

Analysis of induced sputum for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients

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Summary

Background Allergen-specific IgE antibodies have been considered to play an important role in the pathogenesis of atopic asthma. However, studies on allergen-specific IgE antibodies in airway secretion from asthmatic patients are very rare compared with those in serum.

Objectives The present study was undertaken to determine whether induced sputum might provide a useful method for analysing allergen-specific IgE antibodies in airway secretions from asthmatic patients.

Methods Specific IgE antibodies to house dust mite (HDM) antigen were measured in induced sputum from 10 HDM-sensitive asthmatic patients and 12 non-allergic controls by enzyme-linked immunosorbent assay. HDM-specific IgE was regarded as positive when the absorbance value was higher than mean + 2 SD of controls. Their antigen-binding characteristics were determined by immunoblot analysis.

Results HDM-specific IgE was positive in induced sputum from seven of 10 HDM-sensitive asthmatics. The IgE binding to HDM antigen could be inhibited by fluid phase HDM antigen in a dose-dependent manner, not by mugwort antigen. Treatment of induced sputum with dithiothreitol decreased the antigen-specific bindings, and increased the non-specific bindings on the measurement of HDM-specific IgE. These effects were significant in a concentration of dithiothreitol greater than 0.05%. Immunoblot analysis revealed that HDM-specific IgE antibodies in induced sputum recognized the HDM antigens with molecular weights of 42, 34, 32, 25 and 14 kDa. These antigen binding characteristics were similar to those in serum.

Conclusion We conclude that analysis of induced sputum is a useful non-invasive method for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients.

Keywords: induced sputum, specific IgE, asthma, house dust mite

Clinical and Experimental Allergy, Vol. 28, 686–693. Submitted 6 June 1997; revised 11 September 1997; accepted 5 January 1998.

Introduction

Allergen-specific IgE antibodies have been considered to play an important role in the pathogenesis of atopic asthma [1]. However, studies on allergen-specific IgE antibodies from the bronchial mucosa of asthmatic patients are very rare compared with those from serum [2,3]. Although there have been a few reports on measurement of allergen-specific IgE antibodies in airway secretion from asthmatic patients,

the detection method is not well standardized yet [2,3]. This may be at least partly due to difficulty in obtaining airway secretion from asthmatic subjects. The other factor might be that total IgE concentration in airway secretion from asthmatic patients is very low relative to that in serum [4].

Bronchoalveolar lavage (BAL) and bronchial lavage (BL) methods have been used to study biochemical constituents of airway secretion, but their use has been limited by discomfort, inconvenience, and risks [5,6]. Although examination of sputum is a less invasive alternative [7], sputum cannot always be produced spontaneously. Recently, examination of sputum induced by inhalation of

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Table 1. Characteristics of HDM-sensitive asthmatic patients

Subject no.	Sex	Age (years)	FEV ₁ *	PC ₂₀ † (mg/mL)	HDM-specific IgE‡ (IU/mL)	Medication§
1	F	28	96	4.12	9.29	BA, IS, P
2	F	29	75	1.98	4.27	BA, IS
3	M	46	78	0.53	0.69	BA
4	M	42	61	0.36	1.68	BA, IS, P
5	F	28	106	2.20	7.27	BA
6	F	36	91	1.21	7.28	BA, IS
7	M	19	59	0.47	42.76	BA, IS, P
8	F	25	117	0.40	37.24	BA, IS
9	F	32	75	0.27	19.57	BA, IS
10	F	31	88	0.54	23.88	BA, IS, P

* FEV₁ corrected for race, sex, age, and height. † The concentration of methacholine solution, which after inhalation, provokes a fall of 20% in FEV₁. ‡ Serum *D. farinae*-specific IgE was measured by AlaSTAT kit (Diagnostic Product Corporation, Los Angeles, CA, USA). § BA, Inhaled beta-agonist; IS, inhaled steroid; P, oral prednisolone.

aerosolized hypertonic saline was developed for investigation of airway inflammation in asthma [8,9] showing results that are valid, reliable [10] and responsive [11], and that yield more concentrated airway secretions than those obtained by bronchoscopy [12].

Most previous studies, using induced sputum from asthmatic patients, have focused on inflammatory cells and their mediators [8–12], with few attempts being made to analyse allergen-specific IgE antibodies in induced sputum from these patients. To determine whether induced sputum samples might provide a useful method for studying allergen-specific IgE antibodies in airway secretions from asthmatic patients, we measured specific IgE antibodies to house dust mite (HDM) antigen in induced sputum from asthmatic patients and analysed their antigen-binding characteristics.

Methods

Subjects

Induced sputum samples were collected from 10 HDM-sensitive asthmatic patients who showed a positive skin-prick test to HDM (*Dermatophagoides farinae*: Bencard Co, UK). Positive skin test was defined when the mean weal diameter of allergen was 3 mm greater than that of the negative control (normal saline). All patients had typical clinical history of asthma and airway hyperresponsiveness to methacholine. Airway hyperresponsiveness to methacholine was determined by a method described previously [13]. Subject characteristics are shown in Table 1. Twelve healthy non-atopic and non-asthmatic controls consisting of medical students and medical staff at Ajou University Hospital (five male and seven female) with a mean age of 35 years were recruited. All subjects were non-smokers. This study was approved by the Medical Ethical

Committee of Ajou University Hospital and all subjects gave informed consent.

Induction of sputum and serum sampling

Sputum was induced using a previously described method [9,14]. Immediately before the sputum expectoration, each subject was pretreated with 200 µg salbutamol, administered by means of a metered dose inhaler. The subjects then inhaled nebulized sterile 3% saline solution for 20 min through an ultrasonic nebulizer (Omron Co., Japan). After being instructed to spit out the saliva in the mouth first, the subjects were asked to cough and expectorate sputum into a clean plastic container. In six asthmatics, saliva samples were taken before induction of sputum. Venous blood was sampled from each subject at the same time of sputum induction. Sera was collected and stored at –20°C.

Sputum and saliva processing

Collected sputum and saliva samples were immediately processed. The volumes of saliva and induced sputum were determined, and equal volumes of phosphate buffered saline (PBS) were added. The samples were then mixed by vortex mixer for 1 min and centrifuged for 20 min at 3000 rpm. The supernatants were aspirated and frozen at –20°C.

Measurement of HDM-specific IgE antibodies

HDM-specific IgE antibodies in induced sputum were measured by enzyme-linked immunosorbent assay (ELISA) as previously reported [15]. Microtitre plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA, USA) were coated with 2 µg of *D. farinae* antigen (kindly

provided by Dr Weiss at Diagnostic Products Corporation, Los Angeles, CA, USA) in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. For the evaluation of non-specific binding, bovine serum albumin was coated with 2 µg/well in parallel with pairs of wells coated with *D. farinae* antigen. In the first step, plates were washed three times with PBS containing 0.05% Tween 20 and incubated with 3% bovine serum albumin in PBS for 1 h. Washings were conducted between each step. The plates were incubated with duplicated samples (50 µL) of induced sputum and saliva at 1:2 dilution overnight at 4°C. In the second step, the plates were incubated with biotinylated affinity-purified goat anti-IgE (Vector Laboratories, Inc., Burlingame, CA, USA) at 1:500 dilution (1 µg/mL) for 2 h at room temperature. In the third step, the plates were incubated with peroxidase conjugated streptavidin (Sigma Chemical Co., St. Louis, MO, USA) at 1:1000 dilution (0.5 µg/mL) for 1 h at room temperature. In the final step, the substrate solution consisting of 0.04% (w/v) *o*-phenylenediamine dissolved in 24.3 mM citric acid, 51.4 mM NaH₂PO₄ (pH 5.0) and 0.03% H₂O₂, was added. After 30 min, the reaction was stopped by adding 2.5 N H₂SO₄. Absorbance at 490 nm was measured by a microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). In this condition, there was no significant non-specific binding to the bovine serum albumin coated wells in induced sputum samples from controls and asthmatic patients. The levels of HDM-specific IgE antibodies in induced sputum were expressed as sample absorbance minus background (diluent buffer only instead of induced sputum sample) absorbance. In each plate, serial dilutions of a serum with high level of *D. farinae*-specific IgE antibodies were included. Coefficients of intraplate variation ($n=6$) and interplate variation ($n=6$) were below 15%. Serum *D. farinae*-specific IgE antibodies were measured by AlaSTAT kit (Diagnostic Product Corporation, Los Angeles, CA, USA).

ELISA inhibition

ELISA inhibition was performed using a pooled induced sputum sample from three asthmatic patients. The pooled sputum sample was incubated overnight with different concentrations of *D. farinae* and mugwort pollen antigens or buffer only at 4°C. The sample and antigen mixtures were centrifuged and supernatants were then tested for residual HDM-specific IgE antibodies by ELISA. The results of the inhibition study of HDM-specific IgE antibodies were expressed as the percentage of inhibition, calculated with the following formula:

per cent inhibition =

$$\frac{[\text{IgE buffer only} - \text{IgE antigen inhibition}]/\text{IgE buffer only}}{\times 100}.$$

Effect of dithiothreitol (DTT) on the measurement of HDM-specific IgE antibodies

To evaluate the effect of DTT on the measurement of HDM-specific IgE antibodies, serum and induced sputum samples from an asthmatic patient sensitized to HDM antigen were mixed with equal volumes of various dilutions of DTT (Sigma Chemical Co, St Louis, MO, USA) in PBS and incubated overnight at 4°C. The measurement of HDM-specific IgE antibodies was done by ELISA as above.

Immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously [16] with 14% homogeneous gel. The 9.0 × 8.0 cm mini-gel (Novex, San Diego, CA, USA) was used, and the sample gel was divided into two lanes by the use of a special comb; one 0.35 cm wide for molecular weight marker standards (Novex, San Diego, CA, USA) and another 8.0 cm wide for a sample application. The gel was loaded with 100 µg of *D. farinae* antigen and electrophoresed under unreduced conditions. After electrophoresis, the HDM proteins on the gel were electroblotted onto a polyvinylidene difluoride membrane (Novex, San Diego, CA, USA). Then, the membrane was blocked in 10 mM Tris, 0.15 M NaCl, 10% bovine serum albumin (pH 7.4) for 2 h. Serum and sputum samples from four HDM-sensitive asthmatic patients and three controls were incubated at a 1:2 dilution with the membrane overnight at room temperature using a special incubation device (Multiscreen device; Bio-Rad, Hercules, CA, USA). After three washes, the membrane was incubated with affinity-purified and biotinylated anti-IgE (Vector Laboratories Inc., Burlingame, CA, USA) at 1:500 dilution (1 µg/mL) for 3 h at room temperature. After washing, the membrane was incubated with alkaline phosphatase conjugated streptavidin (Sigma Chemical Co., St Louis, MO, USA) at 1:1000 dilution (0.5 µg/mL) for 1 h at room temperature. Finally, after new washes, the membrane was incubated with the substrate solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; Sigma Chemical Co., St Louis, MO, USA). The reaction was stopped after 10 min by rinsing the membrane in distilled water.

Statistics

Data were expressed as mean ± standard deviation (SD). The Mann-Whitney *U* test was used to assess the differences between groups. Spearman's rank analysis was used to evaluate the correlation between data. Wilcoxon signed-rank test was used to compare HDM-specific IgE levels in paired induced sputum and saliva from asthmatic patients.

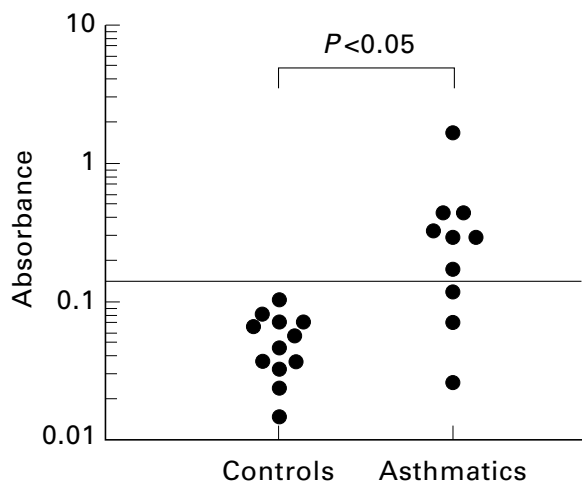


Fig. 1. Levels of HDM-specific IgE antibodies (expressed as absorbance values) in induced sputum from 12 controls and 10 HDM-sensitive asthmatics. The horizontal line indicates mean absorbance + 2 SDs of induced sputum samples from 12 controls.

Results

Measurement of HDM-specific IgE antibodies in induced sputum

When HDM-specific IgE levels in induced sputum were expressed as absorbance values, they were significantly higher in HDM-sensitive asthmatics (0.391 ± 0.477) than controls (0.068 ± 0.030) ($P < 0.05$, Fig. 1). When the absorbance of sample was higher than mean + 2 SDs of absorbance values

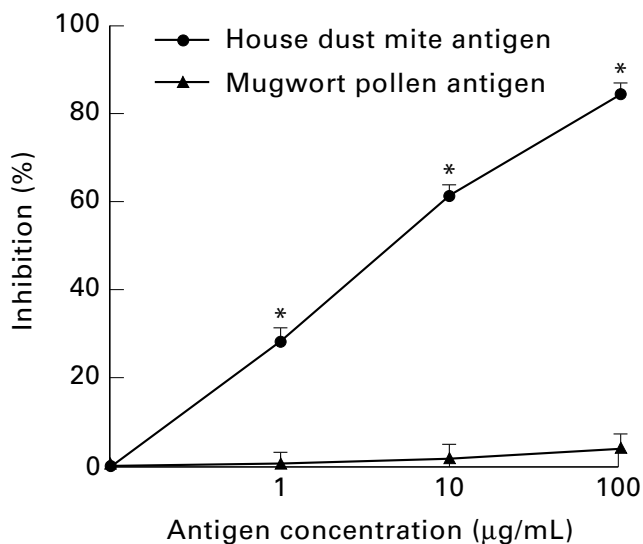


Fig. 2. ELISA inhibition of HDM-specific IgE antibodies with addition of HDM and mugwort pollen antigens. Values are presented as the mean and standard deviation of quadruplicated measurements. *Significantly inhibited by HDM antigen compared with mugwort antigens ($P < 0.05$).

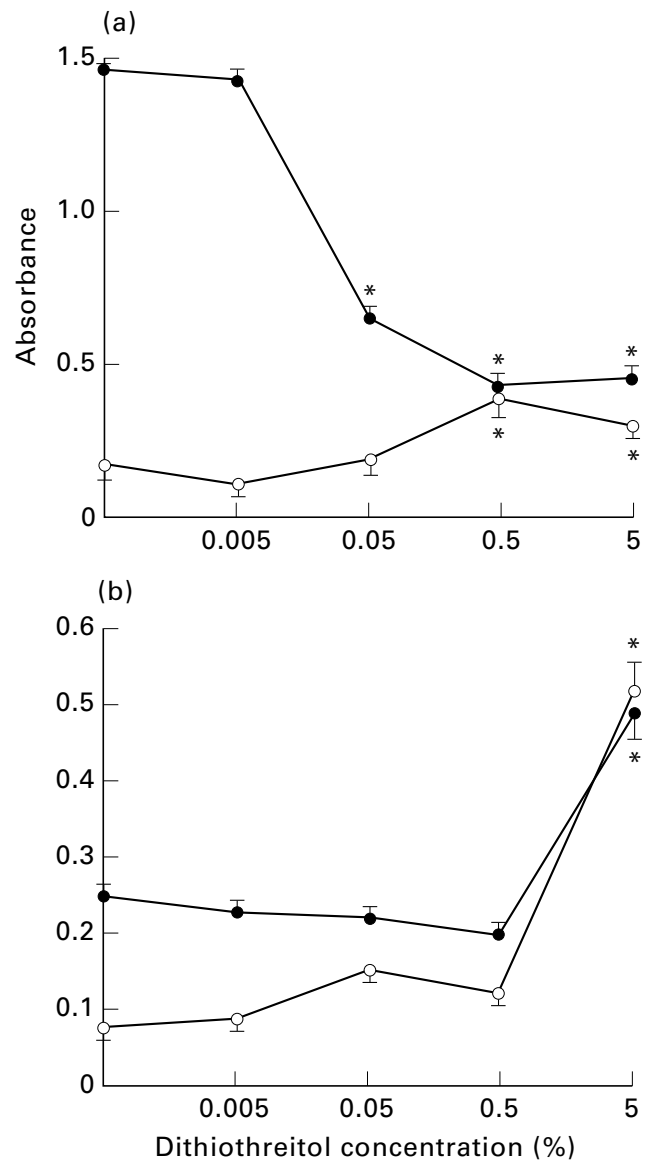


Fig. 3. Effect of reducing agent (dithiothreitol) on the measurement of HDM-specific IgE antibodies in serum (a) and induced sputum (b) from a HDM-sensitive patient expressed as specific binding to HDM antigen coated wells (●) and non-specific binding to bovine serum albumin coated wells (○). Values are presented as the mean and standard deviation of quadruplicated measurements. * Significantly different from the corresponding baseline values.

from 12 controls, it was regarded as a positive result for HDM-specific IgE antibodies. HDM-specific IgE was positive in induced sputum from seven of 10 HDM sensitive asthmatics.

ELISA inhibition of HDM-specific IgE antibodies

The specificity of the HDM-specific IgE antibody bindings was determined by comparing the ability of fluid phase

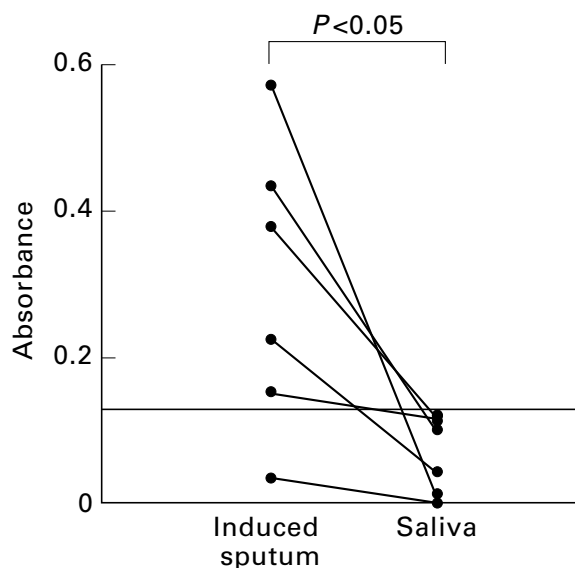


Fig. 4. Levels of HDM-specific IgE antibodies in induced sputum and saliva from six HDM-sensitive asthmatics. The horizontal line indicates a cut off value derived from mean absorbance + 2 SDs of induced sputum samples from 12 controls.

HDM antigen and irrelevant antigens from mugwort pollen to inhibit the binding of a pooled sputum sample. Soluble HDM antigen significantly inhibited the specific IgE bindings in a dose-dependent manner with minimal inhibition by mugwort antigen ($P < 0.05$, Fig. 2).

Effect of DTT on measurement of HDM-specific IgE antibodies

Treatment of serum and induced sputum with DTT decreased the antigen-specific bindings, and increased the non-specific bindings on the measurement of HDM-specific IgE antibodies. These effects were significant in a concentration of DTT greater than 0.05% ($P < 0.05$, Fig. 3).

Levels of HDM-specific IgE antibodies in induced sputum and saliva from the six HDM-sensitive asthmatic patients

In six HDM-sensitive asthmatic patients, the levels of HDM-specific IgE antibodies in induced sputum were significantly higher than those in saliva ($P < 0.05$), and none of the saliva samples had positive HDM-specific IgE antibody bindings (Fig. 4).

Correlation of HDM-specific IgE levels between serum and induced sputum from HDM-sensitive asthmatics

In HDM-sensitive asthmatic patients, there was a correlation in HDM-specific IgE levels between serum and induced sputum ($P = 0.05$, Fig. 5).

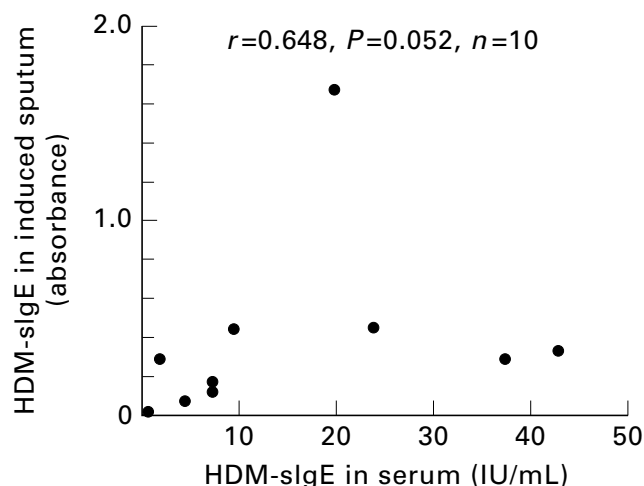


Fig. 5. Correlation of HDM-specific IgE (sIgE) levels between serum and induced sputum in HDM-sensitive asthmatics.

Correlation between asthma severity and HDM-specific IgE levels in serum and induced sputum

Degree of airway obstruction expressed as FEV₁ (%) values which were measured before sputum sampling did not significantly correlate with HDM-specific IgE levels in serum ($r = 0.11$, $P = 0.76$) or those levels in induced sputum ($r = -0.16$, $P = 0.62$). Degree of bronchial hyper-responsiveness expressed as methacholine PC₂₀ values did not significantly correlate with HDM-specific IgE levels in serum ($r = -0.20$, $P = 0.55$) or those levels in induced sputum ($r = -0.22$, $P = 0.32$).

Immunoblot analysis

IgE-binding bands could be observed in three of four induced sputum samples from HDM-sensitive asthmatics, and none from three controls. HDM-specific IgE antibodies in induced sputum recognized the HDM antigens in molecular weights of 42, 34, 32, 25 and 14 kDa. These antigen binding characteristics were similar to those in serum (Fig. 6).

Discussion

This study confirms the presence of allergen-specific IgE antibodies in airway secretion from asthmatic patients using ELISA and immunoblot analysis. For demonstration of the presence of allergen-specific IgE antibodies in a certain sample by solid phase immunoassay, it is widely recommended that several criteria be fulfilled [17]. First, the possibility of non-specific bindings by the individual samples should be checked in newly developed assay, as various factors within antigen, sample (such as reducing agent) and

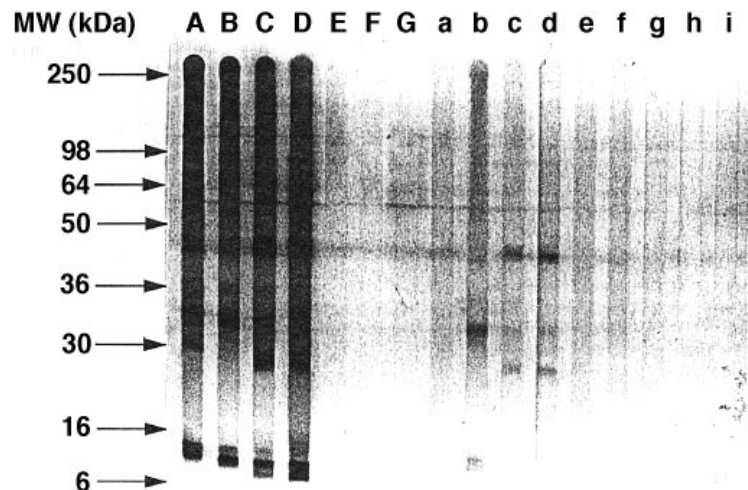


Fig. 6. Immunoblot analysis of HDM-specific IgE antibodies in induced sputum and serum samples. A–D, serum samples from HDM-sensitive asthmatics. E–G, serum samples from controls. a–d, induced sputum samples from HDM-sensitive asthmatics (matched samples to serum samples of A–D). e–g, induced sputum samples from controls (matched samples to serum samples of E–G). h and i, diluent only as negative control.

assay procedure may affect the non-specific bindings [18]. The antigen-specific antibody binding of the sample should be significantly greater than non-specific binding of the same sample when tested using an irrelevant antigen under the same conditions. Secondly, it is acceptable for the level of allergen-specific antibody binding to be at least greater than mean plus two standard deviations of samples from non-atopic controls. Thirdly, allergen-specific antibody binding should be inhibited by the same allergen by at least 80%, and not by any irrelevant antigen. Previous studies on allergen-specific IgE antibodies in airway secretion did not completely verify these criteria [2,3]. In this study, we demonstrated the presence of allergen-specific IgE antibodies in airway secretion from asthmatic patients by ELISA based upon the above three criteria and confirmed it by immunoblot analysis.

The method for measurement of allergen-specific IgE in airway secretion from asthmatic patients has not been standardized yet. Previous reports [2,3] expressed their data on allergen-specific IgE in airway secretion from asthmatic patients as absorbance or cpm value. There are very low concentrations of IgE antibodies in airway secretion from asthmatic patients [4]. In this study, when compared with serial dilutions of a serum with a known amount of HDM-specific IgE antibodies, estimated level of HDM-specific IgE in sputum sample with highest absorbance value was below 0.5 IU/mL. Because the parallelism between standard and most induced sputum samples could not be proven in this study due to the extremely low concentration of allergen-specific IgE in induced sputum, the level of HDM-specific IgE was expressed as absorbance value in this study. Further studies are needed to standardize the method for measurement of allergen-specific IgE in airway secretion.

IgE antibody in airway secretion has shown to be locally produced in bronchial mucosa [19,20]. Use of BAL fluid for detecting allergen-specific IgE antibodies in airway secretion seems to be inadequate because IgE antibodies in BAL fluid are most likely to be passively diffused out of circulation due to easy exchange of immunoglobulins through alveolar spaces [2]. For this reason, use of bronchial lavage (BL) fluid was recommended for the study of locally produced allergen-specific IgE antibodies in bronchial mucosa from asthmatic patients [2]. Bronchoscopy cannot easily be applied repeatedly because of its invasiveness and risk in procedure. Recent studies suggested that the examination of induced sputum may be an effective, non-invasive method to obtain airway secretion from asthmatic patients [10], and may yield more concentrated samples including higher concentration of inflammatory markers than those obtained by bronchoscopy [12]. Cellular characteristics and inflammatory markers in induced sputum correlated more closely with those in BL than in BAL [12]. In this study, we could detect the allergen-specific IgE antibodies in induced sputum without further concentration. These results suggest that induced sputum is a useful method for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients.

Induced sputum is a mixture of lower respiratory secretion and saliva [21]. There are two methods for analysing fluid component of induced sputum. One is by extracting mucus plugs from sputum samples to minimize salivary contamination [10]. The other is by analysing the entire induced sputum sample and by comparing it with results from saliva [9]. In this study, the latter method was used because selection of mucus plug provided more viscous samples, and smaller amounts of specimens for fluid phase

analysis. The levels of allergen-specific IgE antibodies in induced sputum samples from asthmatic patients were significantly higher than those in salivary samples. This result suggests that allergen-specific IgE antibodies in induced sputum may originate from subglottic airways.

For the study of cellular and biochemical components in induced sputum, most studies [8–12] used DTT to decrease the viscosity of sputum samples because it breaks the disulfide bonds of mucinous proteins present in sputum and this processing is essential for cellular analysis of sputum. However, this process can potentially affect antigen-binding capacity of IgE antibodies by splitting inter-heavy-light chain disulfide bond [22]. In this study, addition of DTT to sputum and serum samples decreased the allergen-specific IgE bindings and increased non-specific bindings. However, these effects were significant in a concentration of DTT greater than 0.05% which is a much higher concentration than the final concentration currently used for processing of sputum [9,10]. In addition, the sputum sample was incubated with DTT overnight at 4°C in this study and this condition is different from the sputum processing condition of most investigators in that induced sputum is incubated for 15–30 min at 37°C [9,10]. Another method for reducing viscosity of sputum sample is to obtain the sol phase of sputum by ultracentrifugation [23]. However, measurements of the IgE concentrations in the separate sol and gel phases of sputum were not significantly different from the values in the whole homogenized sputum specimen [19]. In this study, whole induced sputum was mixed with equal volume of PBS and the supernatant was separated after centrifugation. No difficulty in processing was noted.

IgE antibodies in respiratory exocrine fluids have same physiochemical and antigenic characteristics with IgE antibodies in serum [24]. However, the antigen-binding characteristics of allergen-specific IgE antibodies in respiratory secretion have not been fully defined. In this study, allergen-specific IgE antibodies from both induced sputum and serum recognized similar proteins. This result is comparable to the previous report showing that antigen-binding characteristics of grass-pollen specific IgE antibodies in tears from patients with rhinoconjunctivitis were similar to those in sera [25].

Allergen-specific IgE antibodies have been considered to play an important role in the pathogenesis of atopic asthma [1]. However, there has been poor quantitative correlation between allergen-specific IgE antibodies and clinical severity or airway hyperresponsiveness and degree of airway inflammation [26]. This might be due to the fact that most previous reports measured allergen-specific IgE antibodies in serum only and compared them with the clinical parameters [27]. Allergen-specific IgE antibodies in nasal secretion could more rapidly change with avoidance of allergen

and more accurately predict current symptoms differently from those in serum [28,29]. In this study, we could not find any significant correlation between allergen-specific IgE in induced sputum and degree of airway obstruction or bronchial hyperresponsiveness in atopic asthmatic patients. Further study will be needed to elucidate the pathogenetic significance of allergen-specific IgE antibodies in airway secretion from asthmatic patients.

In conclusion, this study confirms the presence of allergen-specific IgE antibodies in airway secretion from atopic asthmatic patients. In addition, the induced sputum can be a useful non-invasive method for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients. This approach may improve our understanding of the significance of allergen-specific IgE antibodies in immunopathogenesis of bronchial asthma.

Acknowledgements

This study was funded by Core Research Grants to Dr D.-H. Nahm from Korea Science and Engineering Foundation. The authors would like to thank Professor Heather Yu for critical revision of this manuscript.

References

- 1 Gelber LE, Seltzer LH, Bouzoukis JK et al. Sensitization and exposure to indoor allergens as risk factors for asthma among patients presenting to hospital. *Am Rev Respir Dis* 1993; 147:573–8.
- 2 Crimi E, Scordamaglia A, Crimi P, Zupo S, Barocci S. Total and specific IgE in serum, bronchial lavage and bronchoalveolar lavage of asthmatic patients. *Allergy* 1983; 38:553–9.
- 3 Diaz P, Galleguillos FR, Gonzalez MC, Pantin CF, Kay AB. Bronchoalveolar lavage in asthma: the effect of disodium cromoglycate (cromolyn) on leukocyte counts, immunoglobulins, and complement. *J Allergy Clin Immunol* 1984; 74:41–8.
- 4 Deuschl H, Johansson SGO. Immunoglobulins in tracheobronchial secretion with special reference to IgE. *Clin Exp Immunol* 1974; 16:401–12.
- 5 Kirby JG, Hargreave FE, Gleich GJ, O'Byrne PM. Bronchoalveolar cell profiles of asthmatic and non-asthmatic subjects. *Am Rev Respir Dis* 1987; 136:379–83.
- 6 Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137:62–9.
- 7 Gibson PG, Girgis Gabardo A, Morris MM et al. Cellular characteristics of sputum from patients with asthma and chronic bronchitis. *Thorax* 1989; 44:693–99.
- 8 Pin I, Gibson PG, Kolendowicz R et al. Use of induced sputum cell counts to investigate airway inflammation. *Thorax* 1992; 47:25–9.
- 9 Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126–31.

- 10 Pizzichini E, Pizzichini MMM, Efthimidis A et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid phase measurements. *Am J Respir Crit Care Med* 1996; 154:308–17.
- 11 Claman DM, Boushey HA, Liu J, Wong H, Fahy JV. Analysis of induced sputum to examine the effects of prednisone on airway inflammation of asthmatic subjects. *J Allergy Clin Immunol* 1994; 94:861–9.
- 12 Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995; 152:53–8.
- 13 Chai H, Farr RS, Froehlich LE et al. Standardization of bronchial inhalation challenge procedures. *J Allergy Clin Immunol* 1975; 56:323–7.
- 14 Nahm DH, Park HS. Correlation between IgA antibody and eosinophil cationic protein levels in induced sputum from asthmatic patients. *Clin Exp Allergy* 1997; 27:676–81.
- 15 Subba Rao PV, McCartney-Francis NL, Metcalfe DD. An avidin-biotin microELISA for rapid measurement of total and allergen-specific human IgE. *J Immunol Method* 1983; 57:71–85.
- 16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- 17 Bernstein IL, Storms WW. Practice parameters for allergy diagnostic testing. *Ann Allergy Asthma Immunol* 1995; 75:553–625.
- 18 Dolen WK, Williams PB, Koepke JW, Selner JC. Immunoassay of specific IgE: low level assays require measurement of allergen specific assay background. *Ann Allergy* 1992; 69:151–6.
- 19 Ishizaka K, Newcomb RW. Presence of γ E in nasal washing and sputum from asthmatic patients. *J Allergy* 1970; 46:197–204.
- 20 Tada T, Ishizaka K. Distribution of γ E forming cells in lymphoid tissues of the human and monkey. *J Immunol* 1970; 104:377–87.
- 21 Pizzichini E, Pizzichini MMM, Ethimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996; 9:1174–80.
- 22 Takatsu K, Ishizaka T, Ishizaka K. Biologic significance of disulfide bonds in human IgE molecules. *J Immunol* 1975; 114:1838–45.
- 23 Groeneveld K, Eijk PP, Van Alphen L, Jansen HM, Zanen HC. Haemophilus influenzae infections in patients with chronic obstructive pulmonary disease despite specific antibodies in serum and sputum. *Am Rev Respir Dis* 1990; 141:1316–21.
- 24 Newcomb RW, Ishizaka K. Physicochemical and antigenic studies on human γ E in respiratory fluid. *J Immunol* 1970; 105:85–9.
- 25 Hoffmann-Sommergruber K, Ferreira FD, Ebner C et al. Detection of allergen-specific IgE in tears of grass pollen-allergic patients with allergic rhinoconjunctivitis. *Clin Exp Immunol* 1996; 26:79–87.
- 26 Platts-Mills TAE, Sporik RB, Wheatley LM, Heyman PW. Is there a dose-response relationship between exposure to indoor allergens and symptoms of asthma? *J Allergy Clin Immunol* 1995; 96:435–40.
- 27 Takeda K, Shibasaki M, Takita H. Relation between bronchial responsiveness to methacoline and levels of IgE antibody against Dermatophagoides farinae and serum IgE in asthmatic children. *Clin Exp Allergy* 1993; 23:450–4.
- 28 Sensi LG, Piacentini GL, Nobile E et al. Changes in nasal specific IgE to mites after periods of allergen-exposure avoidance: a comparison with serum levels. *Clin Exp Allergy* 1994; 24:377–82.
- 29 Otolani C, Miadonna A, Adami R, Restuccia M, Zanussi C. Correlation of the specific IgE in serum and nasal secretions with clinical symptoms in atopsics. *Clin Allergy* 1981; 11:249–56.