

Upregulation of ADAM8 in the Airways of Mice with Allergic Bronchial Asthma

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Abstract Recent microarray analyses revealed that a disintegrin and metalloproteinase (ADAM) 8 (ADAM8; also called CD156) is one of the asthma candidate genes. However, the function of ADAM8 and its localization in the airways are still poorly understood. In the present study, the changes in the expression and localization of ADAM8 in the airways of a mouse model of allergic bronchial asthma were investigated. Male BALB/c mice were sensitized and repeatedly challenged with ovalbumin antigen to induce asthmatic response. After the final antigen challenge, mRNA and protein expressions of ADAM8 were elucidated by quantitative RT-PCR and immunohistochemistry. The mRNA expression of ADAM8 in the airways was significantly increased in this animal model of asthma compared with naive animals. Immunohistochemical examinations revealed that ADAM8 was located in airway epithelia, airway smooth muscles, and infiltrated cells (mainly macrophages) into lung parenchyma. A distinctly stronger immunostaining of ADAM8 was observed in these airway cells of the repeatedly antigen-challenged mice compared with those of the sensitized control animals. An upregulation of ADAM8 in the airways might be involved in the pathogenesis of airway inflammation and/or hyperresponsiveness, characteristic features of allergic bronchial asthma.

Keywords Asthma · Asthma candidate gene · Airway inflammation · A disintegrin and metalloproteinase 8 (ADAM8) · Immunohistochemistry

Introduction

Asthma is one of the most common chronic disorders throughout the world and has been the focus of clinical and public health interventions during recent years. Approximately 300 million people in the world currently have asthma [1]. Asthma involves a complex interaction of airflow obstruction, airway hyperresponsiveness, underlying inflammation, and airway remodeling [2].

Zimmermann et al. [3] performed DNA microarray profile analysis of mice undergoing experimental asthma to elucidate novel pathways involved in the pathogenesis of allergic asthma. To identify allergen-induced genes in the lung that were not specific to a particular experimental regime, they used two different antigens—ovalbumin (OA) and *Aspergillus fumigatus*—for induction of experimental asthma. They demonstrated that 291 genes of the induced transcripts were overlapped between the two experimental asthma models. Among the genes, a disintegrin and metalloproteinase (ADAM) 8 was identified as one of the asthma candidate genes [3, 4]. ADAM8 is one of the ADAMs family, which belong to the zinc protease superfamily. From their unique structure, the ADAMs family has been thought to be involved in diverse physiologic events [5]. They have been implicated in the control of membrane fusion, cytokine and growth factor shedding, and cell migration, as well as processes such as muscle development, fertilization, and cell fate determination [6]. The domain structure of the ADAMs consists of a signal domain,

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a prodomain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain. The metalloproteinase domain, which has consensus HEx-xHxxGxxH site, involves enzymatic activity. ADAM8 also is able to convert membrane-bound CD23 into its soluble form, which might be involved in the regulation of IgE synthesis and the activation of macrophages to release a variety of proinflammatory mediators [7–11].

As described above, the microarray analyses revealed that ADAM8 might contribute to the pathogenesis of asthma [3, 4]. Furthermore, it has been reported that ADAM8 is increased in sputa and endobronchial biopsies of patients with asthma [12, 13]. The ADAM8 mRNA expression is increased with disease progression in asthma. The ADAM8 is strongly induced by allergens in the lung in experimental asthma [14]. However, temporal regulation of the expression and function of ADAM8 and its localization in the airways after antigen exposure are still poorly understood. In the present study, the time-course changes in the expression and localization of ADAM8 in the airways of repeatedly antigen-challenged mice were investigated to determine whether ADAM8 has relevance to allergic bronchial asthma.

Materials and Methods

Animal Treatments

Male BALB/c mice (age 7 weeks; specific pathogen-free; Charles River Japan, Inc.) were housed for appropriate time intervals in the Animal Center of Hoshi University after their arrival. Constant humidity ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$) was maintained with a fixed 12-hour light-dark cycle and free access to food and water. Preparation of a mouse model of allergic bronchial asthma, which has airway hyperresponsiveness (AHR), was performed as described previously [15]. In brief, the mice were sensitized with ovalbumin (OA; 8 μg) together with alum (2 mg) as an adjuvant and were boosted 5 days later (both intraperitoneal injections). Twelve days after the first immunization, the mice were challenged by inhaling aerosolized OA-saline solution (5 mg/ml) for 30 minutes under conscious state. Then the animals were subjected to totally three times repeated antigen challenge every 96 h with the same inhalational challenge method. One, 6, 12, or 24 h after the last antigen challenge, the animals were killed. Age-matched, nonsensitized normal and/or sensitized (saline inhalation instead of antigen challenge) animals were used as controls. All experiments were approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

RNA Isolation and Quantitative RT-PCR

The airway tissues below the main bronchi to the lungs were removed ($n = 5\text{--}7$ in each group) and immediately soaked in oxygenated PBS buffer. The tissues were carefully cleaned of adherent connective tissues and blood vessels. Then the lungs with the main bronchi were quickly frozen in liquid nitrogen and crushed into powders with pestle and mortar. Total RNA was isolated from each frozen sample with a one-step guanidium-phenol-chloroform extraction procedure using TRI reagent. The mRNA levels of ADAM8 were examined by quantitative RT-PCR. Briefly, cDNAs were prepared from the total RNA (1 μg) by using QuantiTect Reverse Transcription kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Then the RT reaction mixture (1 μl) was subjected to real-time PCR analyses using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The thermal cycle profile used was 1) denaturing for 15 seconds at 95°C , 2) annealing primers for 30 seconds at 55°C , and 3) extending the primers for 30 seconds at 72°C . The PCR amplification was performed at 40 cycles with monitoring fluorescence. The primer sets used were: QuantiTect Primer Assay, QT00115563 for ADAM8, QT0019388 for GAPDH (Qiagen Inc.).

Quantitation of Ovalbumin (OA)-Specific IgE in Serum

Blood samples were obtained from the animals used in the above RT-PCR study ($n = 5\text{--}7$ in each group). Serum was obtained from the blood sample by centrifugation at 3,000 g for 10 minutes at 4°C . The OA-specific IgE in serum was measured by ELISA as previously described [16]. For lack of standard OA-specific murine IgE, the OA-specific IgE level in each group was expressed as optical density (OD) at 450 nm.

Immunohistochemistry

Lungs of five animals in each group were fixed in 4% paraformaldehyde in PBS and embedded in Paraplast X-TRATM paraffin (Fisher Healthcare, Houston, TX) as previously described [16]. Four-micrometer sections were obtained from blocks and mounted on silane-coated glass slides, and deparaffinized with xylene and graded ethanol. For the immunohistochemical examination, the sections were immunostained with rabbit anti-ADAM-8 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) by using Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. In brief, before immunostaining, rehydrated sections were pretreated by incubation in 0.5% Triton X-100 in PBS for 10 minutes for permeabilization and were immersed in

0.3% hydrogen peroxide in 100% methanol for 30 minutes to remove endogenous peroxidase activity. The pretreated sections were washed with PBS and incubated in 5% skim milk in PBS for 30 minutes at room temperature. The sections were then rinsed and incubated sequentially at room temperature in the following solutions: 1) primary antibody (diluted 1:100 in PBS containing 1% skim milk) overnight, 2) biotinylated goat anti-rabbit IgG (diluted 1:200 in PBS containing 1% skim milk) for 30 minutes, and 3) avidin-biotinylated peroxidase complexes in PBS for 30 minutes. The bound peroxidase activity was visualized by incubation with 0.7 mg/ml 3,3'-diaminobenzidine tetrahydrochloride-0.02% H₂O₂ in 60 mM Tris buffer (pH 7.5). Sections were rinsed in PBS after each step of the immunostaining procedure. Finally, the sections were counterstained with hematoxylin QS (Vector Laboratories, Inc.), dehydrated, and mounted in permanent mounting medium. For each of the lung specimens, the extent and intensity of staining were graded on a scale of 0–4 + by three blinded observers on three separate occasions using coded slides as previously reported [17]. A 4 + grade implies maximally intense staining, whereas 0 implies no staining.

Statistical Analyses

All data were expressed as mean with standard error. Statistical significance of difference was determined by Bonferroni/Dunn test (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC). A value of $p < 0.05$ was considered significant.

Results

Change in ADAM8 mRNA Expression in the Airways After the Antigen Challenge

As shown in the upper panel of Fig. 1, the sensitized control animals (that received saline inhalation instead of antigen challenge) already had significant increase in serum OA-specific IgE compared with normal control. An increase in the OA-specific IgE level also was observed in the antigen-challenged mice (Fig. 1, upper panel).

The quantitative analyses for mRNA expression of ADAM8 were determined by real-time RT-PCR. Both melting curve analyses and agarose gel electrophoresis of the final amplicons revealed that the PCR amplification generated a single amplicon corresponding to the ADAM8 cDNA under the condition used (data not shown). The lower panel of Fig. 1 shows the mRNA expression levels of the ADAM8 in mouse airways after the repeated antigen challenge. The ADAM8 mRNA expression was

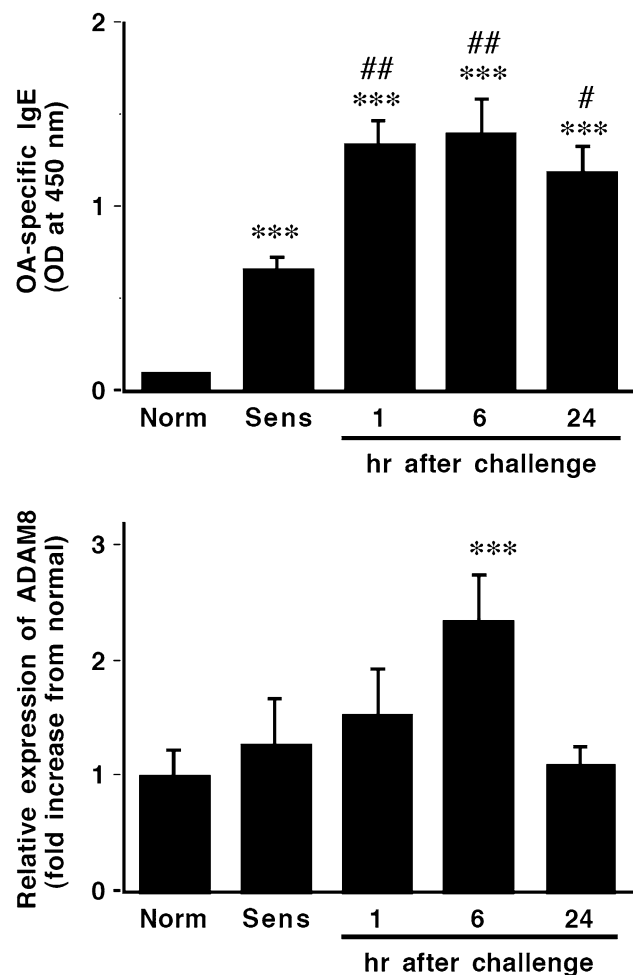


Fig. 1 Changes in ovalbumin (OA)-specific IgE in sera and ADAM8 mRNA expressions in lungs of mice. (*Upper panel*) The serum OA-specific IgE was measured by ELISA. Data represent the mean $\pm$ standard error (SE) from six or seven different animals, respectively. *** $P < 0.001$ vs. normal control (Norm) and # $P < 0.05$, and ## $P < 0.01$ vs. Sensitized control (Sens), which underwent i.p. injections of OA and inhalation of saline instead of OA, by Bonferroni/Dunn test. (*Lower panel*) Total RNAs were extracted from the airways 1, 6, and 24 h after the last antigen challenge and were used for real-time RT-PCR analyses. The levels of ADAM8 mRNA in lungs of each group of animals were expressed as fold increase from those of normal mice. Data represent the means $\pm$ SE from six or seven different animals, respectively. The expression of the ADAM8 mRNA was significantly increased in the airway tissues at 6 h after the last challenge (*** $P < 0.001$ vs. Norm by Bonferroni/Dunn test)

significantly increased at 6 h after the last antigen challenge ($P < 0.001$). However, the increased mRNA expression returned to the control level at 24 h after the antigen challenge.

Localization of ADAM8 in Lung Sections

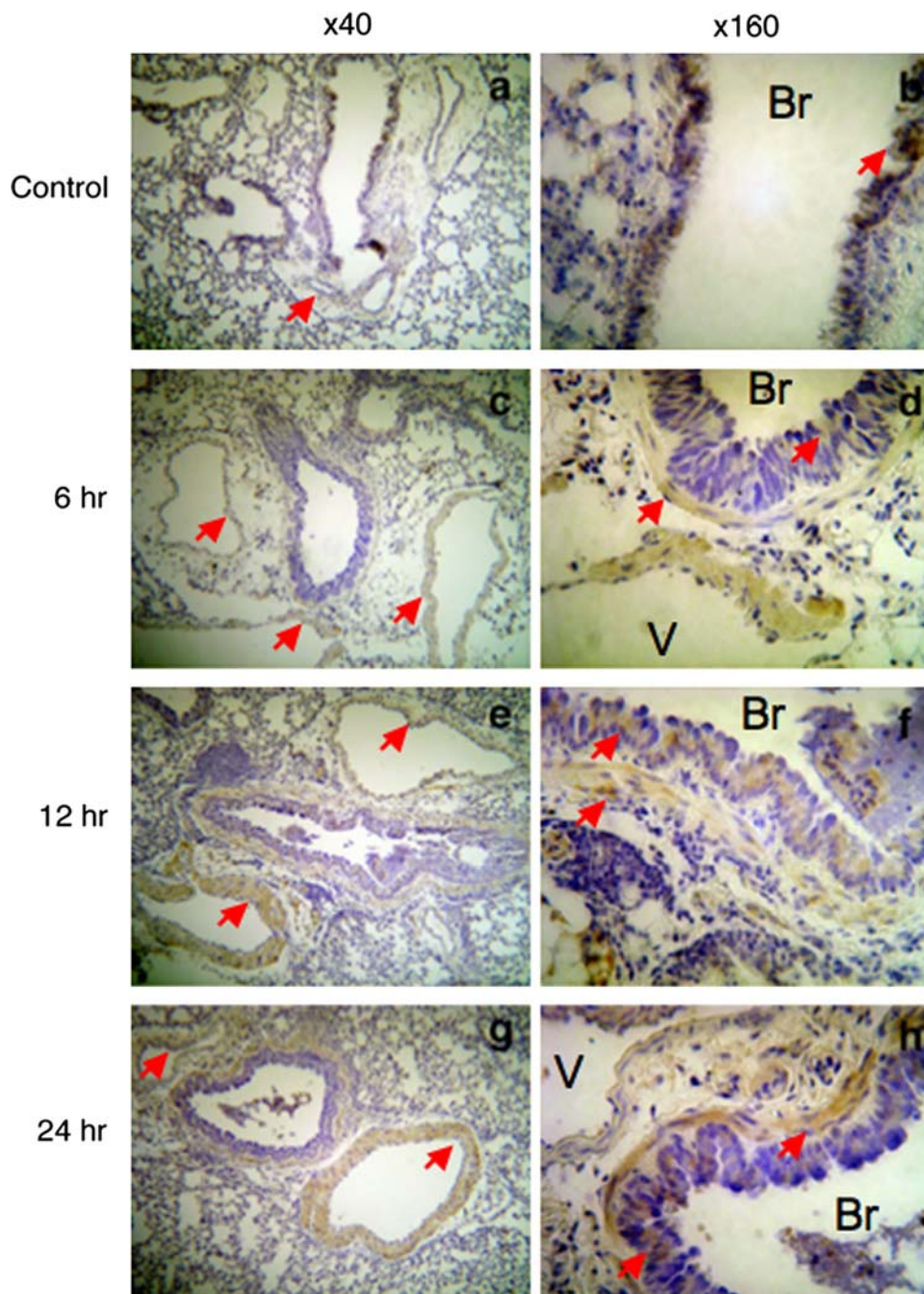
Immunohistochemical examinations revealed an ADAM8 immunostaining in airway epithelia, airway smooth muscles, infiltrated cells (mainly macrophages were stained)

into lung parenchyma, and vascular smooth muscles. The signals were markedly stronger in sections of the challenged group compared with those of the sensitized control group (Fig. 2). Quantitative scores of the immunoreactivities were significantly increased at 12 and 24 h after the antigen challenge (Fig. 3). The results also indicated that protein upregulation of ADAM8 was delayed from the increase in mRNA: the maximal increase in ADAM8 mRNA was observed at 6 h after the last antigen challenge (Fig. 1, lower panel).

Discussion

In the present study, the changes in the expression of ADAM8 in the airways were investigated in a mouse model of allergic bronchial asthma that has marked airway inflammation and bronchial smooth muscle hyperresponsiveness [15]. The mRNA expression of ADAM8 was significantly increased in this animal model of asthma. Immunohistochemical examinations revealed that ADAM8 is located in airway epithelia, airway smooth muscles,

Fig. 2 Immunohistochemistry for ADAM8 in lung tissues of mice. Lung sections of the sensitized control (which underwent i.p. injections of OA and inhalation of saline instead of OA; Control) and 6, 12, and 24 h after the last antigen challenge were examined. The photos shown are representative of five different animals in each group. Immunoreactive ADAM8 was detected in the airway epithelial cells, airway smooth muscle cells, and infiltrated cells (mainly macrophages were stained). Original magnification: **a, c, e,** and **g**, $\times 40$; **b, d, f,** and **h**, $\times 160$. Br: bronchus, V: blood vessel. Arrows in **a, c, e,** and **g**: distinctly stained vascular smooth muscles. Arrows in **b, d, f,** and **h**: distinctly stained airway epithelia and bronchial smooth muscles



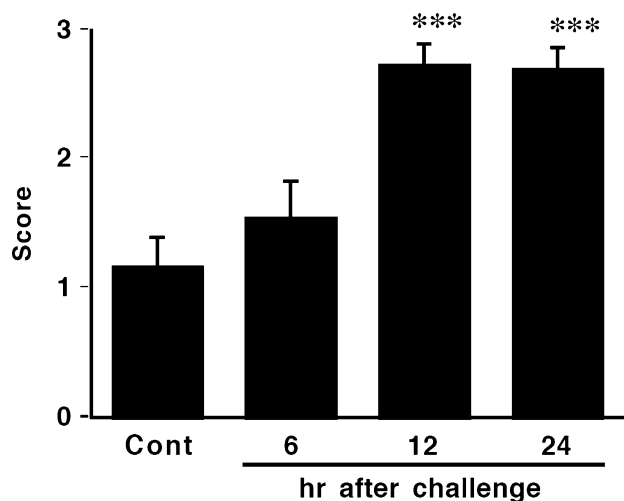


Fig. 3 Extent and intensity of staining with anti-ADAM8 antibody in lung tissues of mice. Lung sections of the sensitized control (which underwent i.p. injections of OA and inhalation of saline instead of OA; Control) and 6, 12, and 24 h after the last antigen challenge were examined. The immunoreactive scores of individual lung specimens were measured by three blinded observers as described in *Materials And Methods*. Data represent the mean $\pm$ standard error from five different animals, respectively. The scores of ADAM8 immunoreactivity were significantly increased in the lungs at 12 and 24 h after the last challenge (*** $P < 0.001$ vs. Normal by Bonferroni/Dunn test)

infiltrated cells into lung parenchyma, and vascular smooth muscles. A distinctly stronger immunostaining of ADAM8 was observed in these airway cells of the repeatedly antigen challenged mice. Thus, upregulation of ADAM8 in the airways may be involved in the pathogenesis of airway inflammation and/or hyperresponsiveness induced by antigen exposure.

ADAM8 was cloned in a monocytic cell lineage and the gene was mapped to human chromosome 10q26.3, an area syntenic to mouse chromosome 7, region F3-F4 [18–21]. Reportedly, ADAM8 may play a role in inflammation by regulating functions of neutrophils [22, 24]. Higuchi et al. [22] produced a mutant ADAM8 transgenic mouse expressing the ectodomain of ADAM8. The expression of L-selectin in neutrophils from peripheral blood leukocytes was more strongly downregulated in the transgenic mice than nontransgenic ones, suggesting that L-selectin in neutrophils from transgenic-mice was shed by sADAM8. A correct regulation of the L-selectin ectodomain processing seems to be necessary for an efficient transendothelial migration of neutrophils [23, 24]. On the other hand, our previous study revealed that main inflammatory cells infiltrated into the airways were eosinophils, but not neutrophils, in this animal model of allergic bronchial asthma [25]. It also has been reported that ADAM8 is associated with the eosinophilic responses and lung inflammation in humans, although the functional relevance is not yet clear [26].

Currently, the expression of ADAM8 mRNA was increased in the airways of mice with allergic bronchial asthma; the peak increment was observed at 6 h after the last antigen challenge (Fig. 1). The time-dependent increase in ADAM8 mRNA is consistent with the previous study [4]. However, it seems that our mouse model, which received three times antigen challenge, showed stronger upregulation compared with the previous report by King et al. [4], in which mice received twice antigen challenges. Antigen exposure to sensitized mice could upregulate the expression of ADAM8 mRNA. It is not yet clear whether the upregulation is a protective reaction or a progressive reaction of allergic responses. Alternatively, the findings that the upregulation of ADAM8 mRNA (6 h after the antigen challenge; Fig. 1) was observed earlier than the peaks of airway inflammation and bronchial hyperresponsiveness (12–24 h after) [16, 25] may suggest its causable role in asthma phenotypes.

Reportedly, immunohistochemical and in situ hybridization analyses revealed that the origin of ADAM8 in lungs is epithelial cells and inflammatory cells but not mesenchymal cells, such as smooth muscle cells and fibroblasts [4, 12, 13]. In the present study, however, the ADAM8 immunostaining also was observed not only in airway epithelial cells and inflammatory cells (mainly macrophages) but also in bronchial and vascular smooth muscle cells (Fig. 2). ADAM8 is initially identified in mouse macrophages and macrophage cell lines by using a differential display approach [18, 19]. The role of the macrophage-derived ADAM8 is not identified now, but ADAM8 is upregulated in response to macrophage activation [18]. On the other hand, this is the first study, to our knowledge, that demonstrated the ADAM8 expression in airway smooth muscle. Although the reason for the discrepancy between the previous and the current studies is not known, our preliminary study using RT-PCR also revealed a distinct expression of ADAM8 mRNA in cultured human bronchial smooth muscle cells (data not shown). Further studies are needed to clarify the roles of ADAM8 in macrophages and airway and vascular smooth muscle cells.

One of the most important roles of ADAMs might be shedding of substrates from membrane surface. Cytokines and their receptors play important roles in the development of airway inflammation. ADAM8 is able to convert membrane-bound CD23 into a soluble form that has been shown to be involved in immunological events, such as regulation of IgE synthesis [9–11]. Notably, soluble CD23 has been shown to be elevated in such diseases as asthma and rheumatoid arthritis, suggesting a possible involvement of ADAM8. Additionally, it is reported that CD23 negatively regulates pulmonary inflammation and airway hyperresponsiveness [27–29]. In contrast, soluble fragments of

CD23 both positively and negatively regulate IgE synthesis in human B cells [30]. Although it has not been elucidated how ADAMs can act on allergic bronchial asthma, it is possible that immunological events are controlled by regulating membrane/soluble CD23 balance by ectodomain shedding.

Conclusions

The current findings revealed an upregulation of ADAM8 in association with the pathogenesis of allergic bronchial asthma. The present study also demonstrated the expression of ADAM8 in airway smooth muscle cells. Although further studies are needed to clarify the detailed relationship between ADAM8 and allergic bronchial asthma, it is of interest that ADAM8 might have functional role in airway smooth muscles—one of the factors for the development of the airway hyperresponsiveness in asthmatics.

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