Chapter 3

To evaluate the prophylactic and therapeutic effect of AEBSF in cockroach allergen induced airway inflammatory disease
3.1. INTRODUCTION

Allergy is a disease of multifunctional etiology. Allergic reaction elicited on allergen exposure to atopic. Allergens possess diverse biochemical properties which facilitate them to induce allergic response. A major group of allergen extracts possess protease activity (Gregory & Lloyd, 2011). Allergens having protease activity are present in house dust mite, insects, molds, pollen etc. (Bhat et al., 2003; Bisht et al., 2004b; Chua et al., 1988; Gupta et al., 2004). Stewart et al. (1994) demonstrated that protease activity of allergen in house dust is important factor for allergic exacerbation. Proteolytic activity of allergens augment allergic responses by multiple ways (Hewitt et al., 1998; Shen et al., 1999a; Kauffman, 2003; Sudha et al., 2009c; Goel et al., 2012d). Proteases facilitate allergic response by enhancing epithelial permeability (Herbert et al., 1995; Wan et al., 1999) activating immune cells, inducing proinflammatory cytokine release, activating signaling through PAR-2 receptors (Schmidlin et al., 2002) and directing Th-2 responses by cleaving CD23 on B-cells and CD25 on T-cells. Protease from allergens may impair innate defense mechanisms in the lung. Proteolytic activity of HDM allergen inactivate the proteins with innate immune functions such as SP-A and SP-D to lower lung defense (Debet al., 2007), for allergic inflammation (Brandt et al., 2008; Madan et al., 2001). Moreover, protease allergen inactivates endogenous protease inhibitor which further enhances inflammation (Kalsheker et al., 1996).

Cockroaches are common source of allergen in house hold. Allergen extract of *Periplaneta Americana* contain multiple IgE binding components including Per a 10, a protease allergen. Per a 10, a major cockroach allergen has serine protease activity. Per a 10 induces allergic immune response in mice and augments the allergic response of adjunct allergen (Sudha et al., 2008, Sudha et al., 2009). Per a 10 bias the dendritic cells towards Th2 immune response by lowering IL-12 secretion (Goel et al., 2012). Additionally, Srivastava et al. (2010) demonstrated that immunotherapy with proteolytically inactive Per a 10 allergic response in Per a 10 sensitized mice.

Serine protease inhibitor has shown immense potential to suppress allergic response, however very few are being studied. Inhibitors targeting specific protease have shown limited therapeutic effect in methacholine or histamine induced bronchial hyperresponsiveness. AEBSF is a serine protease inhibitor of broad specificity and high
affinity and have shown therapeutic effect in purified allergen (ovalbumin) induced airway inflammation (Saw et al. 2015). However natural extract of allergen possess multiple allergen of different biological activity. Additionally presence of protease may lower the therapeutic effect of AEBSF in vivo by blocking its inhibitory property. AEBSF to be used as therapy in allergic diseases need to be further studied for allergen extract containing multiple allergen. Further, it also required to be studied in allergen having protease activity. Therefore, in present study therapeutic effect of AEBSF was evaluated in CE and Per a 10 (purified allergen with protease activity) induced mouse model.

3.2. MATERIALS AND METHODS

3.2.1. Animal ethics, immunization and treatment protocol

BALB/c mice (female), age of 4-6 weeks weighing 18-20 grams were obtained from CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow (India). Mice were acclimatized in experimental conditions for two weeks. Mice were kept under chow diet (ad libitum) and water at 12 hour controlled light: dark cycle. The Institutional ethics committee approved the experimental protocol (Delhi).

Mice were randomly grouped into five. One group of mice was sensitized with CE (10 µg) through i.p. injection on day 0, 7 and 14 and challenged with CE (5 µg) on day 21, 23 and 25. AEBSF was intranasally administered to mice in 10 or 30 µg of doses, (Sigma, St Louis) one hour before or after CE challenge in groups. Another five groups of mice were sensitized and challenged with Per a10 with same protocol. Per a 10 immunized mice were given AEBSF (10 or 30 µg) one hour before or after challenge, in groups. Control group of mice was sensitized and challenged with PBS.

3.2.2. Assessment of airway resistance

Airway resistance of mice was measured using FlexiVent TM ventilator (Scireq, Montreal, Que., Canada). Mice were anesthetized by intraperitoneal injection of Xylazine (10mg/kg) and pentobarbital (100mg/kg). Pressure transducer of ventilator was calibrated with 18 gauge cannula, at a pressure of 0 and 30 cm H2O column. Trachea of mice was exposed and cannula was inserted into trachea and firmly tied with
thread. Mice were ventilated at a speed of 150 breath/ min. Airway resistance of mice were recorded for six increasing doses of methacholine (Newcomb et al., 2008) (2, 4, 8, 12, 16 and 20 mM) and normalized with basal level response of PBS.

3.2.3. Sample collection

Mice were euthanized and visceral organs were exposed to collect samples. Blood was collected to separate sera centrifuged at 400xg for 10 min at 4 ºC. Serum was kept at -20 ºC for immunoglobulin analysis. Bronchoalveolar lavage fluid (BALF) was collected by instillation of cold PBS into the lung. Approximately 1.2 ml of BALF was collected and centrifuged at 400 g for 10 min at 4 ºC. BALF supernatant was separated and preserved at -80 ºC for cytokine analysis. BAL cell pellet was used to evaluate cellular infiltration and intracellular ROS. A part of lung was kept in PBS and stored at -80 ºC for evaluate NF-κB activation. Lung was excised from thoracic cavity and fixed in neutral-buffered formalin for histology.

3.2.4. Cell count and eosinophil peroxidase activity in BALF

The bronchoalveolar lavage cell pellet was resuspended in 100 µl PBS. Ten microliter of cell suspension was taken to enumerate total cell in BALF using trypan blue (Sigma Aldrich Co., St. Luis, USA) hemocytometer (Neuber’s chamber) and light microscope. BAL cell smear was prepared using grease free slides. Slides were stained with Leishman’s stain. Percentage of eosinophils and neutrophils was determined by counting minimum of 200 cells under microscope. Absolute numbers of eosinophils and neutrophils in per ml of BALF were determined by total cell count and percentage count (Mehta et al., 2007).

Eosinophil peroxidase activity in BALF was measured by spectrophotometric method. Briefly, 100 µl of BALF supernatant was taken in microtiter plate in duplicates (Nunc-immuno, Denmark). One hundred microliter of substrate solution containing 1 mM hydrogen peroxide, 0.1 mM ortho-phenylene-diamine-dihydrochloride and 0.1% Triton X-100 in 0.05 M Tris-HCl was added. Plate was incubated for 30 min at 37ºC and reaction was stopped by adding 50 µl of 4 M sulphuric acid. Absorbance was measured at 492 nm using microplate reader (Bio-Rad Laboratories Ltd., UK).
3.2.5. Measurement of intracellular ROS

BAL cell pellets were washed with PBS and incubated with 3.3 µM 2, 7-Dichloroflorescein diacetate (DCFH-DA) (Sigma) in PBS for 10 min at RT in dark. DCFH-DA is membrane permeable dye which was allowed to react with hydrogen peroxide and peroxidases intracellularly for 10 min. BAL cells were washed and fluorescence intensity was measured using cell sorter (Guava Technologies, CA, USA).

3.2.6. Immunoglobulins in sera

Ovalbumin specific IgE, IgG1 and IgG2a were measured in serum samples of mice by ELISA (Mehta et al., 2007). Briefly, 500 ng ovalbumin /well in 100µl of 0.1 M carbonate buffer (pH 9.6) were coated in microtiter plates (Nunc-Immuno, Denmark) and incubated overnight at 4 ºC. Plates were washed two times with PBS and blocked with 3% defatted milk for 3 h at 37 ºC. Plates were washed with two times PBST (0.05% Tween-20 in PBS) followed by two times with PBS. Sera samples were diluted in PBS 1:10, 1:50 and 1:50 v/v for IgE, IgG1 and IgG2a respectively and used in triplicates for estimation. Hundred microliters of diluted sera was added into the plates. Plates were incubated overnight at 4 ºC. Unbound antibodies were removed by washing the plates three times with PBST followed by two times with PBS. For IgG1 and IgG2a plates were incubated at 37 ºC for 3 h with anti-mouse IgG1-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA) or anti-mouse IgG2a-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA) respectively. IgE was estimated by incubating the plate with biotinylated anti-mouse IgE (2 µg/mL, BD Pharmingen) at 25ºC for 90 min followed by washing (two times with PBST followed by one time PBS) and incubating again with streptavidin-peroxidase (1:1000; BD Pharmingen) for 30 min. After washing with PBST five times and two times with PBS, plates were developed using 0.1% ortho-phenylenediamine (w/v in buffer containing 14.4 M citric acid, 4.5 M Na₂HPO₄ and 0.001% H₂O₂ (30%)) and absorbance was read at 492 nm using microplate reader.

3.2.7. Cytokines estimation in BALF

Cytokines IL-4, IL-5 (BD Pharmingen, USA and R & D, MN, USA) and IL-13 (R&D Systems Minneapolis, MN USA) were determined in BALF by ELISA as per as
manufacturer’s protocol. Briefly, 100 µl capture antibody (1:250 v/v) for each cytokine was coated separately in microtiter plates with carbonate buffer (pH 9.6). Plates were kept at 4°C for 12 hour. After washing three times with PBST, plates were incubated with 10% fetal bovine serum (assay diluent) to block at 25°C for 1h. Seven serial dilutions of standards (7.8-500 pg/ml) were prepared in assay diluent and 100 µl of each standard were added to the wells in duplicates. BALF samples were also added into the plates. Plates were incubated for 2 h at 25°C and washed with PBS. After washing with PBST five times, plate were incubated with 100 µl(1:250 v/v in assay diluent) biotinylated detector antibody with avidin-horse radish peroxidise (HRP) at 25°C for 1 h. To develop the plates, 100 µl of tetramethylbenzidine (Sigma Aldrich) substrate solution was added in and incubated at 37°C in dark for 30 min. Reaction was stopped by addition of 4N H₂SO₄ and absorbance was measured at 450nm (wavelength correction at 570 nm) using microplate reader.

3.2.8. Measurement of 8-isoprostane

The level of 8-isoprostane in BALF was measured using enzyme immunoassay kit (Cayman Chemical) following the manufacturer’s instructions. Standards of different dilutions (3.9 to 500 pg/ml) and BALF samples (50 µl/well) were taken in anti- rabbit IgG (mouse) pre-coated microtiter plate. These were mixed with 50 µl of 8-isoprostane tracer (Alkaline Phosphatase linked to 8-isoprostane) and 50 µl of 8-isoprostane antiserum and then plate was incubated at 4°C for 18 h. After washing the plate five times with wash buffer, 100 µl of substrate solution (para-nitrophenyl phosphate) was added to each well and incubated in dark at 25°C with gentle shaking. Absorbance was periodically read at 420 nm until the absorbance of maximum binding wells reached a minimum of 0.3 absorbance unit. Finally, 8-isoprostane level in BALF was calculated on the basis of standard curve obtained by standard concentration against percentage of standard bound upon maximum bound.

3.2.9. Measurement of NF-κB activation in lung

Nuclear proteins from lung tissue was extracted using CelLyticTM NUCLEARTM extraction kit (Sigma, St. Louis, MO, USA). Lung tissue (100mg) were washed with
PBS and homogenized in 1ml lysis buffer containing 50 mM Tris HCl (pH7.5), 15 mM CaCl2, 10 mM MgCl2, 0.1 M of dithiothreitol (DTT), 1.5 M sucrose and protease inhibitor cocktails. Lung homogenate were centrifuged at 11,000 × g for 20 min at 4°C. Cytosolic fraction was discarded and nuclear pellet was again resuspended in 140 µl extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, 0.42M NaCl, 25% (v/v) Glycerol, 0.1M DTT and protease inhibitor cocktails. Mixture was kept at gentle stirring for 30 min and centrifuged at 21,000 × g for 5 min at 4°C. The supernatant containing nuclear proteins were further used for determination of NF-κB levels. Protein content nuclear proteins were determined by bicinchoninic acid assay (BCA) (Stoscheck et al., 1990).

The level of NF-κB activation in nuclear proteins of lung was measured using immunoassay kit (Cayman Chemical) following the manufacturer’s instructions. CFTB buffer (90 µl/well) was added in dsDNA pre-coated microtiter plate. Nuclear proteins of each sample in 10 µl were added in well and incubated for 1h at room temperature. After washing the plate five times with wash buffer, 100 µl of diluted NF-κB (P65) antibody was added per well and incubated for 1 h at RT. After washing five times with wash buffer Goat anti-rabbit secondary antibody was added in each well and incubated for 1h at RT. After washing five times with wash buffer, 100 µl of developing solution was added in each well. Plate was incubated for 30 min in gentle shaking and stop solution was added to stop the reaction. Absorbance was read at 450 nm.

3.2.10. Histology

The lungs were fixed in 10% neutral buffered formalin (pH 6.8) and embedded in paraffin. Embedded lungs were cut into sections of 4 µm thickness. Lung sections were taken in glass slide and deparaffinized and hydrated (passed through xylene and ethanol (100 to 0% in water)). Lung sections were stained with haematoxylin (0.5% w/v) followed by eosin (0.1% w/v). Lung sections were observed under compound microscope (10X) (Nikon eclipse 80i). Inflammation score was determined on basis of peribronchial infiltration of cells and narrowing of airway lumen by experimentally blinded person.
Deparaffinized and hydrated lung section were stained with 0.5% (w/v) of periodic acid and followed by Schiff reagent. Sections were counterstain with hematoxylin. PAS stained lung section was also scored on the basis of goblet cells metaplasia around the airway lumens. For each 20% affected area of lung 1 score was assigned (Such as 1 for <20%, 2 for >20 &< 40%, 3 for >40 &< 60%, 4 for >60 &< 80% and 5 for < 100%) and thus section was scored 1-5.

Hydrated lung section was stained with metachromatic dye toluidine blue (0.1% w/v) and washed with water. Section was again dehydrated and mounted by DPX. Mast cells were counted in the lung section and scored in basis of number of mast cells per microscopic field.

3.2.11. Statistical analysis

All data were analyzed using Microsoft excel and graphs were plotted. Statistical analysis of data was performed using GraphPad Prism and GraphPad Instat software (GraphPad Software, San Diego, CA, USA). Values of parameters were fed into the software in groups. The statistically significant difference was determined using non-parametric test followed by one way ANOVA and Dunnett’s multiple comparison tests. The data were compared between ovalbumin challenged ted and AEBSF treated mice groups. The p value < 0.05 was considered as significant changes (Mehta et al., 2007).

3.3. RESULTS

3.3.1. AEBSF suppressed allergen induced airway resistance

Airway resistance of mice was measured with increasing dose of MCh and normalized with basal response (PBS). Airway resistance of mice was increased with dose of MCh (2-20mM) in CE immunized mice as compared to control (Figure 3.1a). AEBSF administered at 10 or 30 µg dose before CE challenge reduced the airway resistance of mice in dose dependent manner (p ≤ 0.01 or p ≤ 0.001). Same doses of AEBSF administered after CE challenge could also reduce airway response dose dependently (p ≤ 0.01 or p ≤ 0.001).
Figure 3.1: Airway resistances of mice in response to increasing doses of methacholine. Data represents mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Per a 10 immunization to mice showed increased airway resistance in response to MCh stimulation (Figure 3.1b). AEBSF given to mice (10 or 30 µg) before Per a 10 challenge lowered the airway resistance significantly (p ≤ 0.01 or p ≤ 0.001). Same doses of AEBSF administered to mice after Per a 10 challenge also reduced the airway resistance in dose dependent manner (p ≤ 0.01 or p ≤ 0.001). AEBSF showed dose dependent reduction in airway resistance of mice, however best effect of treatment was observed when AEBSF was administered before allergen challenge. The effect of AEBSF in reduction of airway resistance was more pronounced in CE immunized mice.

3.3.2. AEBSF reduced cellular infiltration in lungs

CE immunization of mice increased the number of total cells, eosinophil and neutrophil in BALF. Mice given 10 or 30 µg of AEBSF before CE challenge prevent total cell (p ≤ 0.01 or p ≤ 0.01), eosinophil (p ≤ 0.001 or p ≤ 0.001) and neutrophil (p ≤ 0.01 or p ≤ 0.01) infiltration in lung (Figure 3.2, 3.3 and 3.4). Same doses of AEBSF administered to mice after CE challenge could also significantly reduce the total cell (p ≤ 0.05 or p ≤ 0.01), eosinophil (p ≤ 0.05 or p ≤ 0.01) and neutrophil (p ≤ 0.05 or p ≤ 0.05).

Mice immunized with Per a 10 showed higher cellular infiltration in lungs. AEBSF given to mice in 10 or 30 µg doses significantly reduced the total cell (p ≤ 0.01 or p ≤ 0.001), eosinophil (ns or p ≤ 0.01) and neutrophil (p ≤ 0.01 or p ≤ 0.001) count (Figure 3.2, 3.3 and 3.4). AEBSF administered in 10 or 30 µg doses after challenge could significantly reduce the total cell (p ≤ 0.05 or p ≤ 0.01), eosinophil (p ≤ 0.05 or p ≤ 0.05) and neutrophil (p ≤ 0.05 or p ≤ 0.01) count.

CE immunization to mice increased EPO activity in BALF. Mice treated with 10 or 30 µg of AEBSF either before or after CE challenge significantly lowered EPO activity (p ≤ 0.001 or p ≤ 0.001) (Figure 3.4b).

Per a 10 immunized mice showed increased EPO activity in BALF. AEBSF administration to mice at 10 or 30 µg doses before Per a 10 challenge lowered the EPO activity (ns or p ≤ 0.001). AEBSF treatment given to mice after Per a 10 challenge lowered EPO activity significantly (p ≤ 0.01 or p ≤ 0.01) (Figure 3.4b).

AEBSF administration to mice before Per a 10 challenge was more effective in reduction of cellular infiltration, whereas the effect of AEBSF was more effective in reduction of eosinophil in CE challenged mice.
**Figure 3.2:** Total cell count in BALF of different groups of mice. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Figure 3.3: Neutrophil percentage in BALF. Data represent mean± SEM. *: p<0.05, **: p<0.01 as compared to allergen immunized mice.
Figure 3.4: a) Eosinophil percentage b) Eosinophil peroxidase activity measured in term of OD in BALF. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
3.3.3. AEBSF treatment modulated cytokines

CE immunized mice showed enhanced IL-4 and IL-13 level in BALF (Figure 3.5). AEBSF administered in 10 or 30 µg of doses before CE challenge to mice reduced the IL-4 ($p \leq 0.01$ or $p \leq 0.01$) and IL-13 ($p \leq 0.05$ or $p \leq 0.01$); more effectively when given after CE challenge (IL-4: $p \leq 0.001$ or $p \leq 0.001$ and IL-13: $p \leq 0.01$ or $p \leq 0.001$). Similarly, AEBSF administration in 10 or 30 µg dose to mice before Per a 10 challenge was less effective in lowering of IL-4 ($p \leq 0.05$ or $p \leq 0.05$) and IL-13 ($p \leq 0.05$ or $p \leq 0.05$) as compared to AEBSF given after Per a 10 challenge (IL-4: $p \leq 0.01$ or $p \leq 0.01$) (IL-13: $p \leq 0.01$ or $p \leq 0.01$).

Mice immunized with CE had increased IL-5 in BALF compared to control mice (Figure 3.5). AEBSF at 10 or 30 µg doses significantly reduced the IL-5 given to mice before ($p \leq 0.05$ or $p \leq 0.01$) and after ($p \leq 0.01$ or $p \leq 0.01$) CE challenge. Per a 10 immunization increased IL-5 level in mice, which was significantly reduced on AEBSF treatment at 10 or 30 µg doses given before challenge ($p \leq 0.05$ or $p \leq 0.05$). Same doses of AEBSF given to mice after Per a 10 challenge also lowered IL-5 in BALF ($p \leq 0.05$ or $p \leq 0.01$).

AEBSF administered to mice after allergen challenge was more efficacious in lowering Th2 cytokines. The effect of AEBSF administration in reduction of Th2 cytokines was more effective in CE immunized mice compared to Per a 10 immunized mice.

3.3.4. AEBSF lowers allergen induced IgE and IgG1 in mice

CE immunization increased the allergen specific IgE, IgG1 and IgG2a in serum (Figure 3.6). AEBSF administration at 10 or 30 µg doses before CE challenge significantly reduced IgE ($p \leq 0.05$ or $p \leq 0.01$) and IgG1 (ns or $p \leq 0.05$) in mice. Same doses of AEBSF given to mice after CE challenge also reduced the IgE ($p \leq 0.01$ or $p \leq 0.01$) and IgG1 ($p \leq 0.05$ or $p \leq 0.01$). In Per a 10 immunized mice AEBSF at 10 or 30 µg doses given before challenged suppressed IgE ($p \leq 0.05$ or $p \leq 0.05$) and IgG1 ($p \leq 0.01$ or $p \leq 0.01$). AEBSF at 10 or 30 µg given after Per a 10 challenge to mice also lowered IgE ($p \leq 0.05$ or $p \leq 0.01$) and IgG1 ($p \leq 0.01$ or $p \leq 0.01$).
Figure 3.5: IL-4, IL-5 and IL-13 measured in BALF by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Figure 3.6: Specific IgE, IgG1 and IgG2a in serum measured by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
3.3.5. Allergen induced oxidative stress reduced by AEBSF

CE immunization to mice enhanced the oxidative stress which was observed as 8-isoprostane level in BALF compared to control (Figure 3.7a). Treatment of AEBSF at 10 or 30 µg doses given to mice before CE challenge significantly lowered 8-isoprostane (p ≤ 0.05 or p ≤ 0.001). Same doses of AEBSF administered after CE challenge also reduced 8-isoprostane significantly (p ≤ 0.001 or p ≤ 0.001). Per a 10 immunization to mice increased 8-isoprostane level. Mice given AEBSF (10 or 30 µg) before allergen challenge showed significantly reduced 8-isoprostane (p ≤ 0.05 or p ≤ 0.05). AEBSF given after Per a 10 challenge could also lower 8-isoprostane significantly (p ≤ 0.001 or p ≤ 0.01).

Allergen challenge to mice increased intracellular ROS in BAL cell pellet (Figure 3.7b). AEBSF administered at 10 or 30 µg before CE challenge significantly suppressed ROS level in BAL cell pellet (p ≤ 0.05 or p ≤ 0.001) (Figure 3d). Same doses of AEBSF given to mice after CE challenge also lowered the ROS effectively (p ≤ 0.001 or p ≤ 0.001). AEBSF treatment given to mice at 10 or 30 µg doses before Per a 10 challenge significantly (p ≤ 0.05 or p ≤ 0.05) lowered ROS level. AEBSF could also reduce the ROS synthesis in mice treated after Per a 10 challenge (p ≤ 0.01 or p ≤ 0.01).

3.3.6. AEBSF lowers allergen induced NF-κB activation

CE immunization to mice induced NF-κB activation in lung of mice as seen in nuclear extract of lung homogenate (Figure 3.8). AEBSF administered in 30 µg dose before CE challenge to mice significantly lowered NF-κB activation (p ≤ 0.05). Both the dose 10 or 30 µg given to mice after challenge could reduce the NF-κB activation.

Per a 10 challenge to mice enhanced NF-κB activation in lung. Dose dependent reduction in NF-κB activation was observed in Per a 10 immunized mice treated with AEBSF before or after allergen challenge. AEBSF given after Per a 10 challenge could reduce the NF-κB activation more effectively than given before challenge.
Figure 3.7: Oxidative stress measured in term of a) 8-isoprostane in BALF and b) mean fluorescent intensity in BAL cell pellet. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
3.3.7. Protease inhibitor prevent allergen induced airway inflammation

CE immunization to mice increases lung inflammation as observed in H&E stained lung sections. CE immunization increased in peribronchial infiltration in lung of mice (Figure 3.9) compared to control mice (Figure 3.9). Mice administered with 10 or 30 µg AEBSF before CE challenge showed markedly reduced inflammation (p ≤ 0.05 or p ≤ 0.001). Same doses of AEBSF administered to mice after CE challenge could also significantly lower lung inflammation (p ≤ 0.001 or p ≤ 0.01). Per a 10 immunization also enhanced cellular infiltration in lungs. AEBSF administration 10 or 30 µg to mice before Per a 10 challenge suppressed the lung inflammation (p ≤ 0.05 or p ≤ 0.001). Same doses of AEBSF administered to the mice after Per a 10 challenge significantly reduced lung inflammation (p ≤ 0.01 or p ≤ 0.01).

CE immunization to mice enhanced mucus secretion in lungs (Figure 3.10). AEBSF administered at 10 or 30 µg doses to mice markedly reduced the mucus secretion in lung (Figure 3.10) (p ≤ 0.05 or p ≤ 0.01). Same doses of AEBSF administered to mice after CE challenge lowered mucus secretion in lung (p ≤ 0.05 or p ≤ 0.05). Mice administered with 10 or 30 µg AEBSF before Per a 10 challenge showed reduced mucus secretion (p ≤ 0.05 or p ≤ 0.05). Significant reduction in mucus secretion was also observed in mice administered with same doses of AEBSF after Per a 10 challenge (p ≤ 0.05 or p ≤ 0.05).

CE immunized mice possessed increased number of mast cell in lung (Figure 3.11) as compared to control mice. AEBSF administered at 10 or 30 µg doses before CE challenge markedly reduced mast cell in lungs (p ≤ 0.05 or p ≤ 0.01). Same doses of AEBSF given to mice after CE challenge could also reduce the mast cell accumulation in lung (p ≤ 0.05 or p ≤ 0.01). Per a 10 immunized mice had shown increased number of mast cells in sub-epithelial layer of lungs. AEBSF given before Per a 10 challenge at 10 or 30 µg doses could reduce the mast cell infiltration in lung (p ≤ 0.05 or p ≤ 0.05). Same doses of AEBSF also showed marked reduction in mast cell accumulation when given after Per a 10 challenge (p ≤ 0.05 or p ≤ 0.05).

AEBSF administration to mice reduced lung inflammation with best effect at 30 µg dose given before allergen challenge to mice. AEBSF given to the mice at 30 µg dose before allergen challenge had best effect in lowering of mucus secretion and mast cell infiltration.
Figure 3.8: NF-κB activation measured by ELISA in lung homogenate of mice. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Figure 3.9: H&E stained lung section of mice and inflammation score evaluated in basis of cellular infiltration and narrowing of airway lumen. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Figure 3.10: PAS stained lung section of mice and inflammation score evaluated on the basis of mucus secretion. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
Figure 3.11: Mast cell accumulation in lung and inflammation score evaluated on the basis of mast cell accumulation. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to allergen immunized mice.
3.4. DISCUSSION

Allergen having proteases activity aggravate allergic responses. Proteases may act as an adjuvant and potentiate allergic response of allergens present in the same micro-environment (Sudha et al., 2009). Protease allergen may block the activity of protease inhibitor present at the site of allergic response. In contrast, protease inhibitor provides necessary protection against improper immunological reaction by reducing protease load and maintaining homeostasis. In present study, effect of a serine protease inhibitor AEBSF in cockroach allergen induced allergic airways disease was evaluated in mouse model. The prophylactic and therapeutic effect of AEBSF was evaluated using two different doses.

Allergen exposure to atopic crosslink IgE and degranulate mast cells to release histamine and inflammatory mediators. Histamine induces bronchoconstriction which causes airflow obstruction. Mast cells also release protease during allergic exacerbation which potentiates effect of histamine in bronchoconstriction. Inhibition of protease activity reduces the bronchoconstriction and consequently the airflow obstruction (Chen et al., 2006). Protease inhibitor can further reduce the airway resistance by stabilizing mast cell (He et al., 2004), reducing level of bronchoconstrictor such as bradykinin (by reducing kininogen to bradykinin conversion) (Proud et al., 1988) and reducing hydrolysis of bronchodilator vasoactive intestinal peptide and peptide histidine-methionine (Tam & Caughey, 1990). In present study, AEBSF given to mice reduced methacholine induced airway resistance in CE and Per a 10 immunized mice. Previous study showed that serine protease inhibitor Nafamostate mesilate reduced bronchial hyperresponsiveness. However, some serine protease inhibitors such as MOL-1361 and APC-366 were not effective in lowering histamine /MCh induced bronchial hyperresponse (Krishna et al., 2001; Oh et al., 2002). AEBSF given to mice before allergen challenge was more effectively in the reduction of airway resistance than given after challenge possibly by mast cell stabilizing property of protease inhibitor (He et al., 2004).

Allergen sensitization leads to activation of Th2 immune response. More importantly protease biased immune response by reducing IL-12 secretions from DCs (Goel et al., 2012). Immunization of mice with CE/ Per a 10 induces Th2 cytokines
secretion such as IL-4, IL-5 and IL-13. IL-4 and IL-13 directs IgE class switching inflammation and mucus secretion (Wills-Karp et al., 1998). IL-5 promotes eosinophil infiltration and activation in allergic diseases (Takatsu & Nakajima, 2008). In present study, AEBSF administration to mice effectively reduced Th2 cytokines, cellular infiltration and concomitantly mucus production in lung of allergen immunized mice. Previous, studies have shown that serine protease inhibitor reduced allergen induced cellular infiltration Th2 cytokines and mucus secretion in airway (Ishizaki et al., 2008).

Allergen sensitization to mice enhanced specific IgE and IgG1 level in mice. Cross-linking of IgE and IgG1 on allergen re-exposure degranulates mast cells and induces inflammatory mediator release. CE and Per a 10 sensitization to mice enhanced specific IgE and IgG1 level in mice. AEBSF administration to immunized mice suppressed serum IgE and IgG1 level indicating anti-allergic effect. Our results are in agreement with previous studies shown that protease inhibitor lowered serum IgE and IgG1. Allergen immunization also increased mast cell accumulation in airways. Previously, a study showed that protease inhibitor treatment lower mast cell accumulation in sub-epithelial surface. Protease leads to mast cell infiltration in sub-epithelial surface of airways nearer to smooth muscle (Berger et al., 1999). Previous study showed that Na-Tosyl-Phe chloromethyl ketone a serine protease inhibitor suppressed the mast cells activation and degranulation on antigen stimulation (Nunomura et al., 2008).

Allergen exposure enhances oxidative stress in airway and accumulates excessive ROS. AEBSF administration lowered oxidative stress marker 8-isoprostane in allergen challenged mice. Intracellular ROS level was also lowered in BAL pellet cells of AEBSF treated mice (Izakovicova et al., 2009). Nefamostate mesilate a serine protease inhibitor was also showed reduction in intracellular ROS in allergen challenged mice.

NF-κB is an important transcription factor involved in expression of proinflammatory cytokines in allergic diseases. NF-κB is also involved in activation of inflammatory cells. AEBSF administration effectively reduced activation of NF-κB promising reduction in proinflammatory cytokine and immune cell activation. Our data
are in agreement with previous study showed that serine protease inhibitor treatment to mice lowers NF-κB activation in inflammatory cells (Chen et al., 2006).

The administration of AEBSF was effective in reducing airway resistance and inflammation in CE immunized mice in comparison to mice immunized with purified protease allergen. Moreover suppression of allergic parameters in both the CE and Per a 10 immunized mice on AEBSF administration indicated that the therapeutic effect of AEBSF is independent of protease activity of allergen and targets on endogenous proteases.

AEBSF attenuate airway hyper responsiveness, Th2 cytokines, inflammation and oxidative stress in allergen induced mouse model. The present study suggested that AEBSF has a potential to be used in combination, or as add-on therapy for allergic disease.