Comparison of the allergenic potency of spores and mycelium of *Cladosporium*

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ABSTRACT

The allergenic potency of spore and mycelium extracts of *Cladosporium* was estimated by RAST, RAST inhibition and PCA tests. Spores contained a concentration of allergens higher than mycelia. Results of PCA tests suggested that spores contained specific allergens. However, in a comparative study of extracts from different species of *Cladosporium* animal and human models gave different estimates of the allergenic potency of the different species. In spite of these variations it was shown that extracts from spores of *Cladosporium* contained the highest amount of *Cladosporium* allergens.

Key words: Cladosporium. Allergens. Fungal. Spore. Mycelium. Cross-reactions. Passive cutaneous anaphyllaxis test. Radioallergosorbent test.

RESUMEN

El potencial alergénico de los extractos de las esporas y del micelio de *Cladosporium* ha sido valorado por los métodos de RAST, RAST inhibición y PCA. Las esporas contienen una dosis de alérgenos más elevada que el micelio. Los resultados del ensayo PCA sugieren que las esporas contienen alérgenos específicos. Sin embargo, en un estudio comparativo de los extractos procedentes de diferentes especies de *Cladosporium*, el modelo animal y humano han dado diferentes estimaciones del poder alérgenico entre las distintas especies. Aunque haya variaciones, se ha demostrado que los extractos de las esporas de *Cladosporium* contienen una cantidad más elevada de los alérgenos.

Palabras clave: *Cladosporium*. Alérgenos. Espora. Micelio. Reacción cruzada. Prueba cutánea anafiláctica. Prueba de radioalergosorbencia.

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INTRODUCTION

Cladosporium has been known to be one of the most airborne fungi causing respiratory allergic diseases particularly asthma and rhinitis[1]. *Cladosporium cladosporioides* is the most prevalent species[2]. *Cladosporium herbarum* frequently dominates the outdoor mycoflora and it has been extensively studied[3-5]. The direct implication of this fungus in inhalant allergy is now recognized[6-9]. The lack of standardized extracts, the instability and the variability of their antigenes and allergens composition have been the most problem to progress in understanding fungal allergy. Extracts from different strains of *Alternaria alternata*, *Aspergillus fumigatus*, *Candida albicans* and *Epicoccum nigrum* vary greatly in their allergens composition[10-13]. The variability between different batches of *Cladosporium* extracts seems very high[14,6]. This vari-

ability may be due to genetical heterogeneity of fungal isolates, to extraction procedures, to the propagule used for allergen extraction, and to environment conditions (such as, climatic factors, growth media, degree of exposure). Most studies on allergenic moulds are based on culture filtrate extracts obtained from mycelium, rarely on somatic ones[15-17]). Mycelium is the easiest propagule to grow in vitro. However, it is not the fungal propagule inhaled by the patients whereas fungal allergy results from exposure to spores[18]. Airborne fungal spores can penetrate the lower airway and enter in contact with the mucosa. Moreover, previous studies have demonstrated that spores contain high allergenic potency when compared to the mycelium[19,20]. However, a comparison of *Alternaria alternata* spore and mycelium extract found that mycelium extract have greater potency than that of spore extract on the basis of skin test, RAST inhibition and basophile histamine release[21]. It is now acknowledged that the standardized conditions for optimal growth and sporulation and the preparation of metabolic and somatic extracts for both early and late growth phase fungal culture using the best source material containing the most relevant allergens are recommended[9,22].

In order to choose the best source material for the preparation of *Cladosporium* extracts, the allergenic potency of spore and mycelium extracts of different species of *Cladosporium* has been compared using RAST inhibition and PCA tests. RAST inhibition technique has been used in homologous and heterologous inhibitions between fungal extracts of many species of fungi imperfecti and Basidiomycetes[23-25]. In the case of *Cladosporium*, this method has been coupled to PCA tests which have already proven to be very effecient to study the reaginicity of several *Cladosporium* extracts[26,7].

MATERIAL AND METHODS

Preparation of the fungal extracts

The strain LCP 404 of *C. cladosporioides*, LCP 406 of *C. sphaerospermum* (museum national d'histoire naturelle, Paris, France) and IP1679-87 of *C. herbarum* (Unité de Mycologie, Institut Pasteur, Paris, France) were grown on 2 % malt agar medium at room temperature. The spores (> 95 % pure for *C. cladosporioides* and *C. sphaerospermum*, > 60 % for *C.herbarum*) were harvested with a paint brush after 3 to 4 weeks of growth. The mycelium was obtained in a 2 liters Biolafitte fermenter containing 2 % glucose, 1 % peptone (Prolabo) and 0.1 % Rhodorsil 426 R. After 48h of growth at 25 °C, 700 rpm and 0.5 vvm, the mycelium was recovered by filtration, washed with distilled water and stored at –20 °C.

Spores or mycelia were suspended in 10 mM phosphate buffer saline pH 7.2 (PBS) or 50mM Tris pH 9.0 containing 1mM EDTA and 1 % PVP (TEP) or 50 mM NaHCO3 pH 8.0 (Bic) and disrupted in a glass bead (1 mm) MSK Braun cell homogenizer. Bic total fungal extract was stored freeze dried. PBS and TEP extracts were centrifuged (30 min, 15 000 g) and the supernatants were stored at –80 °C. Protein content was measured using the BioRad method.

Sensitization of guinea pigs and mice and PCA test

Guinea pigs were sensitized using Bic total extracts of spore and mycelium of *C. cladosporioides* as previously described[26]. For mice sensitization spores and mycelium extracts from *C. cladosporioides* were used. Six groups of 5 Female Balb/c were sensitized using TEP soluble extracts as previously described[26]. PCA was performed as described by Ogilvie[27] and modified by Ovary[28]. PCA titer was the lowest dilution of serum giving a positive skin reaction.

RAST and RAST inhibition

Extracts of spores of *C. herbarum* and spores and mycelium of *C. cladosporioides* obtained by cell disruption in PBS were used for RAST and RAST inhibition experiments.

RAST

RAST was performed according to Ceska et al[29]. RAST disks were prepared by incubating overnight at 4 °C CNBr activated cellulose disks in 100 µl aliquots containing increasing concentrations of extracts (5 to 500 µg of proteins/ml depending on the extract). After 2 successives incubations (3 and 1 h) of the disks in 0.1 M Tris pH 8.0 and 3 washes in PBS buffer at room temperture, 50 µl of a pool of sera from positive patients were added. Sera were selected on the basis of positive prick tests and RAST to *Cladosporium* commercial extracts. After 3h incubation, washes and successive incubation with 125 I-rabbit anti-IgE were performed according to Phadebas (Pharmacia) technical informations. IgE uptake was expressed as the percentage of total cpm measured with 50 µl of the same 125 I-anti-IgE.

RAST inhibition

The assay used was adapted from the technique developed by Yman et al[30]. 100 µl of spore or mycelium extract (liquid-phase) containing increasing concentrations of proteins (0,25; 0,5; 1; 2,5; 5 µg/ml) were incubated for 3h at room temperature with 100 µl of the patient sera diluted in PBS (1:1). Cellulose disks coupled to myceliun (500 µg proteins/ml) or spore (5 µg proteins/ml) extracts were added to the serum aliquots adsorbed with the different concentrations of soluble allergens. The allergen concentrations for the coupling and the sera dilution were determined by preliminary experiments as the lowest serum and extract dilutions giving the maximal IgE uptake. IgE uptake was measured as described for RAST. The percentage of inhibition of IgE uptake was plotted against the allergen concentration in the liquid-phase.

RESULTS

Sensitized animals

Table I showed the production of IgE antibodies in mice after sensitization with spore or mycelium extracts. No IgE response was observed after 4 injections of 1 µg proteins per mouse. A primary IgE response was only observed at day 21 when a dose of 50 µg proteins was used. The secondary IgE responses varied according to the concentration of protein used for booster. 10 µg injections induced slowly IgE increase. A titer 30 of IgE-antibodies in the serum was obtained. After 3 injections of 50 µg proteins per mouse, a maximum titer of IgE was obtained. Such sensitization protocol was used for all experiments in mouse.

No cross-reactivity was observed when mice and guinea pigs sensitized to spores of *C. cladosporioides* were challenged with mycelium extracts of the same strain (table II). At the opposite, positive PCA tests were obtained when animals sensitized to spore of *C. cladosporioides* were challenged with spore extracts of *C. sphaerospermum* and *C. herbarum* (table II). These results indicated that spore extracts of different species of *Cladosporium* contained closely related allergens which looked different of the mycelial allergens even when they are extracted from the same strain.

Atopic human patients

The uptake of IgE on the CNBr activated disk was dependant on the concentration used for the coupling of the allergens to the disk (fig. 1). For example, in the case of *C. cladosporioides*, maximal uptake was obtained when the disks were incubated respectively with spores and mycelial extracts containing, respectively 50 µg or 500 µg proteins/ml PBS (fig. 2). A proportional dose dependent immunereresponse was obtained between 0.5 to 5 µg spore extracts and 5 to 500 µg mycelial preparation. Spore and mycelium allergens of *C. cladosporioides* were also compared in RAST inhibition experiments

Figures 2 and 3 showed that RAST inhibition percentages were comprised between 10 % and 80 % according to the protein dose used. Logit transformation of the RAST inhibition curves produced different slopes with the three extract used. *C. cladospo-*

Table I

IgE immune response of 6 groups of female Balb/c mice measured by PCA. The protein dose of spore and mycelial extract injected were 1, 10 and 50 µg. The PCA titers are obtained with the pool of the sera from 5 mices

Days after first injection	Dose of antigen used for sensitization					
	Spore extract			Mycelial extract		
	1	10	50	1	10	50
21	0	0	10*	0	0	10
35	0	30	270	0	10	270
42	0	30	270	0	30	270

*Lowest dilution giving positive skin reaction in PCA test (PCA titer).

Table II

Cross-reactivity between spore and mycelium of different species of *Cladosporium* demonstrated by passive cutaneous anaphylaxis (PCA) test

Antigen used for PCA challenge	Antigen used for sensitization of mice and guinea pig[a]			
	Spore LCP 404		Mycelium LCP 404	
	Rat	Guinea pig	Rat	Guinea pig
Spore LCP 404	270[b]	40	0	0
Mycelium LCP 404	0	0	270	40
Spore LCP 406[c]	90	80	Nd	Nd
Spore IP 1679-87[c]	90	30	Nd	Nd

[a] See material and methods for composition.
[b] Lowest serum dilution giving positive PCA reaction.
[c] Heterologous inhibitions.
Nd: Not done.

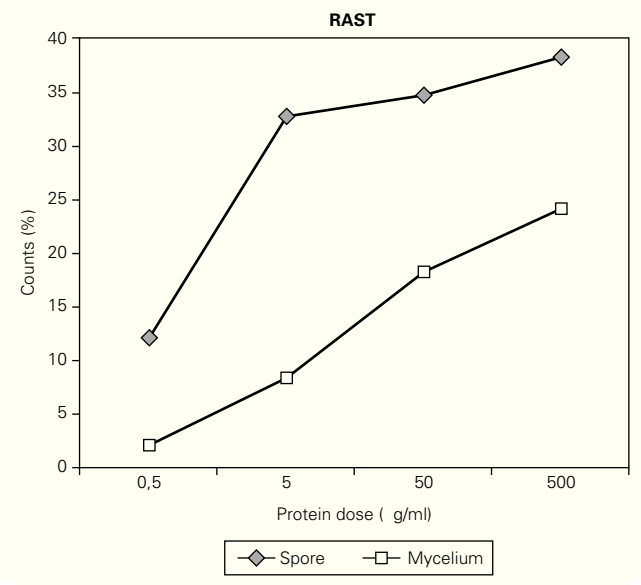

Figure 1.—RAST values obtained after incubation of CNBr-activated disks with different concentration of spore and mycelial extract of *C. cladosporioides*.

rioides spore extract was the most potent inhibitor and *C. herbarum* extract was the lowest (fig. 2). At the lowest protein dose of spore or mycelial extract, the differences in RAST inhibition percentages between the two extracts were more important. At the dose of 5 μg proteins/ml, the spore and mycelial extracts of *C. cladosporioides* showed the same RAST inhibition response. When spores were used as solid phase (fig. 2), 50 % inhibition was obtained with 1 μg/ml of spore extract and 1.75 μg/ml of mycelium extract. When disks coupled to mycelium extracts were used as solid phase, 0.75 μg/ml of spore extract and 1.25 μg/ml of mycelium extract were necessary to obtain 50 % inhibition.

Spore extracts gave the highest degree of inhibition of the uptake of specific IgE antibodies on the cellulose disk when spore but also mycelium were used in the solid phase.

DISCUSSION

A classic question in mould allergy is whether spores or mycelium provide the best source of allergens[7,9]. The results obtained in this study demostrated that spore and mycelium of *C. cladosporioides* have different allergenic potencies; the spore extracts being always much more allergenic than the mycelium. Using PCA tests no cross-reactivity was obtained between mycelium extract and spore allergens from *C. cladosprioides*. A comparable situation was observed in *Alternaria*. Using biochemical and immunochemical techniques, Hoffman et al[20] and Aukrust et al[19] demonstrated that spore extracts were clearly distinct from mycelium extracts. Although spore extracts contained many similar or identical antigens to the mycelium preparation, spore specific antigens and allergens could not be detected in the mycelium extract of *A. alternata*. The results of

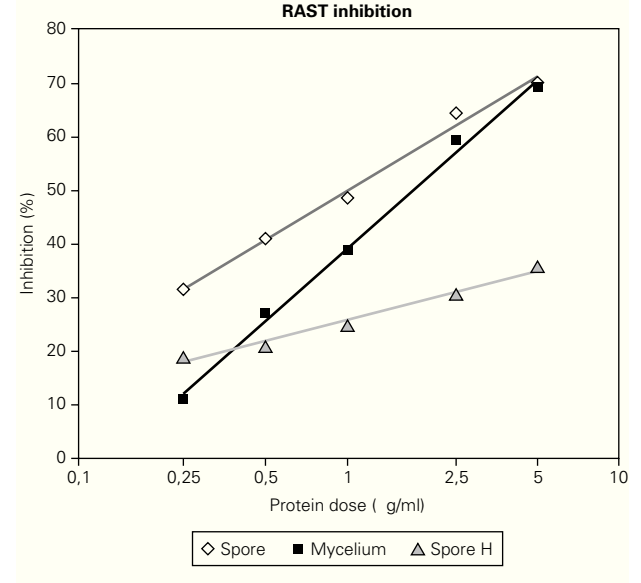

Figure 2.— RAST inhibition curves produced by different protein dose (0,25; 0,5; 1; 5 μg/ml) of spore and mycelium extract of *C.cladosporioides* and by *C. herbarum* spore extract (H) using *C. cladosporioides* as the solid phase.

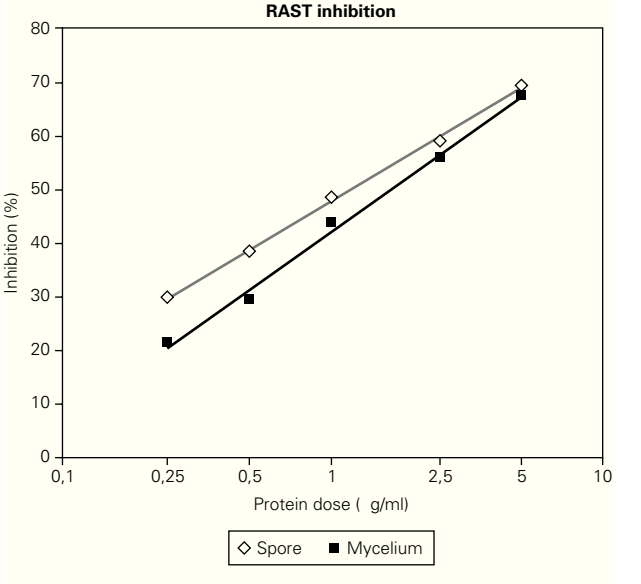

Figure 3.—RAST inhibition experiment with *C. cladosporioides* mycelium preparation as the solid phase and spore and mycelium of the same strain as the the liquid phase at increasing protein dose (0,25; 0,5; 1; 2,5; 5 μg/ml) for serum inhibition.

these studies suggested that mycelium extract are deficient in spore specific allergens and consequently do not seem the most best source material for diagnosis and immunotherapy of mould allergy. However, in certain cases mycelium extracts of *Alternaria alternata* have greater potency than that of spore[21] due probably to a higher concentration of a 31 KDa major allergen[31].

The present study was carried out using RAST, RAST inhibition and PCA tests. It confirmed previous findings[26,7] suggesting that PCA is a very helpful technique to study mould allergy in animal model. A RAST made with partially purified fungal allergens can be comparable to skin test in both sensitivity and specificity[32,33]. RAST inhibition assay have been suggested as a practical way to standardize allergens because it correlates quite well with the major allergen content[22]. However, this test needs reliable solid-phase allergens containing all the allergenic determinants. Such studies should be repeated with different strains and species using different extraction procedures. Moreover, for RAST inhibition one needs a suitable pool of serum containing a representative amount of IgE antibodies directed toward both major and minor allergencic determinants in the extract tested. Changes in the serum pool is unavoidable resulting differences in the measurement of allergens[22]. RAST inhibition has considerable advantages according to the direct RAST because it offers the possibility to compare efficiently different allergen extracts[34,16]. Considerable cross-reactivity was found between *Alternaria* and *Cladosporium*[35] indicating the presence of shared allergenic determinants in these two fungi. Significant correlation between RAST inhibition values of *Aspergillus restrictus* and *Aspergillus fumigatus* was also obtained[16]. Cross-reactivity of IgE-antibodies to allergens reflects the phylogenetic relationship of these organisms. This evidence of common allergenic determinants in these fungi was not clear between select species of Basidiomycetes and Deuteromycetes since a minimal cross-reactivity was observed[36]. Such method should be used more often as a guidance for fungal extract production especially to monitor the homogeneity, the stability and the presence of the major allergen in the different batch extracts.

RAST, RAST inhibition results and PCA tests have demonstrated that spore and mycelium display different allergenic potency. However, discrepancies are found when one looks at the results obtained with spore and mycelium extracts of the same species. RAST inhibition indicated an important community between the two extracts whereas PCA showed an absence of cross-reactivity between the spore and the mycelial extract. The difference with human immuneresponse could result from the way of the sensitization leading to the recognition of different allergens in spore and mycelium. These allergens may be of carbohydrate nature which do not bind to RAST disc such as the β-Galactofuranoic glycoconjugate allergen of *Aspergillus niger*[37].

The immunochemical comparison of conidial and mycelial allergens of *Cladosporium* using an electroblotting technique is currently being undertaken. Cross-inhibition transfers should be performed to look for the presence of shared allergenic components between the spores and the mycelium of *C. cladosporioïdes*.

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