

*Basidiomycete allergens: comparison of three *Ganoderma* species

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High atmospheric concentrations of basidiospores occur in various parts of the world. *Ganoderma* basidiospores are distinctive, easily identifiable in aeroallergen surveys, and widely abundant. Previous studies showed that *Ganoderma* basidiospores cause respiratory allergies. Thus, we investigated various extracts (spore, cap, and/or mycelial) of *G. meredithae*, *G. lucidum*, and *G. applanatum* for allergen components. Analyses included radioallergosorbent test (RAST) inhibition and IgE blots from isoelectric focusing (IEF) and SDS–PAGE. RAST inhibition with spores and caps of *G. meredithae* and *G. lucidum* showed that spores inhibited caps better than caps inhibited spores. Species differences were minor. Coomassie blue (CB) staining of IEF gels detected at least 23 protein bands (pI 3.6–6.6) in caps of *G. meredithae* and *G. lucidum*. *G. meredithae* spore extracts contained 17 of these (pI 3.6–5.0, 6.6). Spores and caps of *G. meredithae* contained 13 and 11 allergen bands, respectively, on IEF blots. SDS–PAGE of *G. meredithae* spore and cap showed one and four bands, respectively, by CB staining, but IgE blots showed 13 bands in cap and 17 in spore. Culture mycelia of *G. lucidum* and *G. applanatum* attained significant and essentially constant RAST activity by day 4. Activity was also present in culture supernatant by day 4. Blots of mycelium and supernatant detected a single allergen in day-8 mycelia and subsequently six allergen bands in day-16 mycelia and eight in day-16 supernatant (one appeared as a doublet). These data show that *Ganoderma* extracts contain a complex mixture of allergens. Differences among species were minor; spores and mycelia are apparently better sources of allergens than caps.

Key words: fungal allergy; mold hypersensitivity.

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Ganoderma species occur commonly worldwide. They grow primarily on senescent or dead trees and are present in most tree habitats, including forests, agricultural windbreaks, parks, and landscaped urban areas.

Spores of *Ganoderma* are sufficiently distinctive to allow reliable identification solely by spore morphology. This allows aerobiologic researchers to segregate *Ganoderma* spores when counting and reporting airborne spores (4, 5, 7, 11, 12, 22, 26). Thus, the distribution and concentration of *Ganoderma* are far better understood than those of most other fungi. Such studies have shown that *Ganoderma* spores are prominent in the air sporas of inner cities (London and Nottingham, UK) and rural areas (Rothamsted, Harpenden, UK) in Europe, Canada (Hamilton and Montreal), New Zealand (Auckland), and the United States (Tulsa, OK) and even in indoor air (5).

Several laboratories have shown, by skin test and/or radioallergosorbent test (RAST), that *Ganoderma* spore extracts are allergenic, 6–43% of subjects reacting (2, 3, 6, 13, 15, 16, 20, 21, 24, 27, 28). *G. lucidum* also induced a reversible, greater than 20% reduction in FEV₁ in a bronchial challenge of a skin test positive asthmatic (23). It is thus well established that *Ganoderma* spores are allergenic and occur in significant numbers in many areas of the world.

The literature on fungal allergy includes information on several *Ganoderma* species, but, to our knowledge, only one report compared species (13) and only one compared source materials (6). Furthermore, there is but a single report on characterization of *Ganoderma* allergens (29). Thus, we sought to characterize further the allergens in *Ganoderma* extracts and to provide a direct comparison of the allergenic activity in different species and source materials.

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Material and methods

Sera

We selected volunteers who had symptoms of respiratory allergy and two or more positive skin tests to common inhalant allergens (atopic). Sera were obtained from subjects who were skin test positive to *G. lucidum* spore extracts. Control sera were obtained from persons who were skin test negative to *Ganoderma*.

Antigen preparation

Spores. Spores were gently brushed from deposits on the fruiting body tops. *G. lucidum* was collected in the New Orleans area. *G. meredithae* was collected from pine stumps at the Stuart seed orchard (United States Department of Agriculture Forest Service) near Bentley, LA.

Caps. The entire pileus (cap), with residual spore load, was cut into approximately 1-cm cubes and pulverized dry in a Waring blender. Pulverized cap was stored under desiccation until used.

In vitro media. Mycelial growth was produced in 2% malt (M) broth (Difco) or ultrafiltered malt broth (UM) which was the filtrate that had passed through an AMICON (Danvers, MA, USA) YM-5 filter. This filtration removed material greater than 5 kDa in order to reduce malt antigen content. Malt broth (M) was selected for routine culture since the fungi grew poorly on Czapek-Dox (CZ-D) broth. Furthermore, RAST results (percent bound) of M, UM, and CZ-D media were similarly minimal (3.7%, 3.2%, and 3.3%, respectively), with a pool of sera from skin test positive subjects.

Mycelium. Cultures of *G. applanatum* (ATCC #64796) and *G. lucidum* (ATCC #64489) were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained on 2% malt agar (MEA) slants. Inoculum was produced by transferring the fungi to MEA petri dishes at room temperature for 4–7 d. Single agar disks (5 mm) were cut from an actively growing colony margin and aseptically transferred to 100 ml malt broth in a 250-ml Erlenmeyer flask. These flasks were placed in a rotary incubator (125 rpm) at room temperature. After approximately 1 week, the resulting spherical colony was dispersed by homogenizing the entire contents of the flask with a 10-s burst in a sterile Waring blender. One milliliter of this mycelial suspension was aseptically transferred to flasks containing 100 ml of fresh, sterile M.

Allergen extraction

Source materials were disrupted in a Braun (Melsungen, Germany) homogenizer with glass beads and 0.125 mol NH_4HCO_3 for 2 min while cooled with escaping liquid CO_2 . The homogenate was centrifuged at 27 000 g for 30 min. The supernatant was recovered, lyophilized, and stored under desiccation until used.

RAST/RAST inhibition

RAST was performed as previously described (19). Briefly, cyanogen bromide (CNBr)-activated paper disks were coated with 1 mg (dry wt) extract per disk, incubated in 100 μl serum, and then labeled with approximately 15 000 cpm ^{125}I -anti-IgE. Bound activity was then determined in a Beckman model 5500 gamma counter. Disks were washed in 0.9% saline three times between incubations. All tests were conducted in duplicate. RAST values (percent bound) were expressed as a percentage of the total activity added that remained bound to the disks.

RAST inhibition was conducted with 50 μl of pooled sera and 50 μl of inhibitor. Inhibitors were diluted serially in phosphate-buffered saline (PBS). Percent inhibition was calculated relatively to a PBS control ($1 - [\text{test \% bound}/\text{control \% bound}] \times 100$).

Electrophoresis

Isoelectric focusing (IEF) was performed in thin-layer horizontal gels (PAGplates, Pharmacia, Piscataway, NJ, USA) with a pH range of 3.6–6.6 according to manufacturer's recommendations. Protein standards (Sigma, St Louis, MO, USA) were included to monitor the pH gradient. Protein samples were dissolved in 1% (w/v) glycine and approximately 1000 μg (dry wt) was loaded per lane.

SDS-PAGE was conducted under reducing conditions in Laemmli vertical slab gels (10% T/2.7% C) with approximately 1000 μg (dry wt) loaded per lane.

Immunoprinting

Passive blots were prepared from the IEF gels onto cyanogen bromide-activated (8), 0.2- μm pore size nitrocellulose, as described previously (17). Resolved proteins in SDS-PAGE gels were transferred electrophoretically to 0.2- μm pore size, CNBr-activated nitrocellulose in a transblot cell (Bio-Rad, Richmond, CA, USA) for 1 h at 200 mA constant current in tris-glycine buffer with 20% methanol.

Stock quenching buffer (containing 5% BSA and 2% Tween-20 in PBS) was diluted 1:5 in deionized water for quenching blotted membranes and diluted 1:2 for use as diluent for sera and isotope. Blotted

Table 1. Percent binding in RAST of 11 sera for *G. meredithae*

Sera	SPT ¹	Spore ²	Caps ³
FB	+	10.7	3.7
WD	+	6.7	5.6
RP	+	2.1	2.6
KS	+	1.2	1.1
KR	+	5.5	3.9
MH	+	4.3	4.0
SD	+	3.3	2.3
CB	+	3.8	1.8
DS	+	4.7	1.5
RH	+	4.4	2.8
LN	+	16.0	5.0
TC	-	0.8	0.8
LZ	-	1.0	0.8
MR	-	1.6	1.2
BA	-	1.4	1.3
JD	-	1.0	1.0

¹ Skin prick test to *G. meredithae*.² Positive = 2.2.³ Positive = 1.7. (Positive = negative mean + 3 SD).

membranes were quenched at room temperature for 1 h, and then overlaid with sera (diluted 1:8) overnight. Blots were washed with PBS/0.01% Tween-20 and labeled with ¹²⁵I-anti-human IgE overnight at approximately 50 000 cpm per lane. Labeled proteins were detected by autoradiography at -80°C until suitable image density was obtained, generally in 2-3 d.

Results

RAST/RAST inhibition

The percent binding in RAST of 11 sera from skin test positive subjects ranged from 1.2 to 16.0 with spores of *G. meredithae*. RAST percent binding with caps ranged from 1.1 to 5.6. RAST percent binding of five sera from skin test negative subjects ranged from 0.8 to 1.6 and 0.7 to 1.3 for spores and caps, respectively (Table 1). For the negative sera, the mean plus 3 SD was 2.2 and 1.7 for spores and caps, respectively. These values were the minimum for a positive reaction in the respective RASTs. Thus, 2 of 11 positive subjects had negative RAST for spores, and 2 of 11 were negative for caps.

Inhibition of *G. meredithae* and *G. lucidum* spore and cap RAST attained 50% for all homologous inhibitors. Spores inhibited spore RASTs more than caps; there was no clear difference for cap RASTs (Fig. 1).

Electrophoresis/blotting

Cap extracts of *G. meredithae* and *G. lucidum* each contained 23 protein bands in IEF. *G. meredithae* spore extract contained 17 bands, but *G. lucidum* spore extract revealed no bands even when 2000 µg of extract was loaded onto the gel. IEF/immunoprints of *G. meredithae* cap and spore with seven sera revealed that 16 bands bound IgE (Fig. 2). The reac-

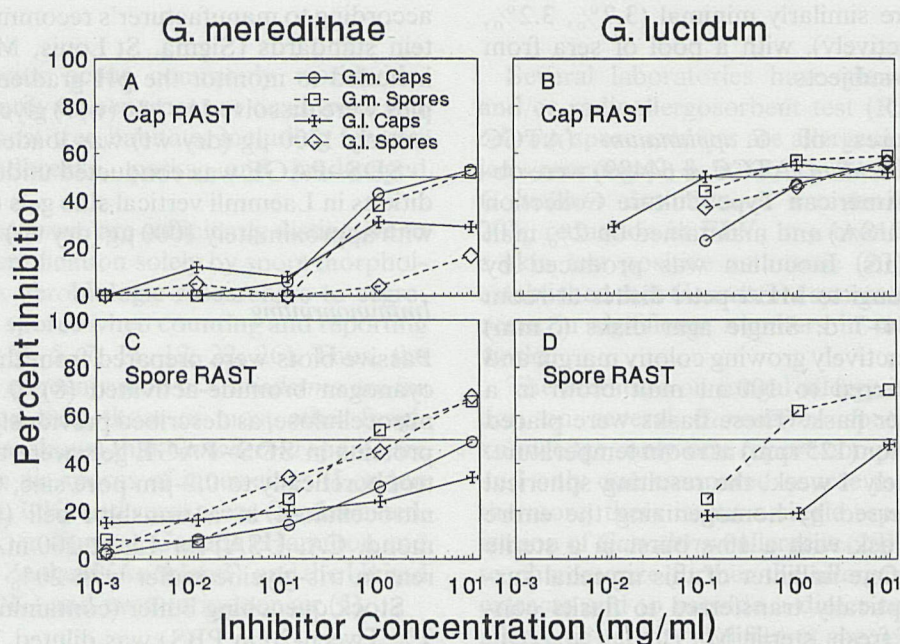


Fig. 1. A and C: RAST inhibition of *G. meredithae* (*G.m.*) cap (A) and spore (C) by *G.m.* and *G. lucidum* (*G.l.*) spore and cap extracts. B and D: RAST inhibition of *G.l.* cap (B) and spore (D) by *G.m.* and *G.l.* spore and cap extracts.

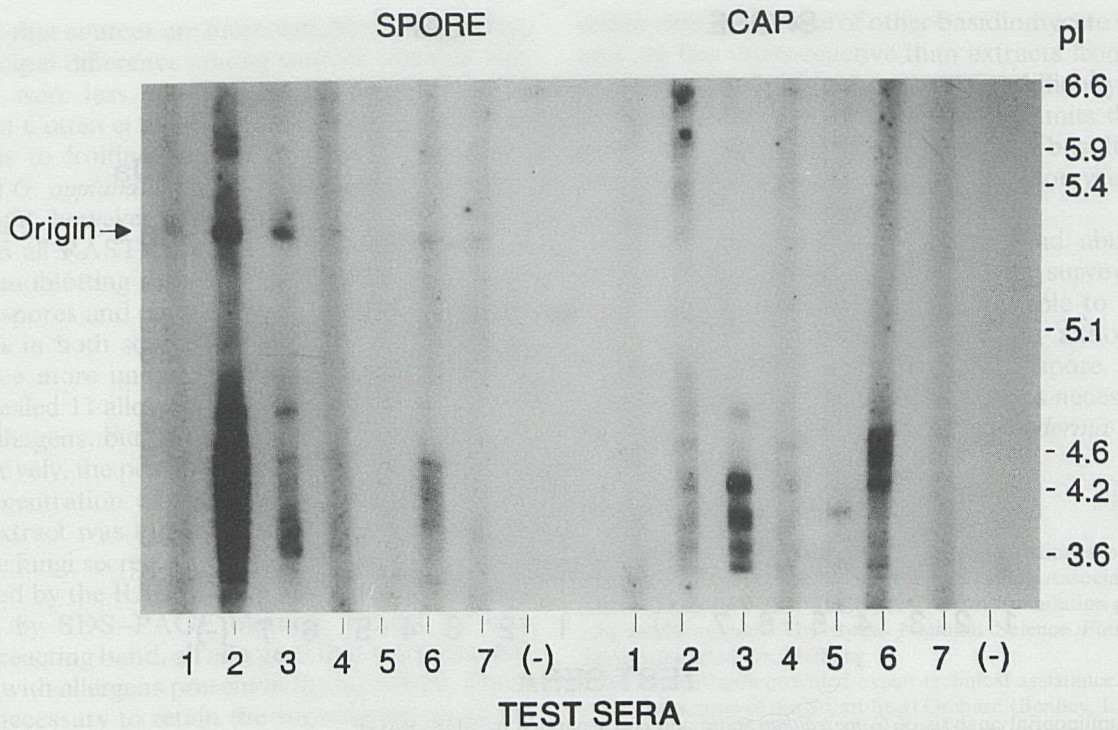


Fig. 2. Immunoprint analysis of *G. meredithae* spore and cap extracts separated by IEF.

tivity of individual bands varied from one to four sera (Fig. 3).

SDS-PAGE of *G. meredithae* spore and cap extracts showed three diffuse bands and one distinct band with caps, and a single distinct band with spores. Immunoprints of the SDS-PAGE gel with

seven positive sera detected 17 IgE-binding bands in spores and 13 bands in caps of *G. meredithae* (Fig. 4). The reactivity of bands varied from one to five sera (Fig. 5).

Mycelial growth

Mycelial production continued to increase for 20 d, although log phase growth appeared to be complete by day 12 (Fig. 6). The RAST activity of mycelia was detectable at the first sampling period, day 4, and remained essentially constant at 5–6% after that. The highest value was at day 16 (6.7%). Supernatant (medium) contained RAST activity also, but at only 50% of the level in mycelia (Fig. 6). A negative sera pool had less than 1% RAST activity for either mycelia or supernatant from all days sampled.

When grown in 2% malt for 16 d, *G. applanatum* produced 705 mg mycelium per 100 ml media (250 ml-flask). *G. lucidum* produced 600 mg in malt and 595 mg in ultrafiltered malt. The RAST activity was 9.1% and 9.0% for *G. applanatum* and *G. lucidum*, respectively, grown in malt, and it was only 7.0% for *G. lucidum* grown in ultrafiltered malt. The RAST activity of supernatants was 7.5%, 5.8%, and 5.7%, respectively. RAST with control (skin test negative) sera was less than 2% for all six sources.

SDS-PAGE/immunoblot of *G. lucidum* mycelia

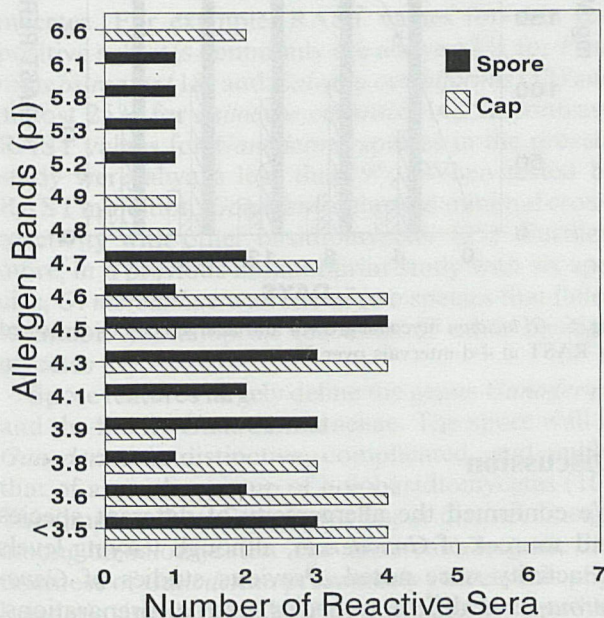


Fig. 3. Reactivity of sera to *G. meredithae* spore and cap allergens separated by IEF.

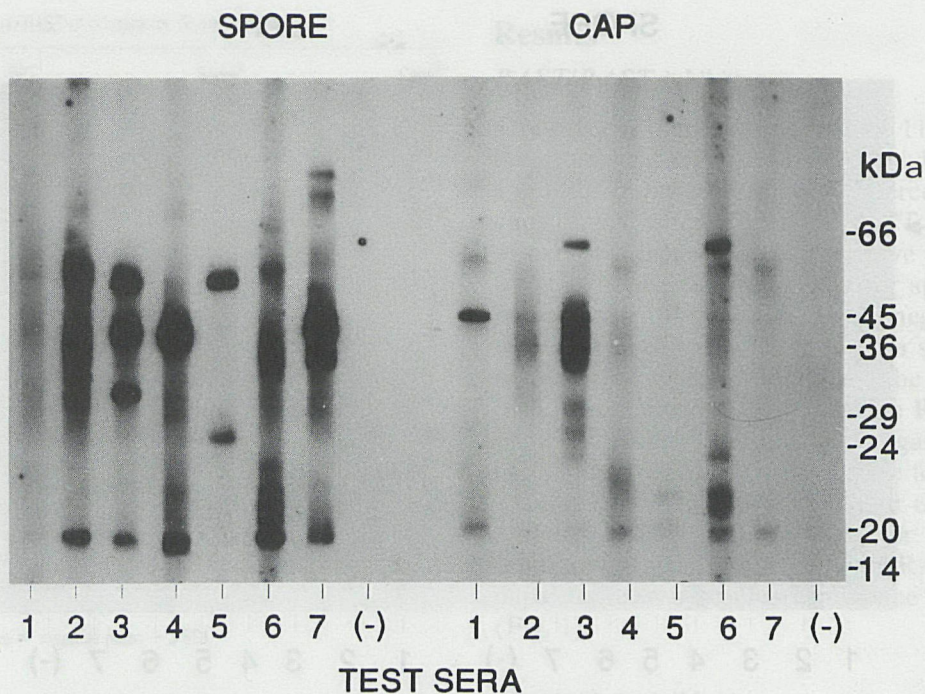


Fig. 4. Immunoprint analysis of *G. meredithae* spore and cap separated by SDS-PAGE.

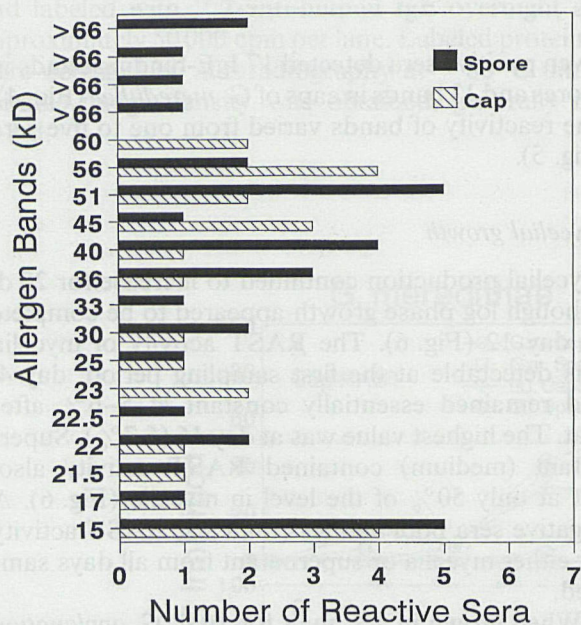


Fig. 5. Reactivity of sera to *G. meredithae* spore and cap allergens separated by SDS-PAGE.

and supernatant from day 8 revealed only a single faint band (41 kDa) in the mycelium. Day-16 mycelia and supernatant contained 6 and 8 bands, respectively. A doublet band was present in supernatant at 50–52 kDa, corresponding to a 53-kDa band in mycelia. A faint 22-kDa band appeared to have no corresponding band in mycelia.

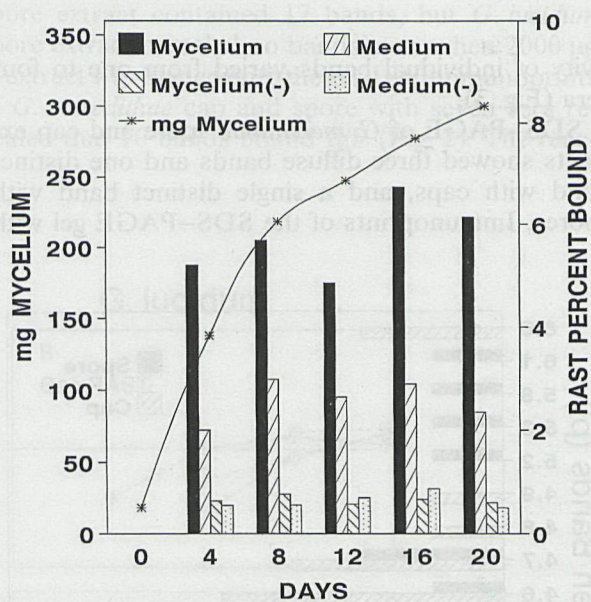


Fig. 6. *G. lucidum* mycelial growth and allergenicity as measured by RAST at 4-d intervals over 20 d.

Discussion

We confirmed the allergenicity of different species and sources of *Ganoderma*, although varying levels of activity were noted. Previous studies of *Ganoderma* used different species, extract preparations, and patient populations; each factor, presumably, could contribute variability. The present comparison

suggests that sources are more variable than species; the principal difference among sources was that cap extracts were less active. This contrasts with the results of Cutten et al. (6), who found more skin test reactions to fruiting body (cap) than to spore extracts of *G. applanatum*. The differences in our study were slight, however, and all spore and cap extracts inhibited all RASTs.

Immunoblotting showed that most allergens were in both spores and caps. The IEF blot showed eight allergens in both sources, five more only in spores, and three more unique to caps. The SDS-PAGE blot revealed 11 allergens in both sources, six unique spore allergens, but just two that were only in caps. Alternatively, the possibility cannot be excluded that the concentration of these unique allergens in the crude extract was too low to detect.

These fungi secreted allergens into the medium, as suggested by the RAST activity in supernatants and showed by SDS-PAGE blotting. Except for one weakly reacting band, all allergens that were secreted aligned with allergens present in the mycelium. Thus, it is unnecessary to retain the supernatant as an allergen source, since the mycelium was superior.

Allergenic similarity between *G. meredithae* and *G. lucidum* should be expected since they both belong to the *G. lucidum* species complex of the genus (1). *G. applanatum* is not in this species complex but has a similar mycelial RAST activity. This suggests that allergenic similarity may extend throughout the genus.

Although *Ganoderma* species compare favorably with other basidiomycetes in skin test surveys (13, 20, 27), their allergenic activity *in vitro* is less than that of other well-characterized allergenic basidiomycetes. For example, RAST values for skin test positive subjects commonly are above 12% for *Pleurotus ostreatus* (18) and *Calvatia cyathiformis* (17) and almost 25% for *Psilocybe cubensis* (14). In contrast, RAST values for *Ganoderma* species in the present study were always less than 9%. When tested by RAST inhibition, *Ganoderma* showed minimal cross-reactivity with other basidiomycetes (25). Furthermore, in a previous immunoprint study with six species, *G. meredithae* was one of two species that failed to inhibit immunoprint reactions of other basidiomycetes (9).

Spore features largely define the genus *Ganoderma* and the family Ganodermataceae. The spore wall in *Ganoderma* is distinctive, complicated, and unlike that of any other group of holobasidiomycetes (10). This implies that *Ganoderma* has a distinct lineage among basidiomycetes. The apparent allergenic distinctness of *Ganoderma* presumably reflects this phylogenetic singularity. Thus, generalizations regarding *Ganoderma* may not apply to other basidiomycetes.

Allergen extracts from *Ganoderma* are less reac-

tive *in vitro* than those of other basidiomycete species and are less cross-reactive than extracts from other basidiomycetes. This increases the difficulty of selecting suitable sera for analysis and limits the utility of *Ganoderma* as a general model of basidiospore allergy. This suggests that other basidiomycetes are more suitable for allergen analysis.

Ganoderma spores are common and abundant, they occur frequently in aeroallergen surveys, and their skin test prevalence is comparable to that of other allergenic basidiospores. Thus, although of limited utility as a model of basidiospore allergy, specific analysis of *Ganoderma* remains necessary to allow diagnosis and treatment of *Ganoderma* allergy.

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References

- ADASKAVEG JE, GILBERTSON RL. Basidiospores, pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. *Mycologia* 1988; 80: 493-507.
- BRUCE RA. Bronchial and skin sensitivity in asthma. *Int Arch Allergy Appl Immunol* 1963; 22: 294-305.
- BUTCHER BT, O'NEIL CE, REED MA, ALTMAN LC, LOPEZ M, LEHRER SB. Basidiomycete allergy: measurement of spore-specific IgE antibodies. *J Allergy Clin Immunol* 1987; 80: 803-9.
- CHATTERJEE J, HARGREAVE FE. Atmospheric pollen and fungal spores in Hamilton in 1972 estimated by the Hirst automatic volumetric spore trap. *Can Med Assoc J* 1974; 110: 659-63.
- COMTOIS P. Indoor mold aerosols. *Aerobiologia* 1990; 6: 165-76.
- CUTTEN AEC, HASNAIN SM, SEGEDIN BP, BAI TR, MCKAY EJ. The basidiomycete *Ganoderma* and asthma: collection, quantitation and immunogenicity of the spores. *N Z Med J* 1988; 101: 361-3.
- DAVIES RR, DENNY MJ, NEWTON LM. A comparison between the summer and autumn air-spores at London and Liverpool. *Acta Allergol* 1963; 18: 131-47.
- DEMEULEMESTER C, PELTRE G, LAURENT M, PANGHELEUX D, DAVID B. Cyanogen bromide-activated nitrocellulose membranes: a new tool for immunoprint techniques. *Electrophoresis* 1987; 8: 71-3.
- DE ZUBIRIA A, HORNER WE, LEHRER SB. Evidence of cross-reactive allergens among basidiomycetes: immunoprint-inhibition studies. *J Allergy Clin Immunol* 1990; 86: 26-33.
- DONK MA. Multiple convergence in the polyporaceous fungi. In: Petersen RH, ed. *Evolution in the higher basidiomycetes*. Knoxville, TN: University of Tennessee Press, 1971: 393-422.
- GREGORY PH, HIRST JM. The summer air-spores at Rothamsted in 1952. *J Gen Microbiol* 1957; 17: 135-52.

12. HASNAIN SM, WILSON JD, NEWHOOK FJ. Fungal allergy and respiratory disease. *N Z Med J* 1985; 98: 342-6.
13. HASNAIN M, WILSON JD, NEWHOOK FJ, SEGEDIN BP. Allergy to basidiospores: immunologic studies. *N Z Med J* 1985; 98: 393-6.
14. HELBLING A, HORNER WE, LEHRER SA. Identification of *Psilocybe cubensis* spore allergens by immunoprinting. *J Allergy Clin Immunol* (submitted).
15. HERXHEIMER H, HYDE HA, WILLIAMS DA. Allergic asthma caused by basidiospores. *Lancet* 1969; 2: 131-3.
16. HERXHEIMER H, HYDE HA, WILLIAMS DA. Allergic asthma caused by fungal spores. *Lancet* 1966; 1: 572-3.
17. HORNER WE, IBANEZ MD, LEHRER SB. Immunoprint of *Calvatia cyathiformis* allergens. I. Reactivity with individual sera. *J Allergy Clin Immunol* 1989; 83: 784-92.
18. HORNER WE, IBANEZ MD, LIENGSWANGWONG V, SALVAGGIO JE, LEHRER SB. Characterization of allergens from spores of the oyster mushroom, *Pleurotus ostreatus*. *J Allergy Clin Immunol* 1988; 82: 978-86.
19. KARR RM, WILSON MR, ANICETTI VR, LEHRER SB, BUTCHER BT, SALVAGGIO JE. An approach to fungal antigen relationships by radioallergosorbent test inhibition. *J Allergy Clin Immunol* 1981; 67: 194-8.
20. LEHRER SB, LOPEZ M, BUTCHER BT, OLSON J, REED M, SALVAGGIO JE. Basidiomycete mycelia and spore-allergen extracts: skin test reactivity in adults with symptoms of respiratory allergy. *J Allergy Clin Immunol* 1986; 78: 478-85.
21. LEHRER SB, McCANTS ML, ALTMAN LC, et al. Basidiospore skin test reactivity in seven different geographical areas of the United States. *J Allergy Clin Immunol* 1990; 85: 228 (Abstr.).
22. LEVETIN E. Studies on airborne basidiospores. *Aerobiologia* 1990; 6: 177-80.
23. LOPEZ M, VOIGTLANDER JR, LEHRER SB, SALVAGGIO JE. Bronchoprovocation studies in basidiospore-sensitive allergic subjects with asthma. *J Allergy Clin Immunol* 1989; 84: 242-6.
24. McCANTS ML, LEHRER SB, BOUSQUET J, et al. Prevalence of basidiospores skin test reactivity in Europe. *J Allergy Clin Immunol* 1990; 85: 249 (Abstr.).
25. O'NEIL CE, HUGHES JM, BUTCHER BT, SALVAGGIO JE, LEHRER SB. Basidiospore extracts: evidence for common antigenic/allergenic determinants. *Int Arch Allergy Appl Immunol* 1988; 85: 161-6.
26. PAWSEY RG. An investigation of the spore population of the air at Nottingham. *Trans Br Mycol Soc* 1964; 47: 357-63.
27. SPRENGER JD, ALTMAN LC, O'NEIL CE, AYARS GH, BUTCHER BT, LEHRER SB. Prevalence of basidiospore allergy in the Pacific Northwest. *J Allergy Clin Immunol* 1988; 82: 1076-80.
28. TARLO SM, BELL B, SRINIVASAN J, DOLOVICH J, HARGREAVE FE. Human sensitization to *Ganoderma* antigen. *J Allergy Clin Immunol* 1979; 64: 43-9.
29. VIJAY HM, COMTOIS P, SHARMA R, LEMIEUX R. Allergenic components of *Ganoderma applanatum*. *Grana* 1991; 30: 167-70.

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