IMMUNE RESPONSE TO N-TERMINAL AND C-TERMINAL DELETION MUTANTS OF ASPERGILLUS FUMIGATUS MAJOR ALLERGEN ASP F 3

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ABSTRACT

The ubiquitous fungus *Aspergillus fumigatus* causes allergic rhinitis, asthma, sinusitis and allergic bronchopulmonary aspergillosis. A number of major allergens from *A. fumigatus* are purified, but their structure-function role in the pathogenesis of disease is not known. Such information is essential for devising alternative therapy of fungal allergic diseases. In the present study, N-terminal and C-terminal deletion mutants of Asp f 3 were constructed and their immunopathological responses studied in a mice model of allergy. Three mutants viz, Asp f 3 (aa 33-168), (aa 1-142), and (aa 23-142) were made by deleting certain amino acids from epitopic regions of full length Asp f 3, a major allergen of *A. fumigatus*. The Asp f 3 and three mutated proteins were expressed in pET vector. The C-terminal deletion mutant Asp f 3 (aa 1-142) induced elevated IFN- γ but low levels of IL-4 by spleen cells. This mutant also showed significant downregulation of peripheral blood eosinophils and lung inflammation in immunized mice. The N-terminal deletion mutant Asp f 3 (aa 33-168) also exhibited an immuno-suppressive effect in terms of IgE production and induction of Th2 cytokine. The results indicate that rAsp f 3 and its deletion mutants induced distinct immune-inflammatory responses in mice on challenge with these proteins. The non-IgE binding deletion mutants of Asp f 3 (aa 1-142 and aa 33-168) could deviate Th2 immune response with a concomitant reduction in airway inflammation and infiltration of inflammatory cells.

KEY WORDS

Aspergillus fumigatus, rAsp f 3, Deletion mutants, Murine model, ABPA

INTRODUCTION

Aspergillus fumigatus (Af), is responsible for a wide range of allergic diseases in humans such as allergic rhinitis, fungal sinusitis, asthma, and allergic bronchopulmonary aspergillosis (1). Studies in both humans and mice models demonstrated that these diseases are characterized predominantly by a Th2 response (2). The protective immune response to Af is mostly due to a CD4⁺ Th1 lymphocyte response. The involvement of Af-specific Th1 cytokines in allergic response raise the possibility of uncovering effective therapeutic modalities that

would promote Th1 immune response and/or T- cell tolerance through regulatory T cells (CD4⁺ CD25⁺) in Af sensitized patients. In recent years, major Af allergens have been identified and purified using molecular cloning and expression protocol (3-6). Asp f 3 has been recognized as a major allergen of Af, which elicited immediate wheal and flare skin reactions and strong IgE binding with a majority of ABPA patients' sera (1, 4, 7). Earlier reports suggest that Asp f 3 with homology to several peroxisomal membrane proteins is actively involved in the pathogenesis of Af mediated diseases (4, 8).

Pharmacological treatment usually alleviates the symptoms of allergic disease, but only allergen immunotherapy affects its course (9, 10). The immune deviation towards T reg cells during specific immunotherapy modulates the allergen specific antibody response and directly or indirectly suppresses mast cells, basophils and eosinophils. The increased IL-10 and TGF- β potently suppress the IgE production and enhance IgG4 and IgA (11). Several approaches such as oligomerization,

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fragmentation and site-specific mutagenesis have been undertaken to produce hypoallergenic variants of common allergens such as Bet v 1, Der p 2 and Lep d 2 (12-14). Further, vaccination with genetically modified recombinant allergens has been shown to prevent progression of allergic disease (15). Targeting pathogenic T-cells use in vaccines consisting of synthetic peptides representing T-cell epitope is the strategy, currently evaluated with encouraging results (16).

Previously, recombinant Asp f 3 and other purified Af allergens demonstrated enhanced IgE response and peripheral blood eosinophilia in a murine model of allergic aspergillosis (17). The lung histology showed infiltration of inflammatory cells, representing lymphocytes, macrophages, eosinophils and neutrophils. Sensitized mice also exhibited varying levels of airway hyperresponsiveness with different antigens. An attempt was made earlier to develop Asp f 3 mutants for use in immuno-modulation of type-I allergy due to Af and related fungi (18). Experiments with these mutants showed remarkable difference in immune response. The mutant Asp f 3 (aa 1-150) showed IgE binding comparable to full-length Asp f 3 (aa 1-168) establishing the presence of conformational epitopes. Using several constructed mutants of Asp f 2, we have noticed differences in the immune responses when the terminal ends as well as other epitopes are selectively deleted (19). In the present study, three deletion mutants of Asp f 3 were evaluated for cell mediated and humoral responses in BALB/c mice. The study is expected to yield Asp f 3 mutants with hypoallergenic properties, but possessing intact T-cell responses of Th1 dominance.

MATERIALS AND METHODS

Asp f 3 and its Deletion Mutants

Asp f 3 was cloned from Af complementary deoxyribonucleic acid (cDNA) library and expressed in pET vector as described earlier (18). Recombinant construct 1 (aa 33-168) was prepared by deleting 32 amino acids at the N-terminal end of Asp f 3 (aa 1-168). In construct 2 (aa 1-142), 26 amino acids were deleted from the C-terminal end in Asp f 3, whereas 22 amino acids from N-terminal and 26 from the C-terminal end were deleted in Asp f 3 for construct 3 (aa 23-142). The fusion proteins, viz., Asp f 3 and three constructs were purified by Ni²⁺ chelate affinity chromatography. The purity and molecular size of these proteins were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The IgE reactivity of these proteins was studied by ELISA using sera from ABPA patients.

Immunization of Mice

BALB/c mice, 6-8 weeks old were obtained from Charles River Labs, Wilmington, MA, USA, and housed in the Veterinary Medical Unit, V A Medical Center, Milwaukee. Mice were immunized according to the protocol of Kurup *et al.* with minor modifications (17, 20). The first two injections were given intraperitoneally (i.p.) at an interval of four days with Asp f 3 and three mutants separately. Fifty microgram protein and 1 mg alum in 200 µl of phosphate buffered saline (PBS) was used for immunizing the mice. The next two injections were given with 50 µg antigen in PBS without alum. Subsequent immunizations were carried out by both i.p. and intranasal (IN) routes using the same antigen (50 µg/50 µl PBS) once weekly for four weeks. The animals were immunized in groups of six mice for each antigen, and control animals received only PBS. The animal experiments were carried out according to the guidelines and protocol approved by Animal Research and Use Committee of the Institute.

Eosinophils in the Peripheral Blood

Animals were bled from tail vein before immunization at 0 and thereafter at 2 week interval and the eosinophils stained using Eosin-Y (Sigma, USA) and enumerated in a hemacytometer as described before (20).

Histologic Studies

The lungs were inflated with 10% buffered formalin to prevent atelectasis and fixed in formalin. The lung tissue was processed and embedded in paraffin. The sections were cut at 5 mm thickness and stained with hematoxylin-eosin and periodic acid Schiff reagent. The inflammatory response and infiltration of cells in the lungs of immunized mice and normal control mice were studied under microscope and graded as described earlier (20,21).

Antibody Estimations in Serum

Total serum IgE was determined using purified anti-mouse IgE monoclonal (Pharmingen, USA) antibody as described elsewhere (20). The IgE was presented as nanograms/ml of serum after converting the values using a standard curve obtained with purified mouse IgE. Antigen specific IgG₁ and IgG_{2a} were estimated by indirect ELISA (17). A serum dilution of 1:50 was used to determine specific IgG₁ and IgG_{2a} antibodies to Asp f 3 and the fragments.

Cytokines in the Culture Supernatants

The cytokine expression by spleen cells was determined after stimulation with rAsp f 3 and or three mutants. Both, IL-4 and IFN-γ in the spleen cell culture supernatants (CS) were analyzed by two-site sandwich ELISA (22). In brief, microtiter plates were coated with capture antibody at 2 μg/ml (rat anti-mouse IL-4 and anti-mouse IFN-γ; Pharmingen, USA). Biotinylated anti-cytokine antibody was used (1 μg/ml) as detection antibody. After washing the plates, 100 μl of streptavidin peroxidase (1:1000 v/v) was added to the wells. The color was developed with the substrate orthophenylene diamine (OPD) and the absorbance was read at 490 nm using an ELISA reader (22). The optical density (OD) values were converted into nanograms/ml or picograms/ml using a standard curve.

RESULTS

Immunobiochemical characterization of rAsp f 3 and its deletion mutants

The amino acid sequence of rAsp f 3 and deletion mutants constructed are shown in Fig. 1. Recombinant Asp f 3 (aa 1-168) exhibited a molecular size in the range of 18 kDa, whereas, deletion mutants Asp f 3(aa 33-168), Asp f 3(aa 1-142) and Asp f 3(aa 23-142) were in the molecular mass range of 10-15 kDa on SDS-PAGE (data not shown).

Antibody responses to rAsp f 3 and deletion mutants

The total serum IgE levels in mice sensitized with recombinant Asp f 3(aa 1-168) and its deletion mutants are shown in Fig. 2A. The mice challenged with deletion mutants Asp f 3(aa 33-168), Asp f 3 (aa 1-142) and Asp f 3 (aa 23-142) showed significantly low serum IgE levels (in the range of 2-3.6 μg/ml) as compared to 6.8 μg/ml for Asp f 3 (aa 1-168) immunized mice. The mean total serum IgE levels in mice challenged with the three deletion mutants and Asp f 3 (aa 1-168) are statistically significant (p<0.01).

IgG subclass antibodies in mice immunized with full length rAsp f 3 and its deletion mutants are shown in Fig. 2B. There was no major difference in the specific serum IgG1 levels among Asp f 3 and the mutants immunized mice. However, the N-terminal deletion mutant Asp f 3 (aa 33-168) exhibited low serum IgG1 (OD₄₉₀ 1.406±0.053) compared to the levels (OD₄₉₀ 2.27±0.017) in sera of mice immunized with full-length rAsp f 3 allergen (p=<0.01). Specific IgG2a levels were comparable in mice immunized with Asp f 3 (aa 1-168) and its deletion mutants (Fig. 2C).

Eosinophils in peripheral blood of immunized mice

The eosinophils level in mice challenged with rAsp f 3 and its three constructs are shown in Fig. 3. Eosinophil numbers were elevated (2-7 times) in sensitized mice compared to normal control. The peripheral blood eosinophilia was least in mice immunized with Asp f 3 (aa 1-142). The difference in eosinophil counts between mice immunized with C-terminal deletion mutant Asp f 3 (aa 1-142) and full length protein rAsp f 3 (aa 1-168) is statistically significant (p<0.006). The N-terminal deletion mutant Asp f 3 (aa 33-168) and both N- and C-terminal deletion mutant Asp f 3 (aa 23-142) also showed significantly low eosinophil levels in peripheral blood of immunized mice compared to rAsp f 3 (aa 1-168) sensitized mice (p<0.05 and p<0.01, respectively).

Fig.1 A. The deduced amino acid sequence of Af allergen Asp f 3 (aa 1-168). B. The schematic representation of three cDNA constructs representing N-, C- and NC-terminal deletion mutants of Asp f 3.

Fig. 2 Antibody response in the sera of mice before (Pre) and after (post) challenge with Asp f 3 and its deletion mutants (A) Total serum IgE (B) *Aspergillus* specific IgG1 and (C) IgG_{2a}. The mean + SE is represented in the figure. The statistical significance between Asp f 3 (aa 1-168) and Asp f 3 mutant immunized groups are shown as (*). p value of <0.05 is considered significant.

Airway inflammation in sensitized mice

The lung sections of mice sensitized with rAsp f 3 and its deletion mutants are shown in Fig. 4A, B, C & D. Extensive inflammation from the center to periphery was observed in mice immunized with full-length allergen and the mutants, Asp f 3 (aa 33-168) and Asp f 3 (aa 23-142). The lung sections of these mice showed significant interstitial inflammation with peribronchiolar and perivascular infiltrates (Fig. 4A, C & D). The inflammatory cells consisted primarily of small lymphocytes, and occasional neutrophils and eosinophils. The epithelial lining is surrounded by slightly hyperplastic dense chronic inflammatory cells. Contrary to this, only a few foci of inflammation were noticed in the lungs of mice immunized with mutant Asp f 3 (aa 1-142) (Fig. 4B). There were no mucus plugs identified in the airways.

IL4 and IFN- γ secretion by spleen cell cultures of mice

The release of cytokines IL-4 and IFN- γ from spleen cell cultures of mice immunized with rAsp f 3 and its deletion mutants was measured by ELISA (Fig. 5A & B). The spleen cell culture supernatants from rAsp f 3 (aa 1-168) immunized mice exhibited more IL-4 secretion i.e. 86 ± 13 pg/ml as compared to cultures from mutants Asp f 3 (aa 33-168) and Asp f 3 (aa 1-142) immunized mice, which were 30 ± 2 pg/ml and 60 ± 9 pg/ml, respectively (Fig. 5A). The difference in IL-4 cytokine secretion by spleen cells of mice with Asp f 3 and N-terminal deletion mutant Asp f 3 (aa 33-168) is statistically significant ($p < 0.006$). Overall, N- and C-terminal deletion mutants of Asp f 3 induced less IL-4 from spleen cells as compared to the full length rAsp f 3. In contrast, mutant of Asp f 3 (aa 23-142) with both N- and C-terminal deletion, induced over three fold increase in IL-4 (300 ± 8 pg/ml) as compared to rAsp f 3 (aa 1-168) immunized mice. The control group mice exposed to PBS alone exhibited only a low IL-4 level (7 ± 0.15 pg/ml).

Among the mutants of rAsp f 3 used in this study, the N-terminal deletion mutant Asp f 3 (aa 33-168) exhibited the lowest IFN- γ in immunized mice (Fig. 5B). But the mice immunized with recombinant Asp f 3 and the other two mutants exhibited more release of IFN- γ with highest level in mice immunized with C-terminal deletion mutant Asp f 3 (aa 1-142). The mean IFN- γ level in mutant Asp f 3 (aa 1-142) immunized mice was 0.957 ± 0.136 pg/ml as compared to 0.457 ± 0.198 pg/ml in Asp f 3 immunized mice ($p < 0.02$). The control mice immunized with PBS showed IFN- γ level of 0.036 ± 0.0001 pg/ml.

DISCUSSION

In the present study, the potential immunomodulatory effect of N- and C-terminal deletion mutants of a major Af allergen, Asp f 3 (aa 1-168) was investigated in BALB/c mice. The activation of allergen specific Th2 cells seems to be causally related to pathophysiology of Af-sensitized diseases. Therefore the knowledge about immune deviation of the allergic response using recombinant Af allergens and their variants is of considerable significance in the management of the disease. The rationale for using a mouse model in present study is that the Th2 response observed with activated Af specific human CD4⁺ T cell is closely comparable to the response detected in mouse (23). Asp f 3 selected here is a well-characterized major allergen, which binds to IgE from more than 80% of Af-sensitized patients (4, 6, 7). Earlier, the IgE binding epitopes of Asp f 3 were identified using sera from patients with ABPA (18). The study suggested that the 12 amino acids at the N-terminal end (aa 1-12) and 8 amino acids at C-terminal end (aa 143-150) contribute significantly towards the conformational constraints for IgE binding.

For the present study, three mutants were designed from rAsp f 3 (aa 1-168) without the possible IgE binding regions and investigated their immunologic response in BALB/c mice. Immunization with Asp f 3 mutants, Asp f 3 (aa 33-168) and Asp f 3 (aa 1-142) showed reduced Th2 immune response with a concomitant decrease in airway inflammation and infiltration of inflammatory cells. However, unaltered full-length rAsp f 3 (aa 1-168) showed more inflammation in airways. Earlier studies with Af antigens demonstrated Th2 response, particularly increased IL-4, regardless of mouse strain and sensitization protocol used (22,24). This cytokine is essential for the induction of Th2 type lymphocytes, which in turn mediate the events leading to allergic lung inflammation and airway hyperreactivity. At the same time, IL-4, along with IL-13 is critical for the production of IgE (25). The isoallergens and hypoallergenic variants that can downregulate IL-4, IL-5, IL-13 and inflammatory responses in allergen

Fig. 3 Eosinophils in the peripheral blood in the different groups of mice immunized with Asp f 3 and its mutants. The eosinophils were enumerated after staining with Discombe's stain. The mean +SE is represented in the figure. The statistical significance between Asp f 3 (aa 1-168) and Asp f 3 mutant immunized groups are shown as (*). p value of <0.05 is considered significant.

Fig. 4 A. Lung histology of Asp f 3 exposed mouse showing moderate peribronchiolar and perivascular inflammatory infiltrate consisting primarily of lymphocytes (magnification x40). Inset shows inflammatory cells in high power x400. B. Lung histology of Asp f 3 (aa 1-142) exposed mouse showing significantly less inflammatory infiltrates adjacent to bronchioles and blood vessels without eosinophilic infiltration (x40). Inset shows high power magnification x400. C. Lung section from Asp f 3 (aa 33-168) immunized mouse showing comparable cellular infiltration and epithelial hyperplasia as in Asp f 3 immunized mouse (x 60). D. Lung sections from mouse immunized with deletion mutant Asp f 3 (aa 23-142) showing distinct cell infiltration and hyperplastic epithelial linings (x40).

Fig. 5 Cytokine production during in vitro stimulation of spleen cells from different groups of sensitized mice with Asp f 3 (aa 1-168). Cells were stimulated with the allergen at 10 µg/ml for five days and supernatants were analyzed by ELISA for IL-4 (A) and IFN-γ production (B). The mean + SE is represented in the figure. The statistical significance between Asp f 3 (aa 1-168) and Asp f 3 mutant immunized groups are shown as (*). p value of <0.05 is considered significant.

sensitized individuals may be effective immuno-modulatory agent for Af mediated diseases (16, 18).

In the present study, mice immunized with the N-terminal deletion mutant, Asp f 3 (aa 33-168) and C-terminal mutant Asp f 3 (aa 1-142) induced low levels of IL-4 in the spleen cell cultures as compared to the mice immunized with complete rAsp f 3 allergen. Also, the total serum IgE in the mice immunized with these mutants was significantly less compared to mice immunized with rAsp f 3. Mice from the different groups sensitized with rAsp f 3 mutants exhibited significant levels of IFN-γ in spleen cell cultures with a two fold more secretion of IFN-γ in mice immunized with C-terminal deletion mutant Asp f 3 (aa 1-142) compared to full length protein. In contrast, both N – and C-terminal mutant Asp f 3 (aa 23-142) exhibited a three fold increase in IL-4 cytokine production compared to the full length protein. The reason for the differences in immune responses of the rAsp f 3 mutants may be due to differences in the mode of antigen processing and presentation to T- cells

by antigen presenting cells. The precise molecular mechanism of antigen-mediated immune deviation is unknown but results of our earlier study with rAsp f 3 mutants showed that the immune deviation occurs in mice treated with non IgE binding deletion mutants (18). Therapy with long synthetic peptides derived from PLA2, a major bee venom allergen, was able to induce T-cell anergy, immune deviation towards Th1 type cytokine response, enhanced IL-10 secretion and PLA2 specific IgG4 production (26).

Earlier, the mice exposed to purified recombinant Af allergens Asp f 1, Asp f 3, and Asp f 4 exhibited inflammatory changes in the lungs and a predominantly Th2 immune response with elevated serum IgE and enhanced number of eosinophils in the peripheral blood and lungs (17). In the present study, the potential effect of low IgE binding Asp f 3 mutants on peripheral blood eosinophil levels was evidenced by 2 to 3 fold decrease in eosinophils in mice immunized with the mutants as compared to rAsp f 3 immunized mice. Remarkably, a C-terminal mutant Asp f 3 (aa 1-142) exhibited least peripheral blood eosinophils compared to full-length rAsp f 3 and other mutants.

In conclusion, a C-terminal deletion mutant Asp f 3 (aa 1-142) appears to be useful in suppressing the Th2 immune response with reduction in airway inflammation, low peripheral blood eosinophils and reduced IL-4 secretion from spleen cells. It also showed enhanced secretion of IFN-γ (Th1) from spleen cells compared to other two mutants and full length rAsp f 3 allergen. Such recombinant constructs may be explored further for immuno-modulation of Af and related mold allergy.

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