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Proteolytically Inactive Per a 10 Allergen of *Periplaneta americana* Modulates Th2 Response and Enhances IL-10 in Mouse Model

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Abstract
Background Purified allergens with reduced IgE reactivity are required to improve the safety and efficacy of allergen-specific immunotherapy (IT).
Objective The present study investigates the efficacy of purified cockroach allergen immunotherapy with proteolytically active and inactive Per a 10 in allergic mouse model.
Methods Balb/c mice were sensitized intraperitoneally with cockroach extract (CE) and purified allergen Per a 10 in separate groups. Mice were treated subcutaneously with phosphate-buffered saline (PBS), CE, active and inactive Per a 10 and challenged intranasally. Antigen specific IgE, IgG1 and IgG2a in serum and cytokines IL-4, IL-13, IFN-γ, IL-10, TGF-β in bronchoalveolar lavage (BAL) fluid and spleen culture supernatant (CS) were estimated by enzyme-linked immunosorbent assay. Lung histology was analyzed by hematoxylin and eosin staining.
Results IT with Per a 10 demonstrated significant reduction in IgE levels in serum, IL-4 levels in BAL fluid, CS, and eosinophilic infiltration in lungs than PBS-treated mice. This was associated with significantly increased IL-10 secretion in BAL fluid and CS. IT with Per a 10 effectively suppressed T-helper type 2 (Th2) response in mice sensitized with Per a 10 than CE group. Further, IT with inactive Per a 10 showed maximum reduction in systemic and airway inflammation and induced maximum IL-10 release in BAL fluid and CS than other antigens.
Conclusions IT with Per a 10 effectively suppressed Th2 response and lung inflammation in Per a 10- or CE-sensitized mice. The beneficial effects of IT with inactive Per a 10 are more pronounced than active Per a 10.

Keywords Animal model · allergen · immunotherapy · *P. americana* · Per a 10 · serine protease

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Abbreviations

SIT	Specific Immunotherapy
CS	Spleen culture supernatant
EDTA	Ethylenediamine tetraceticacid
AEBSF	Aminoethyl benzenesulfonyl fluoride hydrochloride
PMSF	Phenylmethylsulfonyl fluoride
BAEE	*N*-Benzoyl arginine ethyl ester hydrochloride
CE	Whole body cockroach extract
w/v	Weight/volume
SDS	Sodium dodecyl sulfate
PBS	Phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
BAL	Bronchoalveolar lavage

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Introduction

Cockroaches affect human health through food contamination, transmission of bacterial and viral pathogens, psychologic stress, and allergic sensitization [1]. Studies have established a causal relationship between cockroach allergy and asthma [2]. Two species of cockroaches namely *Periplaneta americana* and *Blattella germanica* are important allergen sources world-over and play a major role in etiology of allergic asthma and rhinitis [3, 4]. Cockroach extracts are rich in proteases and induce direct proinflammatory effects on the airway epithelial cells, attributable to serine protease activity [5].

Allergic diseases have become a serious public health concern, leading to great socioeconomic burden [6]. Allergic symptoms can be mitigated by pharmacological treatment but symptoms reoccur rapidly on withdrawal of drugs. Allergen-specific immunotherapy (SIT) prevents progression of disease and has long-lasting effects [7, 8]. SIT trials have demonstrated reduction in clinical symptoms, drug use, and airway hyperresponsiveness (AHR) in patients of rhinitis and asthma [9, 10]. However, there is always a risk of inducing bronchoconstrictive responses and systemic anaphylaxis [11, 12]. Further, accurate diagnosis of sensitization is prerequisite for the correct prescription of specific immunotherapy [13, 14]. The results of diagnosis with ill-defined natural allergen extracts can be erroneous and inadequate to precisely identify the disease-eliciting allergen [15]. In order to optimize the selection of patients for immunotherapy (IT), component-resolved diagnostic tests are recommended for major allergen sources [16, 17]. Animal studies with purified proteins or peptides will be valuable as a preclinical model to improve the outcome of allergen immunotherapy.

Recently, a ~28-kDa major allergen of *P. americana* with proteolytic activity has been reported [18] and designated as Per a 10. The proteolytic activity of Per a 10 exerts an IgE-enhancing effect in mice. Further, inhibition of the proteolytic activity significantly reduced the Per a 10 associated eosinophilic airway inflammation and related manifestations of allergic airway disease [19]. The present study was aimed to investigate the effect of subcutaneous immunotherapy with purified cockroach allergen Per a 10 (proteolytically active and inactive) of *P. americana*.

Methods

Preparation of Whole Body Cockroach Extract and Proteolytically Active and Inactive Per a 10 The whole body cockroach allergen (*P. americana*) extract was prepared 1:50 *w/v* in phosphate-buffered saline (PBS; pH=7.4) containing ethylenediaminetetracetic acid (Sigma Chemicals Co., St. Louis, MO, USA) and phenylmethylsulfonyl fluoride (Sigma) [4]. The major allergenic protein Per a 10 was purified by affinity chromatography as described previously [18]. Briefly, 50 mg of cockroach extract was loaded onto Benzamidine Sepharose 6B column (Amersham Biosciences, Uppsla, Sweden) pre-equilibrated with binding buffer (50 mM Tris; 0.5 M NaCl; pH=8.0). After removing the unbound fraction, column was washed with binding buffer, and the bound protein was eluted with 1 M NaCl containing 50 mM benzamidine hydrochloride (Sigma), pH=8.0. The eluted protein was dialyzed against distilled water at 4°C, aliquoted, lyophilized, and protein-estimated. The purity of the preparation was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) and zymography. The enzymatic activity of Per a 10 was ascertained using the chromogenic substrate, benzoyl-L-arginine ethyl ester (Calbiochem, San Diego, CA, USA), specific for serine protease. To obtain inactive Per a 10, the proteolytic activity was blocked by incubating 1 μg of Per a 10 with 10 mM of aminoethyl benzenesulfonyl fluoride hydrochloride (Sigma) for 30 min at 37°C and dialyzed.

Sensitization, Treatment, and Challenge Female Balb/c mice (n=48) of 6–8 weeks were obtained from National Institute of Nutrition, Hyderabad (India) and randomly divided into eight groups of six mice each. Animal ethics committee of the Institute of Genomics and Integrative Biology (CSIR), Delhi, India approved the study protocol. T-helper type 2 (Th2) response was induced in Balb/c mice, as described previously [20] with a slight modification in two different protocols.

In first protocol, sensitization was carried out intraperitoneally (i.p.) on days 0 and 7 with 10 μg of cockroach extract (CE) containing 200 μg of alum (Fig. 1). IT was performed after 2 weeks of sensitization with three subcutaneous (s.c.) injections of 100 μg of CE (group 1), proteolytically active Per a 10 (group 2), proteolytically inactive Per a 10 (group 3) and PBS (group 4) on days 21, 24, and 27. To check the response of the treatment, mice were challenged intranasally (i.n.) after 5 weeks by 2 μg of CE in all the groups on days 62, 65, and 68.

In another protocol, mice were sensitized i.p. as mentioned above with 10 μg of Per a 10 (proteolytically active) containing 200 μg of alum (Fig. 1). Further, they were treated s.c. with 100 μg of proteolytically active (group 5) or inactive Per a 10 (group 6) or PBS (group 7) on days 21, 24, and 27. Mice were challenged i.n. in all the three groups, with 2 μg of purified active Per a 10. In a control group, naive mice were sensitized (i.p.), treated (s.c.), and challenged (i.n.) with PBS (group 8). On day 69, mice from all the groups were killed, and spleen, blood,

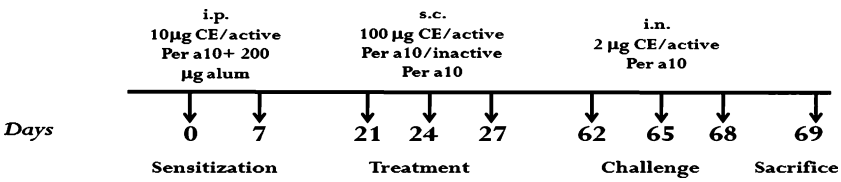


Fig. 1 The protocol for sensitization, treatment, and challenge with antigens and PBS (control) group mice

bronchoalveolar lavage (BAL), and lungs were collected. Before i.n. challenge, mice were lightly anesthetized by 3% isoflurane.

Collection of Bronchoalveolar Lavage Fluid and Blood Mice were killed using sodium pentobarbital (100 mg/kg; i.p.), and blood was collected by cardiac puncture. Sera were separated from blood for immunoglobulin analysis. Bronchoalveolar lavage fluid was collected by infusing 0.5 ml of chilled saline three times in the lungs. Total cell counts in BAL were determined using hemocytometer under light microscope. BAL fluid sample was centrifuged at 400×*g* for 10 min at 4°C. The BAL cell pellets were resuspended in PBS, and cell smears were stained with Leishman stain. BAL cell eosinophils were identified (%) by counting minimum of 200 cells in high magnification (×400). Absolute eosinophil numbers were calculated by multiplying total cell counts by percentage of eosinophil in BAL cell pellets. Cytokines levels in the BAL fluid were determined by enzyme-linked immunosorbent assay (ELISA).

Splenocyte Culture Spleens were collected aseptically, minced and passed through cell strainer (100 µm; BD Pharmingen, San Diego, USA) and washed in sterile PBS to obtain single cell suspension. Splenocytes (5×10^6 cells/ml) were cultured in RPMI-1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 1% sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin; Sigma St Louis, USA) and stimulated with 10 µg/ml of CE and Per a 10, separately. The cells stimulated with phytohemagglutinin (10 µg/ml; Bangalore Genei, India) were taken as positive control, while unstimulated cells were considered as negative control (to deduce basal proliferation). The cells were allowed to proliferate at 37°C in 5% CO_2 incubator for 72 h. The cell culture was centrifuged at 400×*g* for 10 min at 4°C, and culture supernatant was collected to measure cytokines.

Histopathology Lungs were inflated and fixed with 10% neutral-buffered formalin. Lung sections (4 µm) were cut, stained with hematoxylin and eosin (H & E), and scanned using light microscope to evaluate airway inflammation due to cellular infiltration. Histological analysis was performed for peribronchial and perivascular cellular infiltration and graded in a blinded fashion: 0, not present; 1, very slight; 2, slight; 3, moderate; 4, moderate to marked; and 5, marked. An inflammatory reaction affecting <20% of the airways was defined as "1" (<20% airways affected), "2" was defined as 20–40% of the airways affected, "3" 40–60%, "4" 60–80%, and "5" >80% of the airways affected (n=6 in each group) [21].

Determination of Specific Immunoglobulin Levels in Serum CE and active Per a 10 specific serum IgE, IgG1, and IgG2a were determined by ELISA as described previously with a slight modification [19].Briefly, microtiter plates (Maxisorp; NuncTM Immunomodule, Roskilde, Denmark) were coated with 10 µg/ml of CE or 1 µg/ml of active Per a 10 in 0.1 M carbonate buffer (pH=9.6). Nonspecific sites were blocked with 3% defatted milk, washed, and incubated individually with mice sera for IgE (1:10), IgG1 (1:50), and IgG2a (1:50) estimation. After washing, the plates were incubated for 3 h with anti-mouse IgG1-peroxidase and anti-mouse IgG2a-peroxidase (1:1,000 PBS; BD Pharmingen, San Diego, CA, USA). For IgE estimation, the plate was incubated with biotinylated anti-mouse IgE (0.5 mg/ml, BD Pharmingen, 1:1,000) at 25°C for 90 min. Following washing, it was incubated with streptavidin–peroxidase (1:1,000; BD Pharmingen) for 30 min. Color was developed using *o*-phenylene di-amine hydrochloride (Sigma, USA), and the absorbance was read at 492 nm.

Determination of Cytokine Levels in BAL Fluid and Culture Supernatant IL-4 (detection limit; 7.8 pg/ml), IL-13 (detection limit; 62.5 pg/ml), IFN-γ (detection limit; 31.3 pg/ml), IL-10 (detection limit; 31.3 pg/ml), and TGF-β (detection limit; 62.5 pg/ml) levels were determined in undiluted BAL fluid and culture supernatants by ELISA using paired antibodies according to manufacturer's instruction (BD Pharmingen, USA and R & D systems Inc. Minneapolis, MN, USA for IL-13).

Statistical Analysis The data were analyzed by GraphPad Instat software. Levels of immunoglobulins and cytokine were compared using non-parametric, two-tailed Mann–Whitney *U* test in PBS vs CE/Per a 10 (proteolytically active and inactive) treated mice, CE vs Per a 10 (proteolytically active and inactive) treated mice, and active Per a 10 vs inactive Per a 10. $P<0.01$ was considered significant in all cases. Data are expressed as mean ± SD. The sample size for each parameter tested was of six mice.

Results

Cockroach-Specific Immunoglobulin Levels in Serum Mice sensitized with CE (0.325±0.071; $P<0.01$) and Per a 10 (0.356±0.075; $P<0.01$) showed significantly increased levels of specific IgE after sensitization in serum than normal PBS control (0.122±0.043). After completion of protocol, CE-treated mice showed non-significant reduction in specific IgE levels compared to PBS-treated mice ($P>0.01$). Further, immunotherapy with Per a 10 (proteolytically active and inactive) reduced IgE levels effectively compared to PBS-treated mice ($P<0.01$). There was no significant difference in IgE levels between active Per a 10- and CE-treated mice ($P>0.01$). The enzymatically inactive Per a 10 showed significant reduction in specific IgE levels compared to active Per a 10- and CE-treated mice ($P<0.01$; Fig. 2a).

Immunotherapy with Per a 10 (proteolytically active and inactive) in Per a 10-sensitized mice also showed significant reduction in specific IgE levels in comparison to PBS-treated mice ($P<0.01$; Fig. 2b). Most importantly, IT with inactive Per a 10 showed significant reduction in specific IgE level compared to active Per a 10-treated mice in Per a 10-sensitized mice ($P<0.01$; Fig. 2b).

Furthermore, on comparing Fig. 2a, b, IgE levels reduced significantly with active Per a 10 treatment in Per a 10-sensitized mice compared to CE-sensitized mice (#$P<0.01$, Fig. 2b), whereas inactive Per a 10-treated mice showed remarkably significant reduction in IgE levels in active Per a 10-sensitized mice compared to CE-sensitized mice (##$P<0.001$, Fig. 2b). IgG1 and IgG2a levels, however, did not change significantly in all the groups post-IT (data not shown).

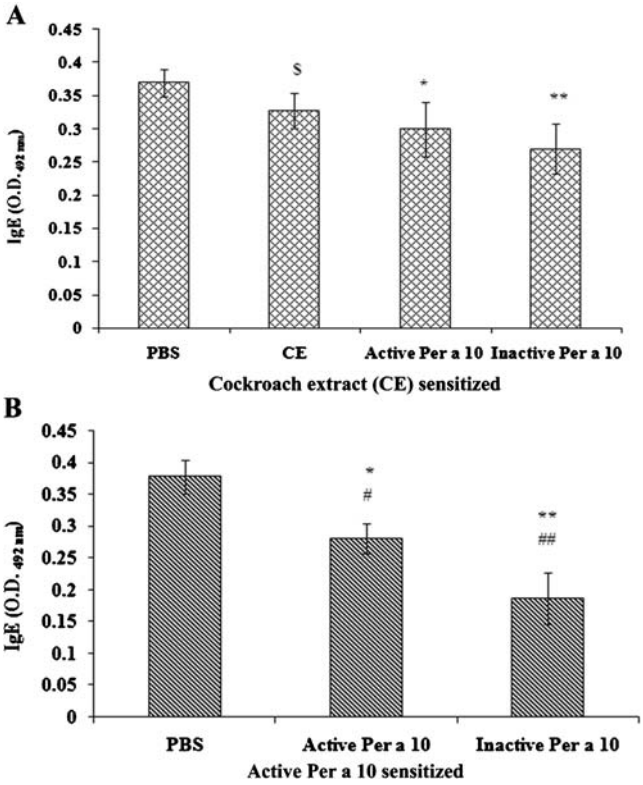


Fig. 2 Serum IgE levels post-immunotherapy with PBS, cockroach extract (*CE*), and Per a 10 (proteolytically active and inactive) were determined by ELISA. **a** CE-sensitized mice, treated with PBS, CE, active Per a 10, and inactive Per a 10 in different groups; **b** active Per a 10-sensitized mice, treated with PBS, active Per a 10, and inactive Per a 10 in separate groups. Results are expressed as mean ± SD of six mice per group. Student's *t* test was used to compare data post-IT in different antigen groups and with PBS control. $P<0.01$ was considered significant. $\$P>0.01$ and $*P<0.01$ compared with PBS; $**P<0.001$ compared with PBS, CE, and active Per a 10, $\#P<0.01$ compared with active Per a 10 treatment in CE-sensitized mice; $\#\#P<0.001$ compared with inactive Per a 10 treatment in CE-sensitized mice

Cytokines in Bronchoalveolar Lavage Fluid and Splenocyte Culture Supernatant In CE-sensitized mice, IT with CE showed substantial reduction in IL-4 levels compared to PBS-treated mice ($P>0.01$). Per a 10 (proteolytically active and inactive) IT showed significant reduction in IL-4 levels than PBS-treated mice ($P<0.01$; Fig. 3a). The active Per a 10-treated mice, however, did not show significant reduction in IL-4 levels compared to CE-treated mice ($P>0.01$; Fig. 3a). But, IL-4 levels showed significant reduction in mice treated with proteolytically inactive Per a 10 compared to active Per a 10- and CE-treated group ($P<0.01$; Fig. 3a). Further, the IL-10 levels showed substantial increase in BAL fluid and culture supernatant post-IT with CE compared to PBS-treated mice ($P>0.01$; Fig. 3a). Immunotherapy with Per a 10 (proteolytically active and inactive) showed significant increase in IL-10 levels compared to PBS-treated mice ($P<0.01$; Fig. 3a). However, Per a 10-treated mice did not show significant increase in IL-10 levels compared to CE-treated group ($P>0.01$; Fig. 3a), and inactive Per a 10 showed maximum increase in IL-10 levels post-IT followed by active Per a 10- and CE-treated mice ($P<0.01$; Fig. 3a) in CE-sensitized mice.

Immunotherapy with Per a 10 (proteolytically active and inactive) in Per a 10-sensitized mice showed significant reduction in IL-4 levels compared to PBS-treated mice ($P<0.01$; Fig. 3b). IL-10 levels showed significant increase in BAL fluid and culture supernatant post-IT with Per a 10 (proteolytically active and inactive) compared to PBS-treated mice ($P<0.01$). Most importantly, IT with inactive Per a 10 showed significant reduction in IL-4 level accompanied by maximum increase in IL-10 level compared to active Per a 10-treated mice ($P<0.001$; Fig. 3b). IFN-γ, IL-13, and TGF-β levels, however, did not change significantly post-IT in all the groups.

Additionally, on comparing Fig. 3a, b, IL-10 levels showed significant increase with active Per a 10 treatment

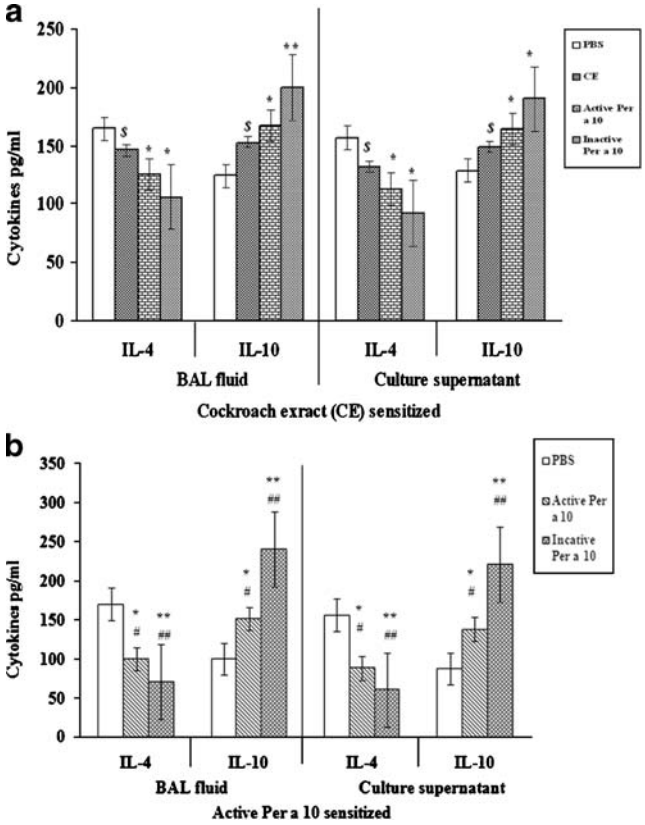


Fig. 3 Cytokine levels in splenocyte culture and BAL fluid. Splenocytes from mice treated with PBS, CE, proteolytically active Per a 10, and inactive Per a 10 were stimulated with CE or active Per a 10. Levels of IL-4 and IL-10 were determined by ELISA in culture supernatant and BAL fluid. **a** CE-sensitized mice treated with PBS, active Per a 10, and inactive Per a 10 in different groups; **b** active Per a 10-sensitized mice, treated with PBS, active Per a 10 and inactive Per a 10 in different groups. Results are expressed as mean ± SD of six mice per group. Student's *t* test was used to compare data post-IT in different antigen groups and with PBS control. $P<0.01$ was considered significant. $\$P>0.01$ and $*P<0.01$ compared with PBS; $**P<0.001$ compared with PBS and active Per a 10. $\#P<0.01$ compared with active Per a 10 treatment in CE-sensitized mice; $\#\#P<0.001$ compared with inactive Per a 10 treatment in CE-sensitized mice

in Per a 10-sensitized mice compared to CE-sensitized mice ($\#P<0.01$; Fig. 3b), whereas inactive Per a 10-treated mice showed highly significant increase in active Per a 10-sensitized mice compared to CE-sensitized mice ($\#\#P<0.001$; Fig. 3b).

Cockroach Immunotherapy Reduces Lung Inflammation - Histological analysis of the lungs in CE-sensitized mice demonstrated significant reduction in peribronchial and perivascular infiltration of eosinophils, in mice treated with active (Fig. 4c) and inactive (Fig. 4d) Per a 10 compared to PBS (Fig. 4a) treated mice. This was supported by a significant decrease in overall inflammation score in mice treated with active and inactive Per a 10 ($P<0.01$; Fig. 4e). CE-treated mice (Fig. 4b) also showed reduction in inflammation compared to PBS-treated but not significant ($P>0.01$). The active Per a 10-treated mice did not show significant difference in inflammation score compared to CE-treated mice ($P>0.01$; Fig. 4e), whereas inactive Per a 10-treated mice demonstrated significant reduction in overall inflammation score compared to CE and active Per a 10-treated mice ($P<0.001$; Fig. 4e). The Per a 10 (proteolytically active and inactive) treated mice also showed a significant decrease in total leukocyte and eosinophil counts in the BAL cell pellet compared to PBS- and CE-treated mice ($P<0.01$) (Fig. 6a).

Histological analysis of the lungs in Per a 10-sensitized mice also demonstrated significant reduction in peribronchial and perivascular infiltration of eosinophils, in mice treated with active (Fig. 5b) and inactive Per a 10 (Fig. 5c) compared to PBS-treated mice (Fig. 5a). This was associated with significant decrease in overall inflammation score in mice treated with active ($P<0.01$; Fig. 5d) and inactive Per a 10 ($P<0.001$; Fig. 5d) than PBS-treated mice. The Per a 10 (proteolytically active and inactive) treated mice also showed a significant decrease in total leukocyte and eosinophil count in the BAL cell pellet compared to PBS ($P<0.01$) (Fig. 6b).

Discussion

Cockroaches are important allergen sources among indoor pests, in most countries especially in the tropics [1]. Over the last 10 years, many cockroach allergens have been identified, sequenced, and produced as biologically active recombinant proteins [22]. However, commercial cockroach extracts used for diagnosis and treatment remain partially standardized. Well-designed controlled studies investigating the role of SIT with purified allergen in patients of cockroach allergy are lacking. Till date, two clinical trials of cockroach immunotherapy have been conducted, leading to beneficial changes in allergic patients [23, 24]. Purified allergens are of increasing importance in terms of component-resolved diagnosis and SIT of allergic diseases. In the present study, we investigated the immune modulation induced by purified major allergen (Per a 10) of *P. americana* in mouse model of allergic airway disease. Immunotherapy was carried out with CE and proteolytically active and inactive Per a 10 in separate groups.

Generally studies with ovalbumin (OVA) IT are well established in mouse model of allergic asthma [25, 26]. But, IT studies with allergen extracts and purified proteins/peptides are few using mouse model [27–29]. In the present study, specific IgE levels reduced significantly post-IT with Per a 10, most effectively with inactive Per a 10 proving

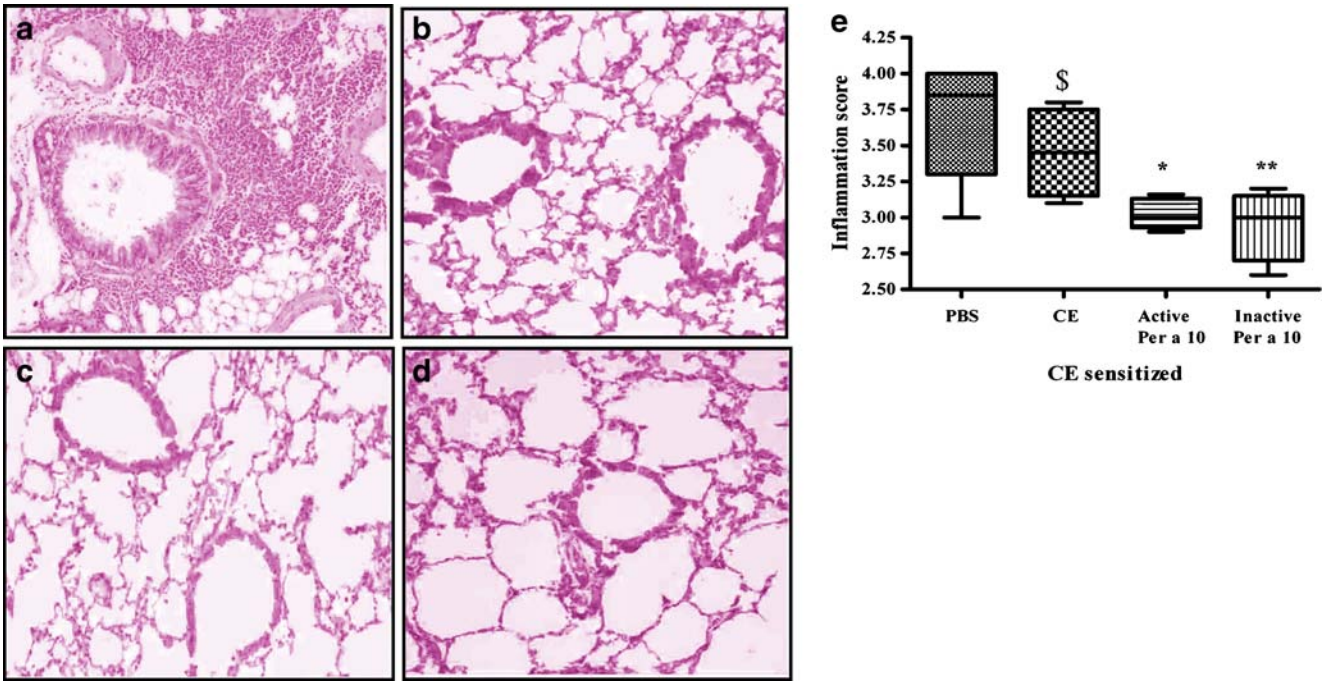
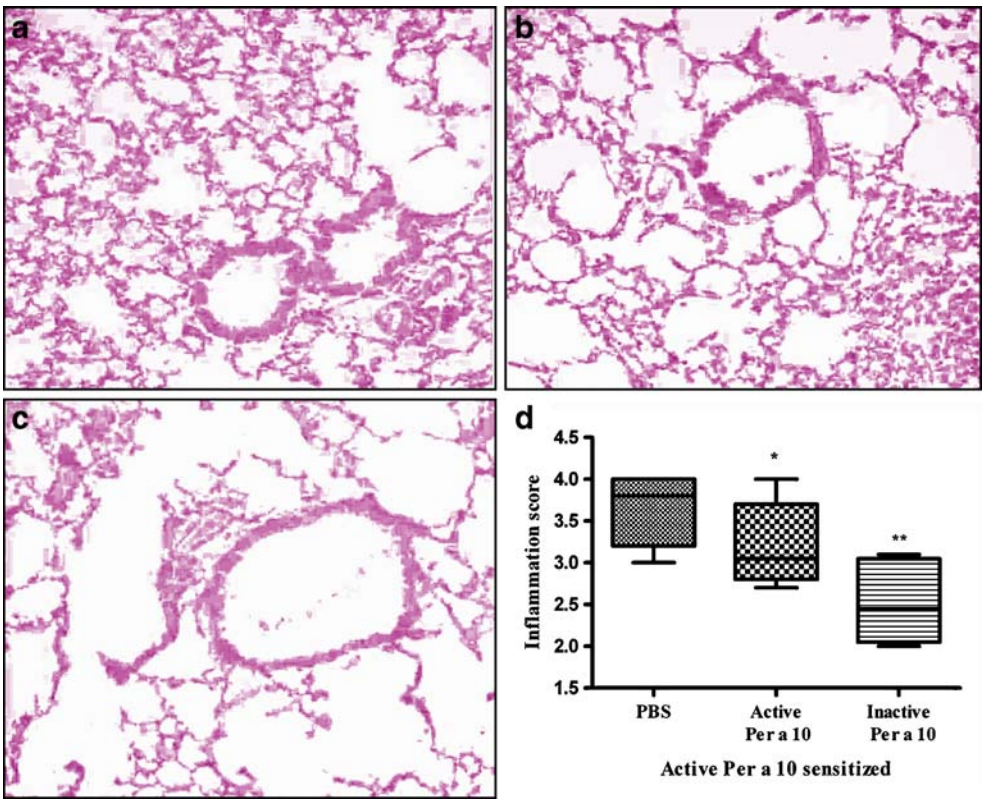


Fig. 4 Lung histology and inflammation scores. Hematoxylin and eosin-stained histological sections of mouse lungs. CE-sensitized and challenged mice were treated with **a** PBS, **b** CE, **c** proteolytically active Per a 10, and **d** inactive Per a 10 in different groups. All images include representative bronchovascular bundles at ×20 magnification. **e** Pulmonary inflammation was then scored on a four-point scale (0–4) by a blinded observer in CE-sensitized mice. $\$P>0.01$ and $*P<0.01$ compared with PBS; $**P<0.001$ compared with PBS, CE, and active Per a 10

Fig. 5 Lung histology and inflammation scores. Hematoxylin and eosin-stained histological sections of mouse lungs. Active Per a 10-sensitized and challenged mice were treated with **a** PBS, **b** active Per a 10, and **c** inactive Per a 10 in different groups. All images include representative bronchovascular bundles at ×20 magnification. **d** Pulmonary inflammation was then scored on a four-point scale (0–4) by a blinded observer in active Per a 10-sensitized mice. $*P<0.01$ compared with PBS; $**P<0.001$ compared with PBS and active Per a 10



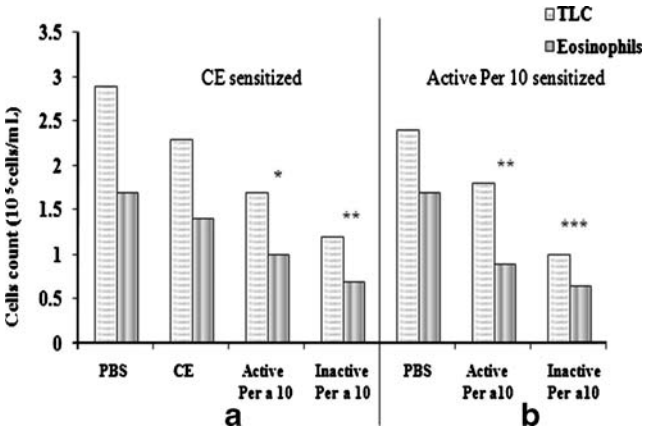


Fig. 6 Total cell leukocyte and eosinophil counts in BAL fluid from different groups of mice. **a** CE-sensitized mice, treated with PBS, CE, active Per a 10, and inactive Per a 10 in different groups. *$P<0.01$ compared with PBS; **$P<0.001$ compared with PBS, CE, and active Per a 10. **b** Active Per a 10-sensitized mice, treated with PBS, active Per a 10, and inactive Per a 10 in separate groups; **$P<0.01$ compared with PBS; ***$P<0.001$ compared with PBS and active Per a 10. Student's *t* test was used to compare data post-IT in different antigen groups and with PBS control. $P<0.01$ was considered significant

efficacy of IT with purified allergen. Similar to our study, there has been down-regulation of specific IgE after IT with OVA [30]. IgG1 and IgG2a levels, however, did not show a significant change post-IT, which is also observed in previous study of OVA IT [25]. The similar dissociation between IgG1 and IgE titer has been observed post-IT in a previous study [31]. The two isotypes IgE and IgG1 are considered to be co-regulated in mouse but increase in IgE response may be attributed to a mechanism unique to IgE isotype switching/synthesis [32].

Airway inflammation is characterized by high level of Th2 cytokines in BAL fluid [33]. OVA IT in preclinical mouse model has demonstrated lowering of Th2 cytokines [20, 25, 30]. In the present study, IL-4 levels reduced significantly post-IT with Per a 10, most effectively with inactive Per a 10 irrespective of antigens used for sensitization. The reduction in IL-4 was more pronounced in Per a 10- than in CE-sensitized mice. Decrease in IL-4 levels and IgE with Per a 10 further indicate that a direct relationship exists between IL-4 and IgE post-IT. IL-4 is reported to play an essential role in the generation of IgE and is a requirement to sustain IgE response [34]. IFN- γ is an important switch factor for IgG2a synthesis by murine B lymphocytes [28]. But, in our study, IFN-γ did not show a significant change post-IT in BAL fluid as well as in culture supernatant. Also we did not find a significant change in serum IgG2a level post-IT. These results indicate that purified cockroach allergen immunotherapy successfully down-regulates Th2 responses but could not affect Th1 response, similar to a study performed with OVA [29].

In humans, SIT is widely used to treat allergic rhinitis and asthma patients [35]. Although the precise mechanisms of IT are still incompletely understood, recent data suggest an important role for IL-10-producing Tr1 cells [35–37]. IT studies against bee venom, house dust mite, and grass pollen suggest that IT exerts its beneficial effect by anergy induction of allergen-specific Th2 cells, or by modulation of the Th2 response toward a Th1 or a regulatory T cell response in [38–40]. In agreement herewith, we also demonstrated that IL-10 plays a crucial role in the suppression of airway manifestation after allergen IT in a mouse model. OVA IT in mice model demonstrated that anti-IL-10R mAb administration at the time of IT and challenge reduced the beneficial effects of IT [20, 36, 37]. In the present study, IL-10 level showed maximum increase post-IT with inactive Per a 10 followed by active Per a 10 and CE. A study on IT with *Chenopodium album* pollen allergen has also shown a significant increase in IL-10 level post-treatment [41]. Unlike IL-10, TGF-β did not follow same pattern in our study. It might be due to TGF-β concentration, which increased considerably following oral administration of antigen in mice [42, 43].

The infiltration of eosinophils and neutrophils in the airways is a prominent feature of asthma and respiratory allergic disorders [44]. A comparative study of intranasal and intradermal OVA IT showed reduction of lung inflammation post-IT [25]. Also sublingual OVA IT in mice model of asthma demonstrated a significant reduction of bronchial inflammation assessed by specific cellular infiltration [45]. In the present study, lung histopathology post-IT with inactive Per a 10 followed by active Per a 10 showed significant decrease in airway inflammation in terms of overall inflammation score. This was associated with significant decrease in total leukocyte and eosinophil counts in BAL cell pellet in inactive Per a 10-treated mice ($P<0.01$). A previous study on OVA IT has reported suppression of AHR, following a prolonged gap between allergen IT and challenge [20]. Our results are consistent with the above study that increasing the period between allergen IT with inactive Per a 10 followed by challenge with active Per a 10 demonstrated significant long-term immunosuppressive effects associated with induction of IL-10.

In the present study, IT with proteolytically active and inactive Per a 10 demonstrated significant down-regulation of inflammatory parameters in mice sensitized to Per a 10 as compared to CE-sensitized mice. Our observation are in corroboration with earlier studies, which suggest that patients sensitized to a particular component in an allergen will respond better to the component-resolved immunotherapy than in polysensitized patients, i.e., sensitized with multiple allergenic fractions of an allergen [46, 47]. Consequently, patients without sensitization to the particular component are less likely to benefit from therapy with the specific component.

In conclusion, the purified cockroach allergen (Per a 10) IT is effective in reducing Th2 inflammatory response (IgE, IL-4, and cellular infiltration in lungs) and in generating immunoregulatory response (IL-10) in mouse model of allergic airway disease. The Per a 10 allergen may be explored for component-resolved specific immunotherapy in Per a 10- or cockroach-sensitized patients. Furthermore, proteolytically inactive Per a 10 (modified allergen) with reduced proinflammatory effects seems better candidate for immunomodulation.

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