

Immunoblot analysis of components of *Penicillium notatum* recognized by human IgE antibodies

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Components of the crude extract of Penicillium notatum recognized by human IgE antibodies (Abs) were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The allergenic components were identified with sera from 19 allergic patients and 20 blood donors. The allergen-Ab complexes were visualized by ¹²⁵I-labeled monoclonal antihuman IgE and autoradiography. A total of 11 allergenic components, ranging in molecular weights (MWs) from 94,000 to 20,000 daltons, were identified. Heterogeneity in the IgE-binding patterns of the serum samples tested was also observed. However, the major allergen appears to be the component with an MW of about 68,000 daltons that was recognized by IgE Abs in 56% of the 39 sera analyzed. Furthermore, the component with an MW of about 64,000 daltons that was recognized by IgE Abs in 46% of the sera analyzed was also considered as an important allergen. Results obtained from this study will be useful in additional characterization of allergens of P. notatum and related fungal species. (J ALLERGY CLIN IMMUNOL 1991;88:802-7.)

Key words: *Penicillium notatum*, allergens, immunoblotting

Various fungi are able to induce human IgE-mediated allergic diseases. Species of *Penicillium* are common air contaminants both indoor and outdoor.^{1,2} The ubiquity and nonseasonal occurrence, as well as the ability of the species to liberate vast numbers of airborne spores, are major factors contributing to the importance of studies and monitoring of *Penicillium* as inhalant allergens.¹⁻⁶ Results from skin tests have also demonstrated that *Penicillium* is one of the molds that most frequently elicits positive skin test reactions in allergic individuals.^{1,7,8} However, little is known about the allergenic components of *Penicillium*. Crude extracts used in clinical practice are not well characterized and standardized. Different results have been reported when preparations from dif-

Abbreviations used

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MW: Molecular weight
Ab: Antibody
RIA: Radioimmunoassay

ferent commercial sources were used.⁹ Thus, it becomes important to identify components of *Penicillium* recognized by human IgE Abs.

In the present study, immunoblotting was performed to analyze components of the crude extract of *Penicillium notatum* recognized by human IgE Abs. *P. notatum* was used in this study because it is frequently used in clinical practice as a skin test reagent and in immunoassays. Results obtained from this study will provide a basis for additional studies on allergens of *P. notatum* and related fungal species.

MATERIAL AND METHODS

Serum samples

One hundred forty-four serum samples from patients with bronchial asthma attending allergy clinics at the Veterans General Hospital, Taipei, were collected. Patients were selected on the basis of reported history of asthma. Additional 517 serum samples obtained from random blood donors

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were also collected. After collection, all the serum samples were stored in aliquots at -70°C . The presence of specific IgE Abs to *P. notatum* in all serum samples was analyzed by RIA. Serum samples tested were added individually into wells of flexible polyvinyl microtiter plates (Costar Corp., Cambridge, Mass.) coated with the crude extract of *P. notatum* (see below). The presence of specific IgE Abs was detected by incubation with ^{125}I -labeled monoclonal anti-human IgE Abs.¹⁰ The radioactivity of each well was then counted in a gamma counter (LKB, Turku, Finland). Forty-two of the 144 serum samples obtained from allergic patients and 43 of the 517 serum samples obtained from blood donors that revealed specific IgE counts >1.5 times the value obtained with cord serum were selected and used in the immunoblot analysis.

The crude extract of *P. notatum*

The ground and freeze-dried preparation of *P. notatum* was obtained from Allergon AB, Engelholm, Sweden. The crude extract of *P. notatum* was prepared by extracting 5 gm of the lyophilized fungi with 100 ml of phosphate-buffered saline (containing 85 mmol/L of NaCl, 2.6 mmol/L of KH_2PO_4 , and 49 mmol/L of Na_2HPO_4 , pH 8.0) for 36 hours at 4°C with constant shaking. After centrifugation, the supernatant was dialyzed against phosphate buffer (10 mmol/L, pH 7.2) for 48 hours at 4°C . After sterile filtration (0.2 μm ; Sartorius, Hayward, Calif.), the extract was lyophilized in aliquots and stored at 4°C . The protein content of the extract was determined by the dye-binding method of Bio-Rad Laboratories (Richmond, Calif.).

Immunoblot analysis

The immunoblot analysis of components of *P. notatum* recognized by IgE Abs in human serum samples was performed essentially as described previously.^{11, 12} Instead of the use of enzyme-labeled anti-IgE Abs, ^{125}I -labeled monoclonal antihuman IgE Abs were used in the present study.¹⁰ Components of the crude extract of *P. notatum* in SDS-PAGE sample buffer (containing 2% SDS and 5% 2-mercaptoethanol), which were boiled for 5 minutes, were separated on a 12.5% SDS-polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membrane (0.45 μm , Millipore, Bedford, Mass.). After the blot was blocked with 1% skim milk and incubated with serum samples, it was incubated with ^{125}I -labeled antihuman IgE Abs (1×10^6 cpm per strip) for 1 hour at room temperature. After an extensive wash with phosphate-buffered saline (150 mmol/L, pH 7.2) containing 0.05% Tween 20, the blot was processed for autoradiography. The blots were exposed at -70°C with x-ray films (Kodak, Rochester, N.Y.) and intensifying screens for 72 hours. The results of immunoblotting were recorded by photography.

RESULTS

For the analysis of components of *P. notatum* recognized by IgE Abs, protein components of the crude fungal extract were separated by SDS-PAGE. The Coomassie blue-stained protein profile of the crude extract is illustrated in Fig. 1. At least 30 different

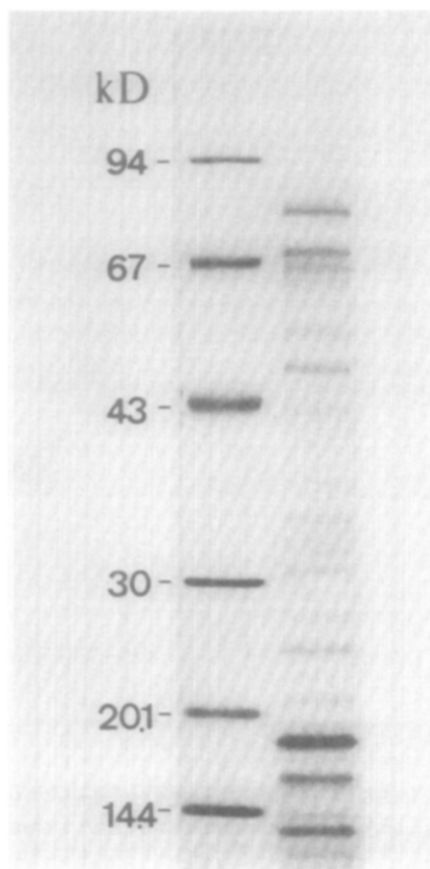


FIG. 1. The Coomassie blue-stained SDS-PAGE protein profile of the crude extract of *P. notatum*. The MW standards (Pharmacia, Uppsala, Sweden) used were phosphorylase b, 94 kd; bovine serum albumin, 67 kd; ovalbumin, 43 kd; carbonic anhydrase, 30 kd; soybean trypsin inhibitor, 20.1 kd; and α -lactalbumin, 14.4 kd.

components with MWs ranging from >100 to about 10 kd in the crude extract were observed. Components with MWs of about 76, 68, 49, 19, 17, and 13.5 kd were predominant.

For immunoblot analysis, serum samples from allergic patients and random blood donors were used. Nineteen of the 42 serum samples obtained from patients and 20 of the 43 samples from blood donors revealed positive results (Figs. 2 and 3, respectively). The components in the crude extract recognized by IgE Abs in different serum samples were identified and tabulated (Tables I and II). For the analysis of results that demonstrated immunoblotting results as smears on autoradiography, shorter exposure time was used to aid identification of IgE-binding components. Heterogeneity in the IgE-binding patterns of the serum samples tested was observed. In total, 11 components of *P. notatum*, ranging in MWs from about 94 to 20 kd, bound to IgE Abs (Table III). Many of the serum samples tested demonstrated IgE-binding activities to

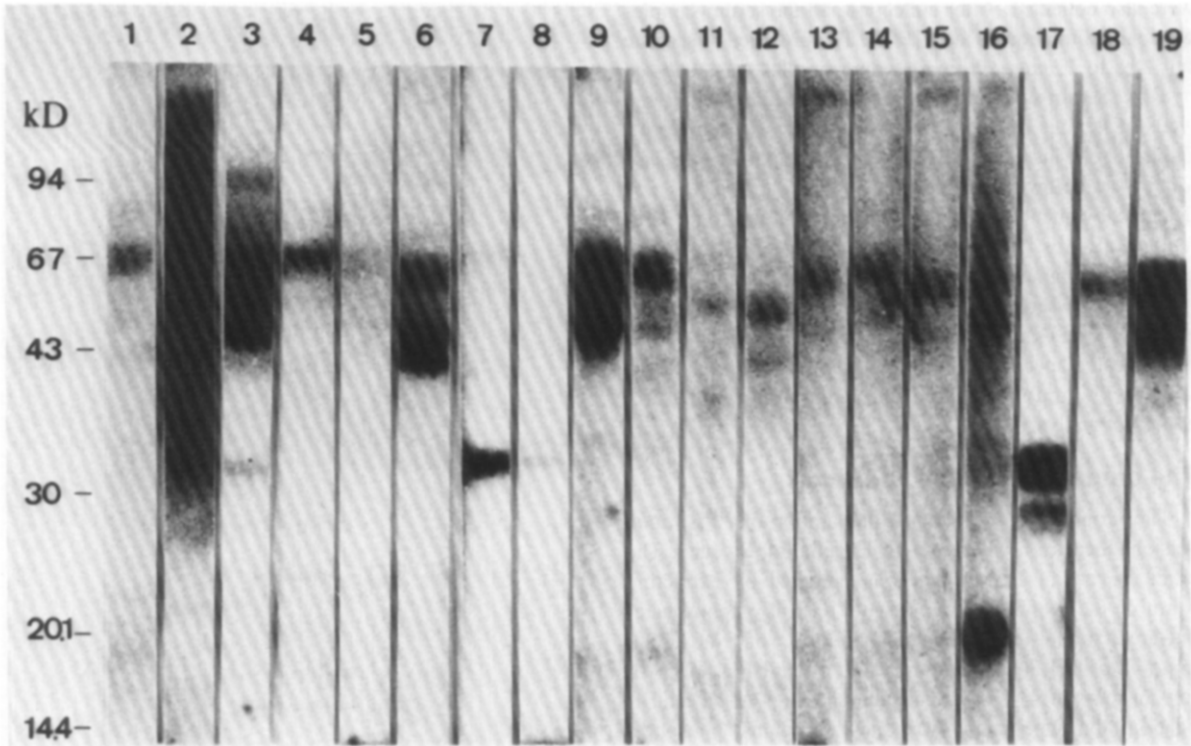


FIG. 2. Identification of IgE-binding components by ¹²⁵I-labeled antihuman IgE Abs after SDS-PAGE of the crude extract of *P. notatum* with sera from 19 allergic patients. The MW markers used were the same as those described in Fig. 1.

TABLE I. Components of *P. notatum* recognized by IgE Abs in sera of 19 allergic patients

Serum No.	Component MW (kd)	IgE binding									
		1 94	2 86	3 68	4 64	5 52	6 49	7 42	8 39	9 33	10 28
1				0							
2		0	0	0	0		0		0	0	
3		0			0	0	0			0	
4				0							
5				0							
6					0		0	0			
7										0	
8										0	
9				0	0		0				
10				0							
11						0					
12						0		0			
13					0						
14				0							
15					0						
16					0		0				
17										0	0
18					0						
19				0	0				0		

TABLE III. Frequencies of specific IgE binding of components of *P. notatum* observed in immunoblots with serum samples from 19 allergic patients and 20 blood donors

Component of <i>P. notatum</i>	MW (kd)	Frequency of IgE binding (%)*	
		Allergic patients	Blood donors
1	94	11	5
2	86	5	10
3	68	42	70
4	64	47	45
5	52	16	15
6	49	26	5
7	42	11	0
8	39	11	30
9	33	26	35
10	28	5	5
11	20	0	15

The number of serum samples with positive immunoblotting activity were 19 for the patient group and 20 for the blood donor group, respectively.

*The frequency of IgE binding was calculated as follows:

$$\frac{\text{No. of serum that demonstrated IgE binding to each component}}{\text{No. of serum samples with positive immunoblotting activity}} \times 100$$

more than one component in the crude fungal extract (Tables I and II). Except for the 28 kd component, different frequencies of IgE binding for each allergenic component with sera from two different groups of individuals were also observed (Table III). The 68 and 64 kd components that demonstrated relatively high frequencies (>40%) of IgE binding in both serum groups could be recognized as important allergens of *P. notatum* (Table III). However, the 68 kd component that demonstrated the highest frequency of IgE binding, being recognized by 22 (56%) of the 39 sera analyzed, may be considered as the major allergen of *P. notatum*. The component with an MW of about 33 kd that demonstrated about 30% IgE-binding frequency in both serum groups may also be recognized as a significant allergen of *P. notatum* (Table III). Furthermore, although only 5% of the sera from the blood donors revealed IgE-binding activity to the 49 kd component of *P. notatum*, five of the 19 serum samples (26%) from allergic patients revealed positive IgE binding to this 49 kd component (Table III). A similar finding was observed for the 39 kd component with IgE-binding frequency of 11% and 30% for the patient and blood donor groups, respectively (Table

III). For all other IgE-binding components, frequencies of <20% were observed in both serum groups (Table III). None of the 19 serum samples from the patients demonstrated positive immunoblotting activity to the 20 kd component of *P. notatum* (Table III). A similar finding was observed for the 42 kd component. The 42 kd component was recognized by IgE Abs in only two of 19 sera obtained from allergic patients (Table III). For negative control, IgE-binding activity was not observed when cord serum samples and sera from allergic patients who demonstrated both negative skin test and specific IgE RIA activity to *P. notatum* were used in the immunoblot analysis (data not presented). The dot observed in the low MW area on strip No. 16 of Fig. 2 was an artifact of the experiment and was not considered as a positive signal.

DISCUSSION

Penicillium is believed to be among the genera of molds most responsible for the induction of allergic respiratory disease.¹ However, it is also one of the least studied genera of molds from the standpoint of antigenic/allergenic activity. For the analysis of components of *P. notatum* recognized by human IgE Abs, immunoblotting was used in the present study. To obtain a large number of serum samples that might demonstrate positive immunoblotting activity, 144 serum samples from allergic patients and 517 serum samples from blood donors were collected. The presence of specific IgE Abs against *P. notatum* in all these serum samples was analyzed by the RIA. Serum samples with specific radioactive counts >1.5 times the value obtained with cord serum were used in the immunoblot analysis. Although there were differences in the IgE-binding frequencies for most of the allergenic components, similar IgE-binding profiles were still observed with these two different groups of sera (Figs. 2 and 3). The observation that different IgE-binding frequencies of different serum groups used in the analysis of the same allergenic extract has been reported before.¹³ Since about 10% of the general population is considered to suffer from allergic diseases, the 43 of the 517 serum samples from blood donors (8%) that were selected and used in the immunoblot analysis may in general be considered as sera from individuals who probably suffer from allergic disorders. Although data of immediate skin test were not available from blood donors, it was available for 14 (Nos. 1 to 14, Fig. 2) of the 19 allergic patients with positive IgE-immunoblotting activity. Except for four patients (Nos. 4, 8, 10, and 11, Fig. 2), eight of the 14 patients demonstrated positive immediate skin test to *Penicillium* with the allergenic extract supplied by Taiwan Allergy Center. Another two of the 14

patients (Nos. 7 and 9) demonstrated positive immediate skin test to mixed mold extracts, including *Penicillium*. Except for the different evaluation systems used, the discrepancy observed between results of the skin test and the immunoblot analysis may be that different allergenic extracts were used in different assay systems. In general, sera with high-titer *Penicillium*-specific IgE Abs, as determined by the RIA, was more likely to demonstrate IgE binding in the immunoblotting studies than sera with lower titers. The discrepancy observed between results of RIA and the immunoblot analysis may be explained by the fact that some heat-labile and detergent-susceptible epitopes might have been denatured in the SDS-PAGE system used in the immunoblot analysis, resulting in negativity in immunoblotting analysis. Furthermore, the use of 2-mercaptoethanol in sample preparation might also have contributed to the discrepancy observed.

Like other studies of immunoblot analysis,¹⁴⁻¹⁶ heterogeneity in IgE-binding patterns with different serum samples was also observed in the present study. However, the component with an MW of about 68 kd that was recognized by IgE Abs in 56% of the 39 immunoblotting-positive sera analyzed could be considered as the major allergen of *P. notatum*. The 64 kd component with an IgE-binding frequency of 46% of the 39 sera analyzed may also be considered as an important allergen. Furthermore, it should also be noticed that some patients may only develop high-titered IgE Abs against components different from these two important allergens, as demonstrated in patients Nos. 7 and 17 in Fig. 2.

In conclusion, 11 components of *P. notatum* recognized by IgE Abs in 39 serum samples were identified by immunoblot analysis in the present study. Components with MWs of about 68 and 64 kd that demonstrated relatively high IgE-binding frequencies were considered as important allergens of *P. notatum*. Based on this preliminary result, a monoclonal Ab (P40) against the 68 kd allergen has been generated in our laboratory and was used in the characterization of this 68 kd allergen and in the analysis of antigenic/allergenic cross-reactivity among different gen-

era of fungi and among different species of the genus *Penicillium*.

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