
The prevalence of IgE antibody reactivity against the alkaline serine protease major allergen of *Penicillium chrysogenum* increases with the age of asthmatic patients

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Background: *Penicillium* species are prevalent airborne fungi. However, the prevalence of allergic sensitization to *Penicillium* antigens and the true impact of these ubiquitous fungi on atopic respiratory disorders remain to be determined.

Objective: The purpose of this study was to analyze the prevalence of immunoglobulin (Ig)E and IgG antibodies against *Penicillium chrysogenum* (Pen ch 13), the alkaline serine protease major allergen of *P. chrysogenum*, in asthmatic patients of different age groups.

Methods: Pen ch 13 was purified from a culture medium of *P. chrysogenum*. The reactivity of IgE and IgG antibodies to Pen ch 13 in the serum samples of 212 asthmatic patients was analyzed by immunoblotting methods.

Results: Sixty-nine (33%) of the 212 sera analyzed showed IgE and/or IgG immunoblot reactivity to Pen ch 13. Significant differences in the prevalence of IgE and/or IgG antibody reactivity to Pen ch 13 were found among eight different age groups of 212 asthmatic patients. The frequency of IgE-binding reactivity to Pen ch 13 increased significantly with the age of the patients. It was 7% for the group less than 10 years old and 42% for the group older than 70 years old. In addition, a significant difference between the prevalence of IgE (7%) and IgG (33%) antibodies against Pen ch 13 in the group aged 10 or less was also found.

Conclusions: Our study demonstrates that IgE and IgG antibodies specific for Pen ch 13 were detected in approximately one-third of the 212 asthmatic patients analyzed. Our results suggest that allergic sensitization to Pen ch 13, and possibly to other airborne *Penicillium* species, is more common in older asthmatic patients.

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INTRODUCTION

Fungi are ubiquitous in our environment. Sensitization to molds has been identified as a cause of human allergic disorders.^{1,2} *Penicillium* and *Aspergillus* species are prevalent indoor airborne fungi. Positive immediate skin reactivity to *Penicillium* components has been detected in 22 to 68% of atopic subjects.^{3–5}

Results from our previous studies showed that among the sera of 70 adult asthmatic patients, 17 (24%) had immuno-

globulin (Ig)E immunoblot reactivity toward components of *Penicillium chrysogenum* (also known as *P. notatum*).⁶ A 34-kDa major IgE-binding component (88% frequency of IgE binding) of this prevalent *Penicillium* species has been identified as an alkaline serine protease (Pen ch 13) after amino acid sequence analysis, cDNA cloning, and observation of its enzyme- and histamine-releasing activities.^{6,7} In addition, proteins belonging to the same alkaline serine protease family have also been identified as major allergens of another seven prevalent *Penicillium* and *Aspergillus* species.⁸ IgE cross-reactivity among group 13 major serine protease allergens from different fungal species has also been detected by enzyme-linked immunosorbent assay (ELISA) inhibition studies.⁷ The results suggest that group 13 allergens may play an important role in clinical mold allergy.

In analyzing human sensitization to aero-allergens in patients with asthma, age was identified as a contributing factor.^{9–12} Using the Pharmacia Enzyme CAP procedure (Pharmacia Biotech AB, Uppsala, Sweden), Nolles et al¹¹ analyzed specific IgE against *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, and *P. chrysogenum* in 137 atopic children age 5 months to 14 years. Their results showed that sensitization to fungi is prevalent in childhood,

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with an age-dependent distribution reaching maximum values at 7.7 to 7.8 years, followed by a decline for fungal sensitization with increasing age.¹¹ Using the same CAP system, Tsai and Chen¹² analyzed 1,070 asthmatic patients age 3 to 70 years. For *Dermatophagoides pteronyssinus*, the highest incidence was 96.8% in the group age 11 to 20 years.¹² For *P. chrysogenum*, however, the highest incidence was 18.9% for the group above 60 years old. For *Candida albicans*, the highest incidence was 27.3% in those who were 51 to 60 years old.¹²

The aim of this study was to analyze the prevalence of sensitization to *Penicillium* fungi among asthmatic subjects in different age groups using a purified group-13 fungal allergen, Pen ch 13. Immunoblotting was used to analyze IgE and IgG antibodies against Pen ch 13 in sera from asthmatic patients. In addition, reactivity of IgG4 antibodies against Pen ch 13 in IgE-positive serum samples was also analyzed. Our results revealed that IgE binding to Pen ch 13 was detected less frequently in children with asthma than in adults with asthma.

METHODS

Serum Samples

Serum samples were collected from asthmatic patients (age 3 to 94 years old) attending the allergy clinics at the Veterans General Hospital in Taipei and the Cathay General Hospital in Taipei. These patients had a history of reversible airway obstruction and were diagnosed in accordance with the statement provided by the American Thoracic Society.¹³ After collection, sera were stored in aliquots at -70°C .

Purification of the Pen ch 13 Allergen

Purification of the Pen ch 13 allergen was performed as previously described.⁷ *P. chrysogenum* strain CCRC 30568 (obtained from the Food Industry Research & Development Institute, Hsinchu, Taiwan) was cultured for 5 days at 25°C in a soybean liquid culture medium. The culture supernatant was collected and the protein components were fractionated through ammonium sulfate (40 to 80%) precipitation and centrifugation. The pellet was dissolved in distilled water and dialyzed against 50 mmol/L Tris-HCl, pH 7.5, before loading onto a diethylamino ethyl (DEAE) cellulose column (DE SeptraSorb, Sepragen Corporation, Hayward, CA) pre-equilibrated in the same buffer. The column was then eluted with a step gradient of 0.1, 0.2, and 0.3 M NaCl. The collected fractions were pooled according to their immunoblot reactivity to monoclonal antibodies (MoAbs) PCM8 and PCM10 against fungal serine protease allergens.¹⁴ The pooled fractions were then dialyzed against 10 mmol/L sodium phosphate (pH 6.8) and further purified on a CM Sephadex C50 (Pharmacia Biotech AB) cation-exchanger that was equilibrated with 10 mmol/L sodium phosphate (pH 6.8). Pen ch 13 was eluted off the column with the same buffer and identified according to its positive immunoblot reactivity to MoAbs PCM8 and PCM10. The purity of the allergen preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). The concentration of the protein preparation was determined by a dye-binding assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

The N-terminal amino acid sequence of the purified protein was determined by Edman degradation as previously described.⁷ The purified protein preparation was subjected to SDS-PAGE, then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm ; Millipore, Bedford, MA). Protein bands were visualized by Coomassie blue staining, excised from the membranes, and inserted into an ABI Procise Model 491 sequencer (Applied Biosystems, Foster City, CA) for automated cycles of Edman degradation according to the manufacturer's instructions.

Gel Electrophoresis and Immunoblotting

Immunoblot analyses of the purified Pen ch 13 allergen with human IgE, IgG, and IgG4 antibodies were performed essentially as described.⁶ Briefly, allergen Pen ch 13 was isolated by SDS-PAGE and transferred onto PVDF membranes. The blots were blocked with 1% skimmed milk and incubated individually with diluted human serum samples (5-, 50-, and 20-fold dilution for IgE, IgG, and IgG4 binding, respectively). After washing, the blots were incubated individually with enzyme-labeled secondary antibodies. These secondary antibodies are horseradish peroxidase-conjugated mouse antihuman IgG monoclonal antibody (2,000-fold dilution; Pharmingen, San Diego, CA), alkaline phosphatase-conjugated mouse antihuman IgE (800-fold dilution), and mouse antihuman IgG4 (1,000-fold dilution) monoclonal antibodies (Pharmingen). The mouse antihuman IgG monoclonal antibody reacts with the heavy chain of human Ig subclasses IgG1, IgG2, IgG3, and IgG4. The blots were washed and developed in a carbonate buffer solution (0.1 M NaHCO_3 , 1.0 mmol/L MgCl_2 , pH 9.8) containing 5-bromo-4-chloro-3-indolyl-phosphate and *p*-nitro blue tetrazolium chloride, or in an acetate buffer solution (50 mmol/L, pH 5.0) containing 3-amino-9-ethyl-carbazole and hydrogen peroxide. Antigen-antibody reactions were indicated by purple or red stain depositions and photographed. Sera from 15 healthy volunteers (20 to 50 years old) were used as a control. Low molecular weight standards (Pharmacia) were used to estimate the molecular mass of proteins.

Statistics

For statistical analysis, the χ^2 or Mantel-Haenszel χ^2 test was used. Significant differences were considered if $P < 0.05$.

RESULTS

Purification of Pen ch 13 Allergen

For the purification of Pen ch 13 allergen, ammonium sulfate precipitation of proteins in *P. chrysogenum* culture medium followed by DEAE ion exchange chromatography was performed. A 34-kDa component, which reacted with MoAbs PCM8 and PCM10, and a 30-kDa protein were recovered from the flow through fractions of the DEAE column. The

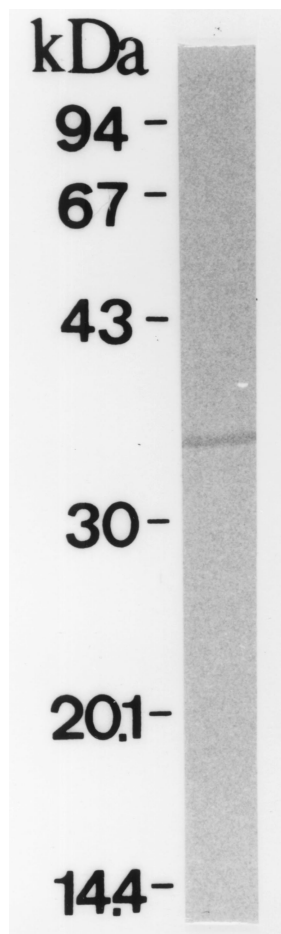


Figure 1. The Coomassie blue-stained SDS-PAGE protein profile of the purified Pen ch 13 allergen. The molecular mass standards (Pharmacia) used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa).

34-kDa immunopositive component was separated from the 30-kDa immunonegative protein using a CM Sephadex C50 cation-exchanger (Pharmacia). The purity of the 34-kDa protein preparation is shown in Figure 1. The first ten N-terminal amino acid residues of the purified protein were determined

(ANVVQRNAPS) and found to be identical to those of the 34-kDa major IgE-binding component in crude extract of *P. chrysogenum*.⁶

IgE and IgG Immunoblot Reactivity to Pen ch 13

IgE immunoblot reactivity to Pen ch 13 was found in 35 (17%) of the 212 serum samples tested (Table 1). The age distribution of the patients is also shown in Table 1. The frequency of IgE binding to Pen ch 13 rises significantly with the age of the patients (Mantel-Haenszel χ^2 test, $P < 0.01$).

Among 212 sera samples tested, 60 (28%) had IgG immunoblot reactivity to Pen ch 13 (Table 1). The prevalence of IgG immunoblot reactivity to Pen ch 13 was 33% and 29% for age groups ≤ 10 and 11 to 20 years old, respectively. For patients more than 20 years of age, an increase in the IgG-positive rate with age was also observed ($P < 0.01$, Mantel-Haenszel χ^2 test).

Results in Table 1 also demonstrate that, among 35 IgE-positive patients, 26 (74%) showed IgG immunoblot reactivity to Pen ch 13. Significant differences in the prevalence of both IgE- and IgG-binding activities to Pen ch 13 were found among asthmatic patients of different age groups ($P < 0.01$, χ^2 test). In addition, 143 patients (67%) showed negative IgE and IgG immunoblot reactivity to Pen ch 13 (Table 1). Significant differences in the distribution of negative antibody responses to Pen ch 13 were also observed between different age groups ($P < 0.05$, χ^2 test).

When IgE and IgG antibody responses to Pen ch 13 were compared within each age group, significant differences were found only in the group aged ≤ 10 years (χ^2 test, $P < 0.05$). Significant differences between IgE and IgG antibody responses to Pen ch 13 were not observed in the other groups. In contrast, IgE-binding reactivity to Pen ch 13 was not found in sera from 15 healthy volunteers. Weak IgG immunoblot reactivity to Pen ch 13 was found in 2 of the 15 controls (data not shown).

The immunoblots generated with IgE-positive serum samples are shown in Figure 2. In addition to an increase in the frequency of IgE binding, a more intense IgE reactivity to Pen ch 13 was also found among older asthmatic patients (Fig 2). In this study, IgG4 immunoblot reactivity of these 35 IgE-positive samples was also analyzed. Our results showed that most of the IgE-positive serum samples had IgG4-bind-

Table 1. Frequencies of IgE and/or IgG Immunoblot Reactivity to Pen ch 13 in Sera of Asthmatic Patients from Different Age Groups

Age group	No. of sera	IgE (+)	IgG (+)	IgE(+)IgG(+)	IgE(-)IgG(-)
≤ 10	27	2 (7%)	9 (33%)	1 (4%)	17 (63%)
11-20	31	3 (10%)	9 (29%)	2 (6%)	21 (68%)
21-30	25	2 (8%)	3 (12%)	1 (4%)	21 (84%)
31-40	36	3 (8%)	7 (19%)	1 (3%)	27 (75%)
41-50	25	4 (16%)	5 (20%)	3 (12%)	19 (76%)
51-60	21	4 (19%)	6 (29%)	4 (19%)	15 (71%)
61-70	21	6 (29%)	7 (33%)	5 (24%)	13 (62%)
> 70	26	11 (42%)	14 (54%)	9 (35%)	10 (38%)
Total	212	35 (17%)	60 (28%)	26 (12%)	143 (67%)

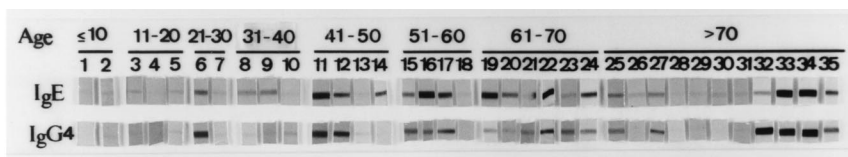


Figure 2. IgE and IgG4 immunoblot reactivity to Pen ch 13 in sera from asthmatic patients of different age groups.

ing activities to Pen ch 13 (Fig 2). IgG4-binding to Pen ch 13 was not detected in the sera of 15 healthy volunteers (data not shown).

DISCUSSION

Evidence associating asthma with indoor exposure to fungi has been reported.¹⁵ *Penicillium* species are among the most prevalent indoor airborne fungi. A 34-kDa major IgE-binding component of *P. chrysogenum* (Pen ch 13) was previously identified and characterized as an alkaline serine protease.^{6,7} Our results, shown in Table 1, indicated that IgE and/or IgG antibodies against Pen ch 13 were detectable in 69 (33%) of 212 asthmatic patients. This result correlates with a previous finding that IgE reactivity to a 34 kDa-component of *P. chrysogenum* has been detected in 15 (21%) of 70 sera samples from patients with asthma.⁶ However, in this study, we also found that the frequency and intensity of IgE-binding reactivity to Pen ch 13 increased with the age of the patients.

Sensitization to fungi has been reported to vary with age.⁹⁻¹² Kauffman et al¹⁰ noted that the rate of sensitization was highest in the youngest age group and declined rapidly with age. In the 14- to 19-year-old group, sensitization to *Alternaria* and *Cladosporium* are 20 and 18.8%, respectively. Above 30 years of age, the prevalence of skin test positivity declines to below 6.5%. It has been suggested that younger children may be less proficient in clearing fungi from the airway. The relatively rapid age-related fall in sensitization to fungi could be the result of increasing immunologic memory, which promotes antibody production against fungal antigens.¹⁰ Recently, using the Pharmacia CAP-radioallergosorbent test, Ezeamuzie et al¹⁶ showed a striking preponderance of sensitization among children (3 to 17 years age group) compared with adults in Kuwait. The rates for three molds (*Cladosporium*, *Aspergillus*, *Alternaria*) ranged from 38.3% to 51.3% for the 3- to 17-year-old age group, but declined sharply to 1.1 to 18.7% in older age groups.¹⁶

Our results show that the prevalence of the IgE antibody response to Pen ch 13 increases with age. We obtained values of 7 and 42% for patients from the ≤10 and >70 years old age groups, respectively. In addition, the IgG response to Pen ch 13 was also detected in 54% of 26 patients age 70 years and above. Discrepancies observed might be attributed to the genetic predisposition of various population groups; environmental factors, including the humidity and the growth of fungi and variations in environmental exposure among patients; the fungal antigen analyzed; and the assay methods used in these studies. Recent Pharmacia CAP test results of sera from 1070 asthmatic patients in the Taipei area also showed that the IgE positive rate for *P. chrysogenum* was

highest (18.9%) among patients more than 60 years of age.¹² The high frequency of allergic sensitization to *Penicillium* in the Taipei area could be explained by the high level of humidity and the hot climate. Taipei has a mean relative humidity of 79%, a mean temperature of 22° C, and an average summer temperature of 29° C. The trend toward higher antibody prevalence against Pen ch 13 because of age would indicate continuing antigen stimulation in the older patients. This may also reflect allergen exposure of these subjects to cross-reacting allergens from other fungal species. We reported that group-13 fungal allergens showed extensive allergenic cross-reactivity.⁶⁻⁸

Interleukin-4 has been shown to regulate Ig isotype switching and production of IgE and IgG4 antibodies.^{17,18} Results obtained in this study demonstrated that most of the IgE-positive sera also showed IgG4 immunoblot reactivity to Pen ch 13. In addition, relatively strong IgE and IgG4 antibody activities to Pen ch 13 were also observed in some of the positive serum samples. The apparent lack of interference of high levels of IgG antibodies with IgE antibodies suggests that these antibodies may be directed toward different epitopes, as has been reported for some cat and mite allergens.^{19,20} Earlier studies have also shown coexisting IgE and IgG4 antibodies directed against the same antigens in patients allergic to dust mites, pollen, cat dander, and mosquito bites.^{19,21-25} Results from Jeannin et al²² also showed that 90% of allergic patients had IgG4 antibody against sensitizing allergens. With a recombinant Der p 2 fusion protein, Tame et al²³ found higher levels of IgG4 but not IgG total or IgG1 to three antibodies in allergic children compared with nonallergic controls. Olsson et al²⁵ found that atopic farmers demonstrated correlating IgE and IgG4 levels to rLep d 2, which were significantly higher than those of nonatopic farmers and urban controls. In addition, no IgG4 antibodies were detected in nonatopic farmers despite chronic allergen exposure. Thus, studies on natural exposures suggest a more IgG4-restricted response to aeroallergens among atopic persons versus nonatopic persons.^{21-23,25} Oshika et al²⁶ also found that Der p 2 was strongly recognized by IgG from patients with bronchial asthma, whereas it was less recognized by IgG from healthy individuals. In the present study, among 15 healthy controls, only weak IgG but not IgE or IgG4 antibodies to Pen ch 13 were detected in two serum samples.

The role of allergen-specific IgG subclass antibodies to fungal allergens in the pathogenesis of asthma is poorly understood. An IgG4-dominating response was found after a prolonged exposure to an antigen.^{27,28} Specific IgG4 antibodies can be found after successful immunotherapy²⁹ and in frequently stung beekeepers.²⁷ Patients allergic to grass pol-

len and *Cladosporium* also have an increase of specific IgG4 after failing immunotherapy.^{30,31} A significantly higher IgG4 response to *A. fumigatus* antigen was found in patients with pulmonary aspergilloma and IgE production.³² An intense IgG4 reaction to *A. fumigatus* appeared as a typical marker for allergic bronchopulmonary aspergillosis.³³ This might reflect chronic antigenic stimulation caused by the chronic intrabronchial presence of *A. fumigatus*.³³ Results from Merrett et al³⁴ also showed that IgG4 levels were higher in patients with atopic eczema caused by prolonged exposure to an allergen that initiated an IgE response. Because patients included in the present study did not receive immunotherapy, the IgG4 response detected to Pen ch 13 might relate to prolonged exposure of the patients to airborne antigens. However, the finding that allergic patients had higher IgG4 antibody levels against a sensitizing allergen implies that factors in addition to chronic allergen exposure are necessary to induce this isotype.

It was also reported that atopic patients might develop IgG1 or IgG4 autoantibodies against IgE.^{35,36} However, in the present study, the presence of IgG4 antibodies against Pen ch 13 was clearly demonstrated in two independent experiments. By using serum number 33 as shown in Figure 2, Pen ch 13-bound antibodies were eluted from PVDF membranes with glycine-HCl (0.1 M, pH 2.5) and then neutralized with NaOH. Both IgE and IgG4 activities to Pen ch 13 were detectable in the neutralized solution (data not shown). After incubation of the solution with plates coated with anti-IgE antibodies, only IgG4- but not IgE-binding reactivity to Pen ch 13 was detected (data not shown). Further, removal of total IgE from the serum sample of patient number 33 by immunoprecipitation correspondingly removed all Pen ch 13-reactive IgE antibodies, but a strong IgG4 reactivity to Pen ch 13 could still be demonstrated by immunoblots (data not shown). These results confirm the presence of both Pen ch 13-specific IgE and IgG4 antibodies in this patient. In addition, when serum samples were diluted 5- and 20-fold for the detection of IgE and IgG4 activities, respectively, it was found that samples 6, 12, 15, 23, 27, and 32 had stronger IgG4 than IgE reactivity to Pen ch 13 (Fig 2). This also suggests that allergen-specific IgE and IgG4 antibodies are present in these serum samples.

Our results also showed that 9 of 27 patients age 10 years or less had IgG binding. Two of the 27 patients had IgE binding, including one patient with both IgE and IgG reactivity against Pen ch 13. Significant differences between the prevalence of IgE and IgG antibody responses against Pen ch 13 were observed in this younger age group. This indicates that a humoral antibody response, mainly of IgG rather than IgE, was elicited in approximately one-third of asthmatic children age 10 years or less in response to the exposure of *Penicillium* fungi. However, in the older age groups, both IgG and IgE developed against Pen ch 13. It has been reported that infants <1 year old are able to mount an IgG1 response to dust mites, even though they lack an IgG4 response because of the immaturity of their immune system.³⁷

In addition, the early IgG1 response precedes the formation of IgE antibodies, which does not normally occur in atopic babies until they are >2 years old.³⁸

CONCLUSION

Our study demonstrates that IgE and IgG antibodies specific for Pen ch 13 could be detected in patients with bronchial asthma after natural allergen exposure. In addition, our results show that allergic sensitization to Pen ch 13, and possibly to other airborne *Penicillium* species, may be of greater relevance to adult asthmatic patients than to children.

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