

Identification of Cytokeratin 18 as a Bronchial Epithelial Autoantigen Associated with Nonallergic Asthma

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The allergic response to common environmental agents (allergens) has been regarded as an important mechanism in the development of airway inflammation of patients with asthma. However, allergic sensitization cannot be detected in a significant number of adult patients with asthma. The etiologic mechanism responsible for nonallergic asthma has not yet been identified. The idea of a possible involvement of autoimmunity in the pathogenesis of nonallergic asthma has been proposed by earlier studies. To test for the possible presence of an autoimmune response to bronchial epithelial cell antigens in nonallergic asthma, we examined circulating autoantibodies to cultured human bronchial epithelial cells (BEAS-2B) in sera from patients with nonallergic asthma by immunoblot analysis. IgG autoantibodies to the 49-kD bronchial epithelial cell antigen were detected in 10 of 23 patients with nonallergic asthma (43%), 3 of 27 patients with allergic asthma (11%), 2 of 20 patients with systemic lupus erythematosus (10%), and 3 of 34 healthy volunteers (9%) ($p < 0.005$). The 49-kD autoantigen was purified and identified as cytokeratin 18 by amino acid sequencing. In this study, we identified cytokeratin 18 as a bronchial epithelial autoantigen associated with nonallergic asthma. Further studies are needed to determine the significance of autoimmunity in nonallergic asthma.

Keywords: asthma; autoantibodies; autoantigen

The allergic response to common environmental agents (allergens) has been regarded as an important mechanism in the development of airway inflammation of patients with asthma (1). However, allergic sensitization cannot be detected in a significant number of adult patients with asthma (1–3). Nonallergic asthma usually begins at an older age and is clinically more severe than allergic asthma (3–5). Nonallergic asthma has been referred to as “intrinsic asthma” on the basis of a belief that there must be an etiologic agent in the patient’s own body (3). However, the “intrinsic” etiology has not yet been defined.

From a pathologic viewpoint, asthma can be defined as a chronic inflammatory disorder of the airways characterized by inflammatory cell infiltration and destruction of bronchial epithelium (1). The bronchial epithelium has been suggested as being a target for the inflammatory response in asthma (6). In allergic asthma, the damage to bronchial epithelium was attributed to the inflammatory immune response induced by inhalation of allergen (1). The mechanism responsible for the inflammatory damage of bronchial epithelium in nonallergic asthma cannot be explained yet.

The idea of the possible involvement of an autoimmune mechanism in the pathogenesis of asthma has been proposed by previous studies that demonstrated high incidences of circulating autoantibodies to bronchial mucosa tissue in patients with asthma, especially in patients with nonallergic asthma (7, 8). The presence of circulating IgG autoantibodies to the 55-kD antigen of endothelial cells has also been reported in patients with nonallergic asthma (9). However, the target antigens of these autoantibodies are still unknown.

In this study, we tested for the possible existence of an asthma-associated autoantigen in the bronchial epithelial cell and identified cytokeratin 18 as a bronchial epithelial autoantigen associated with nonallergic asthma.

METHODS

Subjects

We included 23 patients with nonallergic asthma, 27 patients with allergic asthma, 34 age-matched nonsmoking healthy volunteers, and 20 patients with systemic lupus erythematosus in this study (Table 1). All subjects with asthma had a typical clinical history of asthma and a 20% decrease in forced expiratory volume in 1 second (FEV₁) after the inhalation of less than 8 mg methacholine/ml or documented reversibility of FEV₁ greater than 15% after inhalation of bronchodilator. All asthmatic subjects underwent a skin-prick test with 50 common aeroallergens (Bencard Co., Brentford, UK). Patients with asthma were classified as having allergic asthma when the wheal diameter of any one allergen was greater than 3 mm over the negative control (normal saline) and there was a definite clinical history or objective evidence of asthmatic response induced by allergen exposure. Nonallergic asthma was defined as there being no positive skin reaction to any of the 50 common aeroallergens in the presence of a positive histamine control and serum total IgE concentration being within the normal range (less than 180 IU/ml). Patients with occupational asthma were excluded from the study. Twenty patients with systemic lupus erythematosus classified according to the criteria of the American Rheumatic Association were included as control subjects with disease (10). All patients with asthma had not received systemic steroid treatment for the 4 weeks before the study. Eight of 20 patients with systemic lupus erythematosus were receiving systemic steroid treatment at the time of blood sampling. All serum samples from subjects were aliquoted and stored at -20°C . All subjects gave informed consent, and the institutional review board approved this study.

Cell Culture and Protein Extraction

A human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (Manassas, VA). BEAS-2B cells were cultured as described previously (11). Cultured cells were lysed by the addition of lysis buffer containing 10 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 158 mM NaCl, 1 mM ethylene glycol-bis(aminoethylether)-tetraacetic acid, 1 mM Na₃VO₄, 250 $\mu\text{g/ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride.

Immunoblot Analysis

Proteins in cell lysates were separated by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) using an 8% resolving gel (pH 8.8) and a 4% stacking gel (pH 6.8). After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane

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TABLE 1. CHARACTERISTICS OF THE STUDY SUBJECTS

	n	Age (yr)	Sex (F/M)	Total IgE (IU/ml)	FEV ₁ (%)
Healthy control subjects	34	41 (37–47)	15/19	188 (91–285)	108 (103–113)*
Patients with allergic asthma	27	27 (23–31) [†]	9/18	438 (315–562) [‡]	80 (73–88)
Patients with nonallergic asthma	23	39 (33–45)	14/9	72 (49–96)	73 (63–82)
Patients with SLE	20	31 (26–36) [†]	17/3 [§]	NM	NM

Definition of abbreviations: FEV₁ = forced expiratory volume in 1 s; NM = not measured; SLE = systemic lupus erythematosus. Data are expressed as mean (95% confidence interval for mean; lower bound–upper bound).

* Significant difference compared with patients with allergic and nonallergic asthma ($p < 0.05$).

[†] Significant difference compared with healthy control subjects ($p < 0.05$).

[‡] Significant difference compared with healthy control subjects and patients with nonallergic asthma ($p < 0.05$).

[§] Significant difference compared with healthy control subjects and patients with allergic asthma and nonallergic asthma ($p < 0.05$).

(Bio-Rad Laboratories, Hercules, CA). After the transfer, the membrane strips were probed with 1-ml serum samples at 1 in 100 dilution for 2 hours at room temperature. After washes, the membrane was incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) for 2 hours at room temperature. After a final washing, the membrane was stained with a substrate solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; Sigma). To confirm the characteristics of the 49-kD autoantigen after the amino acid sequence analysis, a mouse monoclonal antibody to human cytokeratin 18 (clone no. CY-90, Sigma) and a negative control mouse monoclonal antibody with the same IgG1 isotype (Sigma) were used for immunoblot analysis. Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was used as secondary conjugate, and the results were developed as described in the foregoing text. Commercially available purified bovine cytokeratin 18 protein (Research Diagnostics Inc., Flanders, NJ) was used as a positive control antigen in the experiment using mouse monoclonal antibody to human cytokeratin 18.

Purification and Identification of Autoantigen

For purification of the autoantigen, bronchial epithelial cell lysates were fractionated by ion-exchange chromatography with diethylaminoethyl Sepharose bead (Sigma). Fractions of interest were analyzed by SDS-PAGE and immunoblot analysis and further concentrated with Centrprep-50 (Amicon, Witten, Germany) and subjected to reverse-phase high-performance liquid chromatography (HPLC) using Vydac C18 column (The Separation Group, Inc., Hesperia, CA). Fractions were collected and lyophilized. They were examined by SDS-PAGE and immunoblot analysis. Because analysis of purified protein on polyvinylidene difluoride revealed that the N-terminal amino acid sequence was blocked, the protein was subjected to enzymatic in-gel digestion by trypsin. Trypsin-digested peptide fragments were separated by a micro-HPLC system using a Sephasil C18 reverse-phase column (Amersham Pharmacia Biotech, Uppsala, Sweden). Two fractions of peptide fragments were subjected to amino acid sequencing using the Procise cLC 492 Protein sequencing system (Applied Biosystems, Foster, CA). To compare the amino acid sequences of peptide fragments with known protein sequences, the SWISS-PROT database (Swiss Institute of Bioinformatics, Geneva, Switzerland) was used.

Statistics

The chi-square test was used to compare the frequencies of autoantibodies between groups. For comparison of the study groups with regard to age, total IgE concentrations, and baseline FEV₁, a one-way analysis of variance was used. For comparison of the sex ratio of the study groups, chi-square test was used. Values of p below 0.05 were regarded as statistically significant.

RESULTS

Detection of Circulating Autoantibodies to Bronchial Epithelial Cell Antigens

Circulating IgG autoantibodies to the 49-kD bronchial epithelial cell antigen were detected in 10 of 23 patients with nonallergic asthma (43%), 3 of 27 patients with allergic asthma (11%), 2 of 20 patients with systemic lupus erythematosus (10%), and 3 of 34 age-matched healthy volunteers (9%) ($p < 0.005$) (Figure 1).

Purification and Identification of the 49-kD Bronchial Epithelial Cell Autoantigen

To characterize the 49-kD bronchial epithelial cell autoantigen, this protein was purified by ion-exchange chromatography and reverse-phase HPLC. The purified protein was separated in an 8% Tris-glycine gel (Figure 2) and transferred to polyvinylidene difluoride membrane. Analysis of the N-terminal amino acid sequence could not be obtained, probably due to N-terminal blocking. The purified protein was then subjected to enzymatic in-gel digestion by trypsin, and the peptide fragments were separated by reverse-phase HPLC. The two fractions of peptide fragments were subjected to amino acid sequencing. Amino acid sequences of two peptide fragments were found compatible with human cytokeratin 18 upon database analysis (Figure 2), and the identification of the 49-kD autoantigen as human cytokeratin 18 was further confirmed by immunoblot analysis using the monoclonal antibody against human cytokeratin 18 (Figure 3).

DISCUSSION

In this study, we identified human cytokeratin 18 antigen as a bronchial epithelial autoantigen associated with nonallergic asthma. Cytokeratin 18 is a cytoskeletal protein found primarily in epithelial cells lining respiratory and gastrointestinal tracts, including bronchial epithelial cells and lung (alveolar) epithelial cells (12, 13). Although cytokeratin 18 is a predominantly intracellular protein, its strong expression on cell surfaces has been observed in a certain epithelial tissue (lacrimal gland aci-

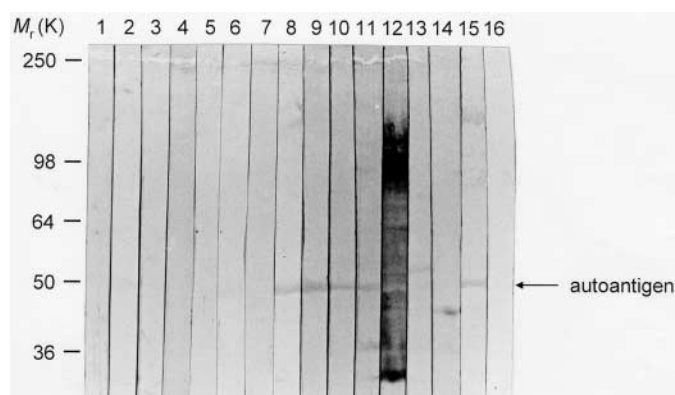


Figure 1. Immunoblot analysis of IgG autoantibodies to human bronchial epithelial cell antigens with human sera. Results from healthy control subjects (lanes 1–3), patients with allergic asthma (lanes 4–7), patients with nonallergic asthma (lanes 8–11), patients with systemic lupus erythematosus (lanes 12–14), a patient with nonallergic asthma as a positive control (lane 15), and dilution buffer only as a negative control (lane 16). Arrow indicates the 49-kD autoantigen.

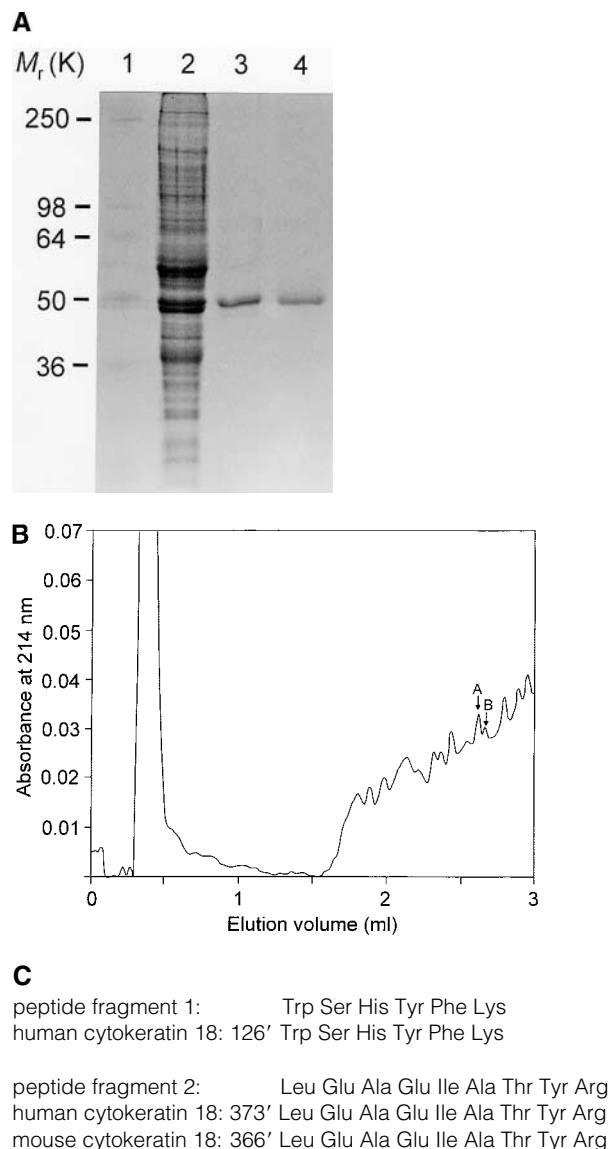


Figure 2. Purification and amino acid sequence analysis of the 49-kD bronchial epithelial autoantigen. (A) Separation of purified 49-kD autoantigen by SDS-PAGE. Protein staining showed molecular weight standard (lane 1), bronchial epithelial cell lysate (lane 2), 49-kD autoantigen purified by ion-exchange chromatography and reverse-phase HPLC (lane 3), and purified bovine cytokeratin 18 protein (lane 4). (B) Chromatogram of the peptide fragments derived from trypsin-digestion of purified 49-kD bronchial epithelial cell antigen separated by reverse-phase HPLC. Two fractions of peptide fragments (peaks A and B) were sequenced. (C) Amino acid sequences of the two peptide fragments were found compatible with human cytokeratin 18.

nar cells) (12, 14). Interestingly, cytokeratin 18 has also been identified as a major diisocyanate-binding protein in human bronchial tissue after inhalation of diisocyanate, which is an important asthma-causing volatile chemical in polyurethane workers (15).

The presence of circulating autoantibodies to cytokeratin 18 could be just a reflection of epithelial damage in patients with chronic severe asthma, considering the recent evidence that the bronchial epithelium is more susceptible to injury in this group (16). Further studies are needed to define the role of the autoimmune response (including T cell response) to cytokeratin 18 in the pathogenesis of nonallergic asthma.

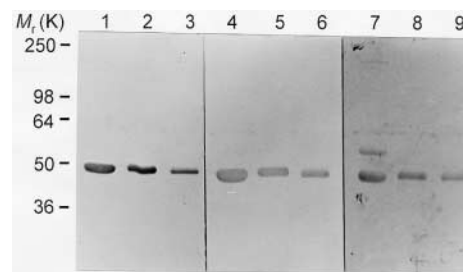


Figure 3. Immunoreactivity of autoantibodies in serum samples from patients with nonallergic asthma and monoclonal antibody to human cytokeratin 18. Antigens from bronchial epithelial cell lysate (lanes 1, 4, and 7), purified 49-kD autoantigen (lanes 2, 5, and 8), and purified bovine cytokeratin 18 (lanes 3, 6, and 9) were subjected to immunoblot analysis. Autoantibodies in serum samples from two patients with nonallergic asthma (lanes 1–3 and 7–9) and monoclonal antibody to human cytokeratin 18 (lanes 4–6) recognized the same 49-kD autoantigen.

The idea of a possible involvement of autoimmunity in the pathogenesis of asthma has been proposed by earlier studies that demonstrated higher incidences of various autoantibodies against antigens in bronchial mucosa, paranasal sinus, lung, endothelial cells, and nuclei in patients with asthma, compared with healthy control subjects (7–9, 17–19). However, these observations could not establish a causal relationship between autoimmunity and asthma, partly due to lack of an identified autoantigen or lack of a logical association between these autoantibodies and airway inflammation. The identification of cytokeratin 18 as a bronchial epithelial autoantigen associated with nonallergic asthma might provide a clue to exploring the autoimmune hypothesis in the pathogenesis of nonallergic asthma.

Increased concentrations of circulating IgG antibodies against bovine cytokeratin 18 antigen have been reported in patients with idiopathic pulmonary fibrosis and autoimmune hepatitis (20, 21), and increased concentrations of circulating IgA antibodies to bovine cytokeratin 18 antigen have also been reported in patients with rheumatoid arthritis (22). These findings suggest that the autoimmune response to cytokeratin 18 antigen may not be specific to nonallergic asthma. However, the possibility that differences in T cell and cytokine responses might result in different clinical features cannot be excluded.

The inflammatory response observed in a significant number of patients with asthma not only involved proximal airways but also lung, nasal mucosa, salivary glands, and even intestinal mucosa tissues (23–26). In some patients, asthma developed into the Churg–Strauss syndrome, a distinct form of systemic vasculitis characterized by eosinophilia, lung infiltration, paranasal sinus abnormality, and neuropathy (27). Rheumatic symptoms were reported to be more common among the patients with asthma with antinuclear antibodies (19). These observations suggest that asthma might be a systemic inflammatory disease in a subgroup of patients with asthma.

A previous report suggested that there were fundamental immunologic differences between allergic and nonallergic asthma in the patterns of T cell activation and cytokine production (28). Asthma might just be a syndrome including various heterogeneous diseases regarding etiology, natural history, and severity. Our study suggests the possible existence of a subgroup of patients with asthma characterized by the presence of autoantibodies to bronchial epithelial antigens.

In this study, we identified cytokeratin 18 as a bronchial epithelial autoantigen associated with nonallergic asthma. Further studies are needed to determine the significance of autoimmunity in nonallergic asthma.

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