

Cur I 3, a Major Allergen of *Curvularia lunata*–Derived Short Synthetic Peptides, Shows Promise for Successful Immunotherapy

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Allergens with reduced IgE binding and intact T cell reactivity are required for safety and efficacy of immunotherapy (IT). *Curvularia lunata* is an important fungus for respiratory allergic disorders having cross-reactive and specific allergens. Previously, we have identified major allergens—namely, Cur I 1 (31 kD, serine protease), Cur I 2 (48 kD, enolase), and Cur I 3 (12 kD, cytochrome c)—from this fungus. Furthermore, Cur I 3 epitope-peptide, P6, showed immunogenicity and higher IgE binding, where cysteine and histidine were observed to be vital for IgE binding. Thus, this peptide and three derivatives with reduced IgE binding were selected for analysis in mice. In the present study, the effect of IT was assessed with Cur I 3, P6, its derivatives (P6.1–6.3), and P10 in a mouse model of allergy. IT with P6.2 and P10 reduced IgE and IgG1 levels significantly ($P < 0.05$), with increase in IgG2a levels as compared to other antigens. There was a significant reduction of IL-4 level associated with increased IFN- γ after IT. Airway inflammation was reduced significantly in terms of eosinophil counts in lung tissue and bronchoalveolar lavage fluid. IT with P6 and P6.2 induced significantly higher IL-10 secretion than baseline after 40 days of treatment. Generally, the effect of IT was more pronounced after 40 days than after 10 days of treatment. In summary, the modified peptide, P6.2, with reduced IgE binding, but intact immunogenicity, showed promise for successful IT.

Keywords: peptide immunotherapy; mice model; airway hyperreactivity; Cur I 3; *Curvularia lunata*

The prevalence of allergy and asthma has increased worldwide. It is an important area of public health concern in industrialized nations. Pharmacotherapy has improved in recent years, but gives symptomatic relief with side effects. Immunotherapy (IT) offers protection against allergen-induced sensitization/symptoms for long periods, even after the cessation of treatment (1). Specific IT induces allergen-specific IgG antibody, reduces recruitment of eosinophils, induces T cell anergy and a shift from T helper type (Th) 2 to Th1 response (1, 2). Recent studies suggest that IT against bee venom, house dust mite, and grass pollen was associated with increased synthesis of regulatory T cells inducing IL-10 secretion (3).

IT by subcutaneous injections with whole mass extracts can induce systemic reaction and/or asthmatic exacerbations in patients (4). The purified allergenic protein(s) may cause an adverse reaction due to cross-linking of IgE on mast cells and

basophils, thereby releasing the mediators. Short synthetic peptides derived from allergen with reduced or no IgE cross-linking capacity, but intact immunodominant T cell regions, have reduced the side effects associated with allergen IT. IT with Fel d 1 peptides administered subcutaneously showed improvement in nasal and lung symptom scores on exposure to cat allergen (5). Clinical studies using short peptides from Fel d 1 and phospholipase A2 have shown promising results, with reduction in Th2 cytokines and late phase reaction on challenge (6–8).

Curvularia lunata (CL) is an important fungal allergen affecting sensitization in 7–28% of patients with allergies worldwide (9, 10). A major allergen (Cur I 3, cytochrome c) of CL was cross-reactive among different fungi and grasses (11). Cytochrome c is demonstrated as an allergen in ragweed, Kentucky blue grass, rye grass, Bermuda grass, and dicotyledons, namely, *Plantago lanceolata* and *Echium plantagineum* (12–15). Previously, B and T cell epitopes of Cur I 3 (108 aa, AAK67492) were deciphered using a combination of bioinformatics and experimental methods (16). An important peptide, P6, with B and T cell epitope properties, may have potential for IT. Small peptides can inhibit IgE cross-linking, and may stimulate T cells that have advantage in IT. The present study was aimed at assessing the Cur I 3, peptide P6, its three mutants (P6.1, P6.2, and P6.3), and P10 for IT in a mouse model of allergy.

MATERIAL AND METHODS

Animals

Female Balb/c mice (4- to 5-wk old, weighing 20–22 g) were procured from NIN (Hyderabad, India) and housed in the animal care facility of the Institute of Genomics and Integrative Biology, Delhi under standard laboratory conditions. The mice were allowed *ad libitum* access to food and water, and were maintained on a 12:12-hour light:dark cycle. The temperature and humidity were maintained at $23 \pm 2^\circ\text{C}$ and $55 \pm 10\%$, respectively. Animals were allowed to acclimatize for 1 week before the start of the experiment. The study protocol was approved by the institutional animal ethics committee.

Chemical Modifications of Peptide P6 and Its Derivative Peptides for Animal Model Studies

The detailed methodology is provided in the online supplement. The peptides before and after modification were more than 95% pure as determined by HPLC (profile not shown).

Experimental Protocol

The IT and sensitization protocol was followed as per Figure 2. The detailed methodology is provided in the online supplement.

Immunoglobulin Analysis

Specific IgE, IgG1 and IgG2a were measured in serum samples of all the mice against respective antigen by indirect ELISA (11). The detailed methodology is provided in the online supplement.

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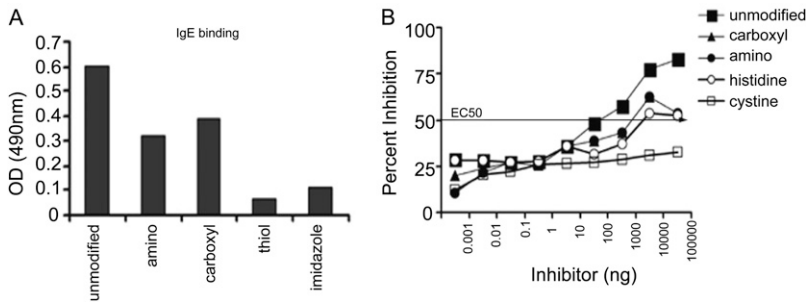


Figure 1. Effect of chemical modifications on P6 peptide. Side chains of different amino acids of P6 were modified using chemical reagents (trifluoroacetate [TFA] for amino group side chains, methanol for carboxyl group side chains, diethylpyrocarbonate for imidazole group, and Elman's reagent for sulphhydryl groups). IgE binding of these variants was measured by (A) ELISA where the chemically modified peptides were coated on the solid phase. (B) Inhibition of IgE binding of modified peptides was assessed by ELISA inhibition where these modified peptides were incubated with hypersensitive pooled patients' sera at different concentrations and processed as ELISA.

Lung Homogenate Preparation and Histopathology

Lung homogenate preparation and histopathology were performed as detailed in the methodology described in the online supplement.

Eosinophil Peroxidase Activity in Lung Homogenate

Eosinophil peroxidase (EPO) activity was measured in lung homogenate supernatant. The assay is based on the oxidation of ortho phenylenediamine by EPO in the presence of H_2O_2 . Briefly, 50 μ l lung homogenate (1:10 vol/vol) was added to wells in duplicate, as was a substrate solution of 3 mM ortho phenylenediamine, 0.05 M Tris (pH 8.0), 0.1% Triton X-100 (Sigma, St. Louis, MO), and 8.8 mM H_2O_2 (Sigma). To differentiate EPO activity from neutrophil and macrophage myeloperoxidases, activity in the absence or presence of the EPO-specific inhibitor, resorcinol (1.5 mg/ml; Sigma) was measured. The reaction was stopped after 30 minutes by addition of 50 μ l of sulfuric acid. The absorbance was measured at 490 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA).

Splenocyte Culture and Cytokine ELISA

The splenocyte isolation, culture, and cytokine analysis were performed as described in the online supplement.

Statistical Analysis

The data were analyzed using Microsoft Excel 2000 (Microsoft Corp., Redmond, WA), GraphPad Prism 5.00 and GraphPad InStat (GraphPad Software Inc., San Diego, CA). A paired t test was used to determine intragroup statistical differences of means between control and treated mice. The sample size for each parameter tested was a minimum of five mice. Probability of significance was assigned by nonparametric ANOVA using the Kruskal-Wallis test. Each parameter was compared in all the groups using Dunn's multiple comparison post test. $P < 0.01$ was considered significant in all cases.

RESULTS

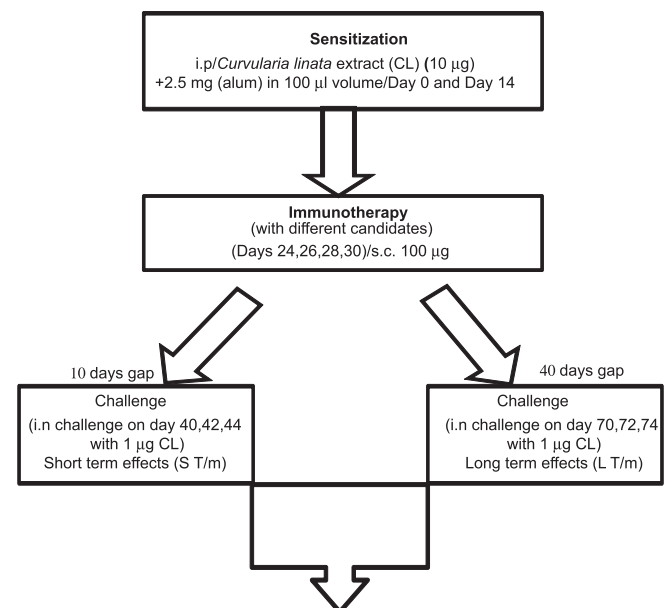
IgE Binding of Modified Derivatives of Peptide P6

Peptide P6 and its derivatives were assessed for IgE binding by ELISA. There was maximum reduction in IgE binding (72%) upon chemically modifying sulphhydryl group indicating the relevance of cysteine (Cys) residues (Figure 1A). It was followed by derivatives with modified imidazole (67%) and hydroxyl/carboxyl (27%). The derivatives with modification of amino group side chains demonstrated minimum reduction in IgE affinity (15%). The maximum IgE inhibition achieved was 32.8% at 100 μ g in Cys-modified P6, followed by histidine (His)-modified P6 (i.e., 37%) (Figure 1B). The chemical modification of the carboxyl group also had affected hydroxyl groups. Cys- and His-modified peptides showed maximum reduction in IgE binding. Thus, three derivatives of P6 were chemically synthesized, namely, P6.1 (Cys⁶ modified to Ala), P6.2 (Cys³ and Cys⁶ each modified to Ala), and P6.3 (His⁷ modified to Ala). The amino acid changes in P6 (TRCAQCHTLK) were (in bold):

P6.1, TRCAQAHTLK; P6.2, TRAAQAHTLK; and P6.3, TRCAQCATLK. Peptide P10 (GLFGRKTGSVA) was used as control.

Serum Antibodies

IT in mice was performed with different antigens, per the protocol described in Figure 2. Specific IgE, IgG1, and IgG2a levels were estimated by ELISA in sera of mice before and after IT with various antigens (Figure 3). Absorbance value of pre-immune serum for each antigen was subtracted from the respective group. Mice immunized and challenged with spore-mycelial extract of CL showed high specific IgE levels (mean optical density, 0.598 ± 0.05) before IT. Specific IgE levels reduced threefold as compared to PBS treated group after IT with CL extract. There was a significant reduction ($P < 0.001$) after 40 days of IT (Figure 3) than 10 days of treatment. IT with P6.2 reduced IgE levels significantly compared with preimmune



BALF and blood were collected from mice for cellular infiltration, EPO activity, antibody titers, cytokine levels and proliferation assays. Lungs were isolated for histopathological analysis

Figure 2. Experimental protocol. Allergic murine model was created by sensitizing mice with 10 μ g *Curvularia* extract with 2.5 mg alum intraperitoneally on Days 0 and 14. The mice were immunized intranasal on Days 17, 19, and 21. After 10 days, subcutaneous immunotherapy (IT) with different antigens was performed on Days 31, 33, 35, and 37. After this, mice from each group were divided into two subgroups: one subgroup each was intranasally challenged after a gap of 10 days after therapy, whereas the other subgroup was challenged 40 days after therapy. The mice from each subgroup were killed to collect bronchoalveolar lavage fluid (BALF), lungs, and blood.

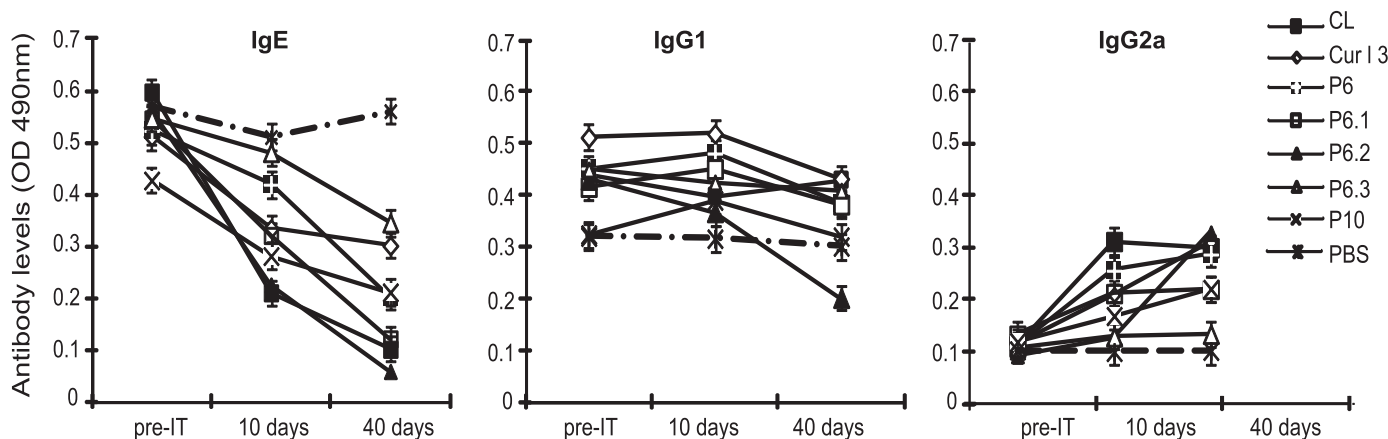


Figure 3. Effect of IT on antibody titers. Specific IgE, IgG1, and IgG2a levels were determined in serum both before and after IT. An ELISA plate coated with *C. lunata* (CL) extract was blocked with 3% defatted milk and (A) 1:10 diluted mouse sera were incubated with antigen. Biotinylated anti-IgE (2 μ g/ml) was used to detect IgE after incubation with streptavidin-peroxidase (1:1,000). (B) 1:50 diluted mouse sera were incubated with antigen and anti-IgG1 peroxidase (1:1,000) was used for detection of serum IgG1. (C) 1:50 diluted mouse sera were incubated with antigen and anti-IgG2a peroxidase (1:1,000) was used for detection of serum IgG2a. In all cases, reaction was developed using ortho phenylenediamine and H_2O_2 . The readings for preimmune sera were subtracted from antibody levels determined. Each reading represents the mean $\pm$ SD of each group resulting from at least two replicates of the experiment. The results with $P < 0.05$ were considered significant.

serum levels ($P < 0.001$). Cur 13, P6, P6.1, and P10 reduced specific IgE levels by twofold after IT as compared with PBS-treated mice.

IT with P6.2 showed a twofold reduction in IgG1 levels after 40 days of treatment. IgG2a levels increased significantly in mice after IT with P6.2 ($P < 0.001$), Cur 13, and CL extract ($P < 0.01$). The P10-treated group also showed a slight increase in IgG2a levels compared with PBS control (Figure 3). PBS-treated mice showed no significant change in IgG2a levels 40 days after IT.

Bronchoalveolar Lavage Fluid Analysis

Protein content in bronchoalveolar lavage fluid (BALF) was measured as nonspecific index of edema. Mice sensitized with ovalbumin showed the highest protein content in BALF, followed by the CL-sensitized group. The control group mice had significantly lower levels of protein content in BALF, showing the minimal extent of plasma exudation.

Before starting IT (pre-IT), a significant increase was recorded in total number of inflammatory cells in antigen-immunized groups compared with control group mice after intranasal challenge. IT with different antigens reduced infiltration of cells by twofold, except for P6.3 (Figure 4). P6.2 showed a threefold reduction in cellular infiltration and eosinophil count compared with control ($P < 0.005$) after 40 days of IT, whereas the PBS-immunized and -treated group showed minimal cellular infiltration. Differential cell count showed that major contributors to inflammatory cells (i.e., eosinophils) were reduced significantly 40 days after IT ($P < 0.001$) in the P6.2-group mice.

Splenocyte Proliferation

Splenocytes isolated from each group of mice were challenged with 1 μ g of CL or Cur 13 for T cell proliferation. Phytohemagglutinin-stimulated cells were taken as positive control, and PBS-stimulated cells were taken as negative control. CL- and Cur 13-stimulated splenocytes from all the groups showed significantly higher T cell proliferation compared with the PBS-treated group (Figure 5). T cell stimulation was reduced significantly ($P < 0.001$) in mice after IT, with P6.2 followed by P6.1 and P10 (Figure 5).

Cytokine Analysis

IL-4, IFN- γ , and IL-10 levels were determined in BALF and splenocyte culture supernatant on antigen challenge, before and after IT. IL-4 levels were higher after challenge with CL than other antigens before the IT (mean optical density, 158.62 ± 22 pg/ml). Ovalbumin-sensitized and -challenged mice also demonstrated high IL-4, whereas the PBS-group mice showed low levels. The cytokine levels and cellular infiltration were significantly higher in BALF after intranasal challenge. There was no significant reduction in IL-4 levels after 10 days of IT ($P = 0.104$). The level of IL-4 with P6.2 and P10 was reduced significantly, and was similar to control after 40 days of treatments (Figure 6). IFN- γ levels were low before IT owing to CL sensitization and challenge. The mice in the P6.2 and P10 IT groups showed significant increases in IFN- γ 40 days after IT (Figure 6).

T regulatory cytokine (IL-10) levels were also determined in culture supernatant. IL-10 level was significantly higher in mice

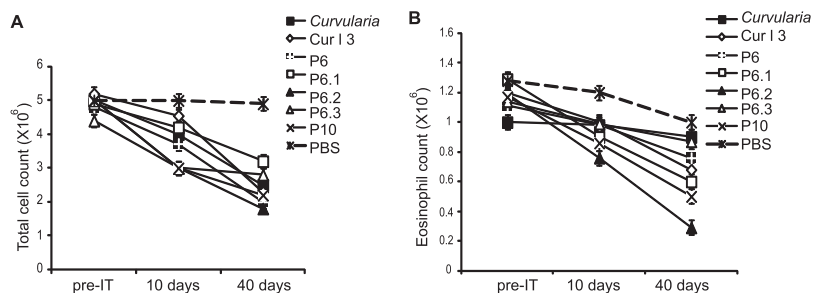


Figure 4. Cellular infiltration in BALF. Aliquots of BALF cell smear were fixed and stained with Leishman stain, and at least 200 cells were counted, analyzed with a hemocytometer under light microscope, to determine (A) total cell counts or (B) eosinophil counts. $P < 0.01$ ($n = 5$ /subgroup) was considered significant.

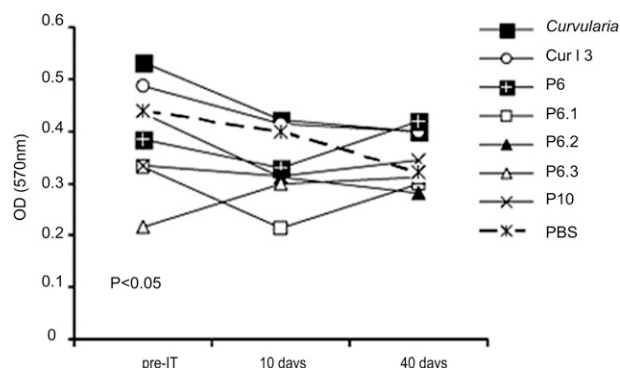


Figure 5. Splenocyte proliferation. Splenocytes isolated from each group were challenged with CL (1 μ g), Cur l 3 (1 μ g), or each of the antigens used for IT. They were cultured for 72 hours in the presence of 10% FCS. Cells were washed with RPMI and incubated for 2 hours with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100 μ g/ml). The cells were lysed in acidic isopropanol solution, and the absorbance read at 570 nm. Phytohemagglutinin (PHA) was used as control for positive stimulation, whereas unstimulated cells gave the basal stimulation level due to FCS. The bar graph presents mean $\pm$ SE values ($P < 0.01$ [$n = 5$ /subgroup] was considered significant).

40 days after IT with P6 and P6.2 as compared to baseline ($P < 0.001$) (Figure 6). Mice receiving IT with P10 also showed increased IL-10 level after IT.

Lung Histology

Hematoxylin and eosin-stained lung sections of mice were analyzed before and after 10 days (Figure 7A) or 40 days of IT (Figure 7B) after challenge with antigen. There was marked disruption of alveolar structure and profound cellular infiltration throughout the lung tissue in ovalbumin or CL-sensitized/-challenged mice before IT. CL-sensitized/-challenged mice in the PBS IT group showed disrupted alveolar structure, with large numbers of mucus-secreting cells and goblet cells in the bronchi. PBS-immunized/-challenged (control) mice showed normal lung structure, and had occasional inflammatory cells in the lung tissue (Figures 7A and 7B). Lung inflammation score was reduced significantly for the group treated with P6.2, followed by P10, P6, Cur l 3, CL, P6.1, and P6.3 after 40 days of IT (Figures 7C and 7D).

The eosinophil peroxidase activity was measured as a marker for activation of eosinophils. The levels are presented as mean

change in absorbance in BALF and lung homogenate of all mice. EPO activity was markedly increased in BALF and lung homogenate of all antigen-group mice before the IT (Figure 7E). Ovalbumin-immunized/-challenged mice demonstrated a significant enhancement in EPO activity compared with PBS ($n = 5$; $P < 0.02$). The mice challenged with CL demonstrated high EPO activity before IT. EPO activity in BALF and lung homogenate of mice was reduced significantly 40 days after IT with P6.2 as compared to 10 days after IT (Figure 7E). EPO activity was also reduced after 40 days of IT with CL, Cur l 3, peptides, and P6 derivatives.

DISCUSSION

IT with allergen extracts/purified proteins is associated with adverse reactions. Numerous strategies have been developed to reduce the allergenicity of vaccines, while maintaining the immune modulation. Such approaches include alteration of the tertiary structure of the allergen by chemical modification (17), gene/protein engineering (18–20), conjugation of bacterial DNA oligomers to purified allergens (21), and generation of allergen multimers (22). Synthetic peptides corresponding to T cell epitopes of the allergen were evaluated in various IT studies (6, 23, 24). These peptides were unable to cross-link IgE on mast cells/basophils, and proved effective candidates for IT (7, 25, 26). T cell epitope peptide from Fel d 1 induced IL-10 secretion in humans and mice, and reduced allergic inflammation (27). C-terminal Cys residues of Asp f 4 allergen were implicated in IgE binding (28). We have previously identified Cur l 3 as a cross-reactive protein in fungi, grasses, and ragweed (11). Furthermore, we have shown that P6 of Cur l 3 has both B and T cell epitopes, and belongs to a conserved region (16). In the present study, Cur l 3-derived peptides, P6, its 3 derivatives (P6.1, 6.3, 6.3), and P10, were evaluated for IT in a mouse model of allergy. In the modified peptides (i.e., P6.1), Cys⁶ was changed to Ala, P6.2 Cys³, and Cys⁶, both replaced with Ala, whereas, in P6.3, His⁷ was changed to Ala. Cys- and His-modified peptides showed significant reduction in IgE binding, but the immunogenicity was intact, as revealed by lymphocyte proliferation assay.

Experimental models of allergic asthma were developed in rodents using ovalbumin (29). Allergen extracts/protein from grass, mite, and cockroach have been used for development of animal models (30–32). *Alternaria* and *Cladosporium* spores induced airway inflammation in the mouse, and were characterized by high numbers of macrophages, neutrophils,

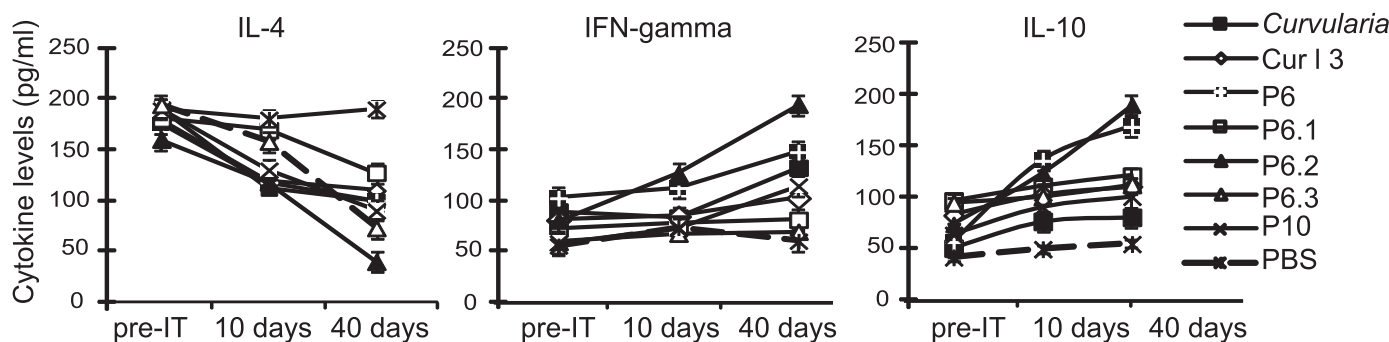


Figure 6. Cytokine analysis in BALF supernatant after antigen challenge before and after IT. (A) IL-4, (B) IFN- γ , and (C) IL-10 levels were determined by sandwich ELISA. Capture antibody (1:250 vol/vol) for each cytokine was coated separately on microtitre plates and incubated overnight. After blocking with 10% FCS, 1:2 vol/vol-diluted BALF samples were added to wells. Biotinylated anti-IL-4, anti-IL-10, or anti-IFN- γ were used to detect specific cytokine levels. The line graph presents mean $\pm$ SE values for each group before and after IT. $P < 0.01$ ($n = 5$ /subgroup) was considered significant.

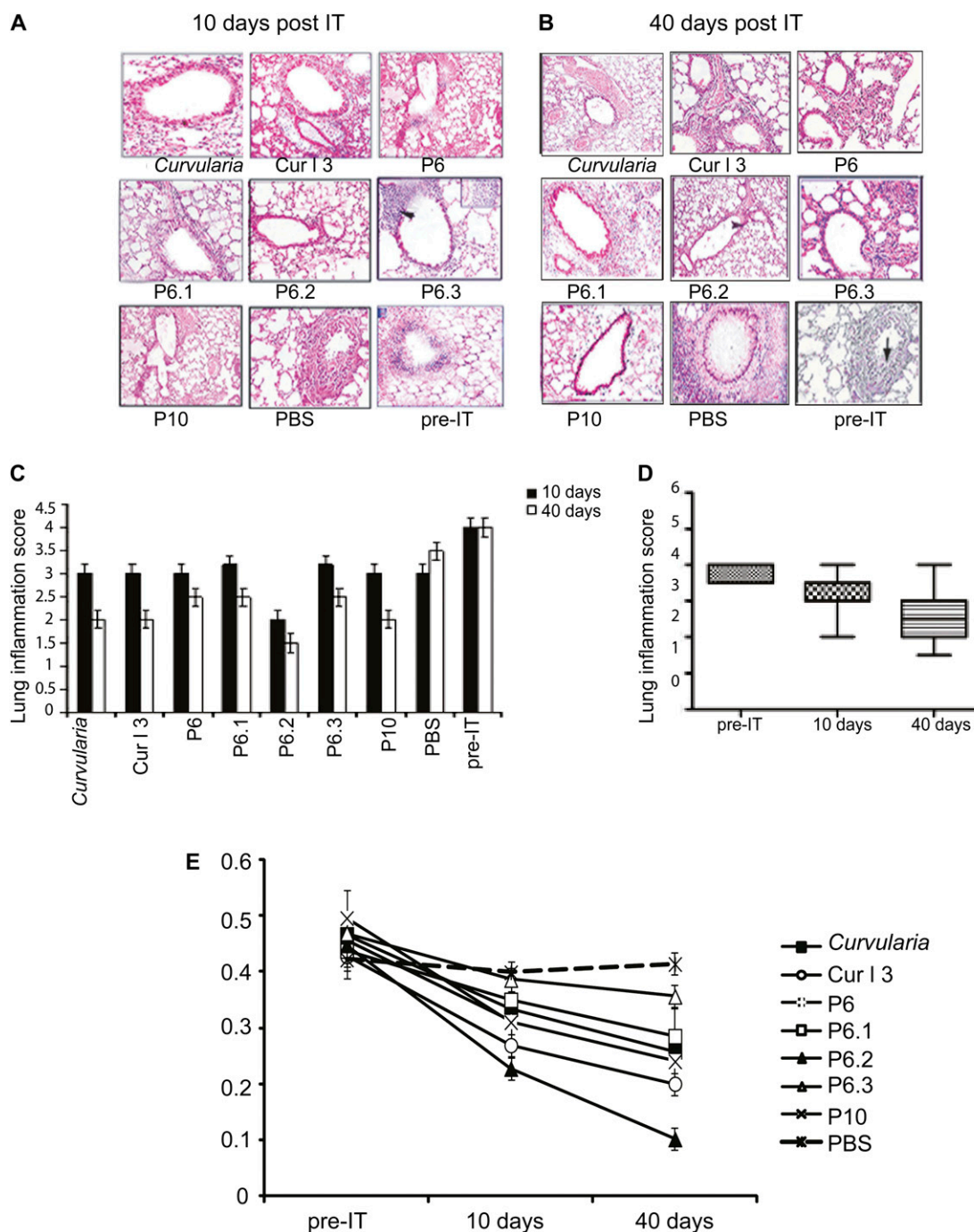


Figure 7. Histopathology of lungs and semiquantitative analysis of airway inflammation. Effect of allergen IT on airway inflammation assessed by lung histopathology after (A) 10 days or (B) 40 days of IT. Lung tissue segments, preserved in formalin, from each mouse were processed for sectioning and embedded in paraffin wax. The sections, cut at 4- μ m thickness, were stained with hematoxylin and eosin and scanned under a microscope. (C) Peribronchial and perivascular inflammation were graded on a subjective scale of 0–4, corresponding to mild, moderate, marked, or severe inflammation, respectively, with an increment of 0.5 if the inflammation fell between two integers. The total lung inflammation was defined as the sum of peribronchial and perivascular inflammation scores. The values are expressed as mean SEM for each group of mice. Total histological score, graded on a scale from 0 to 4: 0, no inflammatory cells; 1, <10%; 2, 10–25%; 3, 25–50%; 4, >50%. For simplicity, these data are represented as a box and whisker plot (D) for lung inflammation score for pre-IT, 10 and 40 days after IT. (E) Eosinophil peroxidase levels in BALF of each mouse was determined by transferring 50 μ l of 1:10-diluted BALF sample to a microtitre plate containing substrate solution comprised of 3 mM OPD, 0.05 M Tris (pH 8.0), 0.1% Triton X-100, and 8.8 mM H₂O₂. The graph presents mean change in absorbance at 490 nm. OD, optical density.

eosinophils, and lymphocytes in BALF (33). Gonzalo and colleagues (34) showed the presence of inflammatory cells in BALF in ovalbumin-sensitized/-challenged mice. In the present study, sensitization/challenge with CL in mice demonstrated significantly increased specific IgE, inflammatory cells (eosinophils), and Th2 cytokine (IL-4). The results were in concordance with a study on *Cladosporium* and *Alternaria* sensitization and challenge (33).

Airway hyperreactivity is associated with infiltration of inflammatory cells, particularly eosinophils (34, 36). Allergenic proteins induced eosinophil activation and release of mediators that caused airway damage and hyperreactivity (37). In the present study, cellular infiltration did not change significantly after 10 days of IT. However, 40 days after IT, total cell count decreased significantly in the P6.2 group, followed by P10-, Cur l 3-, CL-, P6.1-, and P6.3-treated mice. Eosinophils and EPO activity were reduced maximally in the P6.2 IT group, followed by P10 signifying therapeutic potential.

Allergen IT decreases Th2-type antibody titers (IgE and IgG1), with an increase in Th1 antibody, IgG2a (8, 37). Peptide IT modulates immune response by inducing T cell anergy, a switch from Th2 to Th1, and induction of regulatory/suppressor T cells (8, 23). In the present study, P6.2 and P10 reduced IgE and IgG1 significantly ($P < 0.05$), with an increase in IgG2a levels, 40 days after IT. P6.2 and P10 showed better therapeutic potential, with reduction in IL-4 level substantially as compared to P6, P6.1, P6.3, Cur l 3 and CL. This decrease in IL-4 level was associated with increased IFN- γ levels, demonstrating modulation of Th2 to Th1 by P6.2 and P10 peptides. These observations suggest the therapeutic potential of peptide P6.2 (i.e., modified form of P6). Studies show that long-term effects of IT are associated with secretion of IL-10 T-regulatory cytokine (27, 29, 38, 39). Campbell and colleagues (27) showed that epitope therapy induced IL-10 in a mouse model. It has been demonstrated that anti-IL-10R monoclonal antibody reduced the beneficial effects of IT (40, 41). In the present study, P6 and P6.2 stimulated high IL-10 secretion after 40 days after IT. However, P10 was not able to induce higher IL-10 levels.

Earlier studies have shown that lung inflammation and infiltration of cells correlated with antibody titers and cytokines (33–35). In the present study, histopathology showed significant improvement in the airway inflammation after 40 days of IT with P6.2 or P10. Janssen and colleagues (29) observed that ovalbumin IT improved allergic inflammation, airway hyperresponsiveness, and eosinophilia as compared with its peptide having both B and T cell epitopes. Here, IT with peptide P6 peptide induced Th1 response, and proved beneficial in reducing lung and systemic inflammation. Reduction in allergic airway inflammation in Cur l 3 and CL extract IT groups after 40 days could be due to the presence of multiple T cell epitopes. The present study demonstrated that allergic airway inflammation in the mouse model could be reduced effectively by IT, with small synthetic peptides having low IgE binding. The effects of IT were better 40 days after IT than 10 days of IT.

Peptide IT with single T cell epitope results in induction of linked epitope suppression, whereby cells rendered tolerant to one epitope suppress the function of cells specific for other epitopes on the same molecule. Previously, mice sensitized with Fel d 1, challenged with cat allergen extract, and treated with single T cell epitope peptide (Fel d 1^[29–45]) demonstrated significant reduction in airway inflammation and systemic Th2 responses (27, 42). The study also showed that peptide specific T cell population down-regulates an established inflammatory response driven by multiple T cell epitopes. IT results in induction of IL-10 producing “bystander” T cells, apart from the

allergen-specific T cells that result in anergy, and thus reduced T cell–proliferative response. This explanation is in agreement with the present study, where an increase in IL-10 has been seen in both BALF and culture supernatant of the P6.2-treated group.

In conclusion, CL induced airway inflammation and systemic Th2 response in mice, because it contains a large repertoire of allergen components. IT in this mouse model with whole extract, Cur l 3, and short synthetic peptides, P6, P6.1, P6.2, P6.3, and P10, proved beneficial in reducing inflammation and modulating Th2 to Th1 response. Therapeutic potential of short peptides, P6.2, followed by P10 and P6, was more pronounced compared with other antigens after IT. These short peptides may be tested in humans for IT.

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