

Basidiospore Extracts: Evidence for Common Antigenic/Allergenic Determinants

Carol E. O'Neil, Janet M. Hughes, Brian T. Butcher, John E. Salvaggio, Samuel B. Lehrer

Department of Medicine, Tulane Medical Center, New Orleans, La., USA

Abstract. Spore extracts, prepared from *Armillariella tabescens*, *Pleurotus ostreatus*, *Coprinus quadrifidus*, *Amanita muscaria*, *Ganoderma lucidum*, *Psilocybe cubensis*, *Pisolithus tinctorius*, *Scleroderma* sp. and *Calvatia cyathiformis*, were examined for antigenic/allergenic relationships by Ouchterlony and radioallergosorbent testing (RAST) inhibition, respectively. Ouchterlony, using hyperimmunized rabbit sera, demonstrated a high degree of cross-antigenicity among the extracts tested; however, some unique antigens were also present. RAST inhibition, evaluated by comparing extract concentrations which inhibited the RAST by 50% (IC-50), varied with the allergen tested. *P. cubensis* was the most potent inhibitor (IC-50 ranged from 0.034 mg/ml for *A. tabescens* RAST to 0.29 mg/ml for *G. lucidum* RAST). *P. tinctorius* was the least potent inhibitor, failing to reach IC-50 at 10 mg/ml for any basidiospore extract. Evaluation of slopes and intercepts of the dose-response lines demonstrated qualitative and quantitative differences among allergens in these extracts. These results indicate the presence of shared allergenic epitopes, and suggest that representative extract panels could be developed for future use in diagnosis and treatment of basidiospore-sensitive individuals.

Introduction

Basidiomycetes, a major class of fungi consisting of over 25,000 different species, include mushrooms, puffballs, stinkcorns, rusts, smuts, and shelf or bracket fungi [1]. The high atmospheric spore load, up to 29% of the fungal aerospore catch, suggests that basidiomycetes could be important aeroallergens [2-4]. In New Orleans, a correlation between high basidiospore catches and increased incidence of emergency room admissions for asthma suggests a cause and effect relationship [5]. Recently, the importance of basidiospores as allergens has been suggested [6-11]. The prevalence of skin reactivity in atopic individuals ranged from a low of 5% for *Cantharellus cibarius* to a high of 17% for *Scleroderma* sp. [6]. Radioallergosorbent testing (RAST) of sera from these individuals, using selected spore extracts, confirmed the presence of specific IgE antibodies [10]. In general, positive RAST and skin tests, with selected basidiospore extracts, demonstrated a statistically significant association [10].

In individuals with skin and RAST reactivity to

basidiospore extracts, a broad pattern of positive responses is frequently observed, suggesting shared allergenic epitopes among the species [1, 10, 11]. Collection of spores is laborious and time-consuming, and the number of basidiomycete species is extremely large. Thus, it would be advantageous to perform skin-prick testing or immunoassays, using a panel of extracts prepared from representative basidiospores, for diagnosis and, ultimately, for treatment of basidiomycete-induced allergy. The purpose of this study was to examine antigenic/allergenic relationships among spores from selected species of basidiomycetes using Ouchterlony and RAST inhibition.

Materials and Methods

Basidiospore Extract Preparation

Spore collection and extract preparation have been described previously [6]. Briefly, caps from *Armillariella tabescens*, *Pleurotus ostreatus*, *Coprinus quadrifidus*, *Amanita muscaria* or *Ganoderma lucidum* were placed gills down on Whatman No. 1 filter paper sheets and covered to obtain spore prints; spores were then scraped from the paper. Spore prints from *Psilocybe cubensis* were pur-

chased (Florida Mycology Institute, Pensacola, Fla.). Puffballs (*Pisolithus tinctorius*, *Scleroderma* sp. and *Calvatia cyathiformis*) were harvested at maturity, dried at room temperature, and cut in half; the spore mass was removed using a spatula. Purity of samples from mushrooms was greater than 98% and was at least 80–90% for puffballs, with the major contaminant being sterile hyphae.

To prepare extracts, 1 g of spores was homogenized in a Braun homogenizer in 20 ml ammonium bicarbonate buffer (0.125 M, pH 8.1). The homogenate was centrifuged (48,000 g); the supernatant was lyophilized and stored at room temperature under desiccation.

Rabbit Antisera

Antisera against *A. tabescens*, *C. cyathiformis*, *G. lucidum* and *P. ostreatus* were raised using the method developed in our laboratory [12]. For each basidiomycete species, three New Zealand white rabbits were immunized against crude spore homogenate and three were immunized with spore extract. 10 mg/ml of the appropriate antigen, emulsified with complete Freund's adjuvant (1:1), were injected into multiple sites on the back and toepads. Subsequently, rabbits were injected 5 times, at bimonthly intervals, in the back only. Rabbit sera were pooled, concentrated approximately 10 times using ammonium sulfate precipitation (40% saturation), dialyzed exhaustively against phosphate-buffered saline (PBS), and ultrafiltered (Amicon XM50, molecular weight cut off 50,000 daltons).

Ouchterlony [13] was used to screen rabbit sera for the presence of antibodies and to evaluate extracts for the presence of shared antigenic epitopes. Briefly, varying amounts of rabbit antisera were placed into the center well and a range of concentrations of basidiospore extracts was placed into surrounding wells. Following a 48-hour incubation at room temperature, plates were washed extensively with 0.17 M sodium citrate buffer, and examined for precipitin lines.

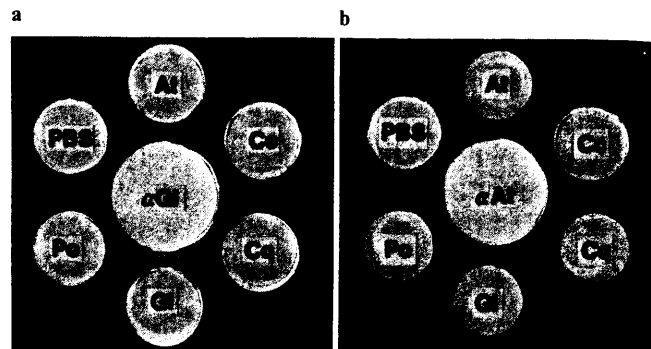


Fig. 1. Representative Ouchterlony plates. **a** Clockwise from top: *A. tabescens* (1,000 µg), *C. cyathiformis* (500 µg), *C. quadrididus* (1,000 µg), *G. lucidum* (500 µg), *P. ostreatus* (500 µg) and PBS; center well: anti-*G. lucidum* (150 µl). **b** Clockwise from top: *A. tabescens* (600 µg), *C. cyathiformis* (800 µg), *C. quadrididus* (600 µg), *G. lucidum* (400 µg), *P. ostreatus* (300 µg) and PBS; center well: anti-*A. tabescens* (150 µl).

RAST Inhibition

Two pools were prepared from equal volumes of five serum samples. Individual samples used in these pools were selected on the basis of high RAST values (% radiolabel bound) obtained from our previous study [10]. Pool I, with average direct RAST values shown in parentheses, was used for testing discs coupled with *A. tabescens* (5.77), *C. quadrididus* (7.65), *P. ostreatus* (7.83) and *C. cyathiformis* (12.81). Pool II was used in testing discs coupled with *G. lucidum* (5.56).

RAST inhibition was performed using the method currently in use in our laboratories [14]. Briefly, *A. tabescens*, *C. quadrididus*, *P. ostreatus*, *C. cyathiformis* or *G. lucidum* (10 mg/ml in borate buf-

Table I. Number of double gel diffusion precipitin arcs obtained with basidiospore extracts and rabbit antisera

Extract	Antisera			
	<i>A. tabescens</i>	<i>P. ostreatus</i>	<i>G. lucidum</i>	<i>C. cyathiformis</i>
<i>G. lucidum</i> ¹	2	1	3	1
<i>C. cyathiformis</i> ¹	1	1	2	1
<i>A. tabescens</i> ¹	3	1	1	0
<i>P. ostreatus</i> ¹	1	3	1	1
<i>P. cubensis</i> ²	0	1	0	NT
<i>C. quadrididus</i> ²	1	2	1	NT
<i>A. muscaria</i> ²	1	1	0	NT
<i>P. tinctorius</i> ²	1	0	0	NT
<i>Scleroderma</i> sp. ²	1	1	1	NT

NT = Not tested.

¹ 500 µg basidiospore extract tested against 50 µl rabbit antisera.

² 400 µg basidiospore extract tested against 45 µl rabbit antisera.

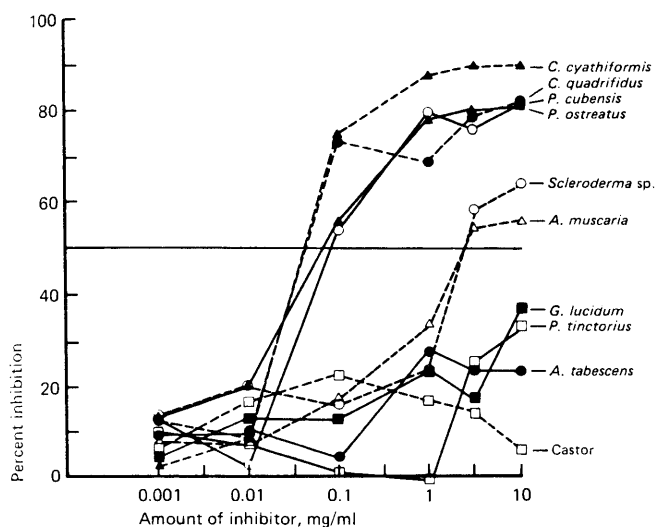


Fig. 2. Inhibition curves for *C. cyathiformis* RAST with basidiospore extracts.

fer, pH 8.0) were coupled to cyanogen-bromide-activated filter paper discs [15]. For the test, 50 μ l of the appropriate serum pool and 50 μ l of increasing concentrations (0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/ml) of the inhibiting antigen, heated castor bean extract (negative control) [16], or PBS were added simultaneously to a basidiospore-extract-coupled disc. RAST inhibition assays were performed in duplicate and were highly reproducible. Statistical evaluation of 150 consecutive pairs of test counts showed that in only 6/150 (4%) did the second count deviate from the first by 20% or more.

For evaluation of results, concentrations of extracts inhibiting RAST by 50% (IC-50) were calculated. Rank order of potency was determined by averaging IC-50 values; if 50% inhibition was not obtained, 10 mg/ml was used in these calculations. In addition, for each extract, the percent inhibition was fitted to the logarithm of concentration using least-squares regression lines; slopes and intercepts were calculated and compared using multiple regression methods [17].

Results

Ouchterlony

Representative Ouchterlony plates, using rabbit anti-*G. lucidum* or anti-*A. tabescens*, are presented in figure 1. Strong precipitin arcs are present in both homologous antigen/antibody systems tested. In fact, all homologous systems demonstrated these arcs. Most of the heterologous antigen/antibody systems also elicited precipitin arcs, suggesting the presence of common antigenic determinants (table I). Normal rabbit sera gave no precipitin arcs (data not shown), suggesting that antibodies were basidiospore-specific.

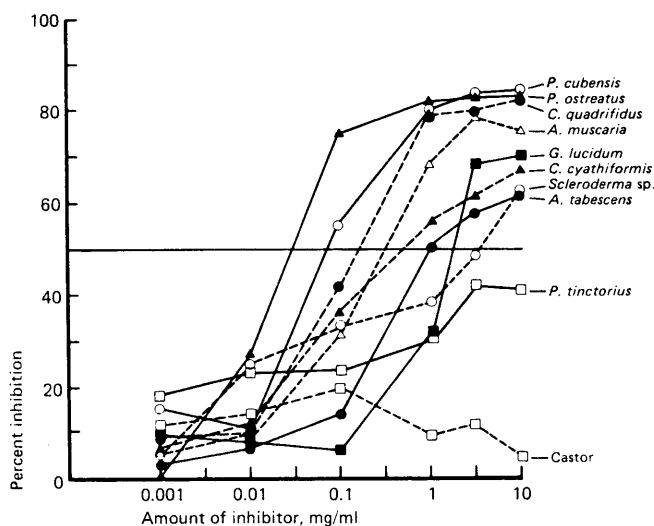


Fig. 3. Inhibition curves for *P. ostreatus* RAST with basidiospore extracts.

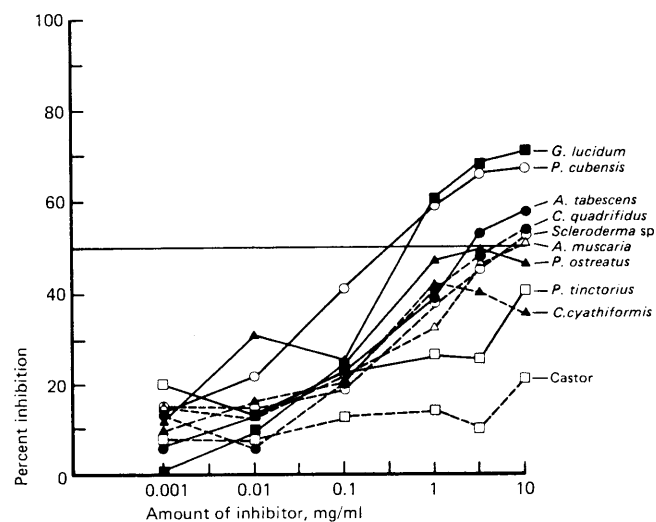


Fig. 4. Inhibition curves for *G. lucidum* RAST with basidiospore extracts.

RAST Inhibition Analysis

RAST inhibition dose-response curves are presented in figures 2-6. Ability of various extracts to inhibit direct RAST varied with the extract tested. IC-50 values are presented in table II and rank order of potency is shown in table III. On the average, *P. cubensis* was the most potent inhibitor; IC-50 values ranged from 0.034 mg/ml for *A. tabescens* RAST to 0.29 mg/ml for *G. lucidum* RAST. The next most potent inhibitor was *P. ostreatus*. *C. quadrifidus*, *C. cy-*

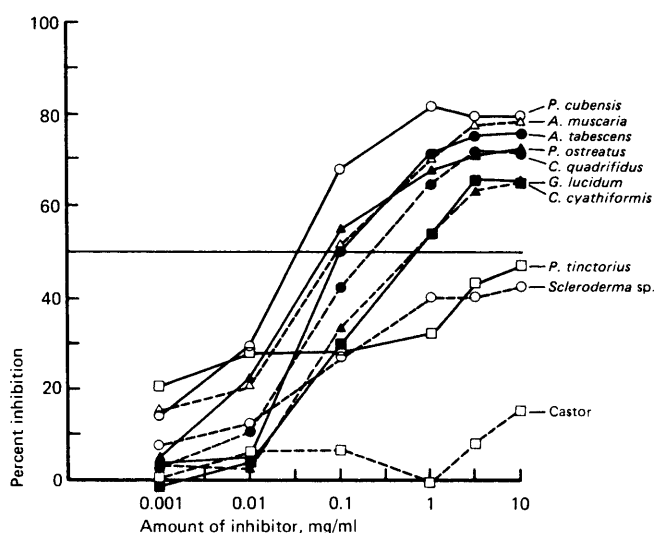


Fig. 5. Inhibition curves for *A. tabescens* RAST with basidiospore extracts.

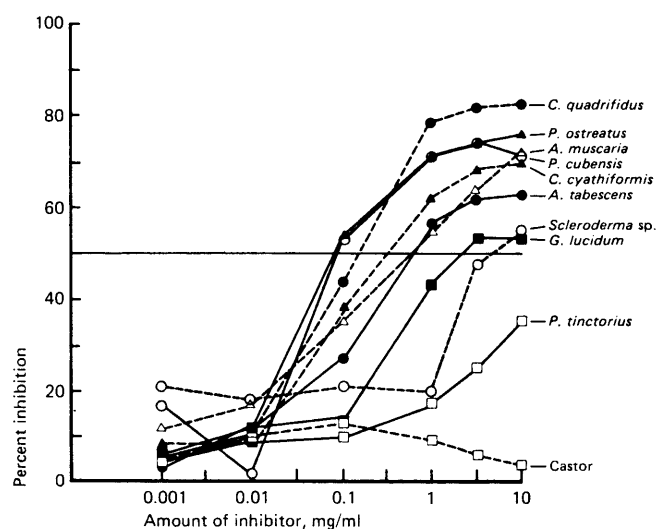


Fig. 6. Inhibition curves for *C. quadrididus* RAST with basidiospore extracts.

Table II. Concentrations (mg/ml) of basidiospore extracts inhibiting RAST by 50%

Inhibiting antigen	Discs coupled with				
	<i>A. tabescens</i>	<i>C. quadrididus</i>	<i>P. ostreatus</i>	<i>C. cyathiformis</i>	<i>G. lucidum</i>
<i>A. tabescens</i>	0.10	0.61	1.00	—	3.30
<i>C. quadrididus</i>	0.34	0.15	0.17	0.42	6.20
<i>P. ostreatus</i>	0.075	0.083	0.095	0.07	1.35
<i>C. cyathiformis</i>	0.60	0.31	0.53	0.043	—
<i>G. lucidum</i>	0.65	3.20	2.35	—	0.50
<i>Scleroderma</i> sp.	—	6.70	5.40	3.40	7.30
<i>A. muscaria</i>	0.087	0.57	0.32	3.55	8.10
<i>P. tinctorius</i>	—	—	—	—	—
<i>P. cubensis</i>	0.034	0.088	0.078	0.081	0.29

— = 50% inhibition was not achieved using a maximal concentration of 10 mg/ml.

Table III. Rank order of potency in RAST inhibition analysis

Rank	Species
1	<i>P. cubensis</i>
2	<i>P. ostreatus</i>
3	<i>C. quadrididus</i>
4	<i>C. cyathiformis</i>
5	<i>A. muscaria</i>
6	<i>A. tabescens</i>
7	<i>G. lucidum</i>
8	<i>Scleroderma</i> sp.
9	<i>P. tinctorius</i>

athiformis, *A. muscaria*, *A. tabescens*, *G. lucidum*, *Scleroderma* sp. and *P. tinctorius*, respectively, had less inhibitory activity. *P. tinctorius*, the least potent inhibitor, did not reach 50% inhibition for any extract tested. Use of the negative control (castor) helped to confirm specificity of the RAST inhibition.

Evaluation of slopes and intercepts of the dose-response curves (regression lines are not shown) demonstrated that some slopes were coincident and some slopes showed significant differences ($p < 0.05$), suggesting both quantitative and qualitative differences in allergens present in these basidiomycete species.

Discussion

Basidiomycetes have long been suspected of inducing respiratory allergy in exposed individuals; however, definitive studies are lacking, primarily because of failure to identify appropriate allergens. Preparations of basidiomycete caps, spores and mycelia have been used for in vivo and in vitro testing, with variable success. Spores probably contain the most relevant allergen(s), since individuals are exposed primarily to spores. Recently, pure spore extracts have been used in studies of basidiomycete-induced allergy and they have been demonstrated to be suitable allergens [2, 6-11, 18, 19]. Results from skin-prick tests and RAST with spore extracts, in sensitive individuals, suggested the presence of shared allergenic determinants among species of basidiomycetes.

Our Ouchterlony results confirm and extend the initial work of Lopez et al. [20]. Using cultured mycelial and somatic preparations and hyperimmune rabbit sera, they demonstrated that both unique and common antigenic determinants were present in selected basidiomycete species. Although that study may not relate directly to the present one, since basidiomycetes grown in vitro do not sporulate, it did suggest cross-reactivity among separate species.

RAST inhibition demonstrated that, although several of the basidiospore extracts did contain shared allergenic epitopes, there was marked variation among species tested. Using results from the RAST inhibition assay as an indicator of potency, the 9 spore extracts appeared to cluster into 3 groups: those with high, intermediate or low inhibitory activity. Dissimilarities among reactivities of individual extracts may result from either quantitative or qualitative differences in allergenic determinants. Analysis of slopes and intercepts from inhibition experiments suggests that either and/or both could be the case.

RAST inhibition and Ouchterlony results cannot be compared directly, since, in this study, these tests evaluate allergenic (human IgE) and antigenic (rabbit IgG) determinants, respectively. Some parallels, however, can be drawn. For example, hyperimmune *A. tabescens* sera reacted strongly with selected spore extracts (fig. 1), suggesting the presence of shared antigenic determinants. These extracts were also effective inhibitors of *A. tabescens* RAST. *G. lucidum* antiserum reacted less strongly in the Ouchterlony test and extracts tested were less effective in inhibiting *G. lucidum* RAST. *P. ostreatus*, on the other hand, gave a

poor precipitin arc when tested with *A. tabescens* antiserum, but was an effective inhibitor. This illustrates the drawbacks of working with rabbit sera.

RAST inhibition, particularly using serum pools, to evaluate the presence of shared allergenic epitopes has recently been criticized. Sera from individuals with high levels of specific IgE directed against one or two components may influence the overall test results. Equally, samples with lower levels of IgE can also affect the test. Use of different serum pools may have accounted for the greater inhibitory effect in our results, as compared with those of Weissman et al. [12].

RAST inhibition does, however, serve as an excellent starting point when evaluating novel allergens or when screening large numbers of previously unstudied materials, such as the basidiospores. Now that we have identified an appropriate patient population, prepared effective basidiospore extracts, established the RAST and RAST inhibition assays for these extracts, and demonstrated that they are immunogenic in rabbits, other tests, including crossed radioimmunoelectrophoresis and Western blot, and immunoprinting can be performed.

The demonstration that basidiospore extracts contain shared antigenic/allergenic epitopes has important economic, clinical and diagnostic implications. Following isolation and characterization of allergens shared by several species of basidiomycetes, panels of test materials could be used for diagnosis and, ultimately, for treatment.

Acknowledgements

These studies were supported by NIH-NIAID grant AI 20331; CEO'N is the recipient of NIH-NHLBI New Investigator award HL 32621. The authors also acknowledge the excellent technical assistance of Margaret A. Reed and Marjorie L. McCants.

References

- 1 Lehrer, S.B.; Aukrust, L.; Salvaggio, J.E.: Respiratory allergy induced by fungi. *Clin. Chest Med.* 4: 23-41 (1983).
- 2 Herxheimer, H.; Hyde, H.A.; Williams, D.A.: Allergic asthma caused by basidiospores. *Lancet* ii: 131-133 (1969).
- 3 Gregory, P.; Hirst, J.: The summer air-spores at Rothamsted in 1952. *J. gen. Microbiol.* 17: 135-152 (1957).
- 4 Hyde, H.A.; Adams, K.F.: Airborne allergens at Cardiff 1942-1959. *Acta allerg* 15: suppl. VII, p. 159 (1960).
- 5 Salvaggio, J.E.; Seabury, J.; Schoenhardt, E.: New Orleans asthma. V. Relationship between Charity Hospital asthma ad-

- mission rates, semiquantitative pollen, fungal spore counts, and total particulate aerometric sampling data. *J. Allergy clin. Immunol.* 48: 96-114 (1971).
- 6 Lehrer, S.B.; Lopez, M.; Butcher, B.T.; Olson, J.A.; Reed, M.A.; Salvaggio, J.E.: Basidiospore reactivity in adults with symptoms of respiratory allergy. *J. Allergy clin. Immunol.* 78: 478-485 (1986).
 - 7 Lopez, M.; Butcher, B.T.; Salvaggio, J.E.; Olson, J.A.; Wright, M.A.; McCants, M.L.; Lehrer, S.B.: Basidiomycete allergy: what is the best source of antigen? *Int. Archs Allergy appl. Immun.* 77: 169-170 (1985).
 - 8 Sprenger, J.D.; Altman, L.C.; O'Neil, C.E.; Lehrer, S.B.; Ayars, G.H.: Skin test reactivity to basidiospores in adults in Seattle with respiratory allergy. *J. Allergy clin. Immunol.* 77: 200 (1986).
 - 9 Giannini, E.H.; Northey, W.T.; Leathers, C.R.: The allergenic significance of certain fungi rarely reported as allergens. *Ann. Allergy* 35: 372-376 (1975).
 - 10 Butcher, B.T.; O'Neil, C.E.; Reed, M.A.; Altman, L.C.; Lopez, M.; Lehrer, S.B.: Basidiomycete allergy: measurement of spore specific IgE antibodies. *J. Allergy clin. Immunol.* (in press).
 - 11 Santilli, J., Jr.; Rockwell, W.J.; Collins, R.: The significance of the spores of the basidiomycetes (mushrooms and their allies) in bronchial asthma and allergic rhinitis. *Ann. Allergy* 55: 469-471 (1985).
 - 12 Weissman, D.N.; Halmepuro, L.; Salvaggio, J.E.; Lehrer, S.B.: Antigenic/allergenic analysis of basidiomycete cap, mycelium, and spore extracts. *Int. Archs Allergy. appl. Immun.* 84: 56-61 (1987).
 - 13 Rose, N.R.; Friedman, H.: *Manual of clinical immunology*; 2nd ed., p. 1105 (Am. Soc. Microbiol., Washington 1980).
 - 14 O'Neil, C.E.; Reed, M.A.; Hughes, J.M.; Butcher, B.T.; Lehrer, S.B.: *Fusarium solani*: evidence for common antigenic/allergenic determinants with other fungi imperfecti. *Clin. Allergy* 17: 127-133 (1987).
 - 15 Ceska, M.; Lundkvist, U.: A new and simple radioimmunoassay for the determination of IgE. *Immunochemistry* 9: 1021-1030 (1972).
 - 16 Lehrer, S.B.; Taylor, J.; Salvaggio, J.E.: Castor bean allergens: evidence for distinct heat-labile and stable entities. *Int. Archs Allergy. appl. Immun.* 65: 69-75 (1981).
 - 17 Kleinbaum, D.G.; Kupper, L.L.: *Applied regression analysis and other multivariable methods* (Duxbury Press, North Scituate 1978).
 - 18 Tarlo, S.M.; Bell, B.; Srinivasan, J.; Dolovich, J.; Hargreave, F.E.: Human sensitization to *Ganoderma* antigen. *J. Allergy clin. Immunol.* 64: 43-49 (1979).
 - 19 Hasnain, S.M.; Wilson, J.D.; Newhook, F.J.; Segedin, B.P.: Fungi and disease. Allergy to basidiospores: immunologic studies. *N.Z. med. J.* 98: 393-396 (1985).
 - 20 Lopez, M.; Salvaggio, J.; Butcher, B.: Allergenicity and immunogenicity of basidiomycetes. *J. Allergy clin. Immunol.* 57: 480-488 (1976).

Received: May 13, 1987

Correspondence to: Dr. Carol E. O'Neil
 Section of Clinical Immunology
 Department of Medicine
 1700 Perdido Street
 New Orleans, LA 70112 (USA)