

Effects of peptide therapy on ex vivo T-cell responses

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Background: Peptide therapy targets T cells directly with short peptides containing multiple T-cell receptor epitopes. Murine studies suggest T-cell anergy as the mechanism of action; however, changes in T-cell cytokine profiles may be more relevant in human beings.

Objective: We sought to study the effects of peptide therapy on ex vivo antigen-specific T-cell responses.

Methods: Antigen-specific T-cell lines were generated from subjects enrolled in a double-blind, placebo controlled, two-dose study of the ALLERVAX CAT therapeutic, containing Fel d 1 peptides (ImmuLogic Pharmaceutical Corp., Waltham, Mass.) ($n = 7, 8,$ and 7 , respectively, for groups receiving placebo, $75 \mu\text{g}$, or $750 \mu\text{g}$). Each subject had three lines propagated before and after receiving peptide therapy; antigens used were cat hair extract, Fel d 1 peptides, and tetanus toxoid (negative control). Proliferative responses and cytokine generation from each line were assessed after two restimulations with antigen and autologous antigen-presenting cells.

Results: The Fel d 1 peptide lines showed a dose-dependent decrease of IL-4 production ($p = 0.02$ and 0.025 , respectively, for the $750 \mu\text{g}$ group vs both the $75 \mu\text{g}$ and placebo groups). IL-4 production from the cat hair allergen extract lines and interferon- γ production from both the Fel d 1 peptide lines and cat hair allergen extract lines showed no statistically significant changes. The control tetanus toxoid lines showed no changes in cytokine production; there were no significant changes in proliferation with any of the antigens in any of the treatment groups. In the clinical arm of the trial, only the $750 \mu\text{g}$ dose of peptides produced a significant response. **Conclusions:** Peptide therapy induces a significant, dose-dependent decrease in peptide-stimulated IL-4 production, consistent with either a shift in T-cell phenotype or peptide-specific T-cell tolerance. (*J Allergy Clin Immunol* 1998;101:506-13.)

Key words: Immunotherapy, IL-4, T cell, allergen, peptide

Despite nearly a century of successful treatment of allergic disease with allergen immunotherapy (IT), the

Abbreviations used

APC:	Antigen-presenting cell
IFN:	Interferon
IT:	Immunotherapy
PBMC:	Peripheral blood mononuclear cell
PHA:	Phytohemagglutinin
TT:	Tetanus toxoid

precise molecular mechanisms underlying this therapeutic modality remain elusive.^{1,2} Changes in humoral immune function occurring during traditional IT include a decline in antigen-specific IgE levels and an increase in antigen-specific IgG levels.³⁻⁶ Changes in cellular immune function suggest diminished lymphocyte responsiveness and the induction of T-cell anergy.⁷⁻⁹ Recently, using mitogen- and/or monoclonal antibody-driven T-cell cultures, shifts in T-cell cytokine responses from a T_{H2} -like to a T_{H1} -like phenotype have been demonstrated during traditional IT.¹⁰⁻¹⁴ However, confirmatory studies with human antigen-driven T-cell lines have not been reported.

T-lymphocyte responses play a key role in the pathogenesis of atopic disease. T_{H2} cells are increased in allergic diseases,¹⁵⁻¹⁷ accumulate at sites of allergen challenge,¹⁸⁻²⁰ and correlate with disease symptoms.²¹ Furthermore, IL-4 and IL-5 produced by these cells can promote class switching to IgE and can stimulate the differentiation, survival, and chemotaxis of eosinophils.²² Thus the ability to downregulate a T_{H2} response could underlie the efficacy of IT. Although traditional IT uses whole allergens that may be recognized by IgE, peptide therapy uses small synthesized peptides corresponding to the T-cell epitopes. These peptides are administered in excess, and they are recognized specifically by the T-cell receptor in the context of major histocompatibility complex class II. Thus peptide therapy reduces the risk of IgE-mediated anaphylaxis and does not require the slow buildup of dose over months or years needed for traditional IT.²³ Although murine²⁴ and in vitro human T-cell line studies²⁵ have implicated T-cell anergy as the mechanism of action of peptide therapy, a previous ex vivo study in human subjects that used peripheral blood mononuclear cells (PBMCs) was inconclusive.^{26,27}

In this study we investigated the effects of peptide

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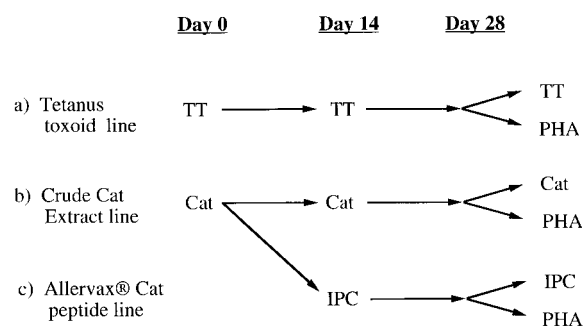


FIG. 1. Culture protocol for ex vivo T-cell lines. PBMCs were placed in culture on Day 0 with appropriate antigen. On day 14, cells were resuspended and restimulated in presence of autologous, irradiated PBMCs and antigen. On Day 28, the cells were resuspended and restimulated in presence of autologous, irradiated PBMCs. At this time, one half of the cells were stimulated with antigen and the other half with PHA.

therapy on T-cell proliferative responses and cytokine generation from antigen-specific, antigen-driven T-cell lines. These lines were generated from subjects before and after treatment with the ALLERVAX CAT therapeutic, a Fel d 1 peptide therapy product, in a phase 2 double-blind, placebo-controlled dose-ranging trial.

We demonstrate an apparent dose-dependent decrease in IL-4 generation from the peptide-driven T-cell lines during peptide therapy without changes in IFN- γ generation or the antigen-driven proliferative response.

METHODS

Clinical study design

The plan of this trial has been previously described and was approved by our Institutional Review Board.²⁸ Briefly, subjects allergic to cats were recruited for a multicenter, prospective, randomized, double-blind, placebo-controlled trial of the ALLERVAX CAT product. Subjects were without underlying medical illnesses, had received tetanus toxoid immunizations within the preceding 3 years, and took no medications for 1 month preceding the study. None of the subjects had undergone immunotherapy, and none received a tetanus booster immunization during the study. Three groups of subjects (placebo, 75 μ g, and 750 μ g) received injections biweekly for 4 weeks. Subjects returned for clinical reevaluation 6 weeks later, with skin test reactivity and cat room challenge scores for allergic rhinitis, asthma, or both as outcome parameters. Thirty subjects enrolled at our center for this trial, and 22 consented to participate in our ex vivo laboratory study. Blood was drawn immediately before the first injection of peptide for the prepeptide therapy T-cell lines and immediately before clinical reevaluation by cat room challenge for the postpeptide therapy T-cell lines.

Generation of T-cell lines

The isolation of PBMCs and generation of antigen-specific T-cell lines were performed as previously described.^{29,30} Briefly, PBMCs were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Inc., Piscataway, N.J.) and resuspended in RPMI (BioWhittaker, Walkersville, Md.) supplemented with 1% penicillin/streptomycin and 5% human AB serum (Gibco/BRL, Gaithersburg, Md.). Platelet contamination of these

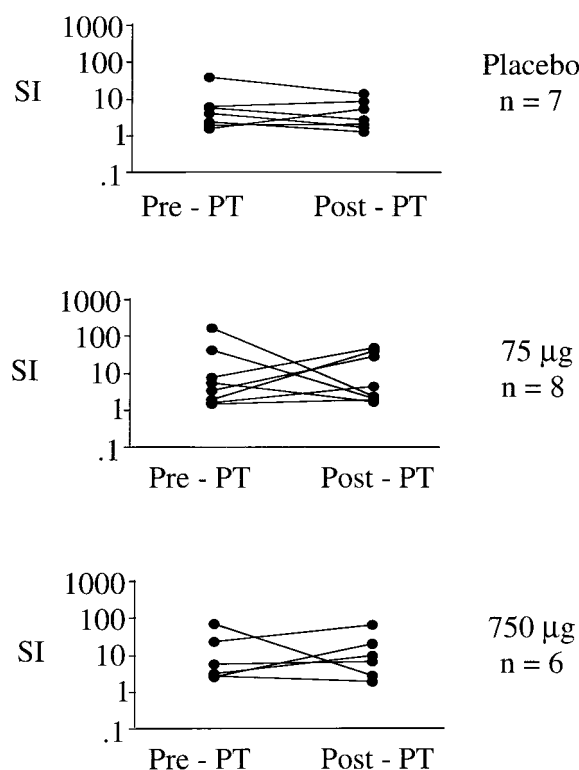


FIG. 2. Fel d 1 peptide-induced proliferation of ex vivo T-cell lines before and after peptide therapy. Day 28 T-cell lines were stimulated with autologous, irradiated PBMCs and antigen. Data are expressed as stimulation indices (cpm stimulated culture/cpm unstimulated culture) for each subject in each study group. PT, Peptide therapy.

preparations was less than 1%, and viability by trypan blue exclusion was uniformly greater than or equal to 99%. These cell preparations were either used directly with antigen in proliferation assays and to generate primary cultures, or they were irradiated for use as antigen-presenting cells (APCs). Each antigen-driven primary culture was maintained by successive, biweekly restimulation in the presence of antigen and autologous APCs at a T-cell:APC ratio of 1:5. The antigens used included standardized cat hair extract (a single lot of 5000 BAU/ml obtained from ALK Laboratories Inc., Milford, Conn., used at a concentration of 9 μ g/ml of Fel d 1 content), the ALLERVAX CAT product (IPC 1 and 2 [an overlapping set of 26 amino acid peptides from the sequence of the Fel d 1 chain 1],³⁰ a gift of ImmuLogic Corp., Waltham, Mass., used at a concentration of 10 μ g/ml for each peptide), and tetanus toxoid (TT) as an irrelevant control antigen (Connaught Laboratories, Swiftwater, Pa., used at a concentration of 0.1 lf/ml). The resulting antigen-specific T-cell lines were studied after two restimulations, because inadequate cell numbers for both proliferation and cytokine generation assays were obtained after the first 2 weeks of culture. The second 2-week culture overcame this problem, while the 2-week interval between restimulations allowed the cell lines to come fully to rest before the next restimulation. Fig. 1 depicts the various cell lines generated for each subject before and after peptide therapy. Primary culture with Fel d 1 peptides resulted in poor stimulation characteristics as assessed by proliferation and cytokine produc-

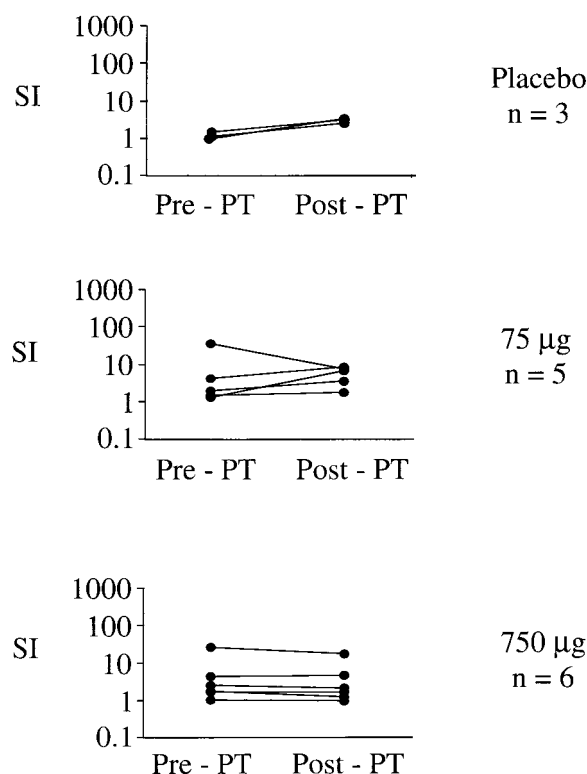


FIG. 3. Cat hair extract-induced proliferation of ex vivo T-cell lines before and after peptide therapy. Day 28 T-cell lines were stimulated with autologous, irradiated PBMCs and antigen. Data are expressed as stimulation indices (cpm stimulated culture/cpm unstimulated culture) for each subject in each study group. *PT*, Peptide therapy.

tion, most likely because of a small number of peptide-specific responder cells in peripheral blood. For this reason, this line was abandoned after the first 11 subjects. However, a primary culture with cat dander followed by restimulation at days 14 and 28 with peptides produced excellent stimulation characteristics. It is this line that possessed the antigen specificity most relevant to the treatment protocol and produced the most significant findings in this study. On day 28, all T-cell lines were divided and used in proliferation and cytokine secretion assays with either antigen or mitogen (phytohemagglutinin [PHA], 2 µg/ml; Sigma Chemical Co., St. Louis, Mo.) in the presence of APCs. Mitogen stimulation parameters allowed for normalization of T-cell responses with greater accuracy than enumeration of responder cells.

Proliferation assays

Proliferation assays were performed as previously described.^{31,32} Briefly, either 2×10^5 PBMCs/well or 2×10^4 oligoclonal T cells with 1.5×10^5 APCs (T-cell:APC ratio of 1:7.5) were cultured in the presence of antigen in 96-well flat-bottom plates. Antigen concentrations were optimized in preliminary studies (data not shown). Final concentrations were as follows: TT = 0.1 Lf/ml; cat dander extract = 0.9, 3, and 9 µg/ml of Fel d 1; and IPC = 3, 10, and 30 µg/ml of each peptide.

Where multiple concentrations were assessed, that which induced the maximal proliferative response from the prepep-

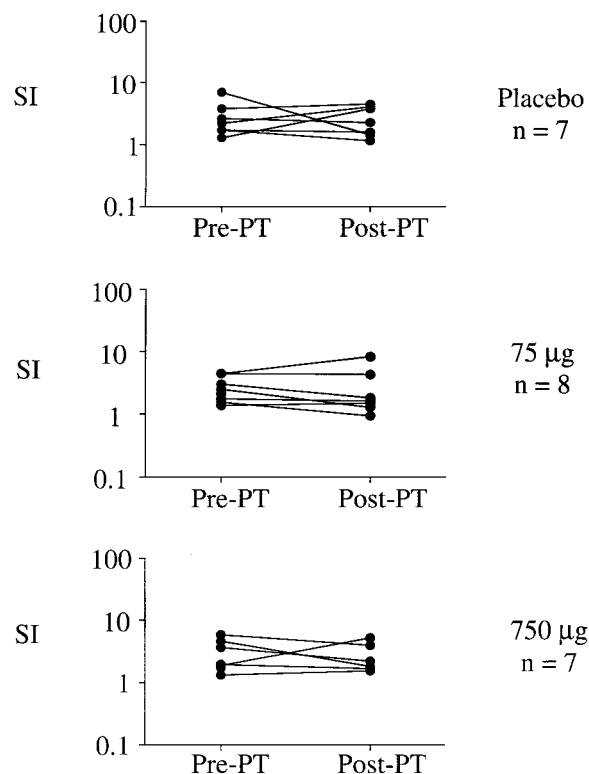


FIG. 4. Cat hair extract-induced proliferation of PBMCs before and after peptide therapy. Data are expressed as stimulation indices (cpm stimulated culture/cpm unstimulated culture) for each subject in each study group. *PT*, Peptide therapy.

tide therapy T-cell line was used for comparative analysis with the postpeptide therapy T-cell line; no significant shift in the antigen dose-response curve was evident with either peptide or cat dander after peptide therapy. No exogenous cytokine was used in these assays. All conditions were performed in triplicate, incubated (72 hours for cat extract; 96 hours for IPC and TT) at 37° C with 5% CO₂, pulsed with 1 µCi of tritiated thymidine for 20 hours, harvested onto glass filter paper on a multichannel harvester (Cambridge Technologies Inc., Watertown, Mass.), and counted on a liquid scintillation β-counter (Beckman Instruments Inc., Fullerton, Calif.).

Cytokine secretion assays

Cytokine protein secretion assays were performed as previously described.³¹ Briefly, 1×10^6 polyclonal cells with 5×10^6 autologous APCs (T cell:APC ratio of 1:5) were cultured in the presence of antigen or mitogen in polypropylene culture tubes. Supernatants from these cultures were collected after a 12-hour incubation. Cellular debris was removed by centrifugation, and the supernatants were stored at -20° C until assayed. Cytokine protein secretion was assessed by ELISA with Cytoscreen Immunoassay kits (Biosource, Inc., Camarillo, Calif.) according to the manufacturer's instructions and World Health Organization standards provided by the company. Dilutions of samples, when necessary, were performed in culture medium. All standards and samples were tested in duplicate. Most samples were used at two different dilutions and compared for internal consistency.

Statistical analysis

Mean and standard error values, as well as *t* test comparisons, were derived by using JMP (SAS Institute Inc.) on a Macintosh Classic computer. Probability (*p*) values are paired, two-tailed Wilcoxon signed-rank tests and two sample Wilcoxon rank-sum tests.

RESULTS

Proliferative responses

Figs. 2 and 3 depict antigen-driven proliferative responses assessed with day 28 T-cell lines before and after peptide therapy for individual subjects in each of the three treatment groups (placebo, 75 μ g, and 750 μ g). Fig. 2 depicts data for IPC peptide-driven cultures, and Fig. 3 depicts data for cat extract-driven cultures. The results for each T-cell line are expressed as a stimulation index (SI), defined as counts per minute of antigen-stimulated culture per counts per minute of unstimulated culture. There were no statistically significant changes with peptide therapy in the proliferative responses to cat extract, IPC peptide, or TT (data not shown) in any of the treatment groups (*p* > 0.2). Moreover, there were also no significant differences among the placebo, 75 μ g, and 750 μ g groups for any of the antigens tested (*p* > 0.2).

Antigen-driven proliferative responses were also assessed with fresh PBMCs isolated at Day 0 before and after peptide therapy. Figs. 4 and 5 depict these responses before and after peptide therapy for individual subjects in each of the three treatment groups for cat extract- and IPC peptide-driven cultures, respectively; TT data is not shown. Once again, there were no statistically significant changes with peptide therapy in the proliferative responses to any of the antigens in any of the treatment groups or among the three treatment groups for any of the antigens tested (*p* > 0.2). Finally, the absolute values of counts per minute from PBMC or T-cell line proliferation assays before and after peptide therapy were statistically unchanged (*p* > 0.2). These data argue against T-cell anergy as a mechanism for the efficacy of peptide therapy.

Cytokine responses

Figs. 6 and 7 depict antigen-driven cytokine secretion assessed with day 28 T-cell lines before and after peptide therapy for each of the three treatment groups (placebo, 75 μ g, and 750 μ g). Fig. 6 depicts data for IPC peptide- and cat extract-driven IL-4 production, and Fig. 7 depicts data for IPC peptide- and cat extract-driven IFN- γ production. To better control for the large variability in absolute cytokine secretion between specific T-cell lines from different individuals, antigen-driven cytokine production was normalized for each individual T-cell line by dividing the antigen-stimulated cytokine production by the PHA-stimulated cytokine production; data are presented as the change in this ratio with peptide therapy for each of the treatment groups (normalized ratio after treatment minus the normalized ratio before treatment). Primary (nonnormalized) values for

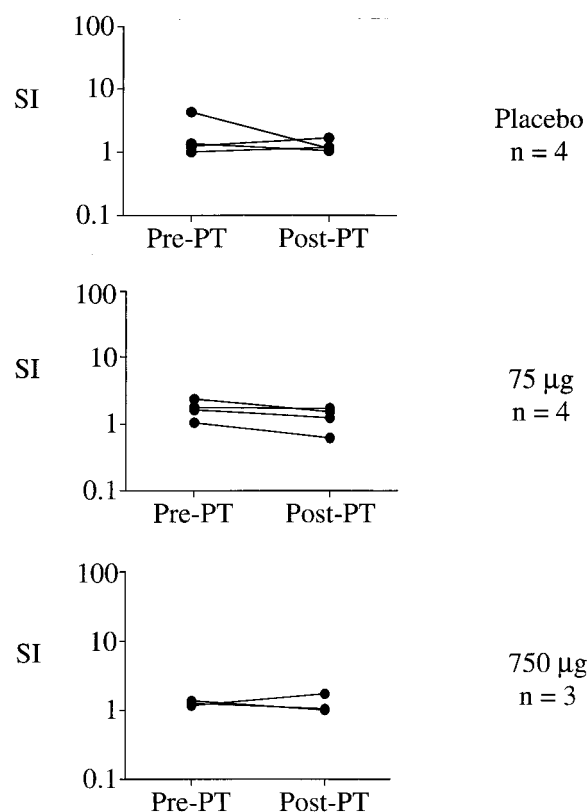


FIG. 5. Fel d 1 peptide-induced proliferation of PBMCs before and after peptide therapy. Data are expressed as stimulation indices (cpm stimulated culture/cpm unstimulated culture) for each subject in each study group. PT, Peptide therapy.

IL-4 and IFN- γ secretion before and after peptide therapy are shown as insets to the respective figures.

Fig. 6, *A* demonstrates a dose-dependent reduction in normalized IL-4 production from the IPC-driven T-cell lines with peptide therapy. The normalized IL-4 production in the 750 μ g group was significantly less than that produced in both the placebo (*p* < 0.025) and the 75 μ g (*p* < 0.02) groups. Although the 75 μ g group showed less IL-4 production than the placebo group, the difference was not statistically significant. Fig. 6, *B* depicts the changes in normalized IL-4 production by the cat extract-driven T-cell lines. Although a dose-dependent decrease in normalized IL-4 production with treatment is suggested, this decrease did not reach statistical significance. The TT-driven T-cell lines showed no changes in IL-4 production with peptide therapy in or between any of the treatment groups.

Fig. 7 depicts the changes in normalized IFN- γ production by the IPC-driven (*A*) and cat extract-driven (*B*) T-cell lines for each of the treatment groups. While dose-dependent decreases in normalized IFN- γ production with treatment are again suggested for both antigens, these decreases did not reach statistical significance, perhaps because of the small number of patients studied. Once again, the TT-driven T-cell lines showed

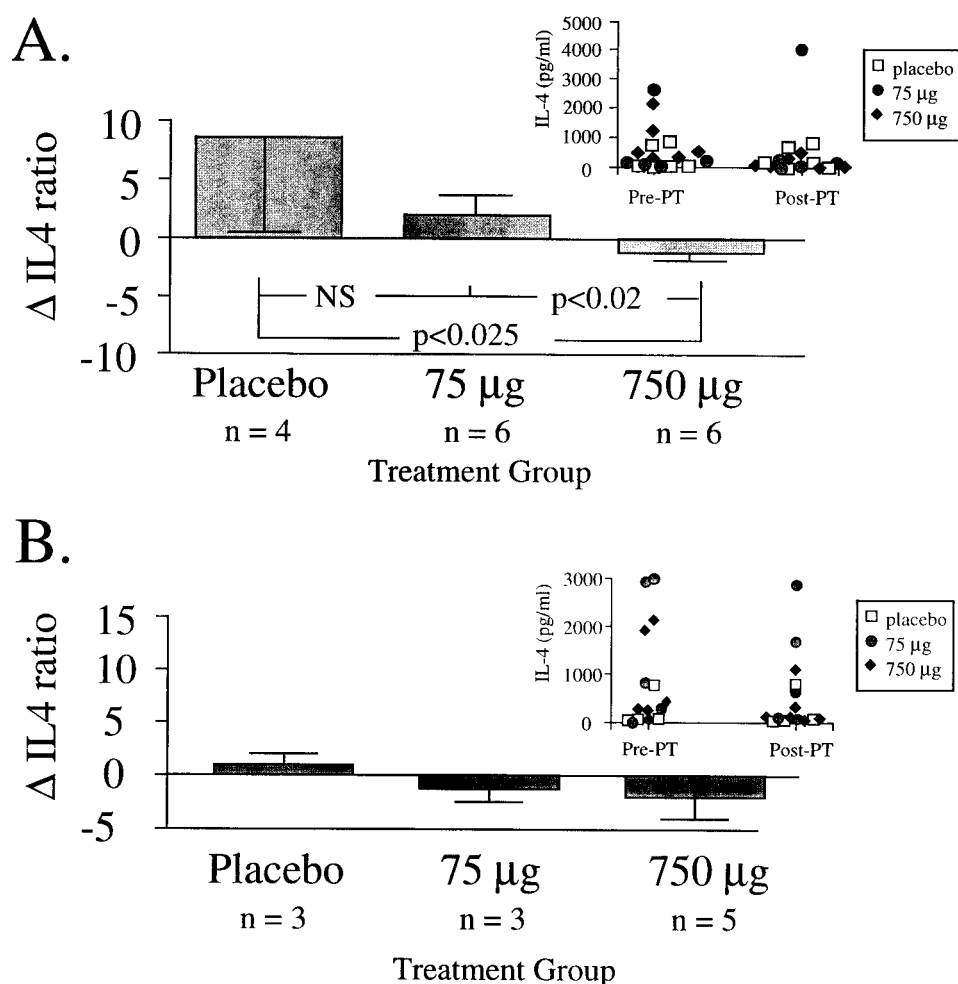


FIG. 6. Change in IL-4 production by ex vivo T-cell lines during peptide therapy. **A**, Day 28 FEL d 1 peptide-derived T-cell lines generated before and after peptide therapy were stimulated with either Fel d 1 peptides or PHA. Results were normalized by dividing peptide-driven IL-4 levels by PHA-driven IL-4 levels. Data are expressed as difference between the pre- and postpeptide therapy ratios for each study group. **B**, Data analogous to top panel with cat hair extract-derived T-cell lines. Primary (nonnormalized) values for antigen-stimulated IL-4 secretion before and after peptide therapy are shown as insets to respective figures. PT, Peptide therapy.

no changes in IFN- γ production with peptide therapy in or between any of the treatment groups.

DISCUSSION

We have investigated the effects of peptide therapy in human subjects on antigen-driven proliferative responses and cytokine production from ex vivo, nontransformed, antigen-specific T-cell lines. Although no differences in proliferative responses to relevant or control antigens before and after immunotherapy were evident in any of the treatment groups, a significant decrease in IL-4 generation after treatment was apparent in the 750 μ g treatment group. This decrease in IL-4 generation was restricted to the peptide-specific lines and corresponded to the dose of peptides that showed clinical efficacy. These data are consistent with, but not conclusive for, a phenotypic shift after peptide therapy. More-

over, aspects of these data argue against anergy as the mechanism of efficacy.

We developed this particular protocol using T-cell lines restimulated twice for the examination of ex vivo responses of antigen-specific T cells from patients undergoing peptide therapy for a number of reasons. First, direct use of PBMCs for study is not tenable, most likely because of the low frequency of responder cells in the periphery and the inability of those cells to establish a conducive microenvironment.²⁶ In fact, neither whole Fel d 1 nor the recombinant peptides were capable of inducing consistent proliferative responses in fresh PBMCs. Moreover, low levels of antigen-driven proliferation and cytokine gene expression from non-T cells in the PBMCs could potentially confound the findings. Second, in contrast to in vitro studies of clonal T-cell responses, ex vivo studies of polyclonal T-cell lines allow

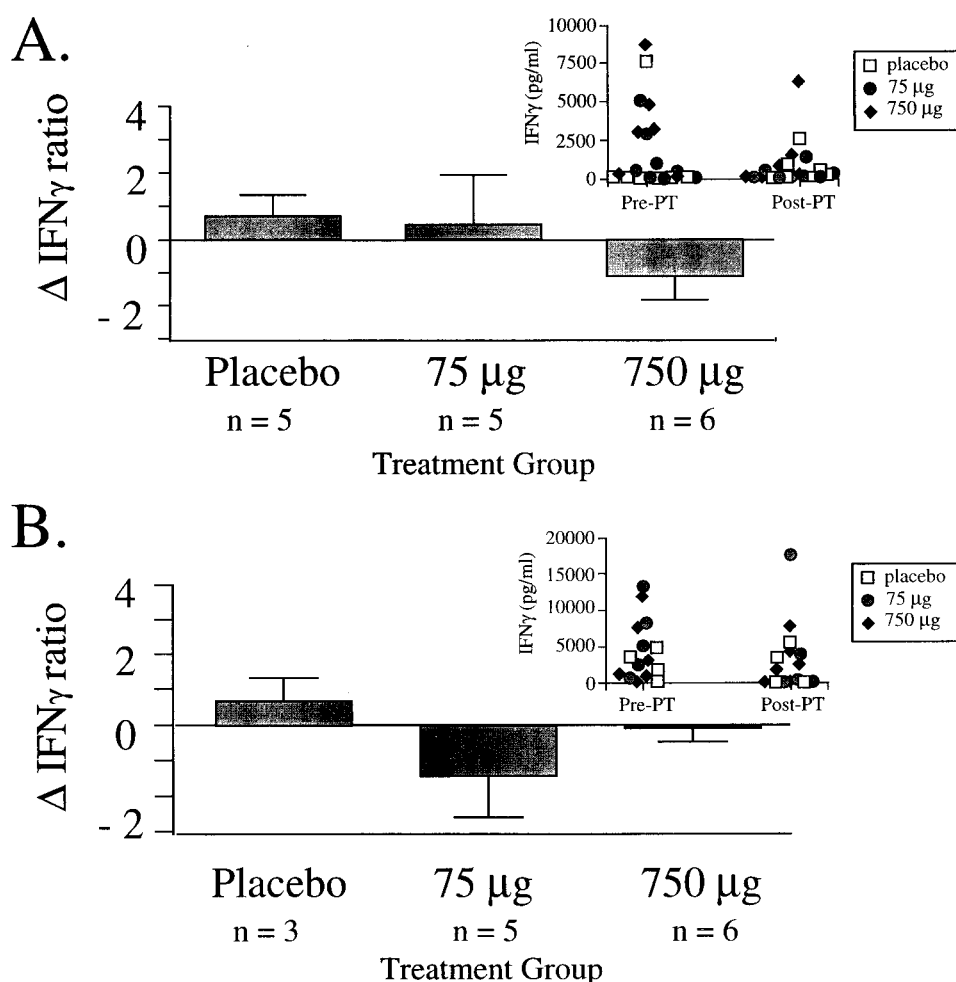


FIG. 7. Change in IFN- γ production by ex vivo T-cell lines during peptide therapy. **A.** Day 28 Fel d 1 peptide-derived T-cell lines generated before and after peptide therapy were stimulated with either Fel d 1 peptides or PHA. Results were normalized by dividing peptide-driven IFN- γ levels by PHA-driven IFN- γ levels. Data are expressed as difference between the pre- and postpeptide therapy ratios for each study group. **B.** Data analogous to top panel with cat hair extract-derived T-cell lines. Primary (nonnormalized) values for antigen-stimulated IFN- γ secretion before and after peptide therapy are shown as insets to respective figures. PT, Peptide therapy.

the measurement of antigen-specific responses while preserving much of the heterogeneity of the in vivo T-cell repertoire. Finally, antigen and autologous irradiated APCs used in these studies, as opposed to mitogens or plate-bound monoclonal antibodies, stimulate T-cell lines in a more physiologically relevant manner, preserving costimulatory signaling pathways.

Several technical issues must be considered in conjunction with this study. First, the precursor frequency of Fel d 1 peptide-specific T cells in the periphery is low, as evidenced by the inability of IPC 1 and 2 to induce consistent, significant proliferation of PBMCs. Even at optimal concentrations, the peptides were less effective stimuli than cat extract or TT. Thus an increased signal-to-noise ratio in all measurements likely made achieving statistical significance more difficult. Second, only a

single time point after peptide therapy was studied, and the kinetics of IL-4 and IFN- γ modulation with IT remain unknown. Jutel et al.¹¹ and Lack et al.¹⁴ studied their patients' T cells days after reaching maintenance, whereas Secrist et al.¹⁰ investigated after years of IT. The first two groups both found increases in IFN- γ production, and only Jutel et al. also found a decrease in IL-4; Secrist et al. demonstrated a decrease in IL-4 only. It is possible that there are distinct time courses for changes in individual cytokines after IT. Third, epitope spreading may be less efficient with peptide therapy than traditional IT, negatively affecting the generation of new T_{H1} clonal specificities. According to this hypothesis, traditional IT causes new, presumably T_{H1}, clones to develop to unusual epitopes of the whole antigen. Peptide-based IT would not contain these epitopes and

would not produce as efficient a phenotypic shift. Finally, the studies demonstrating anergy in the mouse were performed on T cells obtained from nodes and spleen, whereas our T cells were isolated from peripheral blood.²⁴

The decision to use PHA normalization for the cytokine assays was made prospectively on the basis of the observations that the absolute level of cytokine production varies widely between individuals and that by hemocytometer we are unable to quantitate very low cell numbers with sufficient precision and accuracy on sequential cultures to allow valid longitudinal comparisons. PHA stimulation provides a maximal stimulus for cytokine generation, whereas antigen stimulation provides a more selective stimulus that is sensitive to pharmacologic regulation.³² Thus use of the ratio would provide accurate normalization to actual cell number. Moreover, tolerized cells may be expected to produce cytokine under conditions of mitogen stimulation³³; this protocol would allow these cells to be recognized and this effect to be accounted for. Finally, use of the ratio allows the wide range of absolute cytokine values (ranging over nearly three orders of magnitude) to be normalized between individuals.

Murine and in vitro human studies suggest that the mechanism of peptide therapy is T-cell anergy,^{24, 25} whereas in vivo human studies suggest that the mechanism of traditional IT is a shift in T-cell cytokine production.¹⁰⁻¹⁴ However, one might expect different mechanisms of action for peptide therapy and traditional IT because of the differences in the dose, structure, and kinetics of the antigens administered. Briner et al.²⁴ sensitized mice to Fel d 1 antigen then tolerized them with the same ALLERVAX CAT peptides used in this study. The sensitized mice produced IL-2 on stimulation with either Fel d 1 or the peptides, but after tolerization with large doses of peptides, they failed to produce IL-2, IL-4, or IFN- γ . Interestingly, although the mice in the Briner study had been tolerized only with peptide fragments of Fel d 1, stimulation with the entire Fel d 1 molecule did not evoke a T-cell response. Fasler et al.²⁵ demonstrated that Der p 1-specific T-cell clones produced IL-4, IL-5, IL-13, IFN- γ , and TNF- α on stimulation with Der p 1 peptides but did not produce these cytokines on stimulation after tolerization to the peptides. Finally, Smilek et al.³⁴ showed prevention and even reversal of experimental autoimmune encephalomyelitis by using a 14 amino acid peptide with the substitution of only one amino acid from the myelin basic protein used to induce the autoimmune process.

Our hypothesis in this study was that peptide therapy in human beings should cause T-cell anergy as determined by cytokine production and proliferation of our ex vivo T-cell lines. Indeed, we demonstrated a significant decrease in IL-4 production without a change in IFN- γ production from the peptide-stimulated T-cell lines. In addition, epitope spreading was suggested because immunizing with only peptide fragments seemed to produce a dose-dependent attenuation of IL-4 secretion

even in the line stimulated with the cat hair extract. This effect was not generalized to other antigens, because IL-4 generation from the TT-stimulated T cell line was unaffected by peptide therapy. However, the data are not fully consistent with an anergic mechanism. There was no change in antigen-driven proliferation, which might be expected with T-cell anergy. It is possible that tolerized cells were selected out of the T-cell lines and that tolerized cells could have been detected as a decrement to the antigen-driven proliferative response in fresh PBMCs. As previously discussed, it was not possible to eliminate this confounder in this way. Normalization to mitogen-driven proliferation, as performed with the cytokine assays, might have been useful, but cell numbers became a limiting factor. In addition, there was no significant reduction in IFN- γ after treatment. Because it is known that T-cell anergy is reversible by IL-2, and our protocol required the addition of rhIL-2 to the cultures initially, anergy may have been difficult to demonstrate. However, the mounts of rhIL-2 used in this culture protocol are significantly below those required to reverse anergy. Moreover, the proliferation assays had no exogenous IL-2 added. Finally, as demonstrated previously,²⁷ IgE levels did not change.

In conclusion, we herein provide the first report from ex vivo studies that peptide therapy induces a significant, dose-dependent decrease in antigen-stimulated IL-4 production. While not definitive for either mechanism, our results are consistent with either a shift in T-cell phenotype or peptide-specific T-cell tolerance as measured 8 weeks after completion of therapy.

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