ABSTRACT

Background: The lack of well standardized or characterized extracts that contain the relevant allergens of the appropriate fungus is resulting in a high heterogeneity of the commercial preparation.

Material and methods: Immunochemical detection of the allergens composition of spore and mycelium of C. cladosporioides was studied by electroblotting using sera from Cladosporium allergic patients and 125 I- anti-human IgE. A MW range of allergens between 16 to 88 KDa was identified. The most important with a MW of 16, 20, 30, 39, 43, 50, 60 and 88 KDa.

Results: The allergenic composition of spore and mycelium looked very similar. However, partial or total inhibition of the serum with a conidial or mycelial extract demonstrated that the total concentration of allergens (particularily 20 and 60 KDa molecules) was higher in the conidium than in the mycelium.

Conclusions: These results indicated that conidium and mycelium contained the same allergenic determinants but at different concentration in the two propagule. Results with 50 % inhibited sera demonstrated also that the total concentration of allergens was higher in the conidium than in the mycelium.


INTRODUCTION

Over the last years, fungal allergy has played an increasingly important role in allergy1-3. The lack of well standardized or characterized extracts that contain the relevant allergens of the appropriate fungus is resulting in a high heterogeneity of the commercial preparation. The allergens composition of different species of Alternaria alternata, Aspergillus fumigatus, Curvularia lunata and Epicoccum nigrum4-7 vary according to nutritional components growth of media, temperature, incubation period and extraction method. The choice of the propagule, spore or mycelium, selected for the preparation of the best allergenic extracts is another suspected cause of the variability among commercial preparations. RAST inhibition and crossed radioimmunoelectrophoresis (CRIE) have demonstrated that allergen composition of spores and mycelia of Alternaria, Cladosporium, Pleurotus and Psilocybe differ both in quantity and quality8-11. Although spore may contain the most relevant allergens but in some species they are difficult to obtain. A comparison of Alternaria alternata spore and mycelium extract found that mycelium extract have...
greater potency than that of spore preparation on the basis of skin prick test, RAST inhibition and basophil histamine release. However, RAST and CRIE which rely either on the coupling of the allergens to CNBr or on intermediate rabbit antiserum do not allow a chemical identification of the allergens specific of the conidium or the mycelium. Immunoblotting is recognized as the most adapted method to identify fungal allergens. The blotting technique provides biochemical characterization such as MW of the allergens. A nitrocellulose electroblotting technique has been used to compare and characterize the allergens of conidia and mycelia of the most allergenic species of Cladosporium, C. cladosporioides.

MATERIAL AND METHODS

Allergenic extracts

The strain LCP 404 of Cladosporium cladosporioides (Muséum d’histoire naturelle, Paris, France) was grown on 2 % malt agar medium in 20 cm petri dishes. After 3 to 4 weeks of growth at room temperature, the spores were harvested from the surface of dry culture media with a paint brush. The mycelium was grown for 48 h in a 2 liters Biolafitte fermenter containing 2 % glucose, 1 % peptone (Probio labo) and 0.1 % Rhodorsil 426R at 25 °C, 700 rpm, 0.5vvm. Mycelium was recovered by filtration and washed extensively with water. Extraction of spore and mycelium allergens was performed by disruption of fungal cells suspended in 50 mM Tris pH 9.0, 1 mM EDTA and 1 % PVP as previously reported. The disrupted material was centrifuged at 18000 rpm for 30 min and the supernatant was recovered and stored at −80 °C. Protein content was estimated using the Bio-Rad technique (Bio-Rad technical information).

Human sera

Patients allergic to Cladosporium were selected by Dr Lelong (Service de Pédiatrie, Hopital Schaffner, Lens) on the basis of positive skin prick test. A pool of sera from patients (S, M, B, G) in the proportion 2:2:1:1 (V/V) was prepared. This pool had a RAST class 4 measured on CNBr activated cellulose paper disk coupled to C. cladosporioides spore extract.

One volume of diluted sera was added to one volume of allergic extract and the mixture was shaken for 3 h at room temperature. Two concentrations of extracts per 100 µl serum were used, respectively 100 µg and 0.25 µg of spore proteins and 100 µg and 1.25 µg of mycelium proteins. These proteins concentration gave respectively 100 % and 35 to 50 % of RAST inhibition with the pool of sera used. Control sera were obtained from patients not allergic to Cladosporium.

Allergen detection

Electrophoresis

The electrophoresis was carried out using a slight modification of the technique of Laemmli et al. The stacking gel contained 3 % polyacrylamide in 0.125 M Tris-HCl pH 8.8, 0.1 % SDS buffer. The separation gel contained 12.5 % or 15 % polyacrylamide in 0.375 M Tris-HCl pH 8.8, 0.1 % SDS buffer. The upper and the lower reservoir buffer was 50 mM Tris, 372 mM Glycerine, 2 % SDS. The sample buffer contained 625 mM Tris-HCl pH 6.8, 5 % 2-mercaptoethanol, 2.3 % SDS, 10 % Glycerol and 0.001 % Bromophenol blue as tracking dye. All samples containing 80 – 100 µg protein were heated at 100 °C for 3 min. The electrophoresis was run at 30 mA/µl (160 x 1.5 mm) for the stacking gel and at 60 mA/µg for the separation gel. Proteins standards (phosphorylase b [94 KDa], bovine serum albumine [67 KDa], ovalbumine [43 KDa], carbonic anhydrase [30 KDa], soybean trypsin inhibitor [20.1 KDa] and lactalbumine [14.4 KDa]) (Pharmacia) were used to determine the molecular weight of Cladosporium allergens.

Electrophoretic transfer

At the end of electrophoresis run (7 or 8 h), the proteins were transferred from the separation gel to a nitrocellulose membrane 0.45 µm (Schleicher and schuell) by electrophoretic transfer. The membrane sponge pads and filter paper (Wattman n° 3) were previously equilibrated for 5 min in the blotting buffer composed of 25 mM Tris, 192 mM glycine pH 8.3 and 20 % methanol. The nitrocellulose sheet was placed on the top of the polyacrylamide gel. They were subsequently embedded in a double layer of filter paper and sponge pad before being inserted in a trans blot cell (LKB) filled with precooled (± 4 °C) blotting buffer. Blotting was performed at 30 V/Cm during 18 h. After transfer of the proteins, the nitrocellulose membrane was saturated for 1 h at room temperature in PBS-MT.
The blotted gel and a reference unblotted gel were stained with 0.25 % coomassie brilliant blue (CBB) R250 in 45 % methanol and 10 % acetic acid. To estimate the efficiency of the transfer, 0.2 % Ponceau red in 3 % trichloroacetic acid was used to stain proteins after transfer to nitrocellulose.

Immunoblot staining

Nitrocellulose strips (12 x 0.5 cm) were incubated overnight on a shaker at 4 °C with the sera diluted 1:10 in PBS-MT buffer. Adsorbed and non adsorbed positive sera and control negative sera were used. The nitrocellulose strips were washed 3 times for 10 min with PBS buffer containing 0.1 % Tween 20 (PBS-T). The strips were then incubated overnight with 125 I-labelled antibodies anti-IgE (Pharmacia) in PBS-T buffer (50000 cpm/strips) at room temperature. After 6 washings for 10 min with PBS-T, the strips were dried and placed on top of an X-ray film (Kodak-pathé) and exposed in a kodak X-omatic cassette equipped with a DuPont cronex intensifying screen (Laborix) for detection of the 125 I-labelled proteins by autoradiography.

RESULTS

When the spore and mycelium extract of C. cladosporioides were subjected to SDS-PAGE, over 50 bands of proteins with a MW comprised between 10 and 110 KDa were detected after CBB staining (fig. 1). No important differences were noticed in the proteic pattern of spore and mycelial extract. Only 10 to 12 allergenic proteins were identified in the spore and mycelial preparation after transfer to nitrocellulose and incubation with the pool of positive sera and 125 I-labelled anti-IgE (figs. 2, 3). After two days of exposure in the cassette 7 allergens can be identified with a MW of 16, 20, 39, 43, 50, 60 and 88 KDa (fig. 2b, c). A prolonged exposure (up to 10 days) revealed 3 to 5 supplementary allergens with a MW of 30, 35, 41, 72 and 80 KDa (fig. 3c, d, f). Controls were negative with the exception of a slight non-specific labelling at the top of the gel (fig. 2d).

The allergenic composition of spore and mycelium extract looked similar. However, the staining intensities of several bands appear different in these two extracts and two proteins 20 and 60 KDa were detected specifically in the spore (fig. 2).

Partial inhibition of the serum with a mycelial extract (giving 50 % RAST inhibition) reinforce the staining of these 20 and 60 KDa bands (fig. 3c). However, complete inhibition of the serum with 1 mg/ml of mycelial proteins resulted in the disappearance, almost complete for the 60 KDa band, of the spore allergens (fig. 3b). With spore extract giving 50 % RAST inhibition, the labelling of the 20 and 60 KDa bands disappaered completely (fig. 3d). Mycelial allergens transferred to nitrocellulose were also inhibited completely or partially with spore extracts giving respectively 100 % or 50 % RAST inhibition (fig. 3e, f).

These results indicated that conidium and mycelium contained the same allergenic determinants but at different concentration in the two propagule. Re-
sults with 50 % inhibited sera demonstrated also that  the total concentration of allergens was higher in the conidium than in the mycelium.

**DISCUSSION**

This study and previous reports on immunotransfer methods had demonstrated the usefulness of using such techniques to identify fungal allergens. Such method can be efficiently used to control the presence of all the major allergens in a commercial preparation. In *C. cladosporioides* extracts, the same allergens were always detected among various batches of spore production. This result indicate that the extraction procedure and the buffer used are very well adapted to the recovery of *Cladosporium* allergens.

The molecular weight of fungal allergens identified in Alternaria, Aspergillus, Cladosporium, Curvularia, Drechslera, Epicoccum, Fusarium, Ganoderma, Penicillium and *Psilocybe* was generally comprised between 10 and 90 KDa. A considerable allergenic relationship was found between *Cladosporium herbarum*, *C. cladosporioides* and *Alternaria alternata* by the immunoblot technique. A cross-reactivity between *A. flavus* and *P. citrinum* was documented by Yu et al. This evidence of the presence of shared allergenic epitopes in these fungi is different to the sensitivity of the subjects to several fungal extracts. It express similarities of different species with respect to sensitization and IgE human response. Spores and mycelium of *C. cladosporioides* have the same qualitative allergenic composition which differ only quantitatively. Previous PCA experiments in mice and guinea pigs have demonstrated an important immunologic identity among spore extracts of *C. cladosporioides*. 

**Figure 2.**—Identification of IgE-binding proteins by autoradiography after 2 days exposure following SDS-PAGE on 15 % polyacrylamide gel and electroblotting. Strips a shows Ponceau red staining of MW markers. Strips b-c show respectively the transferred spore and mycelium extracts incubated with human sera from *Cladosporium* allergic patients. Strip d was incubated with the sera of non-allergic patients.

**Figure 3.**—Immunoblotting experiments using sera adsorbed with spore and mycelium extracts after SDS-PAGE on 12.5 % polyacrylamide gel. Spores strips (b-d) were incubated with sera adsorbed with mycelium and spore extract at a concentration giving 35 to 50 % (c) and 100 % (b) RAST inhibition for mycelium and 50 % RAST inhibition for spore (d). Mycelium strips (e-f) were incubated with sera adsorbed with spore extracts at concentrations giving similar 50 % and 100 % (e) RAST inhibition values.
C. cladosporioides and C. herbarum whereas no cross-reactivity was observed between spore and mycelium extracts of C. cladosporioides. This result suggest that the method of immunization can be important and may lead to the recognition of different allergenic determinants by human or animal IgE. Observations of this nature emphasize the necessity when one is using an animal antiserum for the identification and standardization of allergens to assess that the animal antiserum is recognizing the same allergenic determinants as human IgE antibodies.

The allergens of C. cladosporioides have a MW range between 16 and 88 Kda. This is similar to other allergens detected in fungi which have the same range of MW. Most of these allergens have a glycoproteic nature, the β-Galactofuranoside glyco-conjugate part; found on conidia and conidiophore of Aspergillus niger, being essential to confer the allergenicity to the molecule whereas the allergenic activity of the Cla h 2 (Ag 54) allergen of C. herbarum was not sensitive to deglycosylation. The 16 Kda and the 20 Kda allergens detected in C. cladosporioides could probably correspond to Cla h 1 and Cla h 2 the major allergens of C. herbarum. Cross-inhibition transfers should be performed to confirm the identity of the two allergens of C. cladosporioides and the Cla h 1 and the Cla h 2 of C. herbarum.

REFERENCES