNF- α (30), IL-13 (31), and GM-CSF (30), as previously described (30). The sensitivity of all assays was about 50 pg/ml.

B cell isolation and B-T cell cocultures for Ig isotype production

Highly purified B lymphocytes (>99% CD20⁺) were isolated from normal spleens, obtained from patients undergoing splenectomy due to trauma, by negative FACS sorting, as previously described (32). Ten thousand anergic or activated T cell clones were cocultured with 10^4 highly purified spleen B cells in the presence or the absence of exogenous cytokines in 96-well round-bottom plates (Flow) in Yssel's medium supplemented with 10% FCS and 10 mg/ml ultrapure transferrin (Pierce Chemical Co., Rockford, NY) in a final volume of 200 μ l. Fourteen days later, the supernatants from triplicate cultures were harvested, pooled, and analyzed for Ig isotype content by ELISA, as described previously (5). The sensitivities of the ELISA, determined with a calibrated standard from Behring (Marburg, Germany), was 0.2 ng/ml.

Determination of intracellular free calcium levels

The concentration of [Ca²⁺]_i was measured according to the calibration method of Chused et al. (33), as described previously (34).

Results

Characterization of Der p 1-specific T helper clones

The three T cell clones used in this study produced high levels of IL-4, IL-5, IL-10, and IL-13 and low levels of IL-2 and IFN- γ

⁴ Abbreviations used in this paper: Der p 1, group I allergen in *Dermatophagoides pteronyssinus* extracts; EBV-LCL, EBV-transformed lymphoblastoid B cell lines; CD40L, CD40 ligand; [Ca²⁺]_i, intracellular free calcium.

Table I. Cytokine production profile of the Der p 1-specific Th2 T cell clones

T Cell Clone	Stimulation	Cytokine Production ^a (ng/ml)			
		IL-4	IL-5	IL-13	IFN- γ
NP-12	Tyr(94)-Arg(104)	25.8	10.7	4.4	1.4
	TPA/A23187	30.0	13.4	4.1	>50
NP-44	Tyr(94)-Arg(104)	>50	>50	3.7	<0.05
	TPA/A23187	>50	>50	5.0	25.5
AR-142	Ala(171)-Gly(182)	28.5	10.6	4.0	0.8
	TPA/A23187	35.6	15.5	3.9	0.8

^a One million T cell clones/ml were stimulated with 1 μ g/ml peptide in the presence of 2×10^6 autologous EBV-LCL. Cytokine production was measured after 48 h of incubation by cytokine-specific ELISA.

Table II. Induction of IgE and IgG4 synthesis by supernatants from the Ag/APC-activated Th2 clone NP-44^a

Culture Conditions	IgE (ng/ml)	IgG4 (ng/ml)
B Cells only	<0.2	<1
B + α CD40	<0.2	<1
B + α CD40 + NP44 s/n	40 $\pm$ 3	56 $\pm$ 26
+ Anti-IL-4	3 $\pm$ 0	<1
+ Anti-IL-13	13 $\pm$ 1	6 $\pm$ 1
+ Anti-IL-4 + anti-IL-13	5 $\pm$ 1	<1
B + α CD40 + IL-4	200 $\pm$ 22	76 $\pm$ 22
B + α CD40 + IL-4 + NP44 s/n	368 $\pm$ 16	245 $\pm$ 41
B + α CD40 + IL-13	4 $\pm$ 0	4 $\pm$ 2
B + α CD40 + IL-13 + NP44 s/n	92 $\pm$ 4	75 $\pm$ 2

^a 2.5×10^4 highly purified (>99%) CD20⁺ FACS-sorted B cells were cultured in the presence of the anti-CD40 mAb 89 (1 μ g/ml), 10% culture-supernatant (s/n) from T cell clone NP-44, stimulated for 16 h with peptide Tyr⁹⁴-Arg¹⁰⁴ (1 μ g/ml) and irradiated autologous monocytes, and cytokines (100 μ g/ml) or neutralizing mAbs (10 μ g/ml), as indicated. The cultures were performed in six replicates. Fourteen days later, supernatants from each of the replicates were harvested, pooled, and assayed for Ig isotype content by ELISA. The values are expressed as the mean and standard deviation of triplicate ELISA measurements and from four independent experiments.

following activation with their specific antigenic peptides, presented by autologous EBV-LCL, indicating that these allergen-specific T cell clones belong to the Th2-like subset (Table I). Addition of culture supernatants of these T cell clones, activated with peptide and APC for 24 h, to highly purified B cells that were costimulated with anti-CD40 mAb resulted in the induction of high levels of IgE and IgG4. The synthesis of these Ig isotypes induced in this culture system was partially blocked by the addition of a neutralizing anti-IL-13 mAb, whereas the presence of a neutralizing anti-IL-4 mAb almost completely inhibited IgE and IgG4 production by the B cells (Table II). The combination of the two mAb was as effective as the anti-IL-4 mAb alone, indicating that endogenous IL-4 production by these Der p 1-specific T cell clones is the main cytokine driving IgE production in this culture system.

Induction of nonresponsiveness in Der p 1-specific T cell clones

Incubation of the three Der p 1-specific T cell clones for 16 h with the relevant Der p 1-derived peptides at a concentration of 50 μ g/ml in the absence of professional APC resulted in a state of anergy or nonresponsiveness. The anergic T cell clones failed to proliferate in response to stimulation with optimal (1 μ g/ml or higher) concentrations of Der p 1 or specific Der p 1-derived peptide, presented by autologous EBV-LCL. In contrast, the same T cell clones that had been preincubated with the irrelevant Der p 1-derived control peptide Ser⁴⁵-Ser⁶⁷ responded normally to sub-

Table III. Induction of anergy in Der p 1-specific Th2 clones

T Cell Clone	Stimulation with Ag/APC ^a			Stimulation with rIL-2	
	Preincubation (μ g/ml)			Preincubation (μ g/ml)	
	0	1	50	0	50
	{ ³ H}TdR Incorporation (cpm $\times 10^{-3}$)				
NP-12	21.1	13.3	3.3	3.1	21.0
NP-44	38.2	15.5	2.7	44.3	58.2
AR-142	25.3	12.4	1.8	4.7	8.3

^a T cell clones were preincubated for 16 h with their specific peptide (see Table I), washed, cultured for 3 days in medium without growth factors. Two $\times 10^4$ T cells were subsequently stimulated with 1 μ g/ml of Der p 1, presented on 4×10^4 autologous EBV-LCL or with 100 U/ml rIL-2 and [³H]TdR was measured after 72 h.

sequent Ag-specific activation (Table III). Significant reductions in proliferative responses were observed when the Th2 cells had been preincubated with peptide concentrations as low as 1 μ g/ml, whereas the cells generally became completely nonresponsive after preincubation with peptides at a concentration of 50 μ g/ml. All anergic T cell clones proliferated strongly in response to exogenous rIL-2 (100 U/ml) to levels generally higher than those of the nonanergic control cells, indicating that the anergic cells were viable and had not lost their capacity to proliferate in response to signals circumventing TCR triggering.

Cytokine production by anergic T cell clones

Next, we analyzed the cytokine production profile of anergic Der p 1-specific T cell clones. It is important to note that stimulation of resting T cell clones with antigenic peptide alone resulted in the production of high levels of IL-4, IL-5, IL-13, GM-CSF, and TNF- α , as well as low levels of IFN- γ (Data not shown), indicating that during anergy induction the cells went through a state of activation. To rule out the possibility of measuring ongoing cytokine production, anergic cells were kept in culture medium for 3 days before restimulation. Preincubation of T cell clones NP-12, AR-142 (Table IV), and NP-44 (not shown) with increasing concentrations of specific peptide before restimulation with peptide and autologous EBV-LCL resulted in a dose-dependent reduction of the production of all cytokines. In particular, the production of IFN- γ , IL-4, and TNF- α was strongly inhibited following preincubation of these cells with specific peptide, whereas the production of IL-13 and GM-CSF, notably in T cell clone AR-142, was less affected.

Although nonresponsive to Ag-specific stimulation, activation of the anergic Th2 clones NP-12, AR-142 (Table IV), and NP-44 (not shown) by TPA and Ca²⁺ ionophore resulted in the production of IL-4, IL-13, GM-CSF, TNF- α , and IFN- γ . These results again indicate that anergic Th2 clones are able to respond to activation signals that circumvent TCR triggering.

Phenotype of anergic T cell clones

The expression of various surface molecules on the anergic Der p 1-specific T cell clones was analyzed by flow cytometry. The expression of CD25 (not shown) was enhanced on the anergic T cell clones, which was consistent with the observation that these cells proliferated in response to exogenous rIL-2. Furthermore, no differences in CD2, CD4, CD28, CD80, and CD86 expression were observed between the anergic T cell clones and their nonanergic counterparts (not shown). Anergic NP-44 cells expressed CD40L at levels comparable to those expressed by NP-44 cells stimulated

Table IV. Reduced cytokine production by Der p 1-specific T cell clones after preincubation with specific peptides^a

T Cell Clone	Pre-Incubation $\mu\text{g/ml}$	Cytokine Production (ng/ml) After Restimulation with									
		Ag/APC					TPA/A23187				
		IFN- γ	IL-4	IL-13	GM-CSF	TNF- α	IFN- γ	IL-4	IL-13	GM-CSF	TNF- α
NP12	0	1.4	30.1	4.4	8.9	2.8	>50	30.2	4.1	8.5	>50
	1	0.3	0.8	1.6	2.4	0.2	>50	12.0	3.5	9.1	10.3
	10	0.2	0.1	1.5	2.1	0.2	>50	11.3	4.9	9.1	20.2
	50	0.2	<0.1	0.9	1.4	0.3	>50	3.0	4.6	10.2	17.1
AR142	0	2.6	>50	6.1	9.0	9.4	22.7	49.0	3.9	7.7	15.0
	1	0.5	14.8	4.6	7.2	1.1	20.6	33.0	4.7	8.2	15.8
	10	0.4	5.7	4.0	7.6	1.0	>50	11.5	4.0	8.7	13.7
	50	0.5	0.3	4.2	6.2	1.0	>50	11.0	4.5	8.9	13.6

^a T cell clones NP-12 and AR-142 were preincubated for 16 h with the indicated concentrations of Tyr⁹⁴-Arg¹⁰⁴ or Ala¹⁷¹-Gly¹⁸², respectively, in the absence of APC. The cells were then washed, incubated for three days in medium, and restimulated with the same peptide and autologous APC (APC/T cell ratio, 2:1) or with TPA (1 ng/ml) and A23187 (500 ng/ml). The cytokine production was measured on 10⁶ T cells after 48 h by cytokine specific ELISA.

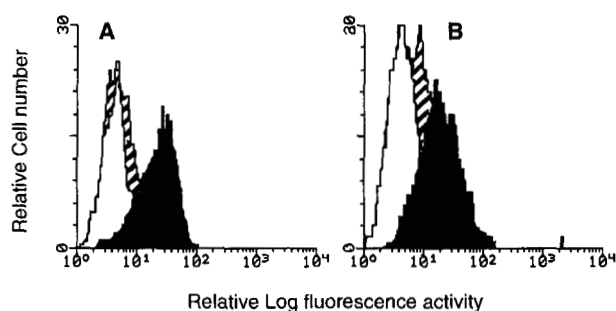


FIGURE 1. Expression of CD40L on a Der p 1-specific T cell clone, following induction of nonresponsiveness. NP-44 T cells, stimulated for 16 h with 1 $\mu\text{g/ml}$ of the synthetic peptide Tyr⁹⁴-Arg¹⁰⁴ in the presence of autologous EBV-LCL (A) or stimulated with 50 $\mu\text{g/ml}$ Tyr⁹⁴-Arg¹⁰⁴ (B) were stained with the anti-CD40L mAb LL48 (striped histograms) and analyzed by flow cytometry. Ag/APC-stimulated cells or anergic cells, incubated for an additional 6 h with TPA (1 ng/ml) and A23187 (500 ng/ml), were stained with LL48 (black histograms) or control mAb (open histograms). The x- and y-axes represent fluorescence (4-decade log scale) and relative cell number, respectively.

with peptide in the presence of autologous APC (Fig. 1). In addition, incubation of anergic and peptide/APC-stimulated T cell clones with TPA and the Ca²⁺ ionophore A23187 for an additional 6 h increased CD40L expression to similar levels, indicating that anergic T cells have no intrinsic defects in the up-regulation of this costimulatory molecule.

Incubation of the T cell clones with increasing concentrations of their specific peptides in the absence of APC resulted in a dose-dependent down-regulation of the TCR/CD3 complex. The expression of CD3 on T cell clone NP-44 was reduced about 10-fold after 16 h of preincubation with 50 $\mu\text{g/ml}$ of Tyr⁹⁴-Arg¹⁰⁴ compared with its expression on untreated NP-44 cells (Fig. 2). Similarly, expression of TCR V β 8 and V β 13 on T cell clones NP-12 and NP-44, respectively, was strongly down-regulated under these conditions (data not shown). However, stimulation of NP-44 cells with 1 $\mu\text{g/ml}$ of Tyr⁹⁴-Arg¹⁰⁴ in the presence of autologous EBV-LCL, which induces optimal proliferative responses, also resulted in a down-regulation of the TCR/CD3 complex. The average decreases in TCR/CD3 complex expression, compared with that of resting T cell clones were 6.4 ± 3.5 - and 5.3 ± 3.2 -fold for anergic and peptide/APC-stimulated NP-44 cells, respectively. The TCR/CD3 expression on peptide/APC-stimulated T cell clones was restored to normal levels generally after 24 to 48 h. On the other hand, the period of reduced TCR/CD3 expression varied among

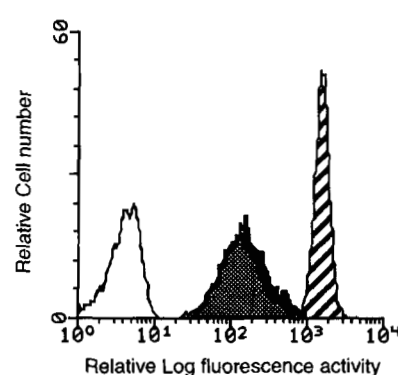


FIGURE 2. Expression of TCR/CD3 complex on a Der p 1-specific T cell clone following induction of nonresponsiveness. T cell clone NP-44, incubated for 16 h in medium alone (striped histograms), rendered anergic with 50 $\mu\text{g/ml}$ of Tyr⁹⁴-Arg¹⁰⁴ (black histogram), or activated with 1 $\mu\text{g/ml}$ of the synthetic peptide Tyr⁹⁴-Arg¹⁰⁴ in the presence of HLA-matched monocytes (gray histogram), was analyzed by flow cytometry for CD3 ϵ expression using the FITC-conjugated anti-CD3 mAb Leu-4a. In addition, cells were stained with FITC-conjugated control mAb (open histograms). The x- and y-axes represent fluorescence (4-decade log scale) and relative cell number, respectively.

the anergic T cell clones. The expression of TCR/CD3 on the anergic T cell clones NP-44 and AR-142 was reduced for up to 2 wk, which was the maximal period that these T cell clones could be maintained in culture in the absence of feeder cells, whereas anergic NP-12 cells re-expressed normal levels of the TCR/CD3 complex after 48 h.

Down-regulation of the TCR/CD3 complex is not associated with nonresponsiveness

Triggering of T cells through the TCR results in a rapid increase in [Ca²⁺]_i levels. To determine whether the decreased proliferative responses and cytokine production observed in the anergic T cell clones were due to impaired signaling through the TCR/CD3 complex or to its reduced expression, levels of [Ca²⁺]_i were measured after different modes of activation. Stimulation of resting NP-44 cells with autologous EBV-LCL that had been pulsed with Tyr⁹⁴-Arg¹⁰⁴ for 24 h resulted in a rise of [Ca²⁺]_i to levels that were maximal after 2.5 min of incubation (Fig. 3A). Similarly, stimulation of NP-44 cells with cross-linked anti-CD3 mAb induced [Ca²⁺]_i fluxes in these cells. In contrast, anergic NP-44 cells did not show a significant increase in levels of [Ca²⁺]_i following stimulation with peptide-pulsed APC or cross-linked anti-CD3 mAb.

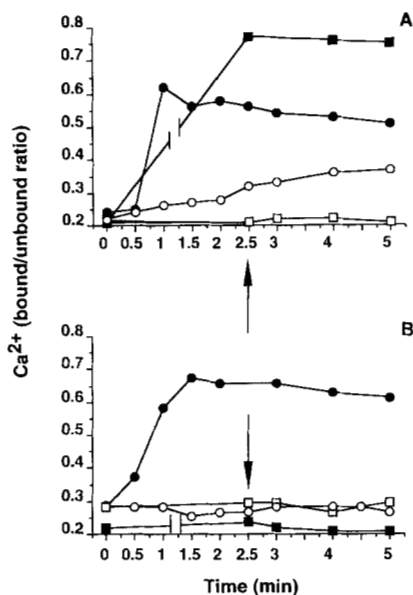


FIGURE 3. Mobilization of intracellular calcium by a Der p 1-specific T cell clone following induction of nonresponsiveness. Levels of $[Ca^{2+}]_i$ in control NP-44 cells (A) and NP-44 cells rendered anergic (B) following incubation with 50 μ g/ml of peptide Tyr⁹⁴-Arg¹⁰⁴ were determined after stimulation with equal amounts of the autologous EBV-LCL NPR ($\square$), with NPR cells pulsed with Tyr⁹⁴-Arg¹⁰⁴ for 24 h ($\blacksquare$), with 10 μ g/ml of the anti-CD3 mAb SPV-T3b ($\circ$), or with 500 ng/ml of the calcium ionophore A23187 ($\bullet$). NP-44 cells and EBV-LCL were centrifuged for 2.5 min to initiate contact, and $[Ca^{2+}]_i$ measurements were started at the time point indicated by the arrow.

Activation of resting and anergic NP-44 cells with the Ca^{2+} ionophore A23187 resulted in the induction of comparable $[Ca^{2+}]_i$ levels (Fig. 3B). These results indicate that signal transduction pathways downstream of TCR activation were perturbed in the anergic NP-44 cells, whereas signaling pathways that circumvent triggering of the TCR/CD3 complex were intact. The nonresponsiveness of these anergic cells to Ag/APC stimulation was not associated with down-regulation of the TCR/CD3 expression, since anergic NP-12 cells that could not be induced to mobilize $[Ca^{2+}]_i$ expressed the TCR/CD3 complex at almost normal levels after 3 days of culture (Fig. 4A). Moreover, despite differences in TCR/CD3 complex expression on the anergic Th2 clones (Fig. 4B), their proliferative responses were equally impaired upon stimulation with specific peptide and APC.

Anergic Th2 clones fail to provide help to B cells for IgE and IgG4 synthesis: restoration by IL-2

Following the observation that anergic Th2 cells expressed detectable levels of CD40L, we tested whether these cells could help B cells with Ig synthesis. As shown in Table V, anergic T cell clones failed to induce the synthesis of IgE and IgG4 when cocultured with highly purified B cells, even in the presence of exogenous rIL-4 or rIL-13. Surprisingly, in this culture system, peptide/APC-activated T cell clones were also unable to induce the synthesis of IgE and IgG4 by B cells, although the addition of rIL-4 or rIL-13 resulted in the production of significant levels of these isotypes. The lack of induction of IgE and IgG4 synthesis by activated NP-44 and AR-142 cells in the absence of exogenous cytokines is probably due to the transient production of IL-4 during T cell activation, which was removed when the T cells were washed before coculture with B cells. Indeed, addition of culture supernatants of peptide/APC-activated NP-44 cells, obtained 16 h after

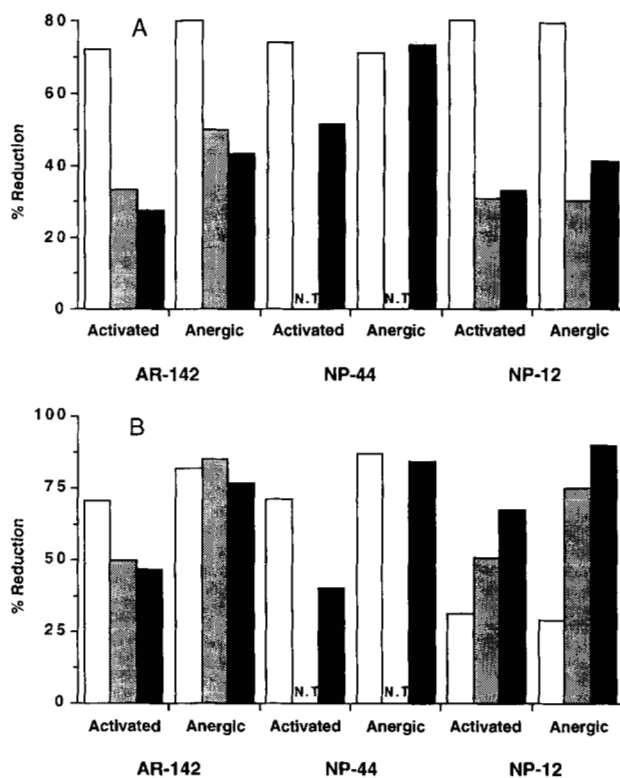


FIGURE 4. CD3 expression and proliferative responses of Der p 1-specific T cell clones following induction of nonresponsiveness. T cell clones AR-142, NP-44, and NP-12 were incubated for 16 h with autologous EBV-LCL pulsed with peptides Ala¹⁷¹-Gly¹⁸² or Tyr⁹⁴-Arg¹⁰⁴, respectively (Activated), with the same peptides in the absence of APC (Anergic), or with medium alone (Resting). The cells were then washed, and on days 1 ($\square$), 3 ($\blacksquare$), and 6 ($\blacksquare$), the expression of CD3 was analyzed by flow cytometry using the anti-CD3 ϵ mAb Leu-4a (A), or the T cell clones were restimulated with their specific peptides in the presence of autologous EBV-LCL and the proliferation was measured 3 days later by [³H]TdR incorporation (B). Results are expressed as the percent inhibition of the values of resting cells. N.T., not tested.

activation, resulted in the induction of high levels of both isotypes in the T-B cell coculture system (Table V). These results show that CD40L expression on anergic T cell clones, at levels comparable to those on their peptide/APC-stimulated counterparts, seems to be insufficient for B cell activation, Ig switching, and Ig production and suggest that additional stimuli are required for productive T-B cell interaction.

Since exogenous rIL-2 was found to induce proliferation of anergic Th2 T cell clones (as shown in Table III), we investigated the capacity of rIL-2 to restore the impaired helper activity of these cells. Addition of rIL-2 to cultures of B cells and anergic NP-44 or AR-142 cells, respectively, resulted in the synthesis of significant levels of IgE and IgG4 (Table V), which were strongly enhanced when combinations of exogenous rIL-2 and rIL-4 and, to a lesser extent, rIL-2 and rIL-13 were added to the cultures. Moreover, levels of IgG4 and IgE production induced by costimulation of B cells by anergic T cells in the presence of both rIL-2 and rIL-4 or rIL-2 and rIL-13 were much higher than those induced by the peptide/APC-activated T cell clones under the same conditions. These data indicate that rIL-2, but not rIL-4 or rIL-13, can restore the helper activity of anergic T cell clones and that IL-4 as well as IL-13 synergize with IL-2 in this culture system in the induction of IgE.

Table V. Restoration of helper T cell function of anergic T cell clones by IL-2

T Cell Clone NP-44 Culture Conditions	Ig (ng/ml) ^a		T Cell Clone AR-142 Culture Conditions	Ig (ng/ml)	
	IgE	IgG4		IgE	IgG4
B cells ^b	<0.2	2 ± 0	B cells	<0.2	2 ± 0
B + NP44 (peptide/APC-activated)	<0.2	2 ± 2	B + AR142 (peptide/APC-activated)	<0.2	2 ± 2
+ IL-4 ^c	47 ± 7	9 ± 1	+ IL-4	35 ± 5	23 ± 5
+ IL-13	5 ± 1	3 ± 1	+ IL-13	8 ± 3	18 ± 2
+ IL-2	6 ± 1	37 ± 13	+ IL-2	13 ± 1	476 ± 62
+ IL-4 + IL-2	52 ± 10	185 ± 42	+ IL-4 + IL-2	74 ± 4	331 ± 38
+ IL-13 + IL-2	9 ± 0	50 ± 13	+ IL-13 + IL-2	55 ± 5	388 ± 32
+ Supernatant ^d	86 ± 5	193 ± 9			
B + NP44 (anergic)	<0.2	3 ± 2	B + AR142 (anergic)	<0.2	<0.2
+ IL-4	1 ± 0	3 ± 0	+ IL-4	<0.2	<0.2
+ IL-13	<0.2	1 ± 0	+ IL-13	<0.2	<0.2
+ IL-2	15 ± 3	73 ± 9	+ IL-2	18 ± 0	41 ± 4
+ IL-4 + IL-2	107 ± 17	725 ± 78	+ IL-4 + IL-2	149 ± 8	391 ± 71
+ IL-13 + IL-2	26 ± 1	623 ± 112	+ IL-13 + IL-2	51 ± 5	107 ± 8

^a Ig isotype content was measured in supernatants at day 14 of culture by ELISA. The values are expressed as the mean and standard deviation of triplicate ELISA measurements and are from three independent experiments.

^b 10⁴ highly purified (>99% CD20⁺) FACS-sorted B cells were co-cultured with Der p 1-specific T cell clones NP-44 (A) or AR-142 (B) T cells which had been stimulated with Ag (activated) or rendered nonresponsive (anergic), as described in *Materials and Methods*.

^c IL-4 (100 U/ml), IL-13 (100 U/ml), IL-2 (100 U/ml).

^d Culture supernatant (10%) obtained from NP-44 cells, stimulated overnight with the peptide Tyr⁹⁴-Arg¹⁰⁴ in the presence of autologous monocytes.

Discussion

Induction of T cell effector function generally requires two sets of signals: TCR ligation by antigenic peptide-MHC complexes and noncognate costimulatory signals, mediated mainly by interactions between CD28 on T cells (35) and their ligands CD80 (36) and CD86 (16) expressed on APC. Synergy between the two signals has been postulated at both the transcriptional and the post-transcriptional level. Indeed, engagement of the TCR in the absence of CD28-mediated signals leads to a nonresponsive state in T cells as a result of rapid degradation of IL-2 transcripts and lack of IL-2 production (reviewed in Ref. 37).

Human T cell clones, due to the constitutive expression of MHC class II molecules, can present peptide Ag to the TCR of the appropriate specificity, resulting in either activation or nonresponsiveness (13, 38). It is demonstrated here that incubation of Der p 1-specific Th2 clones with Der p 1-derived peptides in the absence of professional APC induced a state of anergy during which the cells no longer proliferated in response to restimulation with specific Ag. In addition, the anergic T cell clones failed to produce cytokines or to help with IgG4 and IgE synthesis by B cells, a situation that could be reversed by the addition of exogenous IL-2, but not IL-4 or IL-13.

Most human T cell clones express functional CD80 (39, 40) and CD86 (16) (H. Yssel, unpublished observations) molecules on their surface that are able to trigger CD28-mediated signal transduction events in these cells. It is clear from our study that human T cell clones become anergic following incubation with relevant peptides, regardless of the presence of CD28, CD80, or CD86 on their surface. Addition of a mAb directed at CD28 could not prevent the induction of anergy (data not shown). These results suggest that despite the expression of functional CD80 and CD86 on their surface, T cell clones are not capable of providing the costimulatory signal(s) necessary for T cell activation.

In all Th2 T cell clones used in our study, the induction of nonresponsiveness was accompanied by a 10- to 20-fold decreased expression of the TCR/CD3 complex. Down-regulation of the TCR/CD3 complex *in vitro* has been documented previously on human T cell clones that had been rendered unresponsive by stimulation with influenza virus peptide (41). Furthermore, in a transgenic mouse model, stimulation of autoreactive T cells with au-

toantigen resulted in a dose-dependent down-regulation of the TCR (42). The latter study suggested that tolerance at the single cell level may be the result of multiple interactions of the TCR/CD3 complex with Ag, with each interaction resulting in a more profound state of anergy, and furthermore indicated that down-regulation of the TCR could be a mechanism by which peripheral tolerance can occur *in vivo*.

Based on our results, however, down-regulation of the TCR does not seem to be a hallmark of anergic T cells per se, since this phenomenon was also observed to the same extent in T cell clones that were optimally stimulated with Der p 1 and APC (Fig. 1). More importantly, however, decreased expression of the TCR/CD3 complex alone cannot account for the inability of the anergic T cells to respond to specific Ag, since the down-regulation of the TCR/CD3 complex on anergic as well as peptide/APC-stimulated T cell clones was only partial. In addition, TCR/CD3 expression by one of the T cell clones returned to normal levels after culturing the cells for an additional 3 days, while these cells were still unresponsive to Ag-specific stimulation. Finally, stimulation of anergic Th2 cells with Der p 1 or with immobilized anti-CD3 mAb did not result in the mobilization of [Ca²⁺], regardless of the expression of the TCR/CD3 complex. In contrast, elevated [Ca²⁺], levels were readily demonstrated after activation with TPA and calcium ionophore. This mode of activation, which circumvents TCR triggering, also resulted in normal levels of cytokine production by the anergic Th2 cells. Comparable results have been described by LaSalle et al., who showed that anergic myelin basic protein-specific T cell clones could proliferate and express cytokine mRNA following stimulation with phorbol ester and ionomycin (43). Taken together, our results indicate that the lack of responsiveness of anergic cells is likely to be due to impaired signaling downstream of the TCR/CD3 complex, rather than to a reduced number of TCR molecules on the cell surface. Moreover, the signaling defect in anergic cells seems to occur before protein kinase C activation and [Ca²⁺], release. A recent study by Sloan-Lancaster et al. (44) provided evidence for the latter idea by showing that anergy induced in mouse T cell clones was associated with altered phosphorylation of the TCR/CD3 complex ζ -chain and subsequent lack of association of the tyrosine kinase Zap70 with this complex.

The degree of anergy induced in the Th2 clones was found to be dependent on the amount of added peptide. Stimulation of the cells with increasing concentrations of peptide resulted in a dose-dependent inhibition of the production of all cytokines as well as the proliferative responses. However, we did not observe a differential effect on the various cytokines produced by these cells even at the highest concentration of peptide used. These data are in contrast with a recent report showing that induction of anergy in murine T cells in vitro resulted in a strongly decreased capacity to produce IL-2, but not of IL-4 and IL-5 (45). Likewise, it was reported that murine Th2 T cell clones, rendered anergic by incubation with altered peptides, no longer proliferated in an Ag-specific manner, but were still able to produce IL-4 (21), whereas human T cells obtained from SCID-hu mice that were rendered anergic following in vivo administration of *Staphylococcus enterotoxin B* failed to produce IL-2 and TNF- α , but were still able to produce IFN- γ , GM-CSF, and IL-10 (46). The reason for the discrepancy between the different experimental systems is not clear, but the latter results suggest that different types of T cell unresponsiveness may be achieved depending on the stimulation of the cells. The failure of anergic T cell to proliferate has been shown to be associated with the absence of IL-2 mRNA transcription, resulting in a lack of IL-2 production (47; reviewed in Ref. 37).

Switching of B cells to IgE production requires the presence of IL-4 or IL-13 (5, 12), as well as a T-B cell contact-mediated signal that is largely dependent on the productive interaction of CD40 with the CD40L, which is transiently expressed on activated T cells (48–50). Although anergic T cell clones expressed CD40L to the same extent as Ag/APC-stimulated control cells, they were unable to provide B cell help in the induction of IgE synthesis, even in the presence of exogenous IL-4 or IL-13. Previous studies have demonstrated that levels of CD40L expression on activated murine T cell clones did not correlate with their ability to induce B cell proliferation (51). This finding led to the proposal that productive T-B cell interactions are dependent on either post-translational modification of CD40L or association of CD40L with other surface molecules. It seems, therefore, that optimally activated Th2 clones may express additional adhesion and/or costimulatory molecules that are necessary for productive T-B cell interactions, whereas these putative molecule(s) are likely to be down-regulated on the anergic T cell clones.

The anergic T cells expressed functional CD25 and were highly responsive to the growth-promoting effects of exogenous rIL-2, which is in agreement with results obtained in most murine and human anergy models described to date (21, 43, 52, 53). Importantly, the results of this study demonstrate that the addition of IL-2, but not that of IL-4 and IL-13, not only reversed the T cell unresponsiveness, but also restored the capacity of anergic T cells to help with the production of Ig by B cells. Furthermore, addition of rIL-2 to the cultures induced low levels of IL-4, IL-5, and IL-13 production (data not shown), which together with the restored T cell helper activity probably account for the capacity of rIL-2 to induce low levels of IgE synthesis in the absence of exogenous IL-4 or IL-13.

At present, there is little information available about the ability of peptides to tolerize allergen-specific Th2 cell responses in vivo. In a recent study (54), it was shown that s.c. immunization of mice with two immunodominant peptides of the major cat allergen, Fel d 1, induced tolerance of the responding T cells, which failed to proliferate in response to a subsequent challenge with the intact recombinant Fel d 1. The lack of responsiveness in these animals was accompanied by a reduced production of IL-2, IL-4, and IFN- γ .

Based on the present information, it is tempting to speculate that induction of nonresponsiveness in allergen-specific Th2 cells, re-

sulting in down-regulation or prevention of the IgE-inducing cytokines IL-4 and IL-13 as well as in the failure to provide B cell help, may contribute to successful immunotherapy in allergic patients. Functional inactivation of allergen-specific Th2 cells by allergen-derived peptides may, therefore, provide a way to modulate immune responses associated with atopy. It should be noted, however, that the initial induction phase of T cell nonresponsiveness by allergen-derived peptides results in substantial production of IL-4, IL-5, and IL-13, which has to be taken into account when peptide vaccination is considered for the treatment of allergy.

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