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Original Article

Anti-endothelial cell IgE antibodies in children with bronchial asthma

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Abstract

Background: There has recently been an accumulation of evidence suggesting that endothelial cells (EC) play a crucial role in the pathogenesis of bronchial asthma. We examined the prevalence and isotypes of anti-EC antibodies (AECA) in the sera of children with asthma and determined the antigenic targets associated with AECA reactivity.

Methods: Levels of each class of AECA were determined by cellular ELISA in 156 children with asthma and in 203 control children. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis were performed in samples that contained high levels of AECA.

Results: In the cellular ELISA, the IgE class of AECA was detected significantly more frequently in children with asthma (25/156; 16.0%) than in healthy controls (2/203; 1.0%; $P < 0.01$). There were no differences in the frequencies of detection of IgG, IgA and IgM classes of AECA between patients and controls. The IgE–AECA was more frequently detected in younger children (23/69 *vs* 2/87 for children younger and older than 4 years of age, respectively). There was no correlation between the level of IgE–AECA and that of total IgE or house dust mite-specific IgE. In western blot analysis, IgE antibodies against a component of EC with a molecular mass of 75 kDa were detected in 20 of 25 patients (80.0%) positive for IgE–AECA, but they were less frequently detected in patients negative for IgE–AECA (2/34 (5.9%); $P < 0.01$).

Conclusions: These results demonstrate that a small fraction of asthmatic children has IgE–AECA and that the antigenic target of IgE–AECA is a component of the EC with a molecular weight of 75 kDa.

Key words: anti-endothelial cell antibodies, bronchial asthma, IgE.

Introduction

Airway inflammation characteristic of bronchial asthma is the result of eosinophil-dependent tissue injury.[1,2] Activated eosinophils release a variety of mediators, including major basic protein, eosinophilic cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase, which are damaging to the airway epithelium.[3,4] Eosinophils also produce leukotrienes, which induce airway smooth muscle contraction and airway edema.[5] Because inflammatory cells have to cross the endothelial wall to access the inflammatory site, upregulation of adhesion molecules on endothelial cells (EC) is an important feature.

Upregulation of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and sulfated the sialyl Lewis X glycans on airway endothelium has been observed in patients with asthma.[6,7] Moreover, serum levels of soluble adhesion molecules, including soluble ICAM-1, soluble E-selectin and soluble VCAM-1, are increased in patients with bronchial asthma.[8–10] In fact, it is widely accepted that an increased expression of cell surface adhesion molecules is followed by the release of their soluble forms into the circulation. Because E-selectin expression is restricted to EC, increased levels of soluble E-selectin in asthma means endothelial damage or activation.[11]

Anti-endothelial cell antibodies (AECA) were detected in the sera from patients with a variety of conditions

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associated with vascular injuries.[12] Anti-endothelial cell antibodies play a role in pathophysiology by inducing activation of EC, resulting in upregulation of the expression of endothelial adhesion molecules and secretion of chemoattractants and cytokines.[13–16] The antigenic targets associated with reactivity of AECA have not been clearly defined, but a range of antigenic sites may be involved.[17] Little is known about the distribution of AECA and the antigenic targets of EC in patients with asthma, particularly in children with asthma.[18]

The purpose of the present study was to determine the prevalence and isotypes of AECA in asthmatic children and the antigenic targets associated with reactivity of AECA.

METHODS

Patients

Sera were collected from 156 patients with asthma (84 males and 72 females) aged from 6 months to 15 years (mean (±SD) age 5.0 ± 3.8 years) and 206 normal healthy children (109 males and 97 females) aged from 6 months to 15 years (mean age 7.5 ± 3.9 years). There was no significant difference between the ages of the patients and the control subjects. Asthma was diagnosed on the basis of a history of episodic wheezing. The patients enrolled in the study were in a stable condition, with neither dyspnea nor wheeze. Symptoms of asthma were controlled by medications. Of 156 patients with asthma, 11 patients were receiving treatment with antihistamine drugs only, seven were receiving treatment with oral leukotriene receptor antagonists (LTRA), three were receiving treatment with inhaled disodium cromoglycate (DSCG) and 41 were receiving treatment with theophylline. Twenty-four patients were receiving treatment with theophylline and LTRA, 11 were receiving treatment with theophylline and DSCG, 51 were receiving treatment with theophylline, DSCG and inhaled β_2-adrenergic receptor agonists, three were receiving treatment with theophylline and inhaled beclomethasone (ICS), three were receiving treatment with theophylline, DSCG and ICS, and two were receiving treatment with theophylline, inhaled β_2-adrenergic receptor agonists, DSCG and ICS. None of the patients was on oral corticosteroid or immunosuppressive drugs at the time of blood collection. In some patients, serum was also obtained during exacerbation of asthma. Of 156 asthmatic children, 146 patients (93.6%) were atopic and 10 (6.4%) were non-atopic. Patients were considered as atopic when they had atopic dermatitis, allergic rhinitis, allergic family history, high total IgE titer or high IgE titer specific for mite allergens. Sixty-nine patients (44.2%) had mild disease, 51 (32.7%) had moderate disease and 36 (23.1%) had severe disease. The grade of severity was determined according to the Japanese Guideline for Asthma.[19] All sera were aliquoted and stored at – 20°C.

Cell isolation

Endothelial cells were obtained from human umbilical veins using methods described previously.[20] Recovered EC were grown to confluence in type I collagen (Sigma, St Louis, MO, USA)-coated flasks in RPMI 1640 medium with 20% fetal calf serum, 25 μg/mL endothelial cell growth supplement (Sigma) and 30 U/mL heparin. At this stage, the cells were detached by exposure to trypsin and EDTA and were washed three times with phosphate-buffered saline (PBS).

Cellular ELISA for detection of AECA

Immunoglobulin classes of AECA were determined by a cellular enzyme immunoassay as described previously.[12] Briefly, EC were transferred to 96-well type I collagen-coated flat-bottomed plates at a density of $1–2 \times 10^4$ cells/well and were grown to confluence. In all experiments, a pool of at least three different donors at the first passage was used. After washing twice with PBS, 100 μL of 2% bovine serum albumin (BSA) in PBS was added to each well and the plates were left for 1 h at room temperature to reduce non-specific binding of immunoglobulins. After three washes with PBS, 100 μL serum sample diluted with PBS was added to each well in triplicate and the plates were left for 1 h at room temperature. Several wells containing diluent only were used to provide a background binding level. Sera were routinely diluted 1 : 400 for IgG-AECA, 1 : 300 for IgM-AECA, 1 : 100 for IgA-AECA and 1 : 100 for IgE-AECA because, in preliminary studies, it was found that these dilutions gave the best discrimination between binding activity in the reference serum and that in normal control serum. In each assay, serum that has been obtained from a patient with systemic lupus erythematosus was used as a positive control. Bound immunoglobulin was detected by peroxidase-conjugated goat antihuman class-specific immunoglobulin (MBL, Tokyo, Japan). Results are expressed as a binding index (BI) calculated as:

$$BI = 100 \times (S - B)/(P - B)$$

where S is the absorbance of the sample, P is the absorbance of the positive control and B is the absorbance of background binding.[12] Values are the mean of three replicate determinations. A BI value of more than 3SD above the average BI of the healthy controls was considered positive. Interassay variation was determined by testing seven replicates of a serum sample positive for AECA. The variation coefficients were 9.5, 11.2, 10.5 and 11.5% for IgG, IgA, IgM, and IgE classes of AECA, respectively. The intra-assay variation was evaluated by testing six serum samples positive for AECA. The variation coefficients were 5.5, 7.2, 8.5 and 8.3% for IgG, IgA, IgM, and IgE classes of AECA, respectively.

Western blot analysis for detection of IgE–AECA

Endothelial cells were lysed at approximately 1×10^6 /mL in NP-40 lysis buffer consisting of 0.5% NP-40, 1% aprotinin, 1 mmol/L diisopropyl fluorophosphate, 5 mmol/L iodoacetamide and 1 mmol/L phenylmethane sulfonyl fluoride. After mixing gently, the cells were kept on ice for 30 min and then centrifuged at 1600 *g* for 15 min at 4°C. The supernatant was kept at – 80°C until use. Cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a reducing condition and the separated proteins were transferred to polyvinilidene difluoride (PVDF) membranes (Amersham-Pharmacia, Uppsala, Sweden). The blots were incubated for 1 h at room temperature in PBS with 2% BSA. Strips of PVDF membrane were then incubated for 1 h with sera from the patients and controls that had been diluted with PBS in the same manner as that of cellular ELISA. After four washes with PBS, the strips were incubated for 1 h at room temperature with peroxidase-conjugated goat antihuman IgE-specific antibody (MBL). After a further four washes, bound peroxidase conjugates were detected using a Peroxidase Stain Kit for Immunoblotting (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Statistical significance of differences between groups was tested by the Mann–Whitney *U*-test. The χ^2-test was used to compare proportions between two groups. Spearman's rank coefficient was used to evaluate correlation between two groups. All tests were accepted as statistically significant if $P < 0.05$.

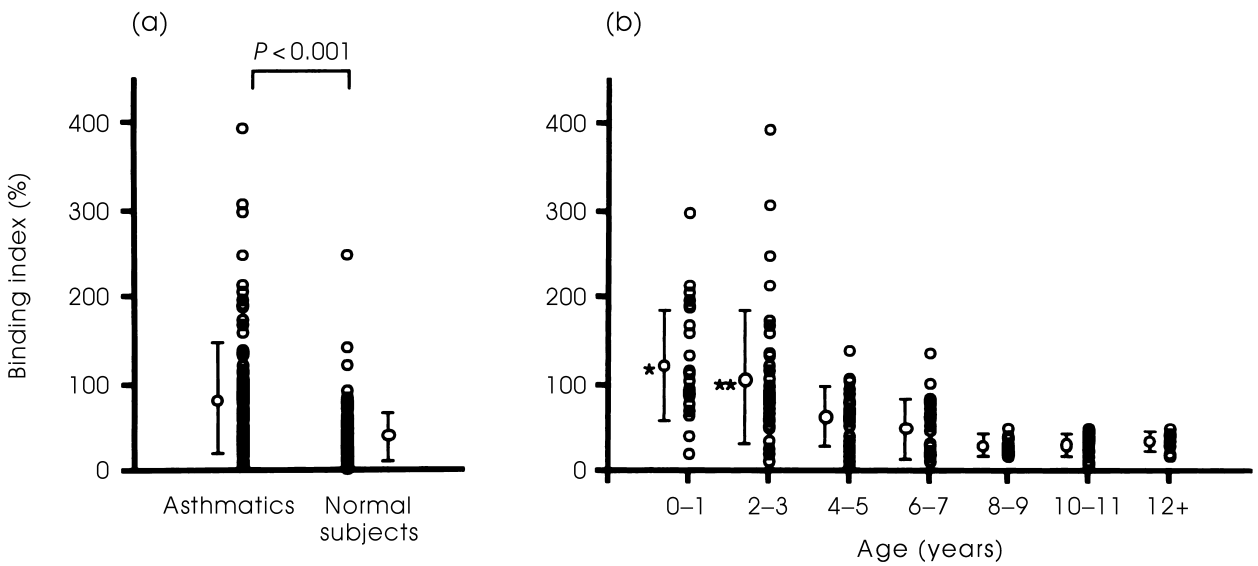

Fig. 1 (a) Comparison of serum levels of IgE–anti-endothelial cell antibodies (AECA) between patients with asthma and those without asthma (normal controls). Serum levels of IgE–AECA were significantly ($P < 0.001$) higher in children with asthma ($n = 156$;72.7 ± 61.6%) than in children without asthma ($n = 206$; 31.5 ± 26.8%). (b) Age distribution of IgE–AECA in children with asthma. Patients aged 0–1 years had significantly higher levels of IgE–AECA than patients aged 2–3 years (118.3 ± 64.4 *vs* 102.6 ± 76.1, respectively; $P < 0.001$) and each age group above 4 years (*$P < 0.0001$). Similarly, patients aged 2–3 years had significantly higher levels of IgE–AECA than each age group above 4 years (**$P < 0.001$).

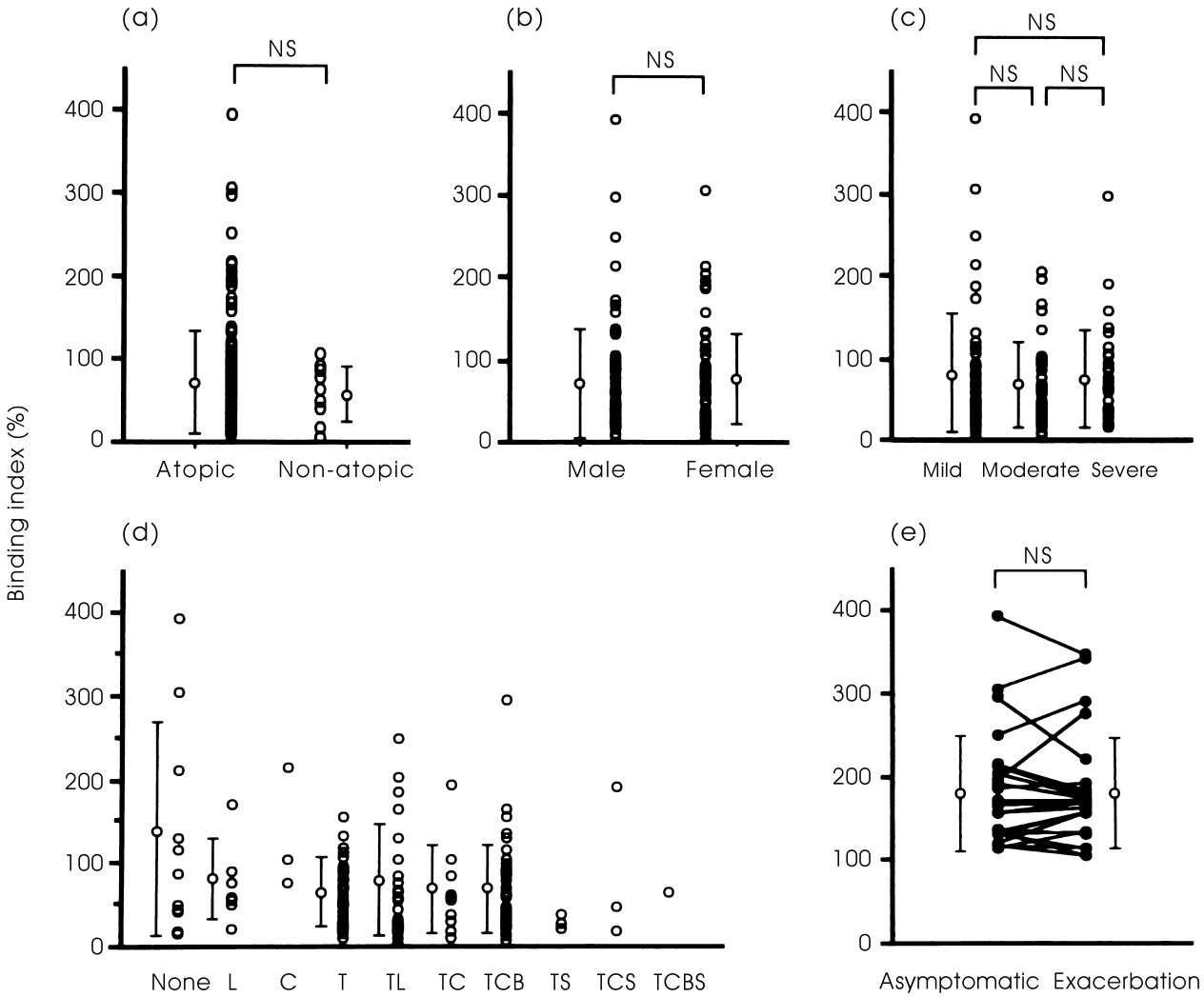

Fig. 2 Relationship between the levels of IgE–anti-endothelial cell antibodies (AECA) and various clinical features of the patients. No significant association was found between the levels of IgE–AECA and other specific clinical features of asthmatic children, including type of asthma (a), sex of the patients (b), severity of disease (c), medications used for the treatment of asthma (d) and exacerbation of disease (e). None, antihistaminic drugs only; L, leukotriene receptor antagonists (LTRA); C, inhaled disodium cromoglycate (DSCG); T, theophylline; TL, theophylline and LTRA; TC, theophylline and DSCG; TCB, theophylline, DSCG and inhaled β_2-adrenergic receptor agonists; TS, theophylline and inhaled beclomethasone (ICS); TCS, theophylline, DSCG and ICS; TCBS, theophylline, DSCG, inhaled β_2-adrenergic receptor agonists and ICS.

RESULTS

Determination of the prevalence of AECA in children with asthma by cellular ELISA

A significantly higher BI titer for IgE–AECA was found in children with asthma than in normal controls (mean (±SD) 72.7 ± 61.6 *vs* 31.5 ± 26.8%, respectively; Fig. 1a). When a BI value greater than the mean + 3SD of normal serum samples was used as the lower limit for detecting positive binding, IgE–AECA was observed in only two of 203 normal serum samples (1.0%). In contrast, 25 of 156 patients with asthma (16.0%) had significantly high levels of IgE–AECA ($P < 0.01$).

We then investigated the relationship between IgE–AECA and various clinical parameters. With regard to patient age, the levels of IgE–AECA were highest in the younger age groups (Fig. 1b). Twenty-three of 69 children with asthma under 4 years of age (33.3%) exhibited positive IgE–AECA. In contrast, significantly high levels of IgE–AECA were detected in only two of 87 asthmatic children over 4 years of age (2.3%). No significant association was

Table 1 Clinical features of children with asthma positive for IgE–anti-endothelial cell antibodies

	Absence of IgE–AECA	Presence of IgE–AECA	*P*
No. patients	131	25	
Age (years)	5.7 ± 3.8	1.8 ± 1.6	< 0.01
Sex ratio (females/males)	60/71	12/13	NS
Atopic dermatitis (*n*)	64/131	14/25	NS
Percentage	48.9	56.0	
Total IgE (IU/mL)	475 ± 560	194 ± 248	< 0.01
Mite-specific IgE (score)	4.0 ± 1.9	2.4 ± 2.0	< 0.01
Detection of anti-75 kDa antibody (*n*)	2/34	20/25	< 0.01
Percentage	5.9	80.0	

Where appropriate, data are given as the mean±SD.
AECA, anti-endothelial cell antibodies.

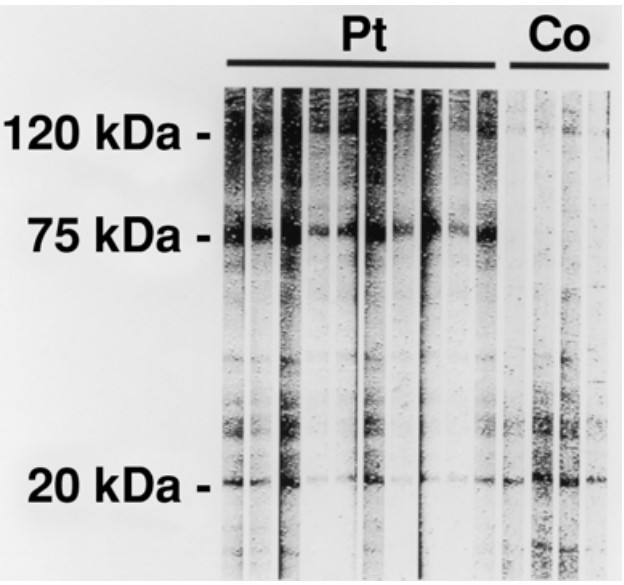

Fig. 3 Binding of serum antibodies to the 75 kDa endothelial cell antigen in asthmatic children. Endothelial cell lysates were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were blotted onto polyvinilidene difluoride (PVDF) membranes. After being blocked with 2% bovine serum albumin, the membrane strips were incubated with diluted sera that contained high levels of IgE–anti-endothelial cell antibodies and those did not. The bound IgE antibodies were detected by peroxidase-conjugated anti-IgE antibody. Binding at 120 and 20 kDa was non-specific, because these bands were detected in all serum samples. PT, patients; Co, controls.

found between the presence of IgE–AECA and other specific clinical features of asthmatic children, including type of asthma (Fig. 2a), sex of the patients (Fig. 2b), severity of disease (Fig. 2c) and medications used for the treatment of asthma (Fig. 2d). We then compared the levels of IgE–AECA within individual patients who showed high IgE–AECA levels when they were asymptomatic compared with levels during acute exacerbation ($n = 25$). The levels of IgE–AECA did not show any changes with exacerbation (Fig. 2e).

The detection of IgE–AECA was evaluated according to the clinical characteristics of the children with asthma (Table 1). The IgE–AECA were detected mainly in younger children with asthma ($P < 0.01$), in patients with low serum IgE levels ($P < 0.01$) and in patients with low serum mite-specific IgE ($P < 0.01$). The serum level of IgE–AECA was not correlated with either total serum IgE or house dust mite-specific IgE.

There were no differences between BI titers for other classes of AECA in children with asthma and normal controls (IgG-AECA: 37.2 ± 7.6 *vs* 36.0 ± 8.7%, respectively; IgA-AECA: 39.0 ± 9.2 *vs* 36.5 ± 7.7%, respectively; IgM-AECA: 43.7 ± 5.9 *vs* 42.9 ± 6.5%, respecitvely). None of the patients or controls exhibited a BI titer of more than the mean + 3SD of normal serum samples for IgG–, IgA– and IgM–AECA

Detection of IgE–AECA to the 75 kDa EC antigen in asthmatic patients positive for AECA

To determine the antigen targets associated with reactivity of IgE–AECA, SDS-PAGE followed by western blot analysis was performed in 25 asthmatic children who showed significantly high serum levels of IgE–AECA by cellular ELISA. Although multiple antigens were detected by serum antibodies of the patients, recurrent binding to human umbilical vein endothelial cells antigens at approximately 75 kDa was evident in samples from 20 asthmatic children positive for IgE–AECA (80.0%; Fig. 3). Anti-75 kDa antigen antibodies were detected in two of

34 asthmatic children with normal levels of IgE–AECA (5.9%) and in none of 25 normal control children (0.0%; $P < 0.01$).

Discussion

The present study demonstrated, for the first time, a high prevalence of IgE class AECA in sera from younger children with asthma, but not in sera from older children with asthma. The IgE–AECA were detected in 33.3% of asthmatic children under 4 years of age, but in only 2.3% of asthmatic children over 4 years of age. Because the serum level of IgE tends to increase with age, no correlation was found between the level of IgE–AECA and that of total IgE or between the level of IgE–AECA and that of house dust mite-specific IgE. This suggests that the reactivity against EC antigens in sera from children with asthma seems to be a specific immune response. The existence of a specific IgE antibody against the 75 kDa antigen of EC in asthmatic children who exhibited increased levels of IgE–AECA further supports this hypothesis.

The distribution of each class of AECA in children with asthma is quite different from that in adults with asthma. Lassalle *et al*. repoted that IgG–AECA were found in approximately one-third of adult patients with asthma, whereas IgE–AECA were detected in only 2.0% of patients.[18] In their study, patients with AECA were mainly non-atopic, had more severe asthma and tended to have aspirin-sensitive asthma. In contrast, in the present study, the IgE class of AECA was frequently detected in younger children with asthma and IgG–AECA was not detected in any asthmatic children. The levels of IgE–AECA were not influenced by other clinical features of asthma, including type of asthma, sex of the patients, severity of disease and medications used for the treatment of asthma. The different distribution of IgG–AECA in children and adults with asthma seems to be a result of differences in the features of asthma. Adult asthma is usually non-atopic and more severe than that in children and it usually occurs in middle age.[21] In contrast, epidemiologic studies have shown that aspirin-sensitive asthma is rarely observed in children.[22,23] Above all, asthma in childhood is closely linked with atopy, which is a genetic predisposition with increased IgE production.[22] It is therefore possible that the difference in immunoglobulin classes of AECA between adults and children with asthma is due to this genetic background.

While AECA are directed against a heterogeneous family of surface endothelial proteins,[24] the results of the present study have revealed that a 75 kDa component of EC is the target of IgE–AECA in asthmatic children. The antigenic targets associated with reactivity of AECA have not been clearly defined, but it has been shown that there is a heterogeneous range of endothelial antigens.[24] However, the frequent detection of anti-75 kDa antigen antibodies in sera that contain high titers of IgE–AECA suggests that the 75 kDa protein on EC is the main target of IgE–AECA in asthmatic children. In adult patients with asthma, IgG–AECA against a 55 kDa antigen shared by platelets and EC has been detected.[18] The difference between the antigenic targets of AECA in children and adults is thought to strongly influence differences in the class and the distribution of AECA. Further investigations are needed to elucidate the nature of the 75 kDa antigen on EC.

The function and role of IgE–AECA in asthmatic children remain unclear. By analogy to the function of IgG–AECA, it is possible that IgE–AECA induce adherence of leukocytes that have receptors for IgE, including basophils, eosinophils,[25] platelets[26] and monocytes,[27] on EC. At the same time, cross-linkage of FcεRI on these cells could lead to activation of these cells, release of mediators and production of various cytokines that directly act on adjacent EC. These speculations are in accordance with the pathophysiology of bronchial asthma in childhood.[28] However, further investigations are needed to determine whether IgE–AECA are involved in the pathogenesis of bronchial asthma.

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