Comparison of *Psilocybe cubensis* spore and mycelium allergens

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Background: *Basidiospores are an important cause of respiratory allergy in mold-sensitive atopic subjects. Collection of the large amounts of spores required for extract preparation is tedious and difficult. A desirable alternative could be mycelium grown in vitro if it is allergenically similar to spores.*
Methods: *Therefore this study compared the allergen contents of* Psilocybe cubensis *spore and mycelium extracts by different techniques with the use of pooled sera from subjects who had skin test and RAST results that were positive to* P. cubensis *spores.*
Results: *Isoelectric focusing immunoprints revealed six common IgE-binding bands at isoelectric points 4.7, 5.0, 5.5, 5.6, 8.7, and 9.3. Two additional bands at isoelectric points 3.9 and 5.7 were detected only in the spore extract. Sodium dodecylsulfate-polyacrylamide gel electrophoresis immunoblots exhibited six common IgE-binding bands at 16, 35, 487, 52, 62, and 76 kd; 20 and 40 kd bands were present only in the spore extract. Although RAST and isoelectric focusing inhibition demonstrated that* P. cubensis *spore and mycelium extracts share many allergens, spores were allergenically more potent than mycelium.*
Conclusion: *The results indicate that mycelium is a useful source of* P. cubensis *allergen, even though several spore allergens were not detected in mycelium. (J* ALLERGY CLIN IMMUNOL *1993;91:1059-66.)*

Key words: *Fungal allergy, basidiospore,* Psilocybe cubensis, *spore allergen, mycelium allergen, in vitro culture*

Basidiomycetes, such as mushrooms, puffballs, bracket fungi, smuts, and rusts, are found in many parts of the world; and their spores are reported worldwide in aerobiology surveys.[1-3] Basidiospores are important fungal aeroallergens that can cause respiratory allergy.[4-6] As with any inhalant allergy, to reliably identify and treat fungi-sensitive individuals, a clinician requires high-quality extracts that contain relevant allergens. Presumably, spores are the primary agent for allergen exposure; therefore, their extracts should provide the best allergen reagent.

Abbreviations used

IEF:	Isoelectric focusing:
PBS:	Phosphate-buffered saline
pI:	Isoelectric point
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Collection of the large quantities of spores required for extract preparation is difficult and time-consuming. Usually, basidiomycete sporulation is seasonal, which makes harvesting inconvenient. Other fungal allergen sources, such as mycelia from fruiting bodies or in vitro cultures, could provide greater quantities of readily available reagent.

Generally, most commercially available fungal allergen extracts for the diagnosis and treatment of mold allergy are prepared from mycelial mats that contain variable spore quantities.[7] Several studies have indicated that spores contain more allergens than mycelium, which has caused concern that all relevant spore allergens may not be present in mycelium. This suspicion has been substantiated for the basidiomycetes *Pleurotus ostreatus*[8] and *Ganoderma* spp[9] and the Fungi Im-

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TABLE I. *P. cubensis*-specific skin prick test, RAST values, and total IgE levels of study subjects

Subjects	SPT	RAST (% bound)	Total IgE (IU/ml)
1*	+	13.5	2600
2*	+	11.7	1650
3*	+	15.0	355
4*	+	15.0	400
5*	+	8.7	290
6†	−	1.4	105
7†	−	1.7	310
8†	−	1.0	60
9†	−	1.0	40
10†	−	2.0	500

SPT, Skin prick test.
*Skin prick test and RAST results were positive.
†Skin prick test and RAST results were negative.

perfecti *Alternaria alternata*[10, 11] and *Epicoccum nigrum*.[12] Although some mycelia may provide good allergen sources, each must have its allergenic similarity to spores established before extracts are prepared for testing and immunotherapy.

Psilocybe cubensis spores have been demonstrated in vivo and in vitro to be important basidiomycete allergens[4-6] that share major allergens within a group of basidiomycete species.[13, 14] Thus *P. cubensis* provides a promising reagent to identify individuals allergic to a range of basidiospores. This study compared the allergen contents of *P. cubensis* mycelium and spores to determine the suitability of substituting mycelium for spores in allergen extract preparation.

METHODS

In vitro growth of mycelium

P. cubensis (culture no. 36459, American Type Culture Collection, Rockville, Md.) was maintained on 2% malt agar plates. A plug (5 mm in diameter) of actively growing *P. cubensis* mycelium on agar was transferred under sterile conditions to a 250 ml Erlenmeyer flask, which contained 125 ml of 2% malt broth or ultrafiltered 2% malt broth or 3.5% Czapek-Dox broth (Difco Laboratories, Detroit, Mich.) and grown for 1 month. The 2% malt broth and the 2% malt broth, which was ultrafiltered (YM-5 filter, Amicon, Danvers, Mass.) to exclude materials greater than 5 kd, had no detectable background IgE reactivity as determined by sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting and had very low RAST background (3.4% and 3.2%, respectively). The mycelial mass was dispersed in a sterile blender for 10 seconds. Culture flasks (250 ml) with 100 ml 2% malt broth were inoculated with 1 ml of the mycelial suspension. Flasks were incubated in a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 23° C and 125 rpm. Mycelia from both culture broths were harvested after 4, 8, 12, 16, and 20 days and rinsed with deionized water before freezing and lyophilization. The lyophilized mycelium was stored at room temperature under desiccation until extracted. Culture supernatants were collected, dialyzed at 4° C overnight (molecular weight cutoff = 3.5 kd) against deionized water, lyophilized, and stored as described above. Dry weight was determined for all extracts and supernatants, and their protein content was measured with "Coomassie plus" assay reagent (Pierce, Rockford, Ill.) according to the manufacturer's instructions.

Preparation of extracts

Extracts were prepared by homogenizing 1 gm of mycelium in 25 ml of 0.125 mol/L ammonium bicarbonate buffer (pH 8.1) for 2 minutes in a Braun homogenizer (Braun Melsungen AG, Melsungen, Germany) cooled with escaping liquid CO_2.[15] The homogenate was centrifuged (27,000 *g*), and the supernatant was collected, lyophilized, and stored at room temperature under desiccation until use. One gram of *P. cubensis* spores (Florida Mycology Institute, Pensacola, Fla.) was defatted with 150 ml ethyl ether for 4 hours, collected by filtration, and air dried overnight. The defatted spores were then extracted with the same procedure as that described for mycelium.

Sera

Sera from five atopic subjects, who had skin test- and RAST results that were positive to *P. cubensis* spore extracts, were selected from a previous study and pooled.[16] RAST values (percent bound) for sera ranged from 8.7% to 15.0% (Table I). Sera from five individuals, who had skin test and RAST results that were negative (percent binding <3.0%) to *P. cubensis* spores, formed a negative control pool. For immunoprinting, the positive pool was diluted fivefold more to attain a mean total IgE value comparable to the negative pool.

RAST

Duplicate CNBr-activated cellulose disks[17] coated with 1 mg dry weight extract per disk were incubated overnight with 100 μl undiluted test serum. After washing with saline solution (0.9%), 100 μl (Kallestad, Chaska, Minn.) of iodine-125-labeled anti-IgE (15,000 cpm/disk) was added before overnight incubation. Disks were washed, and bound ^{125}I was measured in a gamma counter (Gamma 5500, Beckman, Irvine, Calif.). Results were expressed as mean percent binding of total radioactivity added.

RAST inhibition

RAST inhibition was performed by the method of Gleich et al.[18] Fifty μl each of pooled sera and test

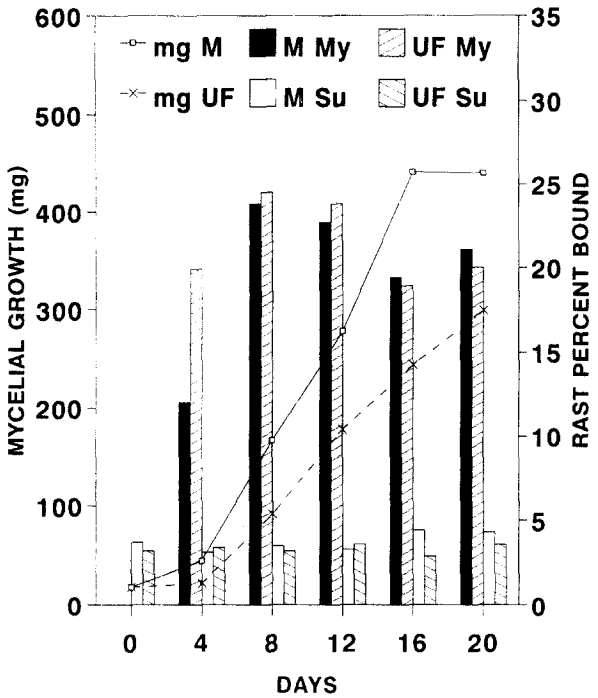

FIG. 1. Weight yield *(lines)* and allergen activity *(bars)* of *P. cubensis* mycelium grown in 2% malt *(M)* and ultrafiltered 2% malt *(UF)*. Yield is milligrams of lyophilized mycelium obtained from 100 ml medium in 250 ml Erlenmeyer flask. RAST values *(percent bound)* of in vitro mycelium *(My)* and culture supernatants *(Su)* were determined after culture in 2% malt or ultrafiltered 2% malt for 4, 8, 12, 16, and 20 days. Day 0 values represent milligrams of inoculum and RAST value of freshly prepared media.

antigen (resuspended spore or mycelium extract in phosphate-buffered saline [PBS], pH 7.2) were simultaneously added to antigen-coated disks and incubated together on a rotator at room temperature overnight; all tests were conducted in duplicate. Bound IgE was determined as described for RAST, and results were expressed in terms of mean percent inhibition of the PBS control by the test antigen.

Isoelectric focusing/immunoprinting

Isoelectric focusing (IEF) was performed in thin-layer polyacrylamide gels (pH range, 3.6 to 9.5), according to the manufacturer's recommendations for power limits and total time (PAG-plates, LKB, Piscataway, N.J.). Gels were loaded with 1000 μg dry weight of extract per lane. Focused proteins were stained with Coomassie brilliant blue R 250 or blotted by passive transfer[19] onto a 0.2 μm pore size nitrocellulose membrane previously activated with CNBr.[20] Unreacted sites on the membrane were blocked with a 40 mmol/L PBS (pH 7.5) that contained 0.4% Tween 20 and 1% bovine serum albumin. Nitro-cellulose membranes were washed in PBS-Tween 20 (0.05%) and overlaid overnight with RAST-positive (diluted 1:8) or RAST-negative sera pool (1:1.6). Bound IgE was labeled with

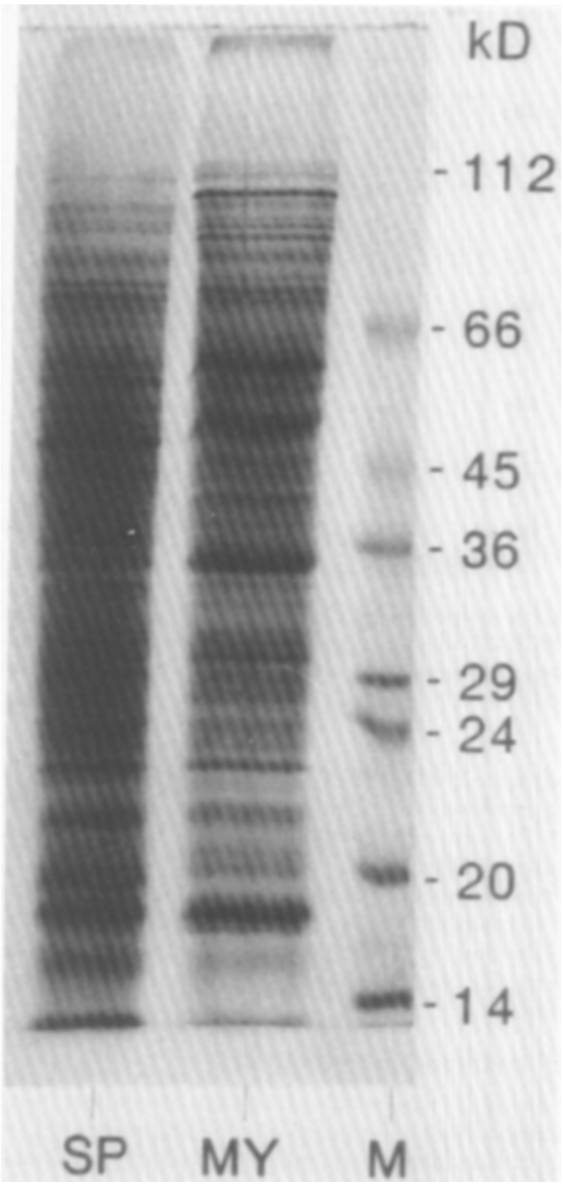

FIG. 2. SDS-PAGE (15%T/2.7%C) analysis of proteins in *P. cubensis* spore *(SP)* and day 16 mycelium *(MY)* extracts. The gel was stained with Coomassie brilliant blue (*lane M* contains markers).

^{125}I-anti-IgE diluted to approximately 50,000 cpm per lane. Autoradiography was performed on x-ray films with intensifier screens (Du Pont Cronex Lightning Plus, Du Pont Co., Wilmington, Del.) at −70° C for 1 to 7 days. Band enumeration was assisted by densitometric analysis (Ultrascan SL, LKB, Bromma, Sweden).

SDS-PAGE/immunoprinting

SDS-PAGE was performed by Laemmli's method.[21] One thousand micrograms dry weight (equivalent to 32 μg protein) per lane of *P. cubensis* extracts were re-

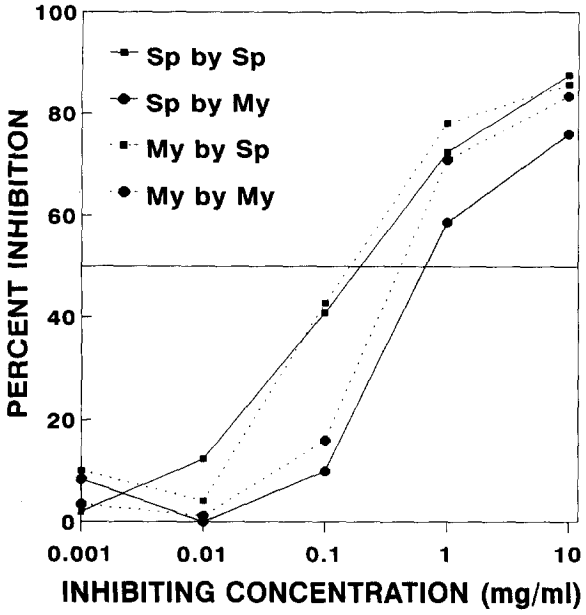

FIG. 3. Inhibition of *P. cubensis* spore *(Sp)* and mycelium *(My)* RAST by spores and mycelium. Though only moderate differences were noted, spores were more potent inhibitors than mycelium.

solved on 15% T/2.7% C acrylamide gels under reducing conditions (5% 2-mercaptoethanol in sample buffer). Proteins were transferred (blotted) electrophoretically to CNBr-activated nitrocellulose[20] in 125 mmol/L Tris buffer that contained 20% methanol with 200 mA of constant current for 1 hour. Detection of specific IgE-binding proteins, as well as staining of proteins, was performed as described for IEF immunoblotting.

IEF immunoprint inhibition

Strips (5 mm wide) of blotted membranes were incubated with 500 μl each of inhibiting antigen (8, 80, and 800 μg/ml protein) and 1:4 diluted sera pool (total volume 1 ml). Remaining activity was detected with ^{125}I-labeled anti-IgE as described.

RESULTS

Growth of *P. cubensis* mycelia

Mycelial growth reached the stationary phase more quickly and attained greater overall mass in 2% malt than in ultrafiltered 2% malt (Fig. 1). Yield per flask in 2% malt (vs ultrafiltered 2% malt) was 45 mg (22 mg) on day 4, 167 mg (92 mg) on day 8, 278 mg (178 mg) on day 12, 441 mg (244 mg) on day 16, and 440 mg (299 mg) on day 20. No substantial growth was observed after 3 weeks in Czapek-Dox broth, a synthetic culture medium. Mycelium and culture fluid extract from day 16, which were grown in 2% malt broth contained 324 μg/ml (total dry weight = 0.440 gm) and 12 μg/ml

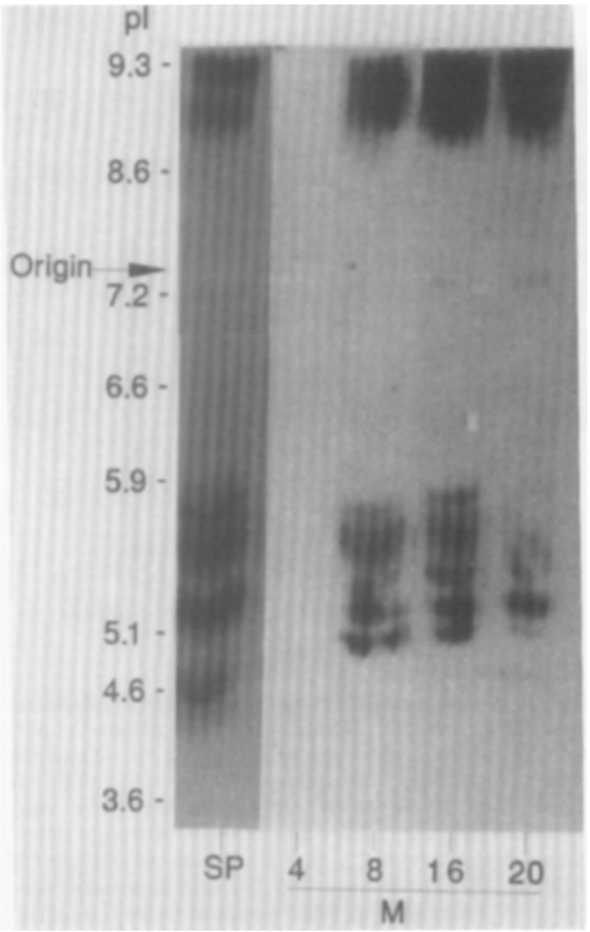

FIG. 4. IEF immunoprint analysis of *P. cubensis* spore *(SP)* and mycelium *(M)* extracts harvested at days 4, 8, 16, and 20. Each lane contained 32 μg of protein in 1 mg of extract. *Arrow* indicates the point of sample application.

protein (total dry weight = 0.753 gm), respectively, when adjusted to equivalent dry weight concentration.

Allergenicity of mycelia

P. cubensis mycelium RAST activity was detected in 2% malt and ultrafiltered 2% malt broth from day 4 to day 20. With pooled sera, spore extract RAST was 30% bound, whereas mycelium on day 4 was 12%; on day 8, 24%; on day 12, 23%; on day 16, 19%; and on day 20, 21%. The percent binding was comparable for both 2% malt and ultrafiltered 2% malt broths, except day 4 RAST values were lower for 2% malt (Fig. 1). Control sera RAST values were between 1.4% and 1.9% for cultures grown in both media. Culture supernatant reactivity with test serum varied from 3.1% to 4.4% in 2% malt and from 2.9% to 3.6% in ultrafiltered 2% malt. Control sera bound be-

tween 1.1% and 1.3% to supernatant-coated RAST disks of both culture media. Fresh broth reactivity with *P. cubensis*-positive pooled sera was 3.7% for 2% malt and 3.3% for ultrafiltered 2% malt. Reactivity of control sera was 1.2% for fresh 2% malt and 1.1% for fresh ultrafiltered 2% malt.

SDS-PAGE analysis of *P. cubensis* spore and mycelium

Although staining of SDS-PAGE gels and immunoprints of mycelium extract were the most intense on day 8, the biomass of mycelium was small. Mycelial yield increased 2.6 times between day 8 and day 16, and there was little change in RAST activity. Therefore day 16 mycelium was compared with spores to assess their similarity. SDS-PAGE (Coomassie blue) indicated that the protein patterns of spore and mycelium (day 16) are very similar (Fig. 2).

RAST inhibition analysis of *P. cubensis* spore and mycelium

Spore RAST was maximally inhibited 87% by spore extract and 76% by mycelial extract (Fig. 3). Maximum inhibition of mycelium RAST was 86% and 83% by spore and mycelial extracts, respectively. The 50% inhibition concentration was 200 μg/ml for spore-inhibiting spore extract and 140 μg/ml for spore-inhibiting mycelium extract. For mycelium, the comparable values were 700 μg/ml and 400 μg/ml for inhibition of spore and mycelium RAST, respectively.

IEF immunoprint analysis

Extracts of 8- to 20-day-old mycelium revealed band patterns similar to those of *P. cubensis* spore extract. The greatest number of proteins were in the pH range of 4.6 to 5.6. The overall staining intensity of protein bands in mycelium was less when compared with spores on a dry weight basis. However, the protein staining was similar when normalized for protein concentration. Autoradiography revealed six IgE-binding bands in mycelia at isoelectric points (pls) 4.7, 5.0, 5.5, 5.6, 8.7, and 9.3 on days 8 to 20 (Fig. 4). *Psi c* Bd9.3 inconsistently appeared in the mycelial extracts. The pl 3.9 and 5.7 allergens were detected only in spore extract. *Psi c* Bd9.3 was only routinely present in immunoprints with fresh mycelial extract and not detected after 2-month storage of extract at room temperature under desiccation (data not shown). The pl 4.7 allergen was not consistently observed in both spore and mycelium extract. The negative control sera pool revealed no IgE-binding bands in spore, mycelium, or supernatant extracts. No protein or IgE-binding bands were detected in mycelium on day 4, although RAST activity was detected.

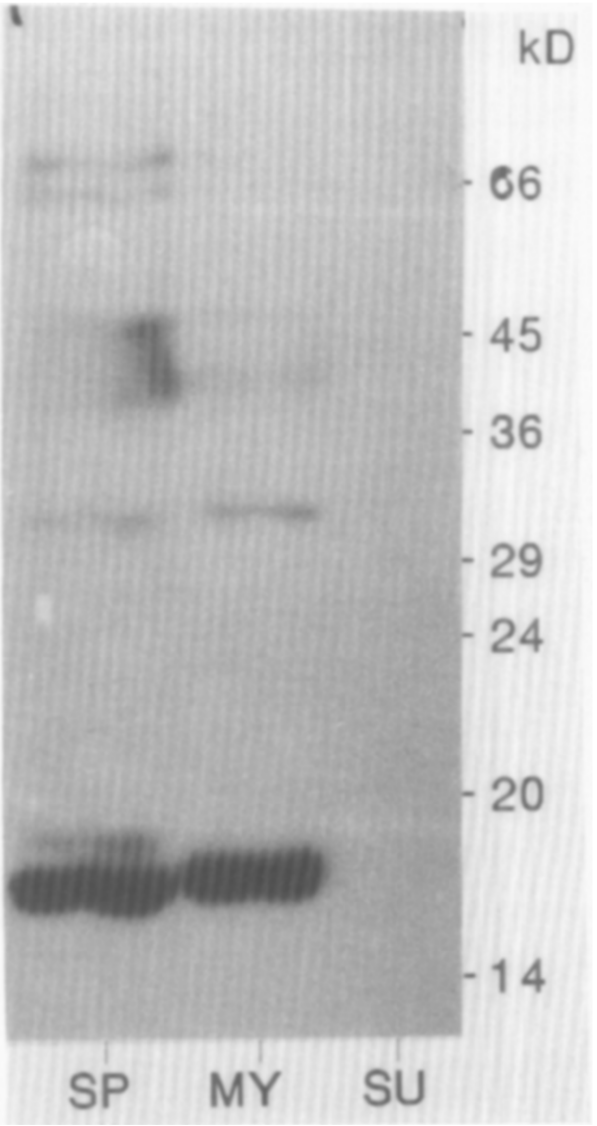

FIG. 5. SDS-PAGE immunoprint analysis of *P. cubensis* spore *(SP)* and mycelium *(MY)* extracts and culture supernatant *(SU)*. Loaded protein was 32 μg/lane for spores and mycelium and 12 μg/lane for supernatant.

SDS-PAGE immunoprint analysis

P. cubensis spore extracts contained at least 35 protein bands between 13 and 112 kd. IgE immunoprints with pooled positive sera revealed eight IgE-recognized bands (16, 20, 35, 40, 48, 52, 62, and 76 kd) (Fig. 5). Mycelial extracts also contained 35 protein bands but only six detectable allergens; the 20 and 40 kd spore allergen bands

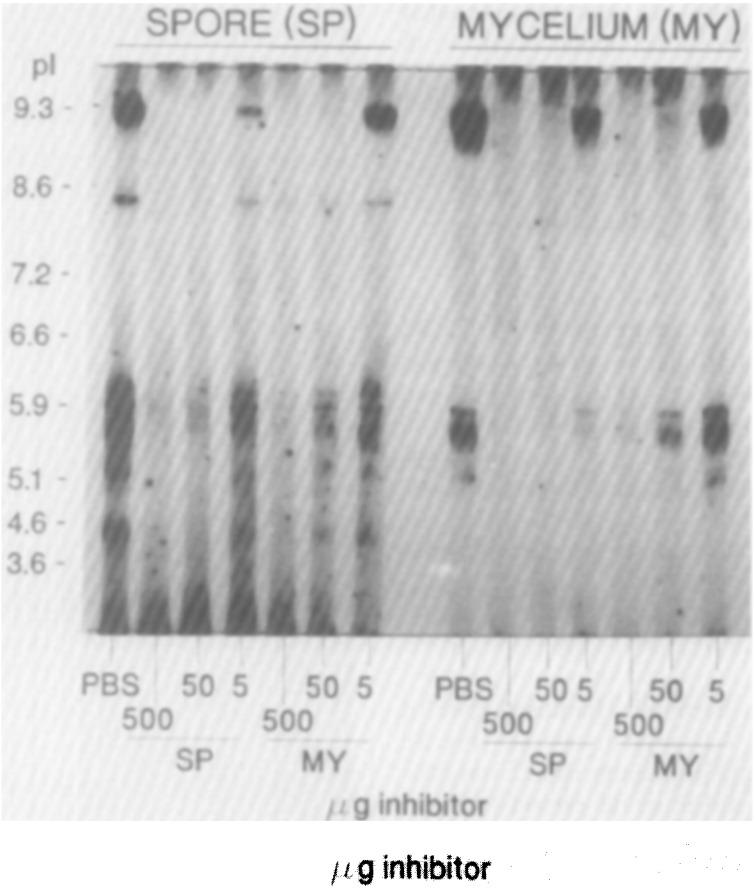

FIG. 6. IEF immunoprint inhibition of *P. cubensis* spore and mycelium extracts. Blots of spore *(SP)* and mycelium *(MY)* were inhibited with increasing quantities of spore and mycelium extracts as indicated. Blots of control strips were inhibited with PBS.

were missing (Fig. 5). Equal amounts (32 μg) of spore and mycelium protein displayed similar profiles; the 16 kd component was the most intense. Culture supernatant, tested at protein concentrations up to 12 μg per lane, contained no detectable protein or IgE-binding bands. Spore, mycelial, and culture supernatant extracts had no reactivity with the negative sera pool.

Immunoprint inhibition

P. cubensis spore extract IgE-binding bands at pI 3.9, 5.0, 5.5, 5.6, 5.7, and 9.3 were inhibited by spore and mycelium antigen in a dose-dependent manner (Fig. 6). Although pI 3.9 and 5.7 bands were not detected in *P. cubensis* mycelium, IgE reactivity to all bands, including those a pI 3.9 and 5.7, was inhibited by both spore and mycelium extracts.

DISCUSSION

Fungal spores are considered the primary agent for allergen exposure, and their extracts are presumed to provide better diagnostic and therapeutic tools than mycelium extracts. Unfortunately, only limited quantities of spores are available. The labor-intensive harvesting of sufficient quantities for extraction is tedious and expensive. Advantages to extracting in vitro mycelium include standardized growing conditions, dependable availability, and better yield. Before clinical use of mycelium extracts is feasible, their antigen-allergen content must be compared with that of spore extracts to establish the degree of similarity.

P. cubensis spores are imporant basidiomycete allergens.[4-6] Previous studies suggest that they might serve as a representative allergen for basidiomycete allergy.[13, 14, 16] *P. cubensis* also cross-reacts with other allergenic basidiomycetes; therefore, it might be an effective diagnostic screen.[13, 14] Both uses of *P. cubensis* require a reliable, abundant source material for extraction, such as mycelium grown in vitro. We compared *P. cubensis* mycelium with spore extracts by RAST, IEF- and SDS-PAGE-immunoprint, and RAST and IEF inhibition to determine how closely the two extracts resemble each other.

RAST to *P. cubensis* mycelium extract was specific and demonstrated considerable reactivity with the positive serum pool; however, mycelium RAST values were not as high as those for spores. Culture medium had no allergenic activity as determined by RAST or immunoprint. Although the maximum protein content of the culture medium was about one third of the value of *P. cubensis* mycelium, the relevant allergens were not present.

Coomassie blue staining of protein bands and IgE binding profiles, after separation by IEF and SDS-PAGE, were similar in both mycelium and spore extract. Although staining and IgE binding intensity were less in mycelium as compared with spores, the difference was minimal when extracts were analyzed on an equivalent protein basis. Thus the variation in allergenic activity appears to be related to extractable protein.

Only two spore-specific allergens were resolved by either SDS-PAGE (20 and 40 kd) or IEF (pI 3.9 and 5.7). Although not the most reactive *P. cubensis* spore proteins, these allergens (two by SDS-PAGE, two by IEF) reacted with more than half of the test sera.[16] No unique mycelium allergens were detected. However, IgE binding to all spore proteins was inhibited by mycelial proteins, which indicates that all spore allergens are present in mycelium. This raises the question of how mycelium, which lack two spore-specific proteins in immunoprints, can completely inhibit immunoprints of spore extract. Possibly, these proteins are present in mycelium at concentrations that are undetectable by blotting methods. Alternatively, the "unique" spore allergens may share allergenic epitopes with other allergen bands detected on the mycelium immunoblots. Such "cross-reactive" mycelial allergens could inhibit immunoprint reactions, even though the "cross-reactive" mycelial allergens differ in molecular weight or pI.

The pI 9.3 band, a major allergen in several basidiomycete species, was present in immunoprints of freshly grown and extracted *P. cubensis* mycelium; however, it was not detected after 8-week storage of the lyophilized extract under desiccation at room temperature, which indicates instability of the allergens. This observation agrees with studies of the pI 9.3 band (*Cal c* Bd9.3) of *Calvatia cyathiformis*, which was shown to be highly labile.[22] Inconsistent detection of the *P. cubensis* pI 4.7 and 8.7 bands may indicate that other allergens are labile or variable in expression.

Previous studies, in which skin prick testing was used, demonstrated the biologic activity of basidiomycete mycelium extracts.[4, 8, 23] In one investigation, the prevalence of positive skin test results in 150 respiratory allergic adults to at least one of the basidiomycete mycelia (27%) or to one or more basidiospore extracts (32%) was remarkably similar; however, spore extracts were more active.[4] Other studies of skin test results with *Alternaria alternata* indicated that spores provide a better source of fungal allergens than mycelium.[10, 11] These investigations underscore the finding that fungal spore extracts are more active than mycelium. Since most fungal allergen extracts are made from mycelial sources, the prevalence of fungal allergy as determined by skin test reactivity might be underestimated.

To date, only a few studies have compared spore and mycelium extracts, and the issue may not be completely resolved. Spores apparently contain spore-specific allergens. Still, mycelium extracts appear adequate for most uses. A recent investigation of 50 children with asthma analyzed the prevalence of skin test sensitivity to basidiospores and determined that all subjects with positive reactions to *P. cubensis* spore (14%) also reacted to *P. cubensis* mycelium extract[24]; no subject responded only to mycelium extract.

RAST inhibition demonstrated that *P. cubensis* spore extract and mycelium extract had very similar RAST inhibitory activity. Spore extracts consistently inhibited RAST better than mycelium, but these differences were slight and may not be clinically important. Spore-specific, but not mycelium-specific, allergens were detected by immunoblotting. Although *P. cubensis* spore extracts are allergenically more potent than mycelium extracts, immunoprint inhibition suggests that all unique spore allergens or allergenic epitopes are also present in mycelium. The far greater availability of mycelium compared with spores outweighs its comparatively lower allergen content. Thus *P. cubensis* mycelium should provide an adequate source material for allergen extraction.

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