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An Investigation of Allergenic Proteins Produced by Moulds on Building Materials

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Key Words

Allergens · Building materials · Immunoblotting · Mould atopy · SPT · Uni-Cap test

Abstract

This paper presents an investigation of allergenic proteins produced by moulds, which react with the blood sera of people living in mould-contaminated dwellings. The production of allergenic proteins by moulds on building materials was evaluated. Mould atopy was confirmed on the basis of personal interviews as well as determination of allergen-specific IgE by an in vivo test (skin prick test (SPT)) and in vitro tests: immunoenzymatic Uni-Cap and immunoblotting. The sera of atopic persons ($n = 4$) reacted with antigens: *Penicillium* (twenty two antigens), *Aspergillus* (four antigens), *Alternaria alternata* (six antigens) and *Cladosporium cladosporioides* (six antigens). Moulds produced a higher number of potentially allergenic proteins when they grew on building materials

containing cellulose (wallpaper, carton-gypsum board) than on a microbiological medium. The immunoblotting test using the blood sera of residents and antigens of moulds produced on the building materials is the most specific method of mould atopy detection. The most important conclusion is that people who live in buildings with visible mould contamination and who display symptoms characteristic of allergic reactions might not be correctly diagnosed with the use of commercially available mould test allergens (SPT, Uni-Cap tests). This might be due to the fact that these test allergens are produced under in vitro conditions rather than conditions similar to in vivo.

Introduction

The moulds *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* sp. are often isolated from buildings. The most frequent strains found in Poland are *Aspergillus versicolor*, *A. niger*, *A. flavus*, *Penicillium chrysogenum*,

Alternaria alternata, *Cladosporium cladosporioides* and *C. herbarum* [1,2]. In conditions of high relative humidity, these moulds are able to grow on building materials of both organic and inorganic origins. In the environment of a building, they produce metabolites that could be harmful to human health, including mycotoxins, volatile organic compounds, and (1→3)-β-D-glucan [3–6]. Their ability to evoke allergic reactions in humans is also documented. Mould allergy affects 5–30% (inhalatory allergy) and 15–50% (skin allergy) of all atopic patients [7–9]. The best-known allergens are the outdoor moulds belonging to *Cladosporium* and *Alternaria* spp. [10,11]. In addition, indoor *Aspergillus* and *Penicillium* spp. moulds are also observed to evoke allergic reactions in sensitive individuals [9,10,12,13]. The occurrence of year-round symptoms can serve as a confirmation of continuous exposure to indoor moulds, but at the same time can be related to allergy to outdoor moulds such as *Alternaria* and *Cladosporium*, which are most abundant during summer time (the concentration of their spores in the air is very high at that time). Mould allergens include proteins present in both the spores and mycelium, with molecular masses of 10–110 kDa [14,15]. Recently, about 150 allergens produced by 80 genera of moulds have been identified [16], among which 70 have been classified by the International Union of Immunological Societies Allergen Nomenclature Committee [17]. These allergens also include proteins produced by moulds present in buildings, such as *Aspergillus* sp. (*A. flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*), *Penicillium* sp. (*P. chrysogenum*, *P. citrinum*, *P. brevicompactum*, *P. oxalicum*, *P. expansum*), *A. alternata* and *C. cladosporioides*.

In the diagnosis of mould allergies, for the detection of allergen-specific IgE (asIgE) antibodies to moulds, in vivo skin tests (skin prick test (SPT)) are used, as well as in vitro tests based on the use of sets of commercial allergen extracts and the patient's serum (for example, immunoenzymatic Uni-Cap tests). For in vitro tests, the immunoblotting method may also be used, based on an immunological reaction between separated proteins (using electrophoresis) and the patient's serum. However this method is not routinely used in diagnostic laboratories.

Allergic response may be due to exposure to moulds growing in buildings, and hence it is important to investigate allergic protein production in the building environment. Because of the great phenotypic variability of moulds, we decided to check whether the profile of proteins produced on a model microbiological medium differs significantly from that produced in building materials.

The goals of this work were twofold:

1. to identify proteins that give a positive reaction with antibodies present in the sera of humans exhibiting allergic symptoms who are exposed to moulds belonging to *Aspergillus* sp. *Penicillium* sp., *A. alternata* and *C. cladosporioides* in their homes; and
2. to compare the methods used routinely in allergy diagnosis and the method of immunoblotting with the use of mould allergen extracts grown on building materials.

The experiments performed were based on building mycology and were a response to the results gathered by allergologists.

The scope of the study included the following:

- Experiments performed in mould-contaminated buildings – isolation and identification of species.
- Medical examination of residents – interviews, SPT.
- Experiments confirming mould atopy (determination of allergen-specific antibodies) – immunoenzymatic test with allergens of common moulds (Uni-Cap test), and immunoblotting using allergens produced by *A. alternata*, *C. cladosporioides*, *Aspergillus* and *Penicillium* – strains isolated from buildings.
- Cultivation of isolated moulds on building materials – wallpaper, carton-gypsum board, gypsum, mortar – and control medium.
- Detection and separation of proteins produced by moulds on the building materials, and determination of their molecular masses.
- Evaluation of the allergenicity of proteins produced on building materials based on the classification of the International Union of Immunological Societies Allergen Nomenclature Committee.

Materials and Methods

Moulds

Moulds were isolated from indoor air using Air Sampler Mass 100Eco (Merck, Germany) and paint-coatings (swab method) in mould-contaminated dwellings (n=4). A description of the dwellings is presented in Table 1.

Air samples were taken in one place in each room in the dwelling (bathroom, kitchen, living room, bedroom) (two samples in each location). The number of mould spores present in the air was represented by the arithmetic mean of all measurements performed in a dwelling. In all rooms with mould contamination, swabs were collected (two

Table 1. Description of investigated mouldy dwellings

Description of dwellings	Numbers of mould-contaminated dwellings*			
	1	2	3	4
Kind of building	Tenement house (built in 1856)	Block of flats (built in 1997)	Tenement house (built in 1935)	Tenement house (built in 1995).
Place of mould growth	Bathroom (walls), kitchen, bedroom (windows frames, corners of the rooms)	Bedroom, living room (corners of the rooms)	Bathroom (walls), living room (corner of the room)	Bedroom, (corner of the room, windows frames, mattress)
Duration of mouldiness	6 years	4 years	15 years	8 years

*Numbers of dwellings are due to numbers of residents.

Table 2. Description of the studied residents

Description of residents	Numbers of residents of mould-contaminated dwellings			
	1	2	3	4
Family history of atopy	Yes	Yes	No	Yes
Sex and age (years)	Female, 10 years	Male, 31 years	Male, 18 years	Female, 27 years

samples in each location). The final number is represented by the arithmetic mean of all repetitions for each dwelling.

Moulds were isolated on MEA medium (Malt Extract Agar, Oxoid) with chloramphenicol (0.1%). After incubation at 25°C for 7 days, the moulds were identified on the Czapek Dox Agar medium (Difco) based on the methods of Samson et al. [18] and Flannigan et al. [12]. In the laboratory experiment, six mould strains isolated from buildings and exhibiting allergenic potential (immunoblotting tests) were used. These strains were characterized by their toxicity [19] and included in the LOCK Collection of Pure Cultures: *A. niger* L0582, *A. flavus* L0583, *P. chrysogenum* L0584, *P. expansum* L0585, *A. alternata* L0586 and *C. cladosporioides* L0587.

Cultivation of Moulds

Building materials – wallpaper, carton-gypsum board, gypsum, mortar – and the control Mo mineral nutrient medium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (5 g/l), $(\text{NH}_4)\text{SO}_4$ (3 g/l), KH_2PO_4 (1 g/l), glucose (20 g/l), yeast extract (5 g/l), pH 5.5) were used to check the production of proteins by the moulds. Building materials were obtained from retail sources. They were cut (carton-gypsum board, wallpaper) to an appropriate size or were prepared by mixing dry powder (gypsum, mortar) with water according to the manufacturer's instructions and then shaped into squares of the desired size (16 cm²) and sterilized at 121°C for 30 min. The control medium and building materials were inoculated with 1 ml of the suspension of mould spores at

a concentration of 1×10^6 – 1.5×10^6 colony forming units (cfu/ml). The spore density was measured using a Thom chamber. At the beginning of the experiment all building materials were supplemented with 0.1 ml of M₀ medium in order to initiate mould growth. The cultures were grown at 25°C for 14 days, the relative humidity of the air being maintained at 95–100%. During incubation, sterilized water was added to each building material sample to keep the moisture at the appropriate level (minimal water activity 0.90–0.95).

Residents

Medical diagnosis was carried out on the residents of the mould-contaminated dwellings. In the research, four subjects (Table 2) living in flats with evident signs of fungal contamination were chosen for the study (persons of whom atopy to indoor moulds was diagnosed on the basis of SPT, medical history and results of the immunoblotting test). The group under medical study consisted of two males and two females aged between 10 and 31 years (Table 2).

The subjects completed a questionnaire that included a history of respiratory and skin symptoms, personal and family history of atopy, housing conditions and area of residence. The medical examinations were carried out during autumn; questions concerning the occurrence of symptoms concerned the whole year. The Regional Bioethical Committee approved the study protocol.

Determination of asIgE Antibodies against Moulds SPT

The SPT was performed on the volar part of the forearm with a standard battery (Allergopharma, Germany) of mixed mould allergens including mould mix 1 containing *A. alternata*, *Botrytis cinerea*, *C. herbarum*, *Culvularia lunata*, *Helminthosporium halodes*, *Fusarium verticillioides*; mould mix 2 containing *Aspergillus fumigatus*, *Mucor mucedo*, *P. chrysogenum*, *Aureobasidium pullulans*, *Rhizopus nigricans*, *Serpula lacrymans*; and the fungal series: *A. alternata*, *A. fumigatus*, *Trichophyton mentagrophytes*, *C. herbarum*, *F. verticillioides*, *H. halodes*, *M. mucedo*, *P. chrysogenum*, *A. pullulans*, *R. nigricans*, *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. The negative control was the allergen diluents and the positive control was 1 mg/ml of histamine dihydrochloride solution. Positive reaction was defined as a wheal diameter of at least 3 mm with no reaction to the diluent and a positive reaction to histamine [20]. Tests for other allergens (dust mite *Dermatophagoides farina*, feathers, pollen, animal fur) were performed simultaneously, as allergic reactions to moulds and to the aforementioned allergens can occur at the same time (they can serve as a carrier for mould allergens).

Immunoenzymatic Method – Uni-Cap test

Specific asIgE antibodies to fungi (*P. chrysogenum*, *C. herbarum*, *A. fumigatus*, *Candida albicans*, *A. alternata*, *Helminthosporium halodes* (mx2), *Alternaria tenuissima* (m6), *Aspergillus fumigatus* (m3), *P. chrysogenum* (m1)) were detected using an immunoenzymatic method – the Uni-CAP system (Uppsala, Pharmacia Diagnostics, Sweden), and the asIgE level >0.35 kU/l was regarded as positive.

Immunoblotting Method

Extraction of Mould Proteins

The biomass (in the form of mycelium with spores) from the control medium as well as the moulded disintegrated building-material samples (using mortar/scissors). Building materials in this form were mixed with phosphate-buffered saline (PBS) buffer (pH, 7.4), and 4 g of glass beads (1 mm of diameter) were added. The samples were shaken at 4°C for 16 h. They were then placed in an ice-bath and sonicated for 5 min at a frequency of 2.5 s and amplitude of 60 kHz. The content was centrifuged at 4°C for 5 min at 12,500 g and the supernatant fraction with the proteins was filtered (0.2 µm, Sartorius). After filtration, the proteins were

precipitated from the solution with 96% ethanol (1:1). The solution was kept at –20°C overnight. The supernatant fraction was then disposed of and the proteinaceous precipitate was dried and dissolved in 1 ml of the sample buffer (0.5 M Tris–HCl, pH 6.8; 20% sodium dodecyl sulphate (SDS); glycerol 10%; β-mercaptoethanol 5%; bromophenol blue 0.1%). The samples were boiled in a water-bath for 5 min, cooled, and stored at –20°C.

SDS-PAGE and Immunoblotting

For sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) [21], 40 µl of the protein samples suspended in sample buffer was used. The electrophoresis was performed with a current intensity of 30 mA for approximately 5 h. Subsequently, the gel was flooded with trichloroacetic acid (TCA) (50%) at room temperature for 30 min, rinsed with distilled water, and stained using the Coomassie Brilliant Blue staining method (Coomassie Brilliant Blue R-250, 0.25%; acetic acid 10%; methanol 45%; overnight at room temperature). The destaining solution (methanol 30%; acetic acid 10%; distilled water 60%) was added at room temperature until the appearance of blue stripes indicating the presence of proteins. The molecular masses of the proteins were determined by the Sigma-Gel computer program (Jandel-Scientific) using molecular masses of standard protein markers in the range of 6.5–200 kDa (Sigma-Aldrich; standard proteins were dissolved in deionized water, 100 µl).

The separated proteins were transferred from the electrophoretic gel to a nitrocellulose membrane (Sigma-Aldrich) by electroblotting using filter paper (Schleicher and Schuell) and transfer buffer (Trizma Base, 4 mM; glycine 0.19 M; methanol 10%; pH 8.3). The transfer was carried out overnight at room temperature and a constant voltage of 20 V. Subsequently, the blocking buffer (3% buffer; dried, defatted milk 0.3%; KH₂PO₄ 2 mM; Na₂HPO₄ 40 mM; NaCl 0.15 M; Tween-20 1%; pH 7.2) was added to the nitrocellulose blot, and the blot was shaken at room temperature for 2 h. The membrane was washed four times for 3 min each in PBS (pH 7.2). For immunological detection, the membranes were incubated for 60 min at room temperature with the patients' sera diluted in the PBS buffer at a ratio of 1:99. The membranes were then incubated with mouse monoclonal anti-human IgE secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich) at a dilution of 1 µl of conjugated antibody in 100 ml of PBS buffer, for 60 min at room temperature. The blot was washed four times for 3 min each in PBS. The binding of the antibodies was detected by the reaction with the conjugate 5-bromo-4-chloro-3-indolyl

phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich). The reaction was stopped by the decanting solution and by washing with PBS. The molecular masses of the proteins were determined by placing the electrophoretic gel with the corresponding distribution of the proteins on the blot with visible bands. The same procedure was used for the determination of molecular masses of proteins as described previously.

Results

The results of the mycological analysis are summarized in Table 3.

In four dwellings of different architectural character (different type and age of building) the amount of moulds present on the walls ranged from 3.3×10^4 (dwelling no. 1) to 7.6×10^6 (dwelling no. 4) cfu/100 cm² and indicated active mould growth, especially in dwellings 2, 3 and 4. Visible mould contamination was present mainly in bedrooms and living rooms, but also in the kitchen (dwelling no. 1) and bathroom (dwellings no. 1 and 3). In these rooms moulds could be found in the corners and on window frames. The period of visible mould contamination ranged from 4 (dwelling no. 2) to 15 (dwelling no. 3) years. Mould concentration in indoor air was at a level of 2.2×10^2 (dwelling no. 3) to 1.3×10^3 (dwelling no. 4) cfu/m³. The following mould species were isolated from the air: *Acremonium strictum*, *A. alternata*, *A. flavus*, *A. niger*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum* and *P. expansum*. Some of these species were also growing on

the walls (*A. flavus*, *A. niger*, *P. chrysogenum* and *P. expansum*).

The medical study (questionnaire, medical interview and SPT) involved four residents of the dwellings described above (Table 4).

Respiratory symptoms were reported by all the subjects. Furthermore, three subjects reported rhinitis, conjunctivitis and skin symptoms. Bronchial asthma was reported by two subjects, while allergic rhinitis confirmed by medical diagnosis was reported by three persons. Residents reported a 3- to 5-year period of manifestation of allergic symptoms (cough, difficulties with breathing) and indicated that these symptoms appeared year-round. Occurrence of year-round symptoms can serve as a confirmation of continuous exposure to indoor moulds, but at the same time can be related to allergy to outdoor moulds such as *Alternaria* and *Cladosporium*, which are most abundant during summer time (the concentration of their spores in the air is very high at that time).

All of the subjects were sensitized to mould mix 1 (*A. alternata*, *Botrytis cinerea*, *C. herbarum*, *Culvularia lunata*, *Helminthosporium halodes*, *Fusarium verticillioides*) and *Alternaria* sp. and one subject was also sensitized to *Aspergillus fumigatus*, *Aspergillus* sp. and *Penicillium* sp. (based on SPT results, Table 4).

The specific asIgE to mix 2 containing the allergens *Penicillium chrysogenum*, *C. herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *A. alternata* and *Helminthosporium halodes* was found in two subjects (residents 1 and 4), and for those persons, the specific asIgE to the fungal series containing *A. alternata*,

Table 3. Mycological analysis of dwellings

Mycological analysis	Numbers of mould-contaminated dwellings			
	1	2	3	4
Number of moulds indoor air (cfu/m ³)	9.5×10^2	7.3×10^2	2.2×10^2	1.3×10^3
walls (cfu/100 cm ²)	3.3×10^4	1.4×10^5	1.9×10^5	7.6×10^6
Mould strains isolated from indoor air (a) and from walls (w) in the residents' buildings	<i>Acremonium strictum</i> (a) <i>Alternaria alternata</i> (a) <i>Aspergillus flavus</i> (a,w) <i>Aspergillus niger</i> (a,w) <i>Cladosporium cladosporioides</i> (a) <i>Penicillium chrysogenum</i> (a,w), <i>Penicillium expansum</i> (a,w)	<i>Alternaria alternata</i> (a) <i>Aspergillus niger</i> (a) <i>Aspergillus flavus</i> (a,w) <i>Cladosporium cladosporioides</i> (a) <i>Penicillium chrysogenum</i> (a,w) <i>Penicillium expansum</i> (a,w)	<i>Alternaria alternata</i> (a) <i>Cladosporium cladosporioides</i> (a) <i>Cladosporium herbarum</i> (a) <i>Penicillium chrysogenum</i> (a,w) <i>Penicillium expansum</i> (w)	<i>Alternaria alternata</i> (a) <i>Cladosporium cladosporioides</i> (a) <i>Cladosporium herbarum</i> (a) <i>Penicillium chrysogenum</i> (a,w) <i>Penicillium expansum</i> (a,w)

Table 4. Allergic reactions to moulds in residents of mould-contaminated dwellings – clinical results

Type of investigation	Numbers of residents of mould-contaminated buildings			
	1	2	3	4
Reported allergic symptoms (personal questionnaires)	Dyspnoea, conjunctivitis, and skin symptoms	Cough, rhinitis, conjunctivitis and skin symptoms	Cough, rhinitis	Cough, wheezing, rhinitis, conjunctivitis and skin symptoms
period of allergic symptoms manifestation	5 years (cough, difficulties with breathing)	3 years (cough)	4 years (cough)	4 years (cough, difficulties with breathing)
Allergic disease confirmed by medical diagnosis	Symptoms appeared year-round	Symptoms appeared year-round	Symptoms appeared year-round	Symptoms appeared year-round
SPT	Bronchial asthma, Allergic conjunctivitis	Allergic rhinitis	Allergic rhinitis	Bronchial asthma, Allergic rhinitis, Allergic conjunctivitis
	Mould mix 1 ¹ positive	Mould mix1 ¹ positive	Mould mix1 ¹ positive	Mould mix 1 ¹ positive
	Mould mix 2 ² negative	Mould mix2 ² negative	Mould mix 2 ² negative	Mould mix 2 ² negative
	Mould series – positive:	Mould series – positive:	Mould series – positive:	Mould series – positive:
	<i>Alternaria</i> sp.	<i>Alternaria</i> sp.	<i>Alternaria</i> sp.	<i>Alternaria</i> sp.
	<i>Aspergillus fumigatus</i>			
	<i>Aspergillus</i> sp.			
	<i>Penicillium</i> sp.			

¹Mould mix 1: *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium herbarum*, *Culvularia lunata*, *Helminthosporium halodes*, *Fusarium verticillioides*.

²Mould mix 2: *Aspergillus fumigatus*, *Mucor mucedo*, *Penicillium chrysogenum*, *Aureobasidium pullulans*, *Rhizopus nigricans*, *Serpula lacrymans*.

Mould series: *A. alternata*, *A. fumigatus*, *Trichophyton mentagrophytes*, *C. herbarum*, *F. verticillioides*, *H. halodes*, *M. mucedo*, *P. chrysogenum*, *A. pullulans*, *R. nigricans*, *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp.

Aspergillus fumigatus, *P. chrysogenum* was estimated (based on the immunoenzymatic Uni-Cap test; Table 5).

Antibodies that reacted with the antigens produced by *Penicillium* sp. (*P. chrysogenum*, *P. expansum*), *A. alternata* and *C. cladosporioides* were found in the sera of all the subjects (immunoblotting test, Table 5). In addition, antibodies against antigens produced by *A. flavus* and *A. niger* were found in the serum of one subject.

Moulds showed differences in the number of proteins produced on the building materials and control media (Table 6). The highest number of proteins found were produced on building materials of organic origin (wallpaper, carton-gypsum board), while the lowest number found was produced on mortar. Several allergenic proteins were also produced on the control media (*P. expansum* and *P. chrysogenum*). The molecular mass of the proteins produced on the building materials was in the range 7–222 kDa, while on the control media, the molecular mass was 32 to 135 kDa. Furthermore, wallpaper, carton-gypsum board, and gypsum were observed to stimulate the production of proteins that were specific only to the given type of building material.

Moulds belonging to *Aspergillus* sp. were observed to produce one to two classified allergens when grown on

building materials; however, on mortar and control media they failed to produce the allergenic proteins. *P. chrysogenum* produced seven classified allergens overall on wallpaper and carton-gypsum board and *P. expansum* produced six allergens on these materials under the conditions of this study. The moulds *A. alternata* and *C. cladosporioides* produced four classified allergens per strain on wallpaper and carton-gypsum board. *P. chrysogenum*, *A. alternata* and *C. cladosporioides* were found to produce two or three classified allergenic proteins on gypsum. As with *Aspergillus* sp., no classified allergenic proteins were found to be produced by *Penicillium* sp., *A. alternata* and *C. cladosporioides* on mortar, although *Penicillium* strains were able to produce classified allergenic proteins on the control medium.

All the proteins used for the immunoblotting test were obtained from the cultures grown on carton-gypsum board or/and on wallpaper. Sensitization to *A. niger* and *A. flavus* was observed in one study patient – these strains produced four antigens on the building materials (34 kDa Asp n18; 105 kDa Asp n14, 34 kDa Asp fl 13, 71 kDa). Furthermore, *P. chrysogenum* proteins of molecular mass from 21 to 89 kDa reacted with one or two sera. Some of these proteins are denoted in the literature as antigens: Pen ch 18, Pen ch

Table 5. Allergic reactions to moulds in residents of mould-contaminated dwellings – laboratory results

Type of investigation	Numbers of residents of mould-contaminated buildings			
	1	2	3	4
Immunoenzymatic method	Mix 2 ¹ positive	Mix 2 ¹ negative	Mix 2 ¹ negative	Mix 2 ¹ positive
Uni-Cap test	Mould series ² m1, m3, m6 positive	Mould series ² not investigated	Mould series ² not investigated	Mould series ² m1, m3, m6 positive
Presence of asIgE to mix 2 ¹ and mould series ²				
Immunoblotting test	<i>A. flavus</i> : 34 kDa [Asp fl 13]; 71 kDa;	<i>P. expansum</i> : 71 kDa; 88 kDa; 93 kDa	<i>P. chrysogenum</i> : 21 kDa; 27 kDa; 32 kDa [Pen ch 18]; 33 kDa [Pen ch 13]; 40 kDa [Pen c 32]	<i>P. chrysogenum</i> : 24 kDa; 85 kDa;
Antigens of moulds that reacted with serum.	<i>A. niger</i> : 34 kDa [Asp n 18]; 105 kDa [Asp n 14];	<i>P. chrysogenum</i> : 46 kDa [Pen c 22w]; 68 kDa [Pen ch 20]	<i>P. expansum</i> : 88 kDa	<i>P. expansum</i> : 16 kDa [Pen ch 33]; 33 kDa [Pen c 13]; 40 kDa [Pen c 32]; 46 kDa [Pen c 22w]; 53 kDa; 64 kDa; 71 kDa; 79 kDa; 88 kDa
	<i>P. chrysogenum</i> : 27 kDa; 34 kDa [Pen ch 13]; 43 kDa; 89 kDa;	<i>A. alternata</i> : 16 kDa [Alt a 1]; 53 kDa [Alt a 10]	<i>A. alternata</i> : 16 kDa [Alt a 1]; 53 kDa [Alt a 10]	<i>A. alternata</i> : 16 kDa [Alt a 1]; 22 kDa [Alt a 7]; 26 kDa [Alt a 13]; 28 kDa; 53 kDa [Alt a 10]; 64 kDa;
	<i>P. expansum</i> : 71 kDa [Pen c 19]; 88 kDa; <i>A. alternata</i> : 16 kDa [Alt a 1]; 53 kDa [Alt a 10]; 64 kDa; <i>C. cladosporioides</i> : 36 kDa [Cla c 9]; 53 kDa	<i>C. cladosporioides</i> : 48 kDa	<i>C. cladosporioides</i> : 11 kDa [Cla h 5]; 22 kDa [Cla h 7]; 36 kDa [Cla c 9]; 45 kDa [Cla h 2]	<i>C. cladosporioides</i> : 45 kDa [Cla h 2]; 48 kDa

¹Mix 2: *Penicillium chrysogenum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *Alternaria alternata*, *Helminthosporium halodes*.

²Mould series: *Alternaria alternata* (m6), *Aspergillus fumigatus* (m3), *Penicillium chrysogenum* (m1).

13, Pen c 32, Pen c 22w and Pen ch 20. Potentially allergenic proteins 32 kDa (Pen ch 18) and 68 kDa (Pen ch 20) were produced by *P. chrysogenum* on gypsum. The *P. expansum* protein of molecular mass 88 kDa reacted with all the examined sera; similarly a protein of molecular mass 71 kDa reacted with three out of four sera. Almost all *P. expansum* proteins were produced on wallpaper. Two proteins of *A. alternata* – 16 and 53 kDa (denoted as Alt a 1 and Alt a 10) – reacted with four sera and were produced on wallpaper and carton-gypsum board. *C. cladosporioides* proteins reacted with one or two sera and were produced on wallpaper, carton-gypsum board and also on gypsum (Cla h 5, Cla h 7, Cla h 2).

Discussion

Medical diagnosis indicated allergy in all the examined subjects who were exposed to moulds in their homes

(symptoms included rhinitis, conjunctivitis, bronchial asthma and skin symptoms). It was assumed that this exposure to moulds could contribute to the development of mould atopy in the subjects. This was confirmed by the in vivo method (SPT) as well as the presence of asIgE antibodies in serum measured by in vitro methods (immunoenzymatic Uni-Cap test and immunoblotting test). The most detailed analysis using the immunoblotting test with respect to the antigens of moulds isolated from the investigated dwellings confirmed the reaction of human sera with four antigens of *Aspergillus*, *Penicillium* (22 antigens), *A. alternata* (six antigens) and *C. cladosporioides* (six antigens). Our results are consistent with those obtained by Kurup et al. [9], who reported that atopic individuals can develop mould sensitization on exposure. In addition, in agreement with Kurien and Scofield [22], it can be concluded from this experiment that the immunoblotting technique constitutes a powerful tool for allergen detection.

Table 6. Protein production by moulds on building materials

Type of investigation	Building materials	Moulds					
		<i>A. niger</i>	<i>A. flavus</i>	<i>P. chrysogenum</i>	<i>P. expansum</i>	<i>A. alternata</i>	<i>C. cladosporioides</i>
Number of proteins	Wallpaper	16	10	24	26	9	20
	Carton-gypsum board	20	12	11	10	7	15
	Gypsum	13	8	16	1	4	12
	Mortar	7	2	6	0	2	4
	Control medium Mo	4	2	7	18	1	4
Number of characteristic proteins*	Wallpaper	7	4	15	12	4	7
	Carton-gypsum board	9	6	6	5	2	2
	Gypsum	5	0	7	1	2	1
	Mortar	0	0	4	0	2	0
	Control medium Mo	0	2	5	6	1	3
Molecular mass of proteins (kDa)	Wallpaper	7–124	16–221	21–118	17–116	12–118	9–222
	Carton-gypsum board	7–124	16–106	20–82	17–74	30–114	9–97
	Gypsum	9–124	16–104	30–113	70	22–130	9–97
	Mortar	22–124	32–48	51–118	-	24–66	12–110
	Control medium Mo	63–124	32–44	61–97	17–116	30	40–135
Number of classified allergens	Wallpaper	1	1	5	4	4	4
	Carton-gypsum board	2	1	3	2	2	4
	Gypsum	1	0	2	0	2	3
	Mortar	0	0	0	0	0	0
	Control medium Mo	0	0	2	4	0	0
Molecular mass of classified proteins (kDa)	Wallpaper	105 Asp n14	34 Asp fl13	33 Pen ch13	32 Pen n18	16 Alt a 1	11 Cla h 5
				34 Pen ch13	33 Pen c13	22 Alt a 7	22 Cla h 7
				40 Pen c32	40 Pen c32	26 Alt a 13	36 Cla c 9
				46 Pen c22w	46 Pen c22w	53 Alt a 10	45 Cla h 2
				68 Pen ch20			
	Carton-gypsum board	34 Asp n18 105 Asp n14	34 Asp fl13	32 Pen ch18	16 Pen ch33	16 Alt a 1	11 Cla h 5
				40 Pen c32	71 Pen c19	53 Alt a 10	22 Cla h 7
				68 Pen ch20			36 Cla c 9
	Gypsum	105 Asp n14	-	32 Pen ch18	-	22 Alt a 7	45 Cla h 2
				68 Pen ch20		26 Alt a 13	11 Cla h 5
							22 Cla h 7
	Mortar	-	-	-	-	-	45 Cla h 2
				-	-	-	11 Cla h 5
	Control medium Mo	-	-	68 Pen ch20	18 Pen c3	-	22 Cla h 7
				97 Pen c30	32 Pen n18	-	45 Cla h 2
					40 Penc32	-	
					46 Penc22w		

¹Classification based on International Union of Immunological Societies Allergen Nomenclature Sub-Committee.

(-) proteins absent; (*) number of specific proteins produced only on particular building materials or control medium.

The immunoblotting results, showing proteins produced on the building materials that gave a positive reaction with the human sera, indicated the intensive production of allergenic proteins by *P. chrysogenum* (11 proteins) and *P. expansum* (10 proteins) (Table 7).

In the present study, significant differences in allergen detection were observed when different techniques were used. The differences were the most noticeable in the case of residents no. 2 and no. 3. In both cases the presence of asIgE against moulds (for example, *P. chrysogenum*) was not detected with the immunoenzymatic method (Uni-Cap test), but it was confirmed in the immunoblotting test. In our opinion these differences were not due to the sensitivity of the two methods, but were rather caused

by their specificities. The immunoenzymatic method, based on spectrophotometric measurement, is more sensitive than the immunoblotting method, which utilizes visual or densitometric measurement. However, the allergen extracts currently used in diagnostics performed with the Uni-Cap test do not include mould allergens produced in buildings.

The moulds *A. alternata*, *C. herbarium* and *A. fumigatus* are the most studied moulds with regard to allergens

Table 7. Allergenic proteins that reacted with blood sera of residents of mould-contaminated dwellings, produced on building materials

Allergenic proteins of moulds that reacted with residents' blood sera (molecular mass in kDa)	Positive immunological reactions with residents' sera (number of investigated sera)	Type of building material on which mould proteins were produced	Classification of protein ¹
<i>A. niger</i>			
34	1	Carton-gypsum board	Asp n18
105	1	Wallpaper, carton-gypsum board	Asp n 14
<i>A. flavus</i>			
34	1	Wallpaper, carton-gypsum board	Asp fl 13
71	1	Wallpaper	–
<i>P. chrysogenum</i>			
21	3	Wallpaper	–
24	4	Wallpaper, carton-gypsum board	–
27	1, 3	Wallpaper, carton-gypsum board	–
32	3	Carton-gypsum board, gypsum	Pen ch 18
33 and 34	1, 3	Wallpaper	Pen ch13
40	1	Wallpaper, carton-gypsum board	Pen c 32
43	3	Wallpaper, carton-gypsum board	–
46	2	Wallpaper	Pen c 22w
68	2	Wallpaper, carton-gypsum board, gypsum	Pen ch 20
85	4	Wallpaper	–
89	1	Wallpaper	–
<i>P. expansum</i>			
16	4	Carton-gypsum board	Pen ch 33
33	4	Wallpaper	Pen c 13
40	4	Wallpaper	Pen c 32
46	4	Wallpaper	Pen c 22w
53	4	Wallpaper	–
64	4	Wallpaper	–
71	1, 2, 4	Wallpaper	Pen c 19
79	4	Wallpaper	–
88	1, 2, 3, 4	Wallpaper	–
93	2	Wallpaper	–
<i>A. alternate</i>			
16	1, 2, 3, 4	Wallpaper, carton-gypsum board	Alt a 1
22	4	Wallpaper, gypsum	Alt a 7
26	4	Wallpaper, gypsum	Alt a 13
28	4	Wallpaper	–
53	1, 2, 3, 4	Wallpaper, carton-gypsum board	Alt a 10
64	1, 4	Wallpaper	–
<i>C. cladosporioides</i>			
11	3	Wallpaper, carton-gypsum board, gypsum	Cla h 5
22	3	Wallpaper, carton-gypsum board, gypsum	Cla h 7
36	1, 3	Wallpaper, carton-gypsum board	Cla c 9
45	3, 4	Wallpaper, carton-gypsum board, gypsum	Cla h 2
48	2, 4	Wallpaper	–
53	1	Wallpaper	–

¹Based on International Union of Immunological Societies Allergen Nomenclature Sub-Committee.

(–) Not classified.

[23,24]. These species occur in residential buildings, though it was concluded during the study that they are less abundant than the moulds commonly encountered in this type of environment belonging to the genera *Penicillium* (for example, *P. chrysogenum*) and *Aspergillus* (for example, *A. versicolor*, *A. niger*). Moreover, *A. alternata* was investigated during the study

and it was concluded that the suite of allergens produced by this species grown in a building environment is different from the allergens produced on control media.

Extracts of the mould allergens *A. alternata*, *C. herbarum* and *A. fumigatus*, which are commonly used in diagnostic tests (SPT, Uni-Cap test), are obtained from natural sources, though some allergens can also be

produced with the help of methods of molecular biology [25, 26]. Very often these allergens are available in the form of mixtures (mix 1, mix 2, mix 3 as presented above). Because of antigen similarity among different fungal genera and species, the risk of cross-reactions is high [10, 27]. It is also important that a method of preparation of allergen standards for diagnostic purposes is still not established. In our study, the importance of the environment from which allergens are extracted for diagnostic purposes has been presented. The development of new techniques for recombinant allergen production will certainly contribute to the manufacture of allergen extracts containing specific epitopes of allergens that are responsible for allergic reactions. These types of extracts are still not used routinely. Moreover, recombinant allergenic proteins of many mould species (including those present in buildings) are not yet available. Considering these matters, the environment from which allergens are isolated should be given high importance. This problem was linked to the negative result for asIgE obtained with the immunoenzymatic method (Uni-Cap) and the positive one obtained with the immunoblotting technique. This confirmed that the commercial extract mix did not contain specific allergens that caused a response in the studied persons. The immunoblotting technique, disregarding its lower sensitivity, is advantageous in this type of study as it makes it possible to detect a broader spectrum of allergens that can cause allergic responses in the study subjects. The results of medical analysis constituted the basis for determining whether all the antigens that react with the investigated human sera could be produced on building materials of organic and inorganic origin as well as on the control media.

It was observed that the protein profiles produced by moulds on different types of building materials and control media were different. The highest number of potentially allergenic proteins was found to be produced by moulds on building materials of organic origin containing cellulose (wallpaper, carton-gypsum board). Although moulds were also able to grow on mortar with a low level of carbon source (0.1 ml medium Mo) (an exception was *P. expansum*), no potentially allergenic proteins were observed on this type of building material. It was also observed that moulds are able to produce proteins specific to a given type of building material. A similar experiment concerning the separation of *Penicillium* and *Aspergillus* proteins via SDS-PAGE, and testing of their allergenic potential, was carried out by Al-Suwaini et al. [28]. In their experiment, the moulds were cultivated on Czapek semi-synthetic liquid medium and the proteins were extracted further using

PBS. For comparison, in our experiment, the number of separated proteins was generally significantly higher and the range of molecular masses of the proteins was different. Our observations were similar to those obtained by Vijay et al. [13], who demonstrated the production of allergenic proteins by *Penicillium* cultivated on BBL medium (Difco). Little et al. [29] also reported the effects of different culture conditions on allergen production by mould belonging to *Aspergillus* sp. These results allow us to conclude that the type of substrate on which the moulds grow strongly could influence their allergenic potential. Although there are studies concerning the dependence of mould growth on different building materials, mycotoxins and microbial volatile organic compound (MVOC) production, to date there are no studies on the differences in the production of proteins when a single mould is grown on different building materials. This problem is very important because the quantity and type of proteins produced can directly influence the quantity and type of allergens produced. Therefore, more attention should be paid to diagnostics, including building materials, as an environment in which moulds grow and produce allergenic proteins. Furthermore, in the case of exposure of people sensitive to the antigens of indoor moulds, building materials are considered to be the natural habitat for the moulds, where they grow and produce allergenic proteins.

Taking into account the profiles of proteins produced by the moulds on building materials, it was observed that the highest quantity of potentially allergenic proteins was produced by *P. chrysogenum* and *P. expansum*.

Out of the 37 mould proteins that reacted with the sera of the investigated subjects, 21 have been described and classified by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee [17]. Data from the literature show that these allergens react with a majority of the positive serum samples, for example Alt a 1 and Alt a 10 [9]. However, the remaining proteins are not classified as allergens, although one protein belonging to this group (88 kDa) seems to be an interesting subject for further examination; it showed positive reaction with all of the examined sera, and hence it is recommended to prove their allergenic potential.

Conclusion

This study confirmed that moulds belonging to *Penicillium* sp. (*P. chrysogenum* and *P. expansum*) produced 22 antigens, while *Aspergillus* sp. produced four antigens, *A. alternata* six and *C. cladosporioides* six

potential allergenic proteins, which reacted with the sera of people living in mould-contaminated dwellings. It would be significant to carry out further experiments to prove the allergenicity of the 88 kDa protein produced by *P. expansum*, which reacted with all sera. The examined moulds were observed to produce a higher number of allergenic proteins when grown on building materials than on the control media. Building materials containing cellulose (wallpaper, carton-gypsum board) favoured the production of allergenic proteins more than the other types of media. Taking into consideration all the data obtained, it would be apt to consider building materials as the growth media for moulds from which allergenic extracts are produced. For the purpose of identification of allergies to indoor moulds, use should be made of

extracts of allergens obtained from moulds occurring in residential buildings and cultivated on building materials in conditions mimicking their natural environment.

Future Research

The results obtained in the study indicate the need for continuation of work in this area. There is a need to perform such experiments with allergens from moulds not related to the building environment and experiments on people who demonstrate atopy or allergy symptoms to allergens other than mould allergens, as well as control experiments with people who are exposed to moulds but do not have symptoms of mould allergy and sensitivity.

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