INTRODUCTION

Asthma is a complex disorder characterized by airway hyperresponsiveness (AHR), pulmonary obstruction and persistent airway inflammation (Elias et al., 2003). The inflammatory response is important for the initiation of tissue repair. The exaggerated response associated with asthma results in excessive oxidant formation and tissue destruction (Comhair et al., 2005a). Airways are unique in exposure to high levels of environmental oxidants and unusually high concentration of extracellular antioxidants. In the resting state, the balance between antioxidants and oxidants is sufficient to prevent the disruption of normal physiologic functions; however, either increase in oxidants or decrease in antioxidants can disrupt this balance (Halliwell and Gutteridge, 1999). This imbalance between oxidants and antioxidant leads to an increase in ongoing cycle of inflammation in the asthmatic airways that ultimately contributes to airway injury (Comhair et al., 2005b). The state of imbalance is collectively referred to as oxidative stress and is associated with diverse airway pathologies (Halliwell and Gutteridge, 1999). Oxidative stress plays an important role in allergic disorders and increased levels of oxidants are considered markers of the inflammatory process (Reynaert et al., 2007). The major oxidants in airways are reactive oxygen and nitrogen species (ROS/RNS). ROS includes superoxide, hydrogen peroxide, hypochlorous acid and hydroxyl radicals (Reynaert et al., 2007). RNS include nitric oxide and its derivatives such as nitrogen dioxide and peroxynitrite (Ricciardolo et al., 2006). Other molecules that can contribute to oxidative stress include protein radicals and lipid peroxide radicals. These molecules collectively cause non-specific damage to cells and extracellular matrix when produced in excess, for example, superoxide is a crucial component of phagocytosis and nitric oxide mediates smooth muscle relaxation in blood vessels as well as airways (Palmer et al., 1987; Dupuy et al., 1992; Ottonello et al., 1995).

ROS are diffused throughout the lung and are a byproduct of normal metabolism. Mitochondria are the largest producer of ROS because electrons leaked from the electron transport chain onto oxygen form superoxide. It is estimated that 1-3% of O₂ reduced in cells may form superoxide in this manner (Halliwell and
Antioxidant activity of GST and mutated GST

Gutteridge, 1999). Other sources of superoxide include cytosolic xanthine oxidase (Quinlan et al., 1997), mitochondrial respiration, and membrane nicotinamide adenine dinucleotide phosphate oxidases such as cytochrome P450 system of the endoplasmic reticulum (Goeptar et al., 1995). Hydrogen peroxide is formed during the dismutation of superoxide and also by glycolate oxidase in peroxisomes. Hydroxyl radical classically formed in the presence of metals and hydrogen peroxide and decomposition from other molecules like peroxynitrite may play a small role (Merenyi et al., 1998). Unlike nitric oxide, there are no well-described independent signaling roles for superoxide in the lung.

RNS are primarily derived from nitric oxide. In the resting state, nitric oxide is considered a signaling molecule. The sources of nitric oxide are three nitric oxide synthases (NOSs): constitutive NOS, found in respiratory epithelium, blood vessels, and nerve endings; inducible NOS, found in respiratory epithelium and activated macrophages and neuronal NOS, found in the nerve plexus of the trachea (Palmer et al., 1987; Ottonello et al., 1995; Guembe and villaro, 1999; Halliwell and Gutteridge, 1999). When nitric oxide is produced in high concentrations, such as with inducible NOS, it can react with oxygen or superoxide to form the highly reactive compounds namely nitrogen dioxide and peroxynitrite. Only inducible NOS are highly upregulated by cytokines such as TNF-α and interleukin-1ß (Radomski et al., 1993). Location determines the function of nitric oxide in the lung. In pulmonary vessels, nitric oxide is a vasodilator. In airway muscles, nitric oxide functions as a bronchodilator, and in airway epithelium, nitric oxide modulates the immune response.

Antioxidants are the primary defense against ROS/RNS. The antioxidant effect can be derived from either enzymatic or non-enzymatic molecules. Antioxidant enzymes include the families of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-S-transferase and thioredoxin. The non-enzymatic antioxidants include low molecular weight compounds such as glutathione, ascorbate, urate, α-tocopherol, bilirubin and lipoic acid. Concentrations of these antioxidants vary depending on both the sub-cellular and anatomic location. For instance, glutathione is 100-fold more concentrated in the airway epithelial lining fluid compared to plasma (Van der Vliet et al., 1999). Other high molecular weight
molecules that might be considered as antioxidants include proteins with oxidizable thiol groups e.g. albumin or proteins that can bind free metal like transferrin. Albumin and transferrin are found in high concentration in serum but are present at a much lower concentration in airway lining fluid (Reynolds and Newball, 1974). Thus, both the lung parenchyma and airways have several antioxidant systems.

In many airway diseases, the balance between ROS/RNS and antioxidants is disrupted because of excess production of ROS/RNS or depletion of antioxidants. For instance, in the airways of patients with asthma, ROS/RNS react with proteins to form amino acid adducts such as nitrotyrosine or chlorotyrosine; with lipids to generate ethane and isoprostanes and with DNA to form base pair adducts such as 8-oxo-2-deoxyguanosine. In diseases such as asthma and cystic fibrosis, inflammation may be the most significant contributor to oxidative stress. Studies have shown that the footprints of oxidative stress correlate with disease activity (Horvath et al., 1998; Montuschi et al., 2000a). ROS generation is associated with the pathogenesis of asthma and increased levels correlate with the disease severity, AHR, lipid peroxidation, production of chemoattractants and enhance vascular permeability and initiate Th2 cytokine release (Barnes, 1990; Crapo, 2003; Nadeem et al., 2003).

The production of oxidants is regulated by a well coordinated and efficient endogenous antioxidant defense system. But individuals with asthma demonstrate diminished levels of ascorbate and α-tocopherol, suggesting both increased ROS/reactive nitrogen species and decreased oxidative capacity (Kelly et al., 1999). There has been considerable interest in the possible modulation of asthma by supplementation of anti-oxidants e.g. hydrophilic and lipophilic antioxidants like vitamin C and E, respectively (Bowler and Crapo, 2002). However, these oral supplements, have failed to demonstrate overall convincing beneficial effects in asthma (Caramori and Papi, 2004; Hubbard and Fogarty, 2004). Previously, a study indicated that higher levels of serum antioxidants such as vitamin C and β-carotene were associated with low risk of asthma (Rubin et al., 2004). Diet increasingly deficient in anti-oxidants has increased population susceptibility with consequent large increases in disease prevalence (Seaton, et al., 1994). Along with this, there is
an increase in prevalence of allergic rhinitis, despite no increase in pollen levels (Seaton et al., 1996).

Epidemiologic studies have reported association between antioxidants and lipid intake and asthma or atopic disease (Devereux and Seaton, 2005). Dietary saturated fat intake has been reported positively associated with bronchial hyperresponsiveness in asthma and in women with atopic sensitization and hay fever (Soutar et al., 1997; Huang and Pan, 2001; Trak-Fellermeier et al., 2004). Several cross-sectional studies have reported beneficial associations between dietary fish intake, asthma and atopic disease (Hodge et al., 1996; Dunder et al., 2001). Investigations in asthma and dietary lipids suggest that asthma and atopy are a consequence of increasing n-6 polyunsaturated fatty acids and decreasing n-3 polyunsaturated fatty acids consumption that effect inflammatory mediators and Th cell differentiation (Devereux and Seaton, 2005).

Members of the glutathione-S-transferase (GST) superfamily are crucial for protecting cells from ROS (Lee et al., 2005). They are known as phase II xenobiotic detoxifying enzymes that conjugate reactive intermediates with glutathione to produce less reactive water soluble compounds and influence the synthesis of eicosenoid like mediators via the modulation of ROS levels (Hayes and Strange, 1995; Fryer et al., 2000; Strange et al., 2001; Lee et al., 2005). The oxidant scavenging and anti-inflammatory properties of catalytic antioxidants like GST can be exploited to modulate the innate immune response to achieve therapeutic benefit in inflammatory disorders. Previously, GST has been shown as a cross-reactive allergen among various fungi (Shankar et al., 2006). Allergenicity of an *Alternaria alternata* GST fragment (Alt a 131-50) was reduced by mutations at positions 21 and 27 (Shankar et al., 2009). The present study was aimed to investigate the antioxidant activity of enzyme allergen GST and mutated GST (mGST) in mouse model of airway disease.
MATERIALS AND METHODS

**DNA constructs:** *Alternaria alternata* full length GST was mutated at residues 21 and 27 to reduce IgE binding. Three step PCR reactions were performed to generate double mutant (mGST) using *A. alternata* GST (accession no. AY514673) as template. Initial PCR was carried out with an internal sequence specific oligonucleotide GS1F as forward and GMR2 as reverse primers (Table 2.1). In another reaction, GMF3 and GS4R primers were used to obtain the PCR product. Finally, products of above two steps were purified from agarose gel using Gel purification kit (Qiagen, Germany), mixed and amplified using oligonucleotide GS1F as forward and GS4R as reverse primers leading to two point mutations: Theronine\(^{21}\) {ACA (T)} to phenylalanine {TTT (F)} and Aspargine\(^{27}\) {AAC(N)} to Isoleucine {ATC (I)}. The PCR condition for all these reactions were taken as 95°C (5min) as initial denaturation, 94°C (1min) denaturation, 62°C (1 min) annealing, 72°C (1min) extension, cycle was repeated for 25 times and final extension was given at 72°C for 7 min. All the PCR amplifications were carried out using 5µl 10mM dNTP mix, 0.5µl Taq polymaresae (1 unit/µl), 5µl Taq buffer (10X), 2µl of each primers (10µM) and 50ng of template in a final volume of 50µl. The final product was cloned in vector pET 22b+ at *Eco RI* / *Xho I* site. The final product (50ng) and pET22b+ vector (100ng) were digested at 37°C for 4hrs using 0.5 µl of *Eco RI* and *Xho I* (20,000 units/µl). Heat inactivated digested products were purified from agarose gel and ligated at 16°C for 16hrs using 0.5µl T4 DNA ligase (10 unit/µl). Ligated product was transformed into DH-5α strain of *E.coli*. Cells were grown in 5ml of LB (Luria Bertini) broth containing 100µg/ml ampicillin overnight at 37°C and harvested by centrifugation at 10,000xg. Plasmid was isolated from the transformed cells. This was digested with *Eco RI* / *Xho I* and positive clones were selected. The clone was sequenced to confirm the insert and mutations.
### Table 2.1 Primers used for generation of plasmid mGST (Alt a 13 T21F_N27I)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Oligonucleotide Sequence*</th>
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<tr>
<td>GS1F</td>
<td>Fwd. 5’-GAATTGATGTCAGACAAACCTCTGAA-3’</td>
</tr>
<tr>
<td>GMF3</td>
<td>Fwd. 5’-AAAGGCTTCGCCACGTCCACACATATTACGTC-3’</td>
</tr>
<tr>
<td>GMR2</td>
<td>Rev. 5’-GACGTAATGTGTGGACGATGAGCATT-3’</td>
</tr>
<tr>
<td>GS4R</td>
<td>Rev. 5’-AAGCTTAACATCCATCGACAGGTAAAATT-3’</td>
</tr>
</tbody>
</table>

*Eco RI site- GAATTC, Xho I site- AAGCTT

**Protein expression and purification:** GST (glutathione-S-transferase) and mGST (mutated glutathione-S-transferase) were expressed and purified as per standard protocol using glutathione-Sepharose-4B resin (GE, Healthcare) based affinity chromatography (Shankar et al., 2006). Briefly, the plasmids containing the inserts were transformed into BL-21 E. coli cells for protein expression. Transformed cells were grown in 5-ml LB tube containing 100 µg/ml ampicillin overnight at 37°C and 500 l of the cells was taken for secondary inoculation in 2 litres of LB containing 100µg/ml ampicillin. The cells were grown at 37°C, when O.D.<sub>600</sub> reached 0.6, the culture was induced with 1mM isopropyl-beta-thio-galactopyranoside (IPTG) for 4 hrs and harvested. The pellet was suspended in 50 mM Tris-HCl (pH-7.5), sonicated (Misonix, Germany) for 5min with 1min pulse on with an intermittent gap of 30 sec off and centrifuged at 13000 x g for 30 min at 4°C. The supernatant was incubated with glutathione Sepharose-4B resin at 4°C overnight. Unbound fraction was collected, column was washed 5 times with PBS and bound protein was eluted using 10 ml of buffer containing 15 mM reduced glutathione in 50 mM Tris-Cl (pH-8.0). The purified protein was dialyzed and lyophilized.

**SDS-PAGE, Immunoblot and Enzyme activity:** The purified proteins were subjected to SDS-PAGE on 12% resolving gel using discontinuous buffer (0.025 M Tris, 0.2 M glycine and 0.1% SDS) system (Laemmli, 1970). Twenty microgram of the purified proteins were boiled for 5 min at 100°C in sample buffer containing β-mercaptoethanol and electrophoresed at a constant voltage of 120 V. The protein bands were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.
SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose membrane in Tris-glycine buffer (25mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using a BIO-RAD transfer apparatus at 200 mA for 2.5 hours (Towbin et al., 1979). The membrane was blocked with 3% defatted milk in PBS for 1 h at 37º C. The membrane was washed with PBS and incubated overnight with 1:10 v/v *Alternaria alternata* hypersensitive patients’ pooled sera at 4°C. The membrane was washed with two to three changes of PBST (PBS containing 0.05% Tween 20) followed by PBS and probed with 1: 1000 v/v diluted anti-human-IgE conjugated with horse radish peroxidase (HRP) (Sigma) for 3 h at 37°C. The unbound antibodies were removed by washing three times with PBST and PBS. The IgE reactive bands were visualized with 3’-3’-diaminobenzidine (DAB) and hydrogen peroxide in 0.05 M sodium acetate buffer, pH 5. The reaction was stopped by washing the membrane with distilled water.

The enzymatic activity of GST and mGST were assayed spectrophotometrically using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1981). To 1ml of reaction buffer containing 20mM reduced glutathione, 10mM Phosphate buffer (pH-6.5) and 100mM CDNB; 1µg of proteins were added. Absorbance was taken at 340nm over a period of 5 minutes and activity was calculated according to the formula-

$$\text{Activity} = \frac{A_{340}(t_2) - A_{340}(t_1)}{(t_2-t_1)}$$

$$A_340$$ absorbance at 340 nm

**Specific-IgE estimation by Enzyme linked immunosorbent assay (ELISA):** Sera were collected from *A. alternata* positive patients (n=12) at the out-patient department, V. P. Chest Institute, Delhi. The detailed history including age, sex, duration of illness, family history, onset of symptoms, seasonality etc. of each patient was recorded and thorough clinical examination and laboratory investigations (blood analysis, chest X-ray, stool, urine analysis, pulmonary function test etc.) were done with patients’ consent. The diagnosis of asthma was ascertained as per guidelines of the American thoracic society (1991) and cases having any two of the symptoms such as sneezing, rhinorrhea, nasal blockage, post nasal drip, etc for the last two years were diagnosed
as rhinitis. The patients selected for sera collection were suffering with allergic rhinitis, bronchial asthma or both. Informed consent of patients was taken prior to sera collection. Human ethics committee of the Institute has approved the study protocol following guidelines of Indian council of Medical Research, New Delhi.

Specific IgE to GST and mGST were quantitated in patients’ sera by indirect ELISA (Voller et al., 1978). Microtiter plate wells were coated with 1 μg of proteins (GST/mGST) in 100 μl of carbonate-bicarbonate buffer (pH 9.6) per well and incubated overnight at 4°C. The unbound antigen was washed off with 0.1 M PBS and free sites were blocked with 3% defatted milk in PBS (200 μl / well) for 1 h at 37°C. This was followed by washing with PBS (200 μl / well) and incubation with Alternaria alternata hypersensitive patients’ sera diluted 1:10 v/v with PBS (100 μl / well) at 4°C overnight. The plate was then washed four times with PBST followed by PBS and incubated with anti-human IgE conjugated with HRP (1:1000 v/v in PBS; Sigma Chemical Co., St Louis, MO, USA) for 3 h at 37°C. The plate was washed four times with PBST followed by PBS to remove unbound antibodies. The bound antibodies were detected using o-phenylenediamine (OPD) in citrate-phosphate buffer pH 5.0. Reaction was stopped after 30 minutes with addition of 50 μl of 5N H₂SO₄ and absorbance was read at 492 nm in an ELISA reader. Sera from normal healthy individuals were used as control.

**Circular Dichroism (CD) of GST and mGST:** CD analysis was performed to compare the structure of GST and mGST using Jasco-715 spectropolarimeter (Jasco Inc, MD). The CD scan was carried out in the far-UV range (190–300 nm) in 1 mm path length cell containing rGST or nGST (0.5mg/ml) in 20 mM phosphate buffer.

**Animal study protocol:** Female Balb/c mice of (6-8 weeks), weighing 18-20 grams were obtained from National Institute of Nutrition, Hyderabad, India. Mice were quarantined for 10 days to get acclimatized in experimental conditions. They were placed in cages at 22-25 °C, with 40-70% relative humidity and controlled 12-hour light: dark cycle. Water and standard chow diet were given. All animal experiments were carried out in the morning to minimize the effects of circadian rhythm. Mice were randomly divided in 5 groups of six mice each. Group 1 mice were sensitized,
challenged and treated with PBS as control. Four groups were given i.p. injection of 10 µg ovalbumin (OVA) with 1 mg alum in 100 µl of PBS on days 1 and 14. The mice were subjected to airway challenge with ovalbumin 4 µg/mice on days 28, 29, 30. After 1h of the challenge, group 2 mice were treated intranasally with PBS and group 3, 4 and 5 with 2 µg GST, mGST and α-lipoic acid, respectively for 3 days (Figure 2.1). Here α-lipoic acid has been used as standard antioxidant. The study protocol was approved by animal ethics committee of the Institute of Genomics and Integrative Biology, Delhi following guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, Chennai, India.

**Collection of bronchoalveolar lavage (BAL) fluid and blood:** At the end of the protocol, after inhalation treatment mice were sacrificed using overdose of sodium pentobarbital (100 mg/kg i.p.). Blood was collected, sera separated and used for analysis of serum immunoglobulins. The trachea was cannulated after the collection of blood. A 19 gauge needle was inserted into the trachea and lungs were lavaged three times with 0.5 ml of chilled saline and volume recovered was approximately 1.2 ml from each mouse. BAL fluid was centrifuged at 400 × g for 10 min at 4°C and supernatant was collected and stored at -70°C for analysis of cytokines and oxidative stress. The cell pellets were resuspended in 1 ml of PBS and the total cell counts were determined using hemocytometer under light microscope and cell viability was determined by adding trypan blue (0.4 %). BAL cell smears were stained with Leishman’s stain and eosinophils were identified (%) by counting minimum of 200 cells in high magnification (× 400). Absolute eosinophils were calculated by multiplying total cell counts by percentage of eosinophil in BAL cell pellets.

**Determination of immunoglobulins and cytokines by ELISA:** Ovalbumin specific IgE, IgG1 and IgG2a were measured in serum by ELISA as described elsewhere (Singh et al., 2006). Briefly, microtiter plates (Nunc, Denmark) were coated with ovalbumin (100 ng/100 µl/well) in 0.1 M carbonate buffer (pH 9.6) in separate wells and incubated overnight at 4°C. The plates were washed with PBS and blocked with 3% defatted milk in PBS, for 1 h at 37°C. The plate was washed with PBS and mice sera diluted to 1:10 (for IgE) and 1:50 (for IgG1 and IgG2a) was added 100µl/well and
Figure 2.1: Immunization protocol: Mice were immunized with OVA on day 0 and 14 (i.p.). The mice were challenged intranasally on day 28, 29 and 30 with OVA and treated with PBS, GST, mGST or α-lipoic acid 1h after each challenge. Mice were sacrificed on day 31 and blood, BALF and lungs were collected.
incubated overnight at 4°C. Pre-immune sera was also added and processed simultaneously. After washing with PBST (0.05% Tween-20) and PBS, the plates were incubated for 3 hours at 37°C with anti-mouse IgG1-peroxidase and anti-mouse IgG2a-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA). For IgE, biotinylated anti-mouse IgE (2 µg/ml, BD Pharmingen, USA) was incubated at 25°C for 90 min. Following washing, the plates were incubated with streptavidin-peroxidase (1:1000, BD Pharmingen, USA) for 30 minutes. After 5-6 washings, the color was developed using OPD and absorbance read at 492 nm.

IL-4, IL-10 and IFN-γ levels were determined in BALF by ELISA using paired antibodies according to manufacturer’s instruction (BD Pharmingen, San Diego). Briefly, capture antibody (100 µl; 1:250 v/v) for each cytokine was coated separately in the microtiter plates in carbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing, the wells were blocked with PBS containing 10% fetal calf serum (FCS) at 25°C for 1 h. After washing, 100 µl of standards (7 serial dilutions) and BAL fluid (1:2 v/v in PBS) was added to the wells in duplicates and incubated at 30°C for 2 h. The plates were washed again and incubated with biotinylated detector antibody (anti IL-4 or anti IL-10 or anti IFN-γ)-labeled with avidin-HRP at 25°C for 1 h. After washing with PBST and PBS, substrate was added and plates were incubated in dark at 37°C for 30 min. The reaction was stopped with 2N H₂SO₄ and absorbance was read at 450 nm. The cytokine concentrations were calibrated from the standards. The detection limit for IL-4 was 7.8 pg/ml and for IL-10 and IFN-γ it was 31.3 pg/ml.

**Histopathology:** Lungs were fixed with 10% neutral-buffered formalin (pH-7.0) and embedded in blocks containing paraffin. The sections of 4 µm were cut and stained with hematoxylin and eosin (H & E) or periodic acid schiff (PAS). Twelve slides were made for each type of staining (2 slides per mice) from every group and these were analyzed using light microscope, for antigen-induced peribronchial and perivascular inflammation. Coded (blinded) samples of lung tissues were made and given to a pathologist. Semi-quantification of inflammation score was made on the scale of 0–4 for
perivascular accumulation of inflammatory cells followed by statistical analysis (Singh et al., 2005). Lung inflammation score was calculated in terms of eosinophil infiltration and mucus secretion. Score 0 was assigned to no or occasional inflammatory cells, score 1 with a few inflammatory cells, score 2 with scattered aggregates of inflammatory cells, score 3 with thin layer of inflammatory cells surrounding the airways and vessels and score 4 with a thick layer of inflammatory cells surrounding the airways and vessels.

**Measurement of GST level and Oxidative stress in BALF:** GST activity in BALF was estimated by 1-chloro-2, 4-dinitrobenzene (CDNB) assay as above (Habig et al., 1981). Oxidative stress was determined by measuring thiobarbituric acid reactive substance (TBARS) concentration spectrophotometrically (Koca et al., 2005). Malanodialdehyde (MDA) and thiobarbituric acid (TBA) react to form a product with maximum absorption at 532 nm. BALF (200 µl) was mixed with 500 µl of 10% w/v trichloroacetic acid to precipitate the protein. The precipitated protein was pelleted by centrifugation at 10,000xg at 4°C for 15min. The collected supernatant was collected and treated with 0.67% TBA in boiling water for 15min. The different concentrations of malanodialdehyde (Sigma, USA) diluted in saline is taken as standard and incubated with 0.67% TBA in boiling water. The mixture was cooled and centrifuged at 1500xg at 25°C for 10 minutes. The supernatant was collected and absorbance was recorded at 532 nm. The malanodialdehyde equivalents in the samples were calculated from the malanodialdehyde standards.

**Statistical analysis:** Data was expressed as the mean±standard deviation. The data was analyzed using GraphPad Prism 5.00 (Graphpad software Inc. USA). The sample size for each parameter tested was six mice. Statistical significance was calculated by paired t-test to determine intra-group differences of means between untreated and treated mice. Probability of significance was assigned by non-parametric ANOVA using Kruskal-Wallis test. Each parameter was compared in all the groups using Dunn’s multiple comparison post test. P<0.05 was considered significant in all cases.
RESULTS

**Protein purification and enzymatic activity:** GST and mGST were expressed as soluble proteins and purified to homogeneity using glutathione-Sepharose 4B resin. The yield of expressed proteins was approximately 1.5 mg/l and 1.0 mg/l for GST and mGST, respectively. They migrated as homogenous proteins at molecular weight of 26 kDa (Figure 2.2a). GST and mGST proteins transferred to nitrocellulose membrane were recognized by *A. alternata* sensitive patients’ pooled sera on immunoblot. The intensity of mGST band was less as compared to GST (Figure 2.2b). GST and mGST showed comparable enzyme activity as 0.906±0.021/min/µg and 0.862±0.034/min/µg, respectively.

**IgE binding with individual patients’ sera:** IgE binding of GST and mGST was determined by ELISA with *A. alternata* allergic patients’ sera (n=12). The patients’ sera showed high specific IgE binding for GST ranging from OD 0.302-0.711 whereas it was reduced for mGST OD 0.107-0.411. Here the mean value of controls was OD 0.068 ±0.01 (Table 2.2). Specific IgE binding for mGST was lower between 30-69% as compared to GST with hypersensitive individual patients’ sera.

**Structural analysis of GST and mGST:** CD spectroscopy measures differences in the absorption of left handed polarized light versus right handed polarized light. CD spectra analysis of GST and mGST showed that the secondary structure of the two proteins was similar with minima at 225 nm. Both the proteins have predominantly α-helices (Figure 2.3). Mutations at residues 21 / 27 have not affected the secondary structure.

**Inflammatory cells in BALF:** Analysis of cellular infiltration in BALF showed a large number of total cells {(30.47±1.87) x10^4} and eosinophils {(13.34±0.78) x10^4} in ovalbumin-immunized and challenged mice (Figure 2.4). Intranasal administration of GST and mGST in ovalbumin immunized mice significantly decreased (p < 0.01) the cellular infiltration. The total cells in mGST treated group was nearly half {(13.70±2.52) x10^4} to that of GST treated group {(25.76±3.25)x10^4}. mGST caused significant
Table 2.2: Specific IgE binding with *Alternaria alternata* positive individual patients’ sera against GST and mGST.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Age/Sex</th>
<th>Clinical History*</th>
<th>Specific IgE values OD_{492nm}</th>
<th>% reduction</th>
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<td></td>
<td></td>
<td></td>
<td>GST</td>
<td>mGST</td>
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<tr>
<td>1</td>
<td>60/F</td>
<td>A</td>
<td>0.399</td>
<td>0.280</td>
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<tr>
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</tr>
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*M-Male, F-Female, A-Asthma, AR-Allergic rhinitis, C-Conjunctivitis, U-Urticaria*
Chapter 2  Antioxidant activity of GST and mutated GST

Figure 2.2a: SDS-PAGE profile of *E.coli* cell lysate and purified protein: Ten µg of protein was resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Molecular weight marker (MWt), Lane 2: *E. coli* cell lysate having GST, Lane 3: *E.coli* cell lysate having mGST, Lane 4: Purified GST, Lane-5: Purified mGST.

Figure 2.2b: Immunoblot of GST and mGST: Ten µg of each protein resolved by SDS-PAGE was transferred to nitrocellulose membrane, incubated with *Alternaria alternata* sensitive patients' pooled sera (1:10) and detected using anti human IgE peroxidase (1:1000).
Figure 2.3: CD analysis of purified GST and mGST: Proteins (1 mg / ml) were taken in 20 mM phosphate buffer and scanned from 190 nm to 270 nm. Minima at 225 nm shows α-helix predominance and the peaks are almost similar for both the proteins.

Figure 2.4: Total cell and eosinophil counts in BALF on treatment of OVA sensitized mice: (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated and (e) α-lipoic acid treated. Total cell count was determined using haemocytometer. One hundred microlitre of BALF was spread on the slide, fixed and stained with Leishman for eosinophil count. Data is presented as mean±SD. n=6 mice per group.
reduction in total cells and eosinophil counts than GST (p < 0.01). However, the effect of mGST treatment was approximately 70% to that of α-lipoic acid, the standard antioxidant (Figure 2.4). Here α-lipoic acid showed significant reduction (p<0.01) in the inflammatory cells compared to PBS treated mice.

**Serum immunoglobulins and cytokines in BALF:** GST specific IgE levels were higher in ovalbumin immunized and PBS treated mice (Figure 2.5). Intranasal GST treatment showed significant reduction in IgE level (p<0.01) as compared to ovalbumin immunized and PBS treated mice. IgE levels decreased significantly by the administration of mGST (p<0.01) as compared to GST. The reduction in IgE level was maximum on treatment with α-lipoic acid. IgG1 levels however did not change on treatment with GST, mGST or α-lipoic acid. Specific IgG2a levels were not detectable in any group.

IL-4 level in the BALF of ovalbumin-immunized and PBS treated mice (120.33±15.27 pg/ml) was more than 7 times higher to that of PBS control mice (16±2.96 pg/ml). mGST treatment decreased IL-4 levels (71±17.32 pg/ml) significantly as compared to ovalbumin immunized and PBS treated mice (p<0.01). GST treatment reduced the IL-4 levels to 107.67±20.82 pg/ml (p<0.05). Here, α-lipoic acid treatment showed maximum reduction in IL-4 levels (56±10 pg/ml).

IL-10 level was higher in ovalbumin immunized and PBS treated mice as compared to the PBS control. IL-10 level(s) however did not change on treatment with GST, mGST or α-lipoic acid (Figure 2.6). IFN-γ levels were also similar in all the treatment groups. The result suggests that GST or mGST are not able to change the cell milieu from Th2 to Th1 but mGST with reduced IgE binding shows potential to reduce Th2 cytokine level in the lungs.

**Lung histology:** Histopathological analysis of lung sections was performed to assess the anti-inflammatory or modulating effect of GST or mGST. In ovalbumin challenged PBS treated group, numerous eosinophils were observed in the lung interstitium around airways and blood vessels along with narrowing of airway lumen. Mice treated with GST or mGST showed improvement in the lung structure in terms of inflammation score as
compared to ovalbumin group (Figure 2.7a). The recruitment of inflammatory cells in the airways was lowest in mGST treated mice (Score-2) as compared to ovalbumin immunized and PBS treated mice (Score-4) or GST treated mice (Score-3). Mice treated with α-lipoic acid showed lowest inflammation and eosinophil infiltration in lungs tissue (Score-1).

In PBS control group, a few PAS stained epithelial cells were observed (Score-0). Contrary to this, epithelial cells were enlarged in ovalbumin immunized mice, along with airway narrowing and mucus plugs with increased goblet cell hyperplasia (Figure 2.7b). The treatment with mGST suppressed the mucus production and decreased the goblet cell hyperplasia.

**Oxidative stress in lung tissues and BALF:** Antioxidant level in BALF and lung tissues was measured in terms of lipid peroxidation (TBARS). Both GST and mGST treatment reduced the TBARS level as compared to PBS treatment group (p<0.01). α-lipoic acid treatment also showed significant reduction (p<0.01) in TBARS level compared to PBS treatment (Figure 2.8). The reduction in TBARS level was maximum in α-lipoic acid group than mGST and GST treatment groups.
Figure 2.5: Serum immunoglobulin levels after intranasal treatment with PBS, GST, mGST or α-lipoic acid: (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated and (e) α-lipoic acid treated. OVA-specific IgE, IgG1 and IgG2a were measured in serum by ELISA. Microtiter plates were coated with OVA, incubated with mice sera for IgE (1:10), IgG1 (1:50) and detected using secondary antibodies, individually. Data is presented as mean±SD. n=6 mice per group.

Figure 2.6: Cytokine profile (IL-4, IL-10 and IFN-γ) in BALF after i.n treatment with PBS or antioxidant: (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated and (e) α-lipoic acid treated. Capture antibody (1:250 v/v) for each cytokine was coated on plate, blocked, incubated with BALF samples (1:2 v/v) and detected by biotinylated anti IL-4 or anti IL-10 or anti IFN-γ. Data is presented as mean±SD. n=6 mice per group.
Figure 2.7a: HE stained sections of the lungs showing eosinophil infiltration: Lungs were fixed with formalin, embedded in paraffin, sections cut and fixed on the slide for staining. (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated (e) α-lipoic acid treated and (f) inflammation score. Inflammatory cells are marked by arrow.
Figure 2.7b: PAS staining of lung tissues showing goblet cell hyperplasia and mucus secretion: Lungs were fixed with formalin, embedded in paraffin, sections cut and fixed on the slide for staining. (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated (e) α-lipoic acid treated and (f) inflammation score. Mucus layer marked by green arrow.
Figure 2.8: Oxidative stress in BALF and lung tissues: (a) PBS control (b) OVA immunized/challenged-PBS treated (c) GST treated (d) mGST treated and (e) α-lipoic acid treated. Oxidative stress in BALF and lung tissues was determined by measuring thiobarbituric acid reactive substance (TBARS) concentration spectrophotometrically. Data is presented as mean±SD. n=6 mice per group.
DISCUSSION

Oxidative stress is involved in the pathogenesis of a variety of airway diseases such as asthma, chronic obstructive pulmonary disorder (COPD), cystic fibrosis etc. Asthmatic patients have enhanced production of reactive oxygen species (ROS) in blood monocytes, neutrophils and eosinophils and its level correlates with severity of the disease (Cluzel et al., 1987; Chanez et al., 1990; Vachier et al., 1992, 1994). They have increased levels of oxidized glutathione in BALF and increased nitric oxide concentration in exhaled air (Vachier et al., 1994). Furthermore, concentrations of natural antioxidants including, GST, glutathione peroxidase, superoxide dismutase, glutathione, vitamin C and E, are reduced in the blood cells, plasma or BALF of asthmatic patients (Brasch-Andersen et al., 2004). The strategies that counterbalance oxidative process may have a chemoprotective role in clinical or restorative medicine (Castro and Freeman, 2001). Epidemiological studies suggest that changes in diet, in particular reduced antioxidant intake contributes to increase in asthma prevalence and severity and indicates the possibility that dietary interventions may improve asthma (Fogarty and Britton, 2000). While oxidant damage is an established consequence of inflammation, the role of antioxidants and ROS in asthmatic airway inflammation is vital (Wood et al., 2003). Individuals having deficiency of GST are prone to asthma (Cho et al., 2004) and changes in its activity can have a repercussion on the disease. The administration of exogenous GST can help in the clearance of different reactive intermediates by GSH in asthmatics. Studies suggest dietary benefit of antioxidant supplements in curbing allergic airway inflammation (Singh et al., 2005; Silva Bezerra et al., 2006). In the present study, intranasal treatment with antioxidants namely GST and mGST was carried out to assess the effect on oxidative stress and airway inflammation in mice model. In addition, α-lipoic acid has been tested as a standard antioxidant. It is a naturally occurring antioxidant used clinically for the treatment of oxidant induced diseases, such as ischemia-reperfusion injury and diabetic neuropathy (Cao and Phillis, 1995; Van Dam, 2002; Ametov et al., 2003; Cho et al., 2004).
In a previous study, GST (Alt a 13) was detected as a major allergenic protein of *A. alternata* (Shankar et al., 2006). Alt a 13\textsuperscript{1-50} was demonstrated to be a immunodominant fragment using patients’ sera and mutations at amino acid positions 21 and 27 reduced its IgE binding (Shankar et al., 2009). In the present study, mutations at 21 and 27 residues in full length GST protein showed reduced IgE binding in the range of 30-69% than GST with individual patients’ sera. The reduction in allergenic potential of mGST indicates the involvement of amino acid 21 and 27 residues in IgE binding. Analysis of CD spectra revealed that mutations have not affected the secondary structure of the protein. Hence, no significant change was observed in enzymatic activity of mGST than GST.

The cytokines and chemokines released by Th2 cells regulate the activity of effector cells such as eosinophils and mast cells as well as induce IgE and other immunoglobulins (Crump et al., 1991; Amrani et al., 1999; Romagnani et al., 2000). Eosinophil is a central effector cell in the inflamed asthmatic airways (Lee et al., 2001). In the present study, GST or mGST treatment reduced serum IgE levels and release of IL-4 in BALF thereby inhibiting the allergic inflammation in mice model. mGST induced better effect than GST may be due to lower allergenicity of protein. Further, mGST significantly decreased the total number of inflammatory cells as well as eosinophils counts (p<0.01) in BALF indicating reduction in airway inflammation. Lung tissues of mGST treated mice exhibited decreased mucus secreting cells and were comparable to α-lipoic acid treated group (p<0.01) as visualized by PAS staining. There was thick mucus layer due to goblet cell hyperplasia in GST and PBS treated groups.

ROS-generation is enhanced in bronchioalveolar lavage cells of stable asthmatics (Kelly et al., 1988) and increases further after antigen challenge (Sanders et al., 1995). A recent study has also demonstrated the generation of ROS in the airways of ovalbumin-induced asthma model (Lee et al., 2006). There are several markers of oxidative stress and a wide range of antioxidants that are altered in asthma (Shanmugasundaram et al., 2001; Corradi et al., 2003; Brasch-Andersen et al., 2004). In the present study, increased production of ROS in terms of thiobarbituric acid reactive substances (TBARS) was
observed in BALF and lung tissue in ovalbumin sensitized mice. Biological specimens contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of anti-oxidants. mGST or GST treatment by inhalation showed reduction in the TBARS level showing reduction in oxidative stress. Despite better anti-inflammatory effect of mGST than GST, both the proteins showed similar antioxidant response.

Inhalation route is preferred for the delivery of drug directly into the affected organ in asthma or other immune-inflammatory disorder. This may cause less side effects, but the dose delivered is low compared to oral and intravenous routes. The standard antioxidants like α-lipoic acid are prescribed through oral route. But, being a small molecule, it could be cleared easily from the system. mGST a large molecule, will be retained in the system for longer time. In addition, it has shown both the properties as an antioxidant and immunomodulator in ameliorating the Th2 response. But mGST may be modified further to enhance its half life or block the protease cleavage site to prevent degradation.

The increase in ROS during asthmatic attack might overwhelm endogenous antioxidant defenses. Airway glutathione is increased in asthmatics, but the ratio of oxidized to reduced glutathione is also increased. The increase in reduced glutathione is considered as an adaptive response; however, antioxidants namely ascorbate and α-tocopherol are decreased and superoxide dismutase activity is diminished in cells from lavage (Smith et al., 1997; Kelly et al., 1999). Oxidant/antioxidant balance in the airways is important for asthma and improvement in the antioxidant status is likely to correct the inflammatory process. Two strategies are suggested to treat oxidative stress in asthma, firstly, reducing exposure to oxidants and secondly, augmenting antioxidant defenses.

In conclusion, the present study demonstrates that mGST administration by i.n. route limits oxidative stress and attenuates the Th2 immunological responses in mouse model of allergic airway disease.