

Original article

Identification of α -enolase as an autoantigen associated with severe asthma

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Background: Approximately 5% to 10% of patients with asthma have severe disease that is not effectively controlled by typical therapies. The existence of an autoantigen associated with severe asthma has been previously reported.

Objective: We attempted to identify the autoantigen.

Methods: Severe asthma was defined as patients having at least 1 severe asthmatic exacerbation requiring an emergency department visit or admission in the last year despite continuous typical therapies. Autoantibodies to airway epithelial cells (A549) were examined in sera from patients with severe asthma by immunoblot analysis.

Results: IgG autoantibodies to the 52-kd airway epithelial cell antigen were detected in sera from 32 of 78 patients with severe asthma (41%), 9 of 83 patients with mild-to-moderate asthma (11%), and 2 of 58 healthy controls (3%; $P < .001$). The 52-kd autoantigen was identified as α -enolase by mass spectrometry analysis and confirmed by using recombinant human α -enolase protein. The detection of IgG autoantibodies to α -enolase was the most significant indicator for distinguishing severe asthma from mild-to-moderate asthma, even after adjusting for the effects of other clinical variables, including age, sex, atopy, and FEV₁ (adjusted odds ratio, 5.2; 95% CI, 2.1-12.9; $P < .001$).

Conclusion: The α -enolase was identified as an autoantigen associated with severe asthma. Further studies are needed to determine the significance of this autoantigen in severe asthma. **Clinical implications:** IgG autoantibodies to α -enolase could be a biological marker for severe asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Asthma, autoantibodies, autoantigen, airway epithelial cell

Asthma is a chronic inflammatory airway disease that can be severe and sometimes fatal.¹ It has been estimated that 5% to 10% of patients with asthma have severe

Abbreviation used

PVDF: Polyvinylidene difluoride

disease that is not effectively controlled by typical therapies, and these patients are at high risk of asthma-related death.¹⁻³ However, there is still little information on the pathophysiological mechanisms responsible for severe asthma.¹⁻³ Severe asthma has several distinct characteristics compared with mild-to-moderate asthma, such as female dominance, less allergic sensitization, more frequent history of aspirin hypersensitivity, and more prominent sputum neutrophilia.² On the basis of these findings, severe asthma has been suggested as a different disease from mild-to-moderate asthma rather than a more severe case of mild-to-moderate asthma.²

It has been repeatedly suggested that autoimmune mechanism might be involved in the pathogenesis of asthma, especially in nonallergic asthma.⁴⁻⁶ The autoimmune hypothesis of asthma is based on the presence of autoantibodies to the target tissue of inflammation (lung and bronchial mucosa),⁶ common genetic association, frequent coexistence with autoimmune diseases,^{7,8} and response to the medications used for systemic autoimmune diseases such as systemic corticosteroid, cyclosporin A, intravenous IgG, and anti-TNF reagents.^{9,10} Churg-Strauss syndrome is a distinct form of systemic vasculitis characterized by pre-existing adult-onset severe asthma and sudden development of pulmonary infiltrates, peripheral blood eosinophilia, paranasal sinus abnormality, neuropathy, and frequent presence of antineutrophil cytoplasmic antibodies.¹¹ The presence of Churg-Strauss syndrome suggests that a common pathogenetic principle could be shared by severe asthma and systemic vasculitis. However, a direct demonstration of a causal relationship between autoimmunity and asthma has not been possible.

Interestingly, IgG autoantibodies to a 55-kd protein in endothelial cells and platelets has been reported to be associated with severe asthma and aspirin-sensitive asthma.¹² Moreover, a proliferative T-cell response to the 55-kd autoantigen correlated with the severity of airway obstruction in patients with asthma.¹² However, the 55-kd autoantigen associated with severe asthma is not identified yet.

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Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication September 7, 2005; revised March 30, 2006; accepted for publication April 3, 2006.

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0091-6749/\$32.00

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doi:10.1016/j.jaci.2006.04.002

TABLE I. Characteristics of the study subjects and results of the autoantibody analysis||

Variables	Mild-to-moderate asthma (n = 83)	Severe asthma (n = 78)	Healthy controls (n = 58)
Age (y)	30.1 ± 13.5*	43.3 ± 15.1†	41.4 ± 15.0
Female sex, n (%)	36 (43.4)	45 (57.7)	25 (43.1)
FEV ₁ , % predicted	84.3 ± 18.9*	69.0 ± 22.4*†	111.7 ± 13.9
Total IgE, kU/L	405.3 ± 367.0*	583.2 ± 796.9*	216.9 ± 316.6
Atopy, n (%)	67 (80.7)	45 (57.7)‡	—
Aspirin hypersensitivity (%)	5 (6.0)	17 (21.8)§	—
Autoantibodies, n (%)			
α-enolase	9 (10.8)	32 (41.0)*†	2 (3.4)
Cytokeratin 18	23 (27.7)*	25 (32.1)*	5 (8.6)
Recombinant α-enolase	12 (14.5)	31 (39.7)*†	4 (6.9)

**P* < .01 for the comparison with healthy controls.

†*P* < .001 for the comparison with mild-to-moderate asthma.

‡*P* = .002 for the comparison with mild-to-moderate asthma.

§*P* = .004 for the comparison with mild-to-moderate asthma.

||Data of age, FEV₁ (% predicted), and total IgE are expressed as means ± SDs.

The airway epithelium has been suggested to be a target for the inflammatory response in asthma regardless of allergic sensitization.¹³ The deposition of IgG antibodies and complement in the bronchial mucosa of patients with asthma who died of an asthmatic attack has been demonstrated.¹⁴ However, the target antigen recognized by IgG antibodies deposited in the airway mucosa of fatal asthma has not been identified. It could be speculated that the unidentified target antigen may be either an infectious agent or a self-antigen.

We previously identified the cytokeratin 18 protein as an airway epithelial autoantigen associated with nonallergic asthma.¹⁵ In this study, we further attempted to identify an airway epithelial cell autoantigen that was recognized by autoantibodies in sera from patients with severe asthma.

METHODS

Subjects

We included 161 patients with asthma referred to our institution from March 1997 to December 2004 and 58 age-matched nonsmoking healthy volunteers in this study (Table I). The smoking status of the patients with asthma was not determined in this study. Each subject with asthma had a typical clinical history of asthma and a 20% decrease in FEV₁ after the inhalation of less than 8 mg methacholine/mL or documented reversibility of FEV₁ greater than 15% of the baseline measurement after inhalation of a bronchodilator. Severe asthma was defined as when patients had at least 1 severe asthmatic exacerbation requiring an emergency department visit or admission in the last year, despite continuous typical therapies including high-dose inhaled corticosteroids (≥1200 μg/d budesonide or equivalent doses of other inhaled corticosteroids) or oral corticosteroids.² Mild-to-moderate asthma was defined as when patients were controlled by a regular dose of inhaled corticosteroid (a maximum of 1000 μg/d budesonide or equivalent doses of other inhaled corticosteroids) and inhaled bronchodilator in the last year without a severe asthmatic exacerbation.² All subjects with asthma underwent skin prick test with 50 common aeroallergens (for the list of 50 aeroallergens, see this article's Table E1 in the Online Repository at www.jacionline.org). A positive histamine control and a negative saline control were also included in each skin prick test. Atopy

was defined when wheal diameter of any 1 allergen was greater than 3 mm over the negative control. Aspirin hypersensitivity was determined by either a typical clinical history or a positive result of a bronchial challenge test with lysine-aspirin.¹⁶ All serum samples from the subjects were stored at −20°C. All subjects provided written informed consent, and the institutional review board approved this study.

Cell culture and protein extraction

The human airway epithelial cell line (A549) was obtained from American Type Culture Collection (Manassas, Va) and was cultured as recommended. Platelets were isolated from human venous blood as previously described.¹² Cells were lysed by the addition of lysis buffer containing 10 mmol/L Tris/HCl, pH 7.2, 2% SDS, 158 mmol/L NaCl, and 10 mmol/L dithiothreitol.

Immunoblot analysis

Proteins were separated by discontinuous SDS-PAGE. IgG autoantibodies to airway epithelial cell proteins were detected in serum samples by immunoblot analysis using polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, Calif) as we have previously described.¹⁵ The immunoreactivity of the serum samples was assessed by 2 independent investigators. Positive standard and negative control serum samples were included in each experiment. From a technical point of view, our immunoblot detection method of IgG autoantibodies to airway epithelial cell proteins was the same as the current Western blot tests for the serologic diagnosis of HIV infection.¹⁷ Notably, most of the faint reactivity in the Western blot test was shown to be false-positive in epidemiologic studies of HIV infection.¹⁸ Thus, the positive detection of IgG autoantibodies to an airway epithelial cell protein in the serum sample was defined as when the reactivity was equal to or greater than that of a positive standard serum sample as described in the recent paper on the immunoblot detection of autoantibodies.¹⁹ Goat specific antibody to human α-enolase (Santa Cruz Biotechnology, Santa Cruz, Calif) and mAb to human cytokeratin 18 (clone CK5, Sigma Chemical Co, St Louis, Mo) were included in each experiment to confirm the colocalization of IgG autoantibodies and the corresponding protein in both 1-dimensional and 2-dimensional immunoblot analysis. The recombinant human α-enolase protein was produced and purified as we have previously described²⁰ and used as a positive control antigen. Isoelectric focusing for the 2-dimensional immunoblot analysis was performed by using a 7-cm linear immobilized pH gradient strip (Amersham Pharmacia Biotech, Piscataway, NJ).

Mass spectrometry analysis of autoantigen

The airway epithelial cell proteins were separated by SDS-PAGE, and the gel was stained by Coomassie blue. The protein band containing autoantigen was localized by comparing the patterns of immunoblot analysis and protein staining. The excised gel slice containing the target protein was digested with trypsin. Liquid chromatography–electrospray tandem mass spectrometry analysis was performed on a quadrupole time-of-flight mass spectrometer (QTOF2; Micromass, Beverly, Ma). Protein identification was accomplished with the Mascot program using the National Center for Biotechnology Information (Bethesda, Md) nonredundant protein database.²¹

Cross-antigenicity of airway epithelial cell and platelet autoantigens

IgG autoantibodies were eluted from a PVDF membrane containing the 52-kd airway epithelial autoantigen to evaluate the antigenic cross-reactivity of 52-kd autoantigens in airway epithelial cells and platelets. Serum samples from patients with severe asthma were incubated overnight with a PVDF membrane strips containing the 52-kd airway epithelial autoantigen or 45-kd cytokeratin 19 protein (as a negative control). After extensive washing, antibodies were eluted by 0.2 mol/L glycine, pH 2.8, and neutralized with a 0.1 volume of 1 mol/L Tris, pH 8.0. The eluted antibodies were used for immunoblot analysis.

Statistics

The χ^2 test was used to compare the sex ratio, atopy, and frequency of autoantibodies between groups. A 1-way ANOVA was used for comparison of the study groups with regard to age, total IgE levels, and baseline FEV₁. A *P* value < .05 was regarded as statistically significant when comparing the 2 groups. For multiple comparisons of 2 groups among the 3 groups, Bonferroni correction was used. Multivariate logistic regression analysis was used to identify clinically relevant and significant indicators for distinguishing severe asthma from mild-to-moderate asthma.

RESULTS

Detection of autoantibodies to airway epithelial cell proteins

IgG autoantibodies to the 52-kd airway epithelial cell antigen were detected in sera from 32 of 78 patients with severe asthma (41%), 9 of 83 patients with mild-to-moderate asthma (11%), and 2 of 58 healthy controls (3%; *P* < .001; Fig 1, A, and Table I). The detection rate of IgG autoantibodies to cytokeratin 18 did not differ between severe asthma (25/78; 32%) and mild-to-moderate asthma (23/83; 28%; *P* = .55; Table I).

Identification of the 52-kd airway epithelial cell autoantigen as α -enolase

The protein band containing the 52-kd autoantigen was excised from a polyacrylamide gel (Fig 2, B) and digested with trypsin. The resulting peptide fragments were analyzed by mass spectrometry. The database analysis showed that sequence and mass data of 9 peptide fragments matched with human α -enolase and the identified peptide sequences covered 29% of the total amino acid sequence of human α -enolase (Table II). In addition, the identification of the 52-kd autoantigen as human α -enolase was further confirmed by 1-dimensional and 2-dimensional immunoblot analysis using goat specific antibody

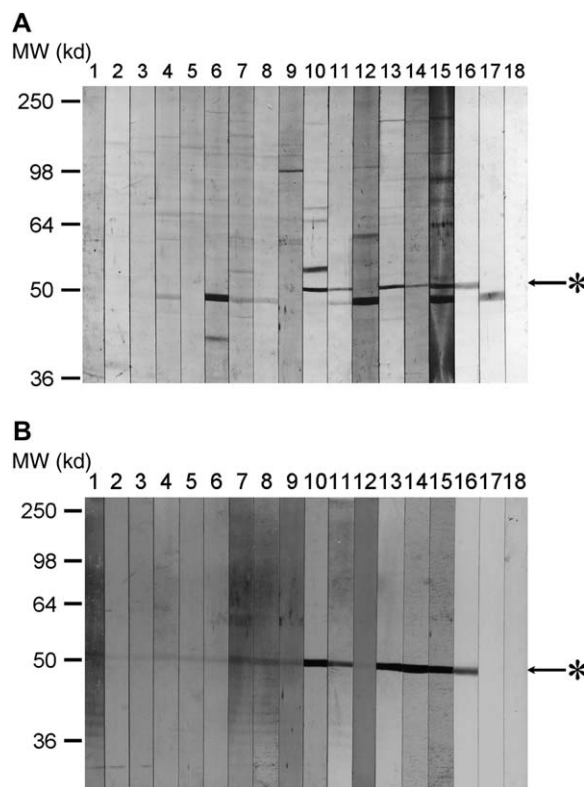


FIG 1. A, Immunoblot analysis of IgG autoantibodies to airway epithelial cell (A549) proteins in serum samples. Results from healthy controls (lanes 1-3), patients with mild-to-moderate asthma (lanes 4-9), patients with severe asthma (lanes 10-15), goat specific antibody to α -enolase (lane 16), mAb to cytokeratin 18 (lane 17), and diluent buffer only (lane 18). *Arrow indicates α -enolase protein. **B,** Immunoblot analysis of IgG autoantibodies to recombinant human α -enolase in same serum samples and configuration as A. *Arrow indicates α -enolase protein. MW, Molecular weight.

to human α -enolase (Fig 1, A, and Fig E1 in the Online Repository at www.jacionline.org). Repeated experiments using serum samples from patients with asthma showed that there was no autoantigen other than α -enolase and cytokeratin 18 among airway epithelial proteins in the molecular weight range between 49 and 52 kD. The detection of IgG autoantibodies to α -enolase in sera from patients with severe asthma was also confirmed by immunoblot analysis using recombinant human α -enolase protein (Fig 1, B). The detection of IgG autoantibodies to recombinant α -enolase and natural α -enolase of airway epithelial cell showed significant agreement (κ = 0.67; *n* = 219; *P* < .001).

Clinical significance of autoantibodies to α -enolase

Multivariate logistic regression analysis showed that detection of IgG autoantibodies to α -enolase was the most significant indicator for distinguishing severe asthma from mild-to-moderate asthma, even after adjusting for the effects of other clinical variables, including age, sex, atopy, and FEV₁ (adjusted odds ratio, 5.2; 95% CI,

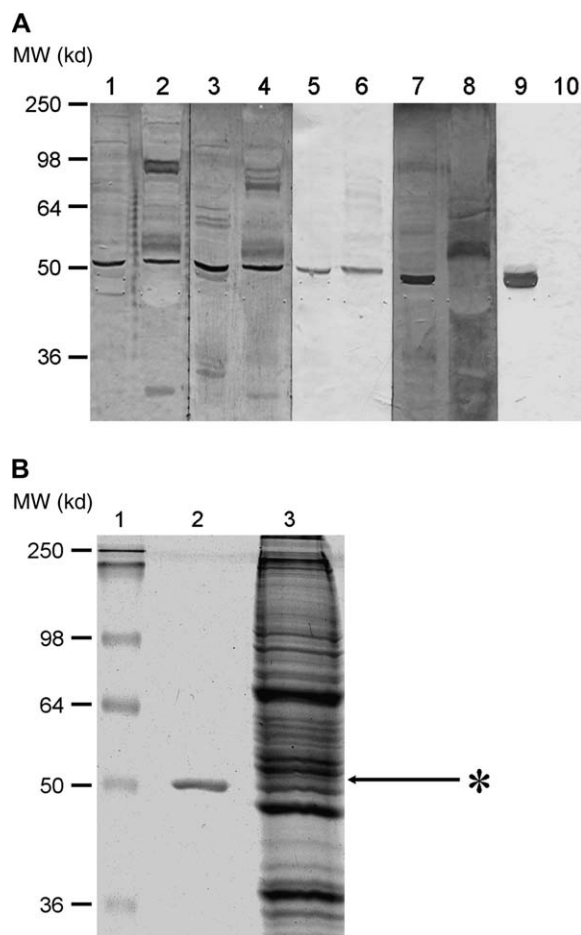


FIG 2. Comparison of airway epithelial cell and platelet autoantigens. **A**, Airway epithelial cell (A549) proteins (lanes 1, 3, 5, 7, and 9) and platelet proteins (lanes 2, 4, 6, 8, and 10) were subjected to immunoblot analysis. IgG autoantibodies in serum samples from 2 patients with severe asthma (lanes 1 and 2; lanes 3 and 4) and goat specific antibody to α -enolase (lanes 5 and 6) recognized the 52-kD proteins in both airway epithelial cells and platelets. IgG autoantibodies in a serum sample from a patient with severe asthma (lanes 7 and 8) and mAb to cytokeratin 18 (lanes 9 and 10) recognized only the 49-kD protein in airway epithelial cells. **B**, Protein staining showed molecular weight standard (lane 1), purified human cytokeratin 18 protein (lane 2), and airway epithelial cell (A549) proteins (lane 3). *Arrow indicates the 52-kD target protein band, which was subjected to mass spectrometry analysis. MW, Molecular weight.

2.1-12.9; $P < .001$). IgG autoantibodies to α -enolase were detected more frequently in patients with asthma with aspirin hypersensitivity (13/22; 59%) than those without (28/139; 20%; $P < .001$). In patients with severe asthma, IgG autoantibodies to α -enolase were detected more frequently in patients with asthma with aspirin hypersensitivity (11/17; 65%) than those without (21/61; 34%; $P = .025$). The detection rate of IgG autoantibodies to cytokeratin 18 did not differ significantly between patients with asthma with aspirin hypersensitivity (7/22; 32%) and those without (41/139; 30%; $P = .82$). IgG autoantibodies to recombinant α -enolase were detected in 31 of 78 patients with severe asthma (40%) and 12 of 83 patients

with mild-to-moderate asthma (15%; $P < .001$; Table I). The detection of IgG autoantibodies to recombinant α -enolase was the significant indicator for severe asthma, even after adjusting for the effects of age, sex, atopy, and FEV₁ (adjusted odds ratio, 3.6; 95% CI, 1.6-8.3; $P = .003$). The detection rate of IgG autoantibodies to recombinant α -enolase was significantly different between patients with asthma with aspirin hypersensitivity (10/22; 46%) and those without (33/139; 24%; $P = .032$). IgG autoantibodies to cytokeratin 18 were detected more frequently in patients with nonatopic asthma (20/49; 40.8%) than those with atopic asthma (28/112; 25.0%) ($P = .04$; see this article's Table E2 in the Online Repository at www.jacionline.org). The detection rate of IgG autoantibodies to α -enolase did not differ between patients with nonatopic asthma (15/49; 30.6%) and those with atopic asthma (26/112; 23.2%; $P > .05$; see this article's Table E2 in the Online Repository at www.jacionline.org). In patients with mild-to-moderate asthma, IgG autoantibodies to α -enolase or cytokeratin 18 were detected more frequently in patients with nonatopic asthma than in those with atopic asthma ($P < .05$; see this article's Table E3 in the Online Repository at www.jacionline.org). In patients with severe asthma, the detection rate of IgG autoantibodies to α -enolase or cytokeratin 18 did not differ between patients with nonatopic asthma and those with atopic asthma ($P > .05$; see this article's Table E4 in the Online Repository at www.jacionline.org).

Comparison of airway epithelial cell and platelet autoantigens

We found that the molecular weight and clinical significance of the 52-kD airway epithelial cell autoantigen were similar to those previously reported for a 55-kD autoantigen commonly expressed in endothelial cells and platelets.¹² The immunoreactivity of IgG autoantibodies to airway epithelial cell and platelet proteins in serum samples from patients with severe asthma was examined to evaluate a possible identities of autoantigens in the 2 cell types. The IgG autoantibodies from 2 patients with severe asthma and goat specific antibody to α -enolase recognized the 52-kD proteins in both airway epithelial cells and platelets (Fig 2, A). IgG autoantibodies eluted from the 52-kD airway epithelial cell autoantigen reacted with a 52-kD platelet protein and a recombinant α -enolase (see this article's Fig E2 in the Online Repository www.jacionline.org).

Long-term follow-up of autoantibodies to airway epithelial cell proteins

Autoantibodies to airway epithelial cell proteins were detected in serially sampled sera from 6 patients with severe asthma. IgG autoantibodies to α -enolase and other airway epithelial proteins were consistently detected in these patients for as long as 4 years (Fig 3). When the serum samples from 3 patients with severe asthma who died of asthmatic attack or infection after multiple severe asthmatic exacerbations were examined, IgG autoantibodies to α -enolase or cytokeratin 18 were detected in 2 patients

TABLE II. Mass spectrometric identification of the 52-kd airway epithelial cell autoantigen as human α -enolase*

Matching peptide no.	Mass (M _r): measured	Mass (M _r): calculated	Peptide sequences (+ modifications)
1	898.22	898.55	TIAPALVSK
2	1142.17	1142.61	IGAENVYHNLK
3	1311.03	1311.57	LMIEMDGTEENK (+2 oxidations of methionine)
4	1405.14	1405.71	GNPTVEVDLFTSK
5	1424.15	1424.72	YISPDQLADLYK
6	1555.13	1555.77	VVIGMDVAASEFFR (+ oxidation of methionine)
7	1803.21	1803.94	AAVPSGASTGIYEALRLR
8	1959.14	1959.92	DATNVGDEGGFAPNILENK
9	2031.23	2031.05	FTASAGIQVVGGDDLTVTNPK

*Liquid chromatography–electrospray tandem mass spectrometry analysis of trypsin-digested peptides of 52-kd autoantigen showed that the sequence and mass data of 9 peptide fragments matched human α -enolase, and the identified peptide sequences covered 29% of the total amino acid sequence of human α -enolase.

2 or 20 months before their deaths (see this article's Fig E3 in the Online Repository at www.jacionline.org).

DISCUSSION

In this study, we identified α -enolase as an autoantigen associated with severe asthma. The autoimmune response to α -enolase could simply be an epiphenomenon reflecting epithelial cell damage that is secondary to chronic airway inflammation, or the autoimmune response to α -enolase could be a pathogenic factor inducing airway inflammation in severe asthma.

The α -enolase is a key glycolytic enzyme with highly conserved amino acid sequences from microbial organism to mammal.²² This enzyme is ubiquitously expressed in nearly all cell types, including epithelial cells, endothelial cells, and hematopoietic cells.²² The expression of α -enolase in primary human bronchial epithelial cells was detected by immunohistochemical staining using specific antibodies.²³ We also detected α -enolase protein in another nonmalignant human bronchial epithelial cell line (BEAS-2B) by immunoblot analysis using IgG autoantibodies from patients with severe asthma and goat specific antibodies to α -enolase (data not shown). Although α -enolase is mainly a cytosolic protein, it is also expressed on cell surfaces and functions as a plasminogen receptor.²² Autoantibodies to α -enolase have been reported in various other chronic inflammatory diseases, including systemic vasculitis, inflammatory bowel diseases, endometriosis, autoimmune hepatitis, and rheumatoid arthritis.^{22,24} This suggests that an autoimmune response to α -enolase is not specific to severe asthma.

In epidemiologic studies, the incidence of asthma is significantly higher in patients with other autoimmune diseases, including celiac disease, rheumatoid arthritis, type 1 diabetes, and endometriosis.^{8,25} Interestingly, the inflammatory response observed in patients with asthma has been shown to involve not only airways but also lung parenchyma,²⁶ blood vessels,²⁷ salivary glands,²⁸

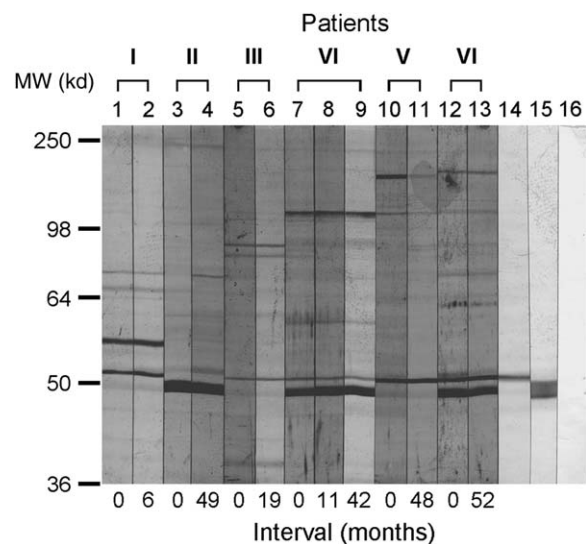


FIG 3. Long-term follow-up of autoantibodies to airway epithelial cell proteins in serum samples of severe asthmatic patients. IgG autoantibodies to airway epithelial cell proteins were consistently detected in serially sampled sera from patients with severe asthma for as long as 4 years. Results from 6 patients (patient I–VI; lanes 1–13), goat specific antibody to α -enolase (lane 14), mAb to cytokeratin 18 (lane 15), and diluent buffer only (lane 16). MW, Molecular weight.

and even intestinal mucosa.²⁹ These observations suggest that asthma could be a type of systemic inflammatory disease in a subgroup of patients with asthma.

In this study, we showed that IgG autoantibodies to α -enolase in serum samples from patients with severe asthma also reacted with a 52-kd platelet protein. IgG autoantibodies to α -enolase were significantly associated with severe asthma and aspirin-sensitive asthma. On the basis of these findings, we suggest that the previously reported 55-kd autoantigen common to endothelial cells and platelets could be α -enolase.¹² The previous study showed that a proliferative T-cell response to the 55-kd autoantigen correlated with the severity of airway obstruction in patients with asthma.¹² Patients with severe

asthma can be clinically improved by anti-CD4 mAb treatment.¹⁰ These findings suggest that a T-cell response to α -enolase could be involved in the pathogenesis of severe asthma.

Low levels of IgG autoantibodies to α -enolase seems to be present in the majority of healthy individuals on the basis of experiments for detecting IgG autoantibodies to recombinant α -enolase protein in this study, as is also observed for the presence of low titer rheumatoid factor and antinuclear antibodies in the majority of healthy individuals.^{30,31} To define the pathogenetic significance of IgG autoantibodies to α -enolase in severe asthma, further studies including the development of an animal model for autoimmune asthma are necessary.

Asthma has been suggested as a manifestation of many different diseases with various etiologies.⁵ In other chronic inflammatory diseases of unknown etiology, such as rheumatoid arthritis and systemic lupus erythematosus, attempts to classify patients on the basis of clinical characteristics and autoantibody assays have been successful.^{32,33} Trials to classify asthma further into subgroups on the basis of autoantibody assays might provide a clue to exploring the pathogenetic mechanism of severe asthma.

In conclusion, our study identified α -enolase as an autoantigen associated with severe asthma. IgG autoantibodies to α -enolase could be a biological marker for severe asthma. Further studies are needed to determine the significance of this autoantigen in severe asthma.

We are indebted to the patients and volunteers for donation of their blood, Professor Yunhwan Lee for reviewing the manuscript, and Jin Young Kim for help with the mass spectrometry analysis. The corresponding author is indebted to Dr Dean D. Metcalfe for his mentorship encouraging this project.

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