

Basidiomycete mycelia and spore-allergen extracts: Skin test reactivity in adults with symptoms of respiratory allergy

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*One hundred fifty adults, with respiratory-allergic disease, and 14 control subjects, without symptoms of respiratory allergy, were skin prick tested with 16 common inhalant allergens, 12 extracts of mycelia from Basidiomycetes grown in vitro, and/or 10 to 15 basidiospore extracts. Eighty-three subjects (58%) had positive skin tests to two or more of the common inhalant allergens. Twenty-seven percent of the study subjects had positive skin reactions to one or more of the Basidiomycete mycelia extracts, and 32% demonstrated positive skin reactions to one or more basidiospore extracts. None of the 14 control subjects had positive skin reactivity to basidiospore extracts. Skin prick reactivity of study subjects to 15 different basidiospore extracts ranged from 5% for *Cantharellus cibarius* to 17% for *Scleroderma* sp. The prevalence of skin test reactivity to basidiospores did not differ significantly from the reactivity to commercial mold extracts of several common species of the Fungi Imperfecti (6% for *Cladosporium herbarum* or *Penicillium notatum* to 13% for *Alternaria tenuis*). These results demonstrate that a significant number of individuals reporting symptoms of respiratory allergy have skin test reactivity to basidiospores and suggest that these spores are important fungal aeroallergens in the New Orleans environment. (J ALLERGY CLIN IMMUNOL 78:478-85, 1986.)*

Although pollens have been widely studied throughout the years as aeroallergens, far less is known about fungal spores, which are present in the atmosphere in concentrations considerably in excess of pollen grains.^{1,2} Several reasons may explain our lack of knowledge of allergic reactions to fungal spores compared with pollen-induced respiratory allergies. First, allergic symptoms related to pollens have been easier to identify because of the seasonal basis of their presence in the atmosphere, whereas fungal spores are often present in the atmosphere over longer, less defined time periods. Second, since so many different species of fungi release spores into the atmosphere, it is virtually impossible to identify a particular one causing allergic symptoms. Finally, it has been relatively easy to harvest the large quantities

Abbreviation used

PBS: Phosphate-buffered saline

of pollen grains necessary to prepare extracts for the testing of patients, whereas pure samples of fungal spores are more difficult to obtain, particularly in amounts necessary for study.

Basidiomycetes are the most morphologically complex of all the fungal classes and include many diverse forms such as mushrooms, brackets, puffballs, rusts, and smuts. Since atmospheric basidiospore concentrations are high in many parts of the world, they are of considerable interest as potentially important spore aeroallergens.³⁻⁵

In previous studies we have demonstrated that extracts prepared from mycelia or culture fluid of selected Basidiomycetes grown in vitro were allergenic in humans and antigenic in the rabbit.⁶⁻⁸ Other studies demonstrated significant skin reactivity to spore extracts from Basidiomycetes.⁹⁻¹³ Since patients are exposed essentially to spores rather than mycelia of Basidiomycetes, we have recently directed our efforts at harvesting spores from Basidiomycetes growing in the wild.¹⁴ These extracts of Basidiomycete mycelia and

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TABLE I. Basidiomycetes selected for study

Basidiomycete type	Growth area	Growth season	Spore size (μm)
Gilled mushrooms			
<i>A. amara</i>	Decaying compost	July to Aug.	5.5 to 7 by 10 to 12
<i>A. muscaria</i>	Pine forests	July to Oct.	7 to 9 by 9 to 12
<i>A. tabescens</i>	Root rot	Sept. to Dec.	4 to 6 by 6 to 7
<i>C. cibarius</i>	Hardwood forests	July to Aug.	4 to 5 by 7 to 9
<i>C. molybdites</i>	Green grassy areas	May to Dec.	6 to 7 by 8.5 to 9
<i>C. quadrifidus</i>	Decaying hardwoods	June to Sept.	4 to 5 by 7 to 9
<i>P. ostreatus</i>	Butt rot, hardwood	Sept. to Feb.	2 to 2.5 by 5.6
<i>P. cubensis</i>	Cow or horse dung	Feb. to Oct.	7 to 10 by 10 to 17
<i>B. merulioides</i>	Mycorrhizal, ash	July to Aug.	6 to 7.5 by 7 to 10
<i>Boletus</i> sp.	Grassy areas	July to Aug.	4 to 9 by 12 to 24
Polypore mushrooms			
<i>G. lucidum</i>	Root rot, hardwoods	June to Dec.	6 to 7 by 10 to 11
<i>I. ludovicianus</i>	Root rot	July to Sept.	3.5 to 4 by 5 to 6
Puffballs			
<i>C. cyanthiformis</i>	Grassy areas	July to Oct.	3.5 by 7.5
<i>P. tinctorius</i>	Mycorrhizal, trees	June to Oct.	8 by 12
<i>Scleroderma</i> sp.	Variety of areas	Aug. to Dec.	6.5 by 10

spores were used to skin prick test adults presenting at our allergy clinics with symptoms of respiratory allergy. Our results indicate that a significant number of potentially allergic individuals have positive skin test reactions to mycelia and spore extracts and suggest that basidiospores are important fungal aeroallergens in the New Orleans area.

MATERIAL AND METHODS
Study population

One hundred fifty adults attending the Charity Hospital of New Orleans or Tulane Medical Center allergy clinics were selected on the basis of perennial rhinitis and/or asthma for study. Fourteen nonatopic adults without symptoms of respiratory allergy served as a control group. Informed consent was obtained, and a questionnaire was administered to all study subjects to obtain information on their respiratory allergies and allergy history. Blood samples were drawn, and all individuals were skin prick tested with 16 common inhalant allergens listed (Hollister-Stier, Spokane, Wash., and Greer, Lenoir, N. C.). Study subjects were divided into two groups. The first group of 74 subjects (group 1) were skin prick tested with 12 metabolic and somatic extracts from six Basidiomycetes grown in vitro and 10 extracts from five different basidiospore preparations. The second group of 76 subjects (group 2) were skin prick tested with extracts prepared from 15 different basidiospore preparations harvested from basidiocarps collected directly under field conditions.

Basidiomycete extracts

Cultures of six Basidiomycetes (*Cantharellus cibarius*, *Coprinus comatus*, *Dacrymyces deliquescens*, *Naematoloma sublateralitum*, *Pleurotus ostreatus*, and *Xylobolus*

frustulatus) were chosen as representative of taxonomic groups that grow in the region of New Orleans. Cultures of each, obtained from the American Type Culture Collection, were grown in dialyzable yeast malt broth for a 2-week period at 24° C. All cultures were then filtered (Whatman No. 1), and after dialysis (6000 to 8000 MW cutoff) against ion-depleted water and lyophilization, the culture filtrate was used as a source of the metabolic antigen. The remaining fungal growth was washed with ion-depleted water and lyophilized. Lyophilized growth was suspended in PBS, pH 7.2, by sonication. After mixing overnight at 4° C, the sonicate was centrifuged (80,000 × g), and the supernatant was concentrated (Amicon Corp., Danvers, Mass., UM 2) and called somatic antigen. The concentrate was recentrifuged, and the supernatant was stored at -20° C. Fractions for testing of the metabolic or somatic antigens were dissolved in PBS in 50% glycerol (diluent), sterile filtered, dry weights were determined, and concentrations were adjusted to 5 mg/ml with sterile diluent.

Basidiospore extracts

Basidiospores were obtained from basidiocarps growing in the New Orleans region as listed in Table I. These Basidiomycetes reflect a variety of types present in Louisiana. They have different growing seasons, ranging from several weeks in the fall to 3 to 4 months in the summer or winter. All Basidiomycetes were identified on the basis of morphology and growth patterns. Within 4 to 6 hours of harvesting, each basidiocarp was set up for spore collection. The mushroom cap was placed, hymenial surface down, on Whatman No. 1 filter paper and covered with either filter paper or aluminum foil to retain high humidity and reduce airflow to obtain the spore print.¹⁵ After 24 to 48 hours, the caps were removed, and the resulting spore deposits (spore prints) were harvested by scraping the filter paper gently

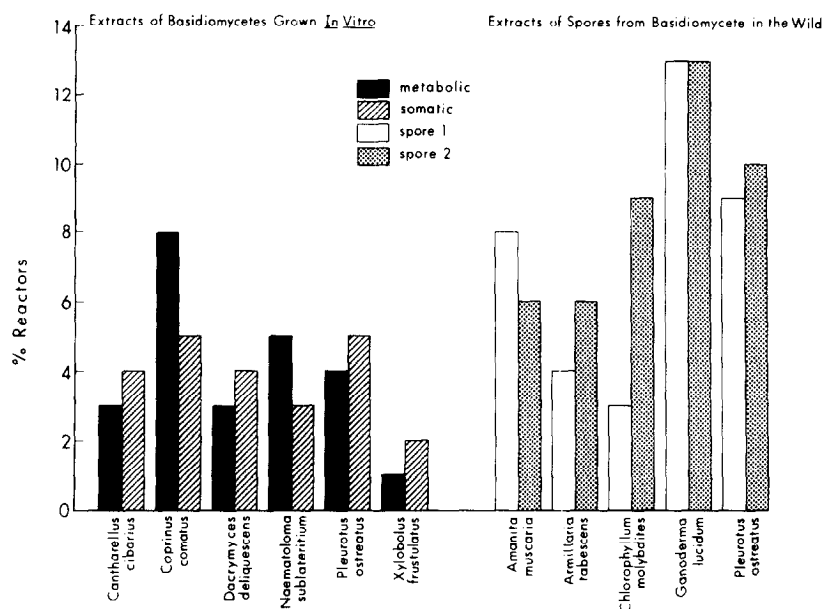


FIG. 1. Skin test reactivity of study subjects to extracts of Basidiomycetes grown in vitro or spores from Basidiomycetes in the wild.

TABLE II. Skin test reactivity of study subjects to common inhalant allergens

Allergen	No.	Reactors	%
House dust mite	150	66	44
House dust	150	57	38
American cockroach	137	41	30
Ragweed	150	45	30
Bermuda grass	150	32	21
Johnson grass	150	30	20
Pecan	150	30	20
Live oak	150	27	18
Cat epithelium	83	15	18
Dog epithelium	83	9	11
<i>A. tenuis</i>	150	19	13
<i>Fusarium solani</i>	150	16	11
<i>Aspergillus fumigatus</i>	150	13	9
<i>Epicozum</i> sp.	150	13	9
<i>P. notatum</i>	150	9	6
<i>C. herbarum</i>	150	9	6

with a spatula. Occasionally, spores bound to the basidiocarp or filter paper and could not be sufficiently removed; they were eluted with ion-depleted water and lyophilized. Spore prints of *Psilocybe cubensis* were purchased (Florida Mycology Institute, Pensacola, Fla.). All spore collection processes yielded >95% pure spores as determined by light microscopy. Identification of each Basidiomycete was confirmed by spore color, size, and shape.

In some cases the fruiting bodies of the Basidiomycetes were not mushrooms but puffballs. These were harvested at maturity, dried at room temperature, and cut in half, and

the spore mass, comprising the interior of the puffball, was removed with a spatula. Light microscopy of this material indicated at least 80% pure spores, with the major contaminant being sterile hyphae.

Spores were homogenized in 0.125 mol/L of ammonium bicarbonate buffer, pH 8.1, with a Braun (B. Braun Instruments, Burlingame, Calif.) homogenizer, in one of two ways. In the first extraction process (a two-step procedure), spores were suspended in ammonium bicarbonate buffer and mixed at 4°C overnight and then centrifuged (70,000 × g). The supernatant was lyophilized (spore 1). Sedimented spores were resuspended in ammonium bicarbonate buffer and homogenized in a Braun homogenizer (2 minutes at 4000 rpm). The homogenate was then mixed at 4°C overnight and then centrifuged. The second supernatant was lyophilized (spore 2). Since subsequent studies indicated that the two-step procedure was unnecessary, the extraction process was revised. Spores were suspended in 0.125 mol/L of ammonium bicarbonate buffer and immediately homogenized in a Braun homogenizer. The homogenate was then centrifuged (70,000 × g), and the supernatant was lyophilized (spore 3).

Before skin prick testing, all spore extracts were tested for toxicity by injecting 1 mg, intraperitoneally, into 6- to 8-week-old female Swiss Webster mice. Mice were observed at 2-hour intervals for the next 6 hours and at 12-hour intervals for the next 48 to 72 hours. Spore extracts having toxicity (e.g., causing reduced physical activity, diarrhea, or death) in mice would have been excluded from use in this study, but none demonstrated toxicity. For skin testing, lyophilized spore extract was suspended in a sterile solution of 50% glycerol in PBS and sterile filtered (0.45 μm). The dry weight of each spore extract was adjusted to 5 mg/ml with diluent.

TABLE III. Skin test reactivity of study subjects to basidiospore extract

Spore preparations	Reactors	%
<i>Scleroderma</i> sp.	13	17
<i>C. quadrifidus</i>	10	13
<i>P. cubensis</i>	9	12
<i>I. ludovicianus</i>	9	12
<i>G. lucidum</i>	8	11
<i>C. cyanthiformis</i>	7	9
<i>B. merulioides</i>	7	9
<i>A. amara</i>	7	9
<i>Boletus</i> sp.	7	9
<i>A. tabescens</i>	7	9
<i>P. ostreatus</i>	6	8
<i>P. tinctorius</i>	5	7
<i>A. muscaria</i>	5	7
<i>C. molybdites</i>	5	7
<i>C. cibarius</i>	4	5

Skin test reaction

All skin prick testing was performed on the volar surfaces of the left and right forearms. Routinely, skin testing with common inhalant allergens was performed on the left forearm, and skin testing with Basidiomycete and/or basidiospore extracts was performed on the right forearm.¹⁶ Glycerol-PBS control was used as a negative control, and histamine (1 mg/ml) was used as a positive control. After a 15-minute interval, any resulting edema (wheal) or erythema was outlined with a felt-tip pen and recorded on transparent tape as a permanent record. The mean of the largest wheal diameter and the diameter perpendicular to it was determined. Any extract inducing a mean wheal diameter 2 mm greater than that of the glycerol-PBS control was considered positive. Results from subjects whose histamine reaction was negative were excluded.

RESULTS

One hundred fifty adults, selected on the basis of symptoms of respiratory allergy were tested in this study. The mean age of this population was 39.2 years, and the percentage of male subjects was 26%. Twenty-seven (26.6%) percent of the individuals reported only symptoms consistent with rhinitis, 36.7% individuals had a history of asthma only, and 36.7% subjects had both. The percentage of atopic subjects (individuals having two or more skin reactions to common inhalant allergens, in addition to their history of respiratory allergy) was 58%.

Immediate wheal-and-flare skin test reactivity of study subjects to common inhalant allergens is summarized in Table II. Reactivity ranged from 6% for *C. herbarum* to 44% for house dust mite. Skin test reactions obtained with commercial mold extracts (from common species of the Fungi Imperfecti) ranged

TABLE IV. Number of positive skin reactors to basidiospore extracts versus number of subjects reacting

No. of positive skin tests to basidiospores	No. of subjects reacting	% Of the total reactors
1	9	37.5
2	2	8.3
3	2	8.3
4	0	0
5	1	4.1
6	2	8.3
7	2	8.3
8	1	4.1
9	3	12.5
10	0	0
11	0	0
12	2	8.3
13	0	0
14	0	0
15	0	0

from 6.0% for *P. notatum* or *C. herbarum* to 13% for *A. tenuis*.

Skin test results obtained with extracts of Basidiomycetes grown in vitro, with both metabolic and somatic antigen fractions, are summarized in Fig. 1. The prevalence of positive skin reactions ranged from 1% with metabolic extract of *X. frustulatus* to 8% for the metabolic extract of *C. comatus*. There was no consistent pattern of reactivity to metabolic or somatic fractions; with some Basidiomycete sp., metabolic extract elicited a greater number of positive skin reactions compared to somatic, whereas with other species, somatic extract appeared to be more reactive than metabolic. In addition to skin testing with extracts of in vitro growth, this group of subjects was also skin prick tested with basidiospore extracts 1 and 2 from spores of *Amanita muscaria*, *Armillaria tabescens*, *Chlorophyllum molybdites*, *Ganoderma lucidum*, and *Pleurotus ostreatus*. Comparison of spore 1 to spore 2 reactivity demonstrated that neither extract was consistently superior. In the case of *A. muscaria*, spore 1 yielded a greater number of positive skin test reactions, whereas spore 2 of *C. molybdites* had greater skin test reactivity. Similar prevalence of reactivity to *G. lucidum* and *P. ostreatus* was obtained with spore extracts 1 and 2. Skin test reactivity was generally less with extracts of Basidiomycete mycelia as compared to basidiospore extracts.

Skin test reactivity of study subjects to the third basidiospore extract is summarized in Table III. These extracts were prepared from spores obtained from 15

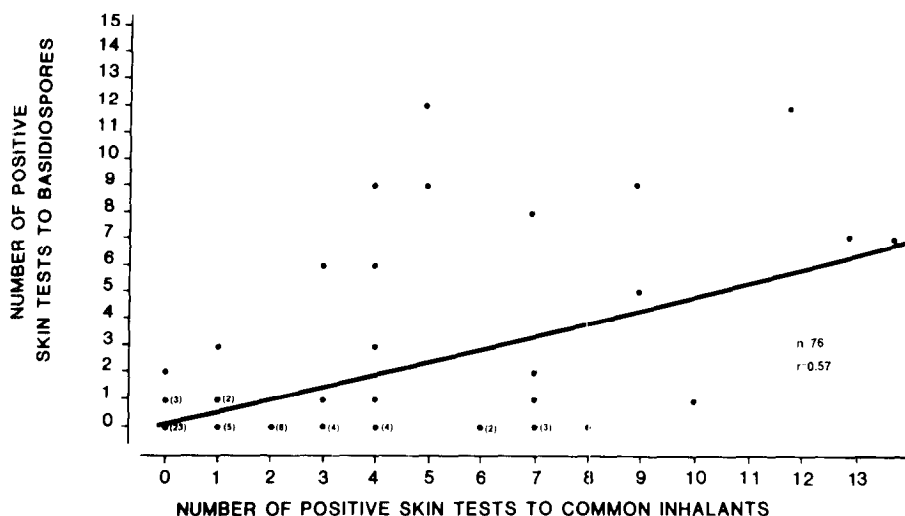


FIG. 2. Relationship of skin prick test reactivity to basidiospores with reactivity to common inhalant allergens.

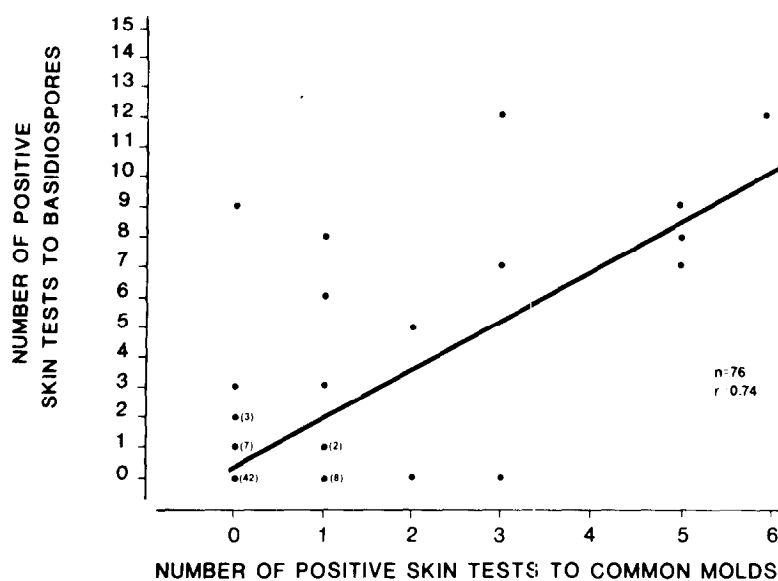


FIG. 3. Relationship of skin prick test reactivity to basidiospores with reactivity to commercial extracts of the Fungi Imperfecti.

species of Basidiomycetes including fleshy gilled mushrooms (*Coprinus quadrididus*, *P. cubensis*, *Agrocybe amara*, *A. tabescens*, *P. ostreatus*, *A. muscaria*, *C. molybdites*, *C. cibarius*, *Boletinus merulioides*, and *Boletus* sp.), polypores (*G. lucidum* and *Inonotus ludovicianus*), and puffballs (*Scleroderma* sp., *Calvatia cyathiformis*, and *Pisolithus tinctorius*). The prevalence of skin prick test reactivity ranged from a low of 5% for *C. cibarius* to a high of 17% for *Scleroderma* sp. The major basidiospore extracts inducing $\geq 10\%$ skin test reactivity include extracts of spores from *G. lucidum*, *I. ludovicianus*, *P. cubensis*,

C. quadrididus, and *Scleroderma* sp. The prevalence of skin test reactivity to basidiospores was similar to or exceeded the reactivity to commercial mold extracts of several common species of the Fungi Imperfecti.

The prevalence of individuals having positive skin test reactivity to at least one of the Basidiomycete mycelia or basidiospore extracts is remarkably similar, ranging from 27% with extracts of mycelia to 32% with spore extracts. Thus, almost one third of our subjects had positive skin reactions to one or more of the basidiospore extracts. Twenty-four subjects had skin reactions to at least one or more basidiospores (Table

IV). Nine subjects reacted to only one basidiospore, whereas four reacted to two or three basidiospores. Of the 11 subjects reacting to five or more basidiospores, two reacted to 12/15 basidiospore extracts.

Figs. 2 and 3 illustrate the relationship between skin test reactivity to basidiospores and skin test reactivity to common inhalants or to commercial extracts of the Fungi Imperfecti. A number of individuals did not react to basidiospores, although they reacted to a number of the common inhalants, indicating that not all atopic individuals react to basidiospores. In subjects reacting to both, there appears to be a relationship between skin test reactivity to common inhalants and to basidiospores. By linear regression analysis of these data, a correlation coefficient of 0.57 was calculated, which was significant at p value of <0.05 . When skin test reactivity of study subjects to commercial mold extracts of the Fungi Imperfecti was compared to that for basidiospores and analyzed by linear regression, a greater correlation coefficient ($r = 0.74$) was demonstrated, which was also significant at p value of <0.05 .

Fig. 4 summarizes skin test reactivity to basidiospores of study subjects, separated according to their respiratory symptoms, into four groups: the control group reporting no respiratory symptoms (none), study subjects reporting rhinitis only, those reporting asthma only, and those with rhinitis associated with asthma. Individuals were classified as positive, if they reacted to one or more basidiospores, and negative, if they reacted to none of the basidiospores. The results indicate that none of the 14 control subjects (non-atopics without respiratory symptoms) had skin test reactivity to basidiospores. Of these study subjects, individuals reporting rhinitis, 5/26 (19.2%) had positive skin reactivity to basidiospores. A higher percentage of individuals with asthma, or rhinitis associated with asthma, had positive skin tests to basidiospores (34.6% and 41.6%, respectively).

DISCUSSION

Although atmospheric sampling and preliminary data suggest that fungal spores may be significant aeroallergens, until now there have not been adequate studies of allergic individuals' reactions to fungal allergen extracts obtained from pure spores. Since extracts of pure spores from Basidiomycetes are now available, we can determine patient reactivity to fungal spores. The results reported here indicate that basidiospores are important fungal aeroallergens in the New Orleans environment. Indeed, the 17% skin test reactivity of subjects to *Scleroderma* sp. exceeded the 13% reactivity obtained with a commercial extract of *A. tenuis*, a species of the Fungi Imperfecti considered

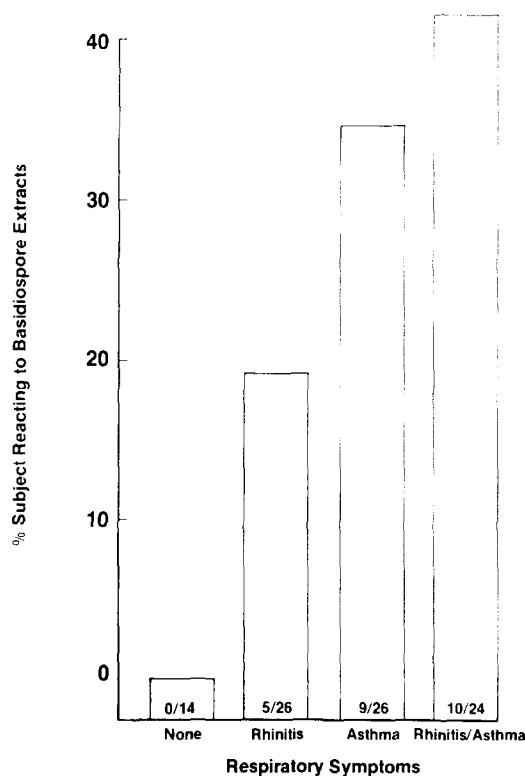


FIG. 4. Skin prick test reactivity to basidiospores of subjects categorized by respiratory symptoms.

to be a major fungal aeroallergen throughout the country. In fact, the prevalence of skin test reactivity to *Scleroderma* sp. was similar to that obtained with pollen of live oak (18%), a major pollen allergen in New Orleans.

Skin test reactivity to spore extracts from individual Basidiomycete sp. was found to vary considerably, from a low of 5% for *C. cibarius* to a high of 17% for *Scleroderma* sp. Variations in skin test reactivity to basidiospores may be due to one of several reasons. First, these variations may reflect differences in basidiospore atmospheric concentrations. Some Basidiomycetes, such as the puffball, produce a large number of spores and thus may contribute more to the spore load than other Basidiomycetes. However, until we have developed methods for identifying individual spore species in the atmosphere, we cannot accurately determine this. Second, since the growing seasons of each Basidiomycete can vary widely, differences in patient reactivity may reflect the time the test is performed in relationship to growing patterns of the Basidiomycetes whose spores are tested. Finally, it is possible that basidiospores may vary in their allergenic potency. Preliminary data have demonstrated that rabbits respond with significant differences when they are immunized with seven different basidiospore prep-

arations, suggesting some basidiospore extracts may be more immunogenic than others.*

The prevalence of skin test reactivity of our patients to basidiospore extracts is less than that reported by Giannini et al.⁹ or Santilli et al.^{11,13} This is particularly perplexing since studies by Giannini et al. were performed in Arizona, where the desert climate is much drier and possibly less conducive to Basidiomycete growth than ours. Since both studies tested spore extracts from *C. molybdites*, this discrepancy is not due to testing different basidiospores. Probably, these differences are due to methods used in testing and measuring skin reactivity, since Giannini et al.⁹ and Santilli et al.^{11,13} tested extracts by both skin and intradermal methods, and in some cases, measured flare reactions, whereas we used only the prick test and only recorded edema (wheal formation) ≥ 2 mm as positive.

A major question, concerning individuals who react to basidiospores, is the relationship between their reactions to spores and their respiratory symptoms. Initial studies suggest that skin test reactors are primarily atopic subjects, as would be expected from earlier studies of other aeroallergens.¹⁶ Our results demonstrate that individuals with respiratory symptoms are more likely to have skin test reactivity to basidiospores, demonstrating that these basidiospores are very important fungal aeroallergens in New Orleans. Furthermore, our results suggest that subjects with asthma alone, or rhinitis associated with asthma, appear to have a higher prevalence of skin test reactivity to basidiospores than subjects with rhinitis alone. This suggests that basidiospores may be important aeroallergens in asthma. Our results support the earlier findings of Santilli et al.^{11,13} who demonstrated that patients with bronchial asthma have a higher incidence of immediate skin test reactivity to basidiospores as compared to patients with rhinitis.

Although our results strongly suggest that basidiospores are important fungal aeroallergens in the New Orleans environment, further investigations are necessary. We must confirm spore hypersensitivity by provocative inhalation challenge and assess patterns of bronchial reactivity to conclusively establish the fact of basidiospore-induced respiratory disease.¹⁷ The role of other immunoglobulin isotypes, such as IgA or IgG antibody, as well as relevant cell-mediated reactions and the role of mediators in basidiospore hypersensitivity, must also be determined. What is the nature and identity of allergens present in spores of Basidiomycetes? This question is particularly impor-

tant, since our preliminary studies indicate that RAST reactivity from sera of skin test positive patients is variable. Because preliminary fractionation of some crude spore extracts can enhance RAST reactivity, we need to fractionate and characterize these allergens in order to develop adequate RAST reagents for detecting antibodies in patients' sera.

In view of the studies needed, and the many difficulties in investigation of mold allergy, the necessity to investigate spores from Basidiomycetes as aeroallergens may be questioned. Currently, the amounts of basidiospores available may not always be sufficient to provide reagents for immunotherapy in the near future. The fact that hundreds of Basidiomycete sp. contribute to the airborne spore load further complicates attempts to identify basidiospores to which patients react. Nevertheless, we believe that it is essential to evaluate and study spores as aeroallergens. The Basidiomycetes are important sources of aeroallergens not only because they produce a significant percentage of airborne spores but also because study subjects are exposed only to their spores, rather than spores and mycelial fragments, as with most species of the Fungi Imperfecti. Thus, we believe that through further analysis of hypersensitivity to basidiospores and identification of spore allergens, well-defined diagnostic and therapeutic reagents for treatment of patients with mold allergy will be developed.

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