IgE-sensitization to cellular and culture filtrates of fungal extracts in patients with atopic dermatitis

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Background: Patients with atopic dermatitis may experience exacerbations of eczema triggered by various inflammatory stimuli. One mechanism may be IgE-mediated reactions to dermatophytes since these patients are more likely to acquire skin infections with dermatophytes and may become sensitized.

Objective: This study investigates IgE-sensitization to fungi in patients with atopic dermatitis and compares the biologic activity of culture filtrates and cellular fungal extracts. The following allergen extracts were provided as culture filtrates and cellular extracts: *Candida albicans*, *Fusarium moniliforme*, and *Penicillium notatum.* In addition, *Pityrosporum ovale* and *Trichophyton rubrum* cultures were included in the test panel.

Methods: Fifteen patients with clinical findings suggesting dermatophytosis and 11 controls were selected. Each subject was tested by leukocyte histamine release and skin prick test to each fungal extract. The extracts were separated and reduced by sodium dodecylsulfate polyacrylamide gel electrophoresis and analyzed by IgE-immunoblotting with sera from all study subjects.

Results: Fourteen patients (93%) reacted to one or several fungal extracts by releasing histamine when challenged in vitro. By immunoblotting experiments, patient sera showed binding to a wide range of components in all extracts. Patient sera recognized allergenic components shared by culture filtrates and cellular extracts but with higher frequent and greater intensity in culture filtrates. Although culture filtrates generated more frequent and potent IgE-reactions than the cellular extracts, the difference was not statistically significant. Biologic potency was similar when evaluated by skin prick tests and leukocyte histamine release.

Conclusion: Patients with atopic dermatitis may develop specific IgE-antibodies to a number of fungi as demonstrated by IgE-immunoblotting. In selected patients, fungi may trigger an IgE-mediated reaction that may contribute to the exacerbation of eczema. Approximately, one-half of the patients, however, produced IgE-antibodies to fungal (glyco)proteins without a significant histamine release or skin test response possibly because of nonspecific interaction with carbohydrate moieties on IgE and poor biologic activity of IgE antibodies directed to cross-reactive carbohydrate determinants of fungal glycoproteins. This warrants caution when interpreting clinical relevance of serologic measurements of fungal IgE-antibodies.

Ann Allergy Asthma Immunol 1998;81:247–255.

INTRODUCTION

Atopic dermatitis is a hereditary inflammatory skin disorder that is associated with other allergic disorders such as asthma and rhinitis. Exacerbation of skin rashes may be strongly influenced by environmental factors, partly because of IgE sensitization and possibly also a T cell response.[1] These patients are known to have a reduced skin barrier and dysregulated T cells facilitating infection with colonizing microorganisms (members and non-members of the skin flora) due to favorable growth conditions. Some members of the skin microflora have been intensely investigated for their potential pathogenic effect in patients with atopic dermatitis. Among these are the gram positive bacterium Staphylococcus and the lipophilic yeast *Pityrosporum ovale*.[2,3] The latter can be isolated from the skin of 80% to 90% of normal individuals.[3] *Pityrosporum ovale* inhabits the greasy areas of the skin and for that reason it is often associated with the "head and neck" type of atopic dermatitis.[4,5] Several studies have demonstrated type I skin reactions and the presence of IgE-antibodies to *Pityrosporum ovale.*[3,5–8] Furthermore, reduction of microbial load through oral treatment with ketoconazole may lead to clinical improvement.[9] Another yeast-like mold *Candida albicans*, which colonizes the mucosal membranes of the body, is also a well-known potential pathogen in atopic dermatitis, and type I-mediated reactions have been reported.[8,10] Several investigators have demonstrated crossreactivity between *Pityrosporum ovale* and *Candida albicans.*[11–13] Crossreactivity has been observed with allergen binding proteins and a number of carbohydrate epitopes; mannans and mannoproteins, both of which are cell wall components of yeast. Additionally, recent studies showed that atopic individuals have an increased risk of persistent infections with *Trichophyton rubrum* and *Penicillium notatum.*[7,14,15] To what extent other members (and non-members) of the skinflora can cause type I reactions is less known.

The aim of this study was to examine a selected group of patients with atopic dermatitis for the presence of

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This study was funded by an unrestricted research grant from Allergifonden af 1981.

Received for publication January 10, 1998.

Accepted for publication in revised form April 14, 1998.

IgE antibodies and record the basophil cell and skin prick test response towards a panel of fungi. In addition, by analyzing patient sera by immunoblotting IgE-binding, (glyco)proteins in extracts were demonstrated.

MATERIAL AND METHODS

Patients

This study was undertaken according to the Helsinki declaration on human investigations and approved by the local ethics committee (protocol KF01-444/93 and KF01-435/93). Informed consent was obtained from all subjects. Fifteen patients fulfilling the Hanifin criteria for atopic dermatitis[16] and with clinical findings suggesting head-and-neck dermatitis or skin infection (honey-colored crusting, extensive serous weeping, folliculitis, and pyoderma) were recruited from the Departments of Dermatology, University Hospital of Copenhagen and Bispebjerg Hospital. The median age was 28 years (range 18 to 60 years).

Heparinized whole blood samples from all patients were tested for basophil histamine release to a panel of fungal extracts. Prior to blood drawing skin prick tests were performed. Five atopic donors with inhalant allergies to pollens and six nonatopic donors with no inhalant allergy to pollens and with no atopic dermatitis served as negative controls. Furthermore, a serum pool consisting of serum from normal individual, and a sample of human myeloma-IgE (greater than 4000 U/mL) were included as negative control sera. All sera were immunoblotted with each fungal extract.

Fungal Extracts

Cellular extracts and culture filtrates of *Candida albicans*, *Penicillium notatum*, *Fusarium moniliforme*, and *Trichophyton rubrum* were delivered lyophilized by Greer Laboratories, Lenoir, NC, and *Pityrosporum ovale* by ALK, Denmark. For skin prick test and leukocyte histamine release the allergens were reconstituted with 50% glycerol and stored at 4 °C. For IgE-immunoblotting, the allergens were reconstituted with distilled water at the day of analysis. Before the patient study was undertaken, fungal extracts were examined for analytical interference or non-specific histamine release; none was observed. In addition, dose-response curves were established in several subjects before including patients in the study.

Skin Prick Test

Skin prick tests were performed according to EAACI guidelines.[17] In brief, a 10 mg/mL histamine solution was included as positive control and a 50% glycerol solution served as negative control. A 1-mm prick lancet was used and results were read after 15 minutes and transferred with at strip of tape to squared millimeter paper. The wheals were measured at the longest axis and perpendicular to it. Then the mean was calculated and used in the data analysis.

Histamine Release Test

The leukocyte histamine release test was performed as previously described with glassmicrofibre-coated microtitre plates that selectively bind histamine.[18,19] Briefly, 25 μL of whole blood was incubated with 25 μL fungal extract (diluted with Pipes-AMC buffer (10 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 140 mM sodiumacetate, 5 mM potassiumacetate, 30 mM Trizma-T-1503, 0.5 mM calcium chloride, and 1 mM magnesium chloride (pH 7.4). Aliquots were run in duplicate and tested with allergen extracts at six 3.5-fold dilution. In addition, all blood samples were tested with a dilution range of anti-IgE (Behringwerke, Germany). After one hour incubation at 37 °C the glassfiber-coated microtitre wells were rinsed and cell debris and proteins were enzymatically removed. Released histamine was coupled to *o*-phthaldehyde and measured spectrophotoflourometrically with a Gilson flourometer. Released histamine was expressed as nanogram histamine per milliliter and calculated on the basis of a histamine standard curve. The histamine release data were evaluated by calculating the area under the curve (AUC) by numeric integration by the formula ½c1 + c2 + c3 + c4 + c5 + c2 + c3 + c4 + c5 + ½c6 where "c" is the histamine released (in ng/mL whole blood) at a given concentration (symbolized by numbers 1 to 6).[20]

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

The fungal extracts were separated in a 16% separation gel with a 5% stacking gel in a discontinuous buffer system according to the method of Laemmli.[21] The assays were performed in a vertical slab gel apparatus (Hoefer Instruments, San Francisco, CA). The fungal extracts were boiled for five minutes with a reducing sample buffer (containing 2-β-mercaptoethanol) and 40 μL of each sample (containing 34 to 100 μg protein) was loaded on a 0.5 cm gel. The gels were stained with Coomassie Brilliant Blue or Periodic Acid Schiff (PAS). For PAS staining, the gel was (1) fixed with a 40% methanol and 7% acetic acid solution for one hour at 4 °C; (2) oxidized in a 1% periodic acid, 3% acetic acid solution for one to two hours at room temperature; (3) rinsed in distilled water at least 6 times for two hours (while stirring); (4) incubated in Schiffs reagent (Merck) in the dark for 50 minutes at room temperature; and (5) rinsed in a 0.5% sodium disulfide solution for one hour and then rinsed in large amounts of water.

For immunoblotting, the same amounts of fungal extracts as indicated above were used per 0.5 cm gel. The molecular weights were estimated using a pre-stained protein standard (14.3 to 200 kD, Amersham International, Burkinghamshire, UK).

IgE-Immunoblotting and Autoradiography

Transfer of (glyco)proteins to nitrocellulose (0.2 mm pore size; Schleicher and Schull) was performed with a semidry electroblotter (JKA-Biotech, Copenhagen, Denmark) at 0.8 mA/cm^2 for 90 minutes in a discontinuous buffer system (anodic buffer 1: 0.3 M Tris with 20% methanol, pH 10.4 and anodic buffer 2: 25 mM Tris with 20% methanol, pH 10.4 and cathodic buffer:

25 mM Tris, 40 mM 6-Amino-*n*-hexanoic acid with 20% methanol, pH 9.4). For IgE-immunoblotting, the nitrocellulose paper was cut into 0.5-cm wide strips and incubated for half hour with TBS buffer (0.05 M Tris, 0.15 M NaCl, 0.005 M NaN_3, 0.5% Tween, pH 7.4) to which another 1% Tween was added for blocking. Sera were diluted 1:10 in TBS and incubated over night at room temperature. Furthermore, one strip from each fungal extract was incubated with human myeloma IgE (4000 U/mL). After the strips had been washed three times in TBS, 1 mL ^{125}I rabbit anti-human IgE (Pharmacia) diluted to 200,000 cpm/mL TBS was added to each strip. After overnight incubation, the strips were washed, air-dried, placed in filmcassettes, and sensitive radiographic films (Hyperfilm-MP, CODE RPN.6, Amersham) were developed at day 1, day 7, and day 14, respectively. The (glyco)proteins detected by IgE-immunoblotting were divided into three categories, weak/visible, moderate, and strong by a visual evaluation. The immunoblot was considered positive if at least one protein band was visible.

Total IgE

The automated HYTEC EIA system using anti-IgE coated paper discs were used to measure total IgE in the sera.[22] The assay is calibrated according to the WHO serum standard (International Reference Preparation 75/502) and values are reported in kU/L. The dynamic range of the assay was 0.5 to 2000 kU/L with a coefficient of variation less than 15%.

Data Evaluation

In nonatopic controls, the skin test extracts gave no positive skin reactions; therefore, a threshold value of 3 mm was chosen. The extracts had not been tested by in vitro histamine release previously and the in vitro potency was not known. The cutoff value was established by incubating blood from nonatopic controls and atopics without eczema with a titrated dilution range of the extracts. The threshold value was found to be greater than 45 ng/mL histamine. The Wilcoxon signed rank test procedure was used to test for matched-pair differences (culture filtrates versus cellular extracts), and Wilcoxon-Mann-Whitney Rank sum test was used when comparing skin test and histamine release with IgE-immunoblotting.

RESULTS

Patients

Patients with clinical signs of skin infection included seven females and eight males with a median total IgE level of greater than 2,000 IU/mL (range 64 to greater than 2,000 IU/mL). Their median skin test wheal size to fungal extracts was 4 mm and ranging from 3 to 7 mm. The histamine control wheal size was 7 mm and ranging from 5 to 10 mm.

None of the controls (11 subjects) reacted to the fungal extracts by skin prick test or histamine release. To assure cell viability a positive anti-IgE-induced histamine release response was confirmed with all blood samples from patients and controls. All fungal extracts were able to induce a basophil histamine release response or skin prick test wheal in the patient group. No significant difference ($P > .05$) was observed in the biologic potency (wheal size or histamine release) between cellular extracts and culture filtrates.

Frequency Distribution of Test Results

Detection of patient sensitization was method dependent. The relative frequency rate of patients (n = 15) sensitized to fungi was 100% (15), 93% (14), and 60% (9) for immunoblotting, histamine release, and skin prick test, respectively. The majority of patients were sensitized to *P. ovale* or *F. moniliforme* (Table 1). Although IgE-immunoblotting data indicated the frequency of patients positive to the cellular extracts was lower than the frequency of patients positive to the culture filtrates, the difference was not significant ($P > .05$).

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

Separation of (glyco)proteins in fungal extracts (Table 2) followed by staining with Coomassie Brilliant Blue or PAS revealed a broad spectrum of glycoproteins of various molecular weights. The PAS staining resulted in a diffuse color concentrated in the high molecular weight area of all extracts. A striking difference was observed when comparing the cellular extracts and culture filtrates, ie, cellular extracts contained more carbohydrate and showed a less distinct banding pattern. This was most pronounced for the *C. albicans* extract. Some of the glycoproteins were more visible on the PAS-stained gel than the Coomassie stained-gel suggesting that these are rich in carbohydrates. Generally, the high molecular weight glycoproteins were better visualized in the Coomassie stained gel, probably because the intensive coloring of the high molecular weight area of the PAS-stained gel was camouflaged by the protein bands.

Table 1. Frequency Distribution of Test Results from 15 Patients with Atopic Dermatitis

Extract	Skin Prick Test Positives	Histamine Release Positives	Immunoblot Positives
P. ovale	9 (60%)	11 (73%)	14 (93%)
T. rubrum	3 (20%)	5 (33%)	10 (67%)
C. albicans (cellular)	4 (27%)	3 (20%)	7 (47%)
C. albicans (culture)	2 (13%)	3 (20%)	9 (60%)
F. moniliforme (cellular)	3 (20%)	4 (27%)	8 (53%)
F. moniliforme (culture)	4 (27%)	9 (60%)	15 (100%)
P. notatum (cellular)	2 (13%)	4 (27%)	4 (27%)
P. notatum (culture)	1 (7%)	1 (7%)	8 (53%)

Table 2. Molecular Weights (in kD) of Fungal Extracts Separated by SDS-PAGE

P. ovale		T. rubrum		C. alb. cel.		C. alb. cul.		P. not. cel.		P. not. cul.		F. mon. cel.		F. mon. cul.	
CO	PAS*	CO	PAS	CO	PAS	CO	PAS	CO	PAS	CO	PAS	CO	PAS	CO	PAS
>200		120				>200		130		130		70		130	
	140	96	96	No visible		175		96		105			62	105	
	110	86	86	bands		135		70		45	45		58		70
96	96	80				125		52		41	41	52		62	62
	86	61				58		45	45	38	38		47		58
	70		54			46	46		41	32	32	39	39		44
	68	46					38		38	28	28		35		43
	54		44				33	35		26			28		41
	52	40	40			30			28		24	>14		39	39
	40	37				29			24		19				35
	37	32					28		22	15					32
36	36		30			27	27		17						30
34	34	28				26			<14					29	29
33	33		25				25							28	
	25	17					23							27	
24	24		16			16	16							26	26
23		15					>14							25	25
22		>14	>14				≪14							15	
	19														>14
	18														

* Abbreviations: CO (Coomasie blue staining) and PAS (Periodic acid Schiff staining) *P. ovale* = Pityrosporum ovale, *C. alb.* = *Candida albicans*, *P. not.* = *Penicillium notatum, F. mon.* = *Fusarium moniliforme*, and *T. rubrum* = *Trichophyton rubrum.*

IgE-Immunoblotting

For further identification of IgE-binding components of the allergenic extracts, immunoblotting was performed with each fungal preparation. Figure 1A-F shows allergograms depicting the most frequent IgE-binding components. Examples of the immunoblots from representative patients are also shown. Components to which less than three patients reacted were not included. To determine the total number of IgE-binding components in the allergen preparation and the number of IgE-binding components shared by culture filtrates and cellular extracts, we compared the number of bands seen on the immunoblots (Table 3). Generally, the frequency of IgE-binding components of the fungal extract was very high (63%). It is evident that patients with atopic dermatitis generate IgE that is directed against a broad spectrum of fungal components (range: 9 to 18 components). The sodium dodecylsulfate-polyacrylamide gel electrophoresis banding pattern resembled the immunoblot. Generally, the same IgE-binding components were present in culture filtrates and cellular extracts, but the staining patterns are more distinct in the culture filtrates. Furthermore, patient sera recognized components shared by culture and cellular filtrates, but with higher frequency and greater intensity in culture extract. One exception is the greater than 200-kD component of *Candida albicans* which was recognized more often in the cellular filtrate. To test for nonspecific binding, extracts were incubated with human myeloma IgE and a serum pool from normal subjects. No binding was observed; however, one control with inhalant allergies and elevated IgE levels showed weak unspecific IgE-binding as described below.

Table 3. IgE-Binding Components

Allergen	Total Number of IgE-Binding Components	Shared IgE-Binding Components	Important IgE-Binding Components
C. albicans (cellular)	13		3
C. albicans (culture)	14	9	7
P. notatum (cellular)	11		2
P. notatum (culture)	14	11	11
F. moniliforme (cellular)	10		3
F. moniliforme (culture)	9	5	4
P. ovale	19		9
T. rubrum	18		11

Candida albicans

The cellular extract and culture filtrate share three components in the high molecular weight area (125, 175, and 200 kD). The cellular extract contains only high molecular weight components. In contrast, the culture filtrate reveals three more important compo-

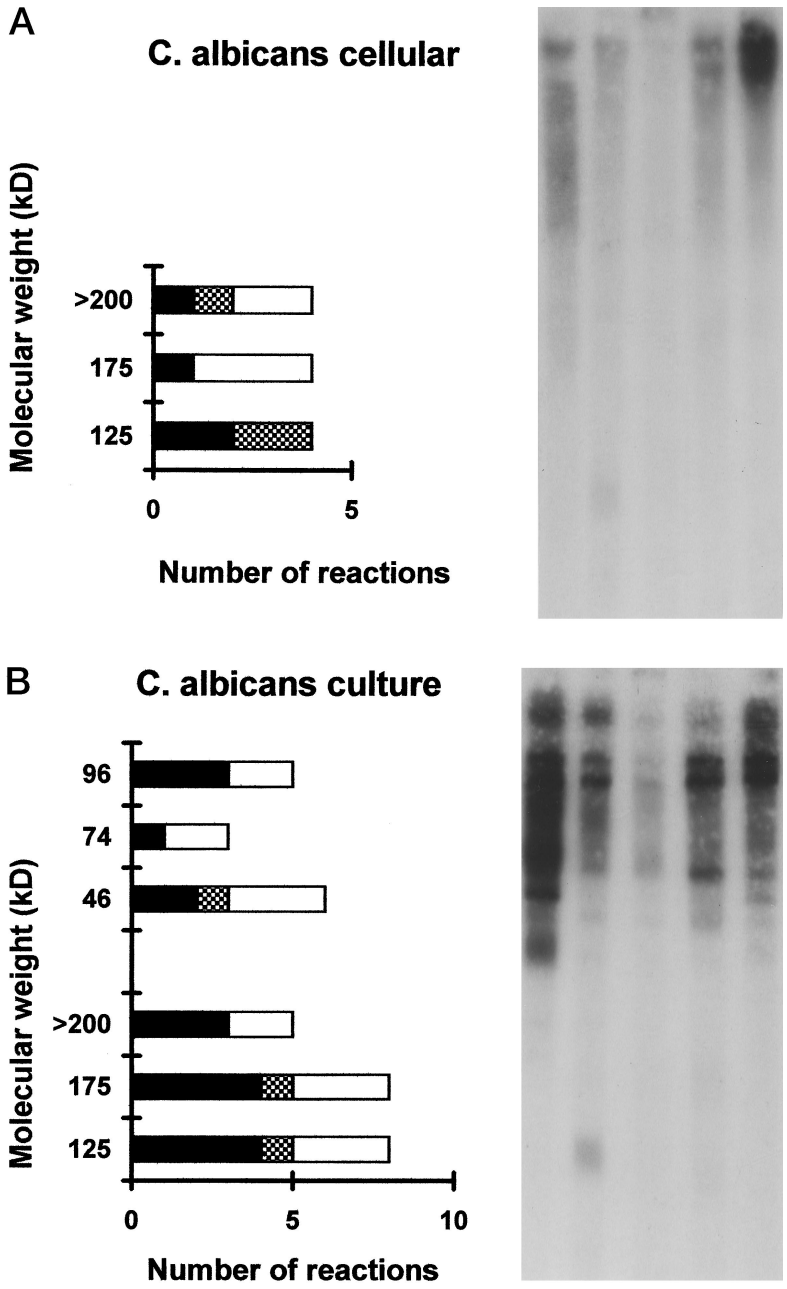

Figure 1. The molecular weights of IgE-binding components in the various extracts are presented as bars (y-axis). The staining intensity is divided into three categories; weak (blank), moderate (hatched) and strong (black). The x axis shows the number of patients reacting to the various components and the staining intensity. The bars shown in the bottom of the figures 1b, 1d, 1f, are components found in both preparations (cellular and culture). Note that components to which less than three sera reacted are not included in the figure. To the right illustrative examples of the patient IgE-immunoblots.

nents (46, 74, and 96 kD). One atopic control recognized a 38-kD and a 52-kD component present in both preparations.

Fusarium moniliforme

F. moniliforme contains nine to ten antigenic components and almost all patients sera recognized a 58-kD and a 41-kD component in both preparations. Most patients reacted to the culture filtrate components. The serum pool from the control group, however, also demonstrated weak binding to these components (41 and 58 kD), suggesting unspecific activity. Only two other important components (32 and greater than 200 kD) were found in the culture filtrate.

Penicillium notatum

The most striking difference between culture filtrates and cellular extract was observed with *Penicillium notatum* where the culture filtrate contained seven IgE-binding components that are not found in the cellular extract. Both preparations share a 52-kD and 90-kD component which bind IgE from three to four patients. Half of the sera recognize a greater than 200-kD component in the culture filtrate. These results suggest that IgE-binding components are more concentrated in the culture filtrates.

Pityrosporum ovale

IgE-immunoblots showed eight important IgE-binding components ranging from less than 14 to greater than 200 kD. The most frequent bands were 110 and greater than 200-kD glycoproteins reacting with the majority (more than 50%) of the patient sera.

Trichophyton rubrum

The IgE-immunoblots revealed eight important IgE-binding components ranging from 22 to greater than 200 kD. The most frequently observed bands were 54, 110, and 125 kD which were recognized by almost half of the patient sera.

DISCUSSION

The clinical importance of fungi as a trigger of IgE-mediated reactions in the skin is uncertain. This study demonstrates that 14 out of 15 patients with atopic dermatitis and clinical findings of dermatophytosis or infection react to one or more fungal species by basophil histamine release testing. A lower number of positive reactions (nine patients) were recorded by skin testing. We confirm previous findings that *Pityrosporum ovale* frequently

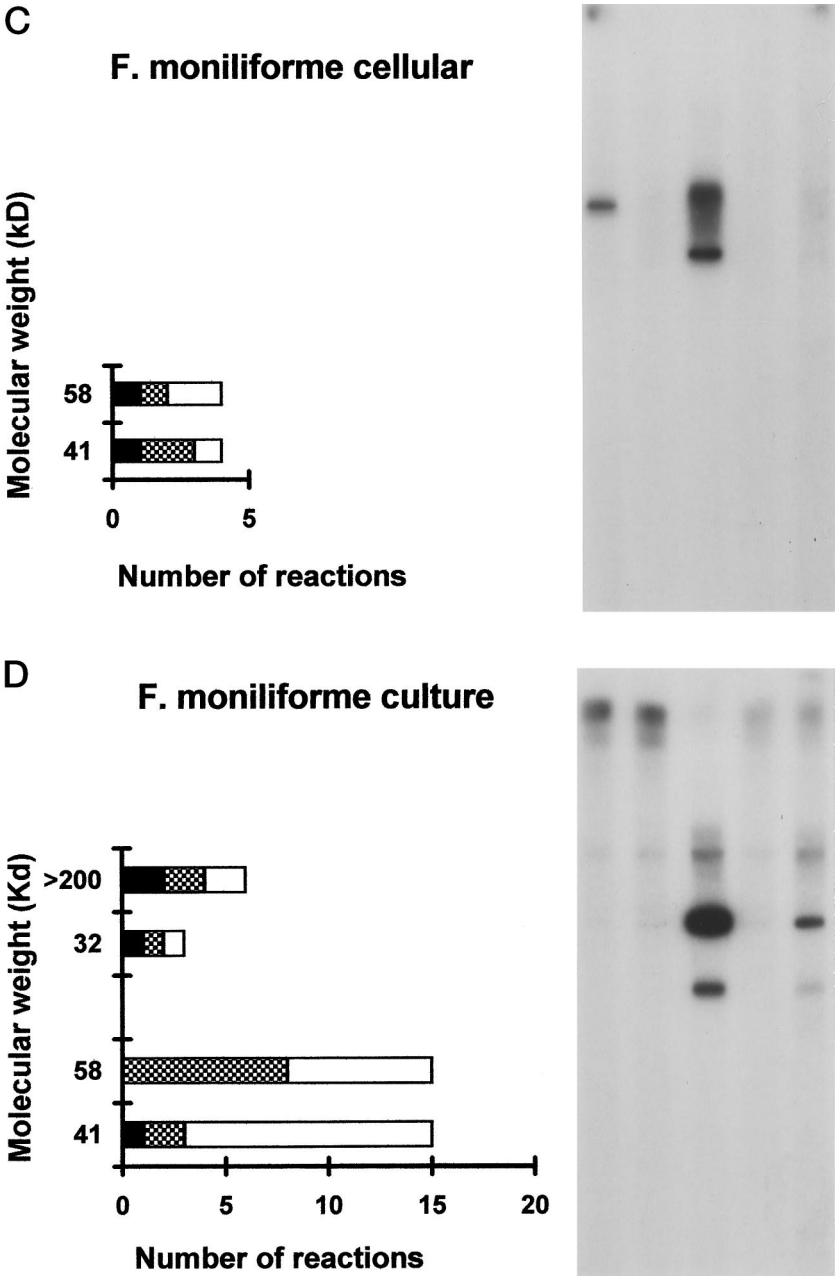

Figure 1 *continued*

sensitizes patients with atopic dermatitis, however, *T. rubrum, P. notatum,* and *C. albicans* may be important as well.

Patients negative by skin tests and histamine release demonstrated IgE-binding to a wide range of components in the immunoblot, hence the relative frequency of positive IgE-responses increased from 33% by histamine release to 63% by IgE-immunoblotting. The most striking example of this occurred with the *Fusarium moniliforme* extract where almost all sera recognized a 41-kD and 58-kD component. Probably, the binding was unspecific because the control group also demonstrated weak IgE-binding to these components. The discrepancy between the test results may be explained by different immunologic mechanisms and the methods used to detect IgE-sensitization. Patients with elevated IgE levels often have multiple weakly positive RASTs against crossreactive and noncrossreactive allergens. In addition, the reduction procedure employed by sodium dodecylsulfate-polyacrylamide gel electrophoresis may change the conformation, affinity, and epitope density of the allergens, eg, reduction of extracts in immunoblots may reveal new epitopes or epitopes with lectin-like properties that are covered when the allergen is folded in its natural state.[3,5,10,12] Also, it was shown in studies employing basophil histamine experiments, RAST inhibition, and immunodot assay that nonspecific binding of IgE to allergens was due to nonspecific carbohydrate interaction or related to a difference in the conformational structure of IgE.[23,24] Alternatively, binding of IgE to crossreactive sugar residues are detected in immunoblots, however, the same IgE antibodies have poor biologic activity in basophil histamine release and skin tests.[23] Tests dependent on the conformational structure of the IgE bound to mast cells or basophils may therefore better reflect the functional importance of the IgE-binding proteins.

Protein components of high molecular weight (>100 kD) constituted one-third of IgE-binding molecules. The staining pattern suggests that many of these components are heavily glycosylated proteins. Possibly, some of these components were part of larger aggregates before they were reduced and separated. Clinically, they may be functionally unimportant since they may be too large to crosslink IgE and may even interfere with the binding of smaller allergenic components sharing the same epitopes.[12]

Previously, *Candida albicans* and *Pityrosporum ovale* have been well characterized by immunoblotting techniques.[3,5,10–12] Our staining pattern of *Candida albicans* is consistent with previous findings.[8,10] We also found diffuse staining in the high molecular weight area and the presence of a

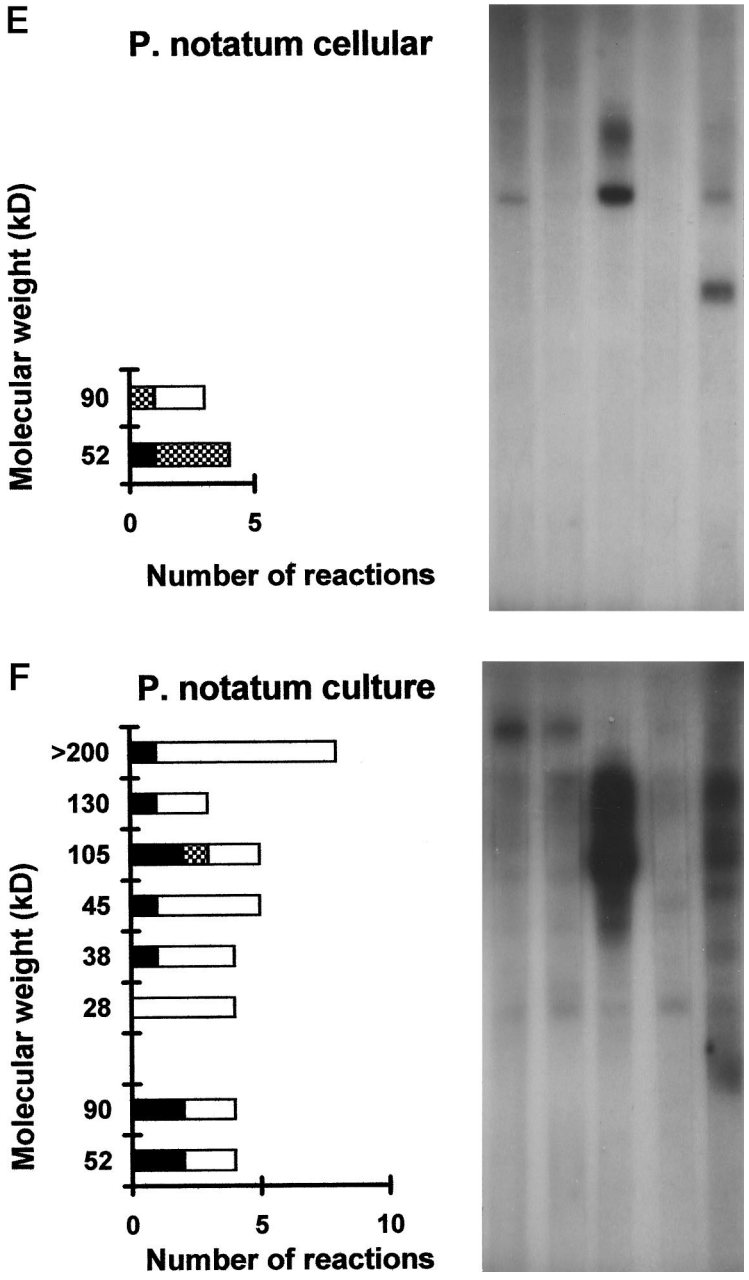

Figure 1 *continued*

46-kD component, previously identified as enolase.[10,25] Only one other study identified IgE-binding components in the high molecular weight area (125 kD and 175 kD).[10]

Pityrosporum ovale often shows considerable variation in IgE-binding components. Until now, the largest IgE-binding component reported had a molecular weight of 94 kD.[5] In contrast, we found important IgE-binding components at 110 and greater than 200 kD. In many studies the extract was not reduced before separation with sodium dodecylsulfate-polyacrylamide gel electrophoresis and this may explain why the high molecular weight components were not identified. Generally, this may explain the differences in the molecular weights of important allergens identified in this study compared with others. Furthermore, considerable variation between strains, growth conditions and batches may explain why it is so difficult to obtain reliable and comparable results.

F. moniliforme demonstrates significant cross-reactivity with other Fusarium species and with Alternaria, Penicillium, Aspergillus, Cladosporium, and Stemphylium.[26,27] Previously, immunoprint analysis with sera from Fusarium-positive patients also showed allergenic proteins between 10 and 70 kD with a 14-kD protein common being predominant.[26] We, however, were not able to show that this extract contained the 14-kD protein band. This fact and the unspecific activity we noted suggests that further work is needed to optimize this fungal extract.

When comparing previous data of immunoblotting with *Penicillium notatum* to our data, similar observations are made. Generally, a wide range of IgE-binding components were found, but of different molecular weights than those found in this study.[14,15]

The second objective of this study was to examine whether culture filtrates differ from cellular extracts. Culture filtrates contain metabolites and cell wall components (eg, deteriorated during budding), whereas cellular extracts predominantly contain cell walls and probably less metabolites (some may be caught between cells or exist as intracellular granules at the time of harvesting). We found a tendency, however, not statistically significant, towards more frequent and pronounced reactions to culture filtrates compared with cellular extracts. The metabolites may be more concentrated in the culture filtrates or possess a greater IgE sensitizing ability than cell wall components. Also, the cell wall components in the cellular filtrates may be too large to crosslink IgE. Despite the obvious differences both preparations contain many shared components.

In conclusion, by IgE-immunoblotting a high frequency of patients with atopic dermatitis were found to have IgE-antibodies to a wide range of com-

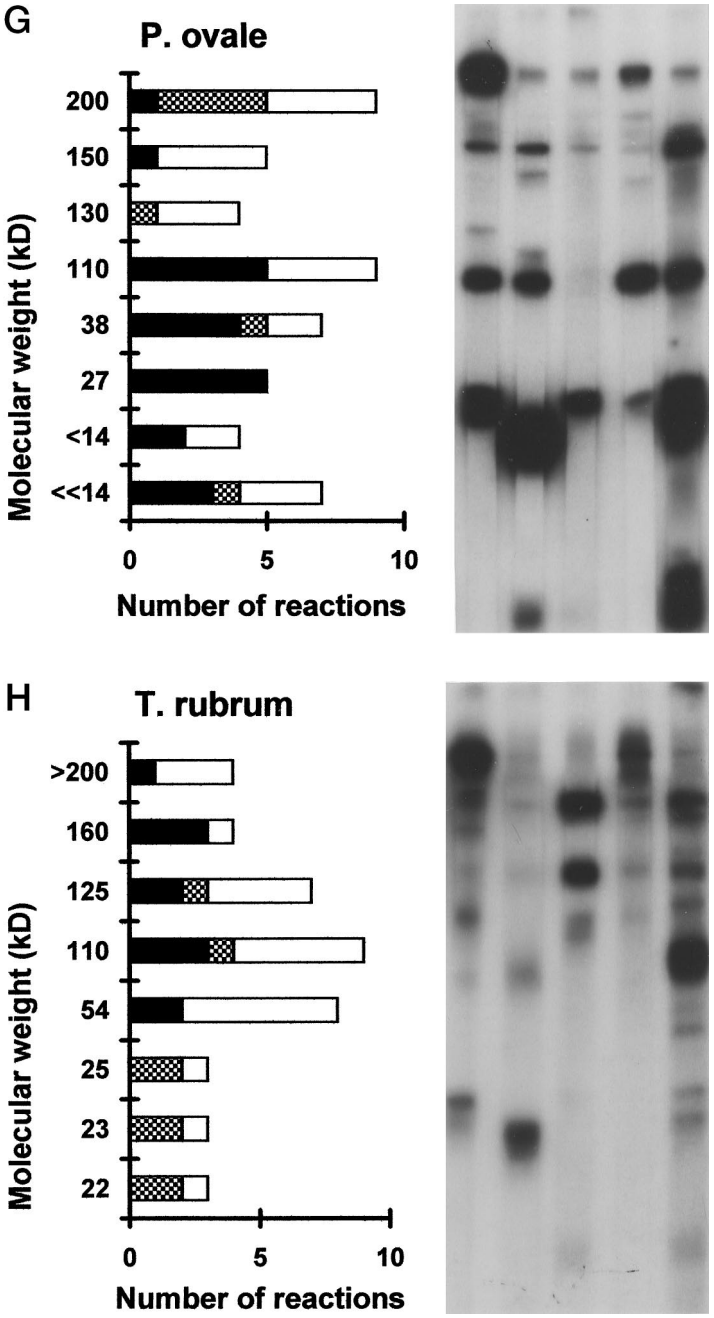

Figure 1 *continued*

ponents in fungal extracts. Skin prick and basophil histamine release tests suggest, however, that in almost one-half of patients the IgE-binding (glyco)proteins had poor biologic activity. In selected patients with atopic dermatitis skin infections with fungi, *P. ovale, T. rubrum, C. albicans*, and to a lesser extent *F. moniliforme* and *P. notatum* may exacerbate eczema. Although IgE-immunoblot data indicated that the culture filtrates may be more suitable for allergy testing than cellular extracts, it was not confirmed by skin prick test or leukocyte histamine release.

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