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MORPHOLOGY AND ALLERGENIC PROPERTIES OF BASIDIOSPORES FROM FOUR *CALVATIA* SPECIES

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ABSTRACT

Basidiospores of *Calvatia cyathiformis* have been identified in aerobiological studies, and spore extracts have demonstrated significant skin and radioallergosorbent test reactivity in sensitive patients. Although fruiting bodies of *Calvatia craniiformis, C. rubroflava,* and *C. gigantea* are also relatively common, basidiospores of these species cannot be specifically identified from the atmosphere. The present study compared the morphology and antigenic properties of basidiospores from these four *Calvatia* species. Electron microscopy showed that they all have globose spores 3–5 μm in diameter but each species has unique spore ornamentation. Only *C. cyathiformis* spores were sufficiently distinctive by light microscopy to be identifiable on air sampler slides. These spores occurred in the Tulsa atmosphere on 68% of the days during September and October. The allergenic properties were compared using radioallergosorbent test and radioallergosorbent test inhibition. Results indicate that *C. rubroflava* and *C. craniiformis* are potentially important aeroallergens. Protein patterns in these species differed from one another and from *C. cyathiformis*; however, common proteins were also present. Spore extracts of *C. cyathiformis* from two states were similar by isoelectric focusing but differed in allergenic activity. *C. gigantea* gave indiscernible protein patterns. These studies indicate that although *Calvatia* species demonstrate a number of similarities, there are distinctive structural and allergenic properties.

Key Words: aerobiology, allergens, basidiospores, *Calvatia*

Since the 1930s fungal spores have been recognized as major allergens (Feinberg, **1935**). Over the years, numerous studies have confirmed the importance of the imperfect fungi as a cause of both asthma and allergic rhinitis. As early as 1952, Gregory and Hirst (**1952**) suggested airborne basidiospores as possible allergens; however, it has only been in the past 10 to 15 yr that clinical evidence has been accumulating on the incidence of basidiospore-induced allergic disease. Recent studies have shown that various members of the hymenomycetes and gasteromycetes are significant allergens (Giannini et al., **1975**; Tarlo et al., **1979**; Hasnain et al., **1984**, **1985a**, **b**; Lopez et al., **1985**; Santilli et al., **1985**; Burge, **1986**; Lehrer et al., **1986**; Butcher et al., **1987**; Weissman et al., **1987**; Davis et al., **1988**; Ibanez et al., **1988**; O'Neil et al., **1988**; Horner et al., **1989**). Aerobiological studies have also shown that high concentrations of basidiospores exist in the atmosphere at various locations around the world (Rubulis, **1984**; Hasnain et al., **1985a**; Misra, **1987**; Levetin, **1990**, **1991**).

Calvatia cyathiformis (Bosc) Morg. is a common grassland puffball in North America (Zeller and Smith, **1964**). Basidiospores have been identified in aerobiological studies since the size, color, and ornamentation of the spores make them distinctive and recognizable from atmospheric samples (Levetin, **1990**, **1991**). Basidiospore extracts of this species have demonstrated significant skin and radioallergosorbent test (RAST) reactivity in sensitive patients (Lehrer et al., **1986**; Butcher et al., **1987**; Ibanez et al., **1988**; Horner et al., **1989**). Spore allergens from *C. cyathiformis* are well characterized relative to most basidiospore allergens, and they broadly cross react with spore allergens from the Agaricales and Aphyllophorales (O'Neil et al., **1988**; De Zubiria et al., **1990**).

Although fruiting bodies of *Calvatia craniiformis* (Schw.) Fr., *C. rubroflava* (Craigin) Morg.,

and *C. gigantea* (Batsch *ex* Pers.) Lloyd are also relatively common, spores of these species have not been identified from the atmosphere nor has their allergenicity been examined. The present study was undertaken to determine the airborne concentration of *C. cyathiformis* spores, to assess cross-reactivity of *Calvatia* allergens among related species and to find a basis for visual identification of other *Calvatia* spores that may be allergenically important but previously overlooked from air samples.

METHODS AND MATERIALS

Air sampling.—The atmosphere in Tulsa, Oklahoma was monitored with a Burkard Volumetric Spore Trap which was located on the roof of a building on The University of Tulsa campus with the intake orifice at 12 m above ground (Levetin, **1990**). The sampler was set for 7-day sampling onto Melenex tape which had been coated with a thin film of Lubriseal (Thomas Scientific, Swedesboro, New Jersey). The tapes were changed weekly and cut into 1-day segments which were mounted on microscope slides, stained with glycerin-jelly containing basic fuchsin, and microscopically examined. The concentrations are expressed as daily averages in spores/m^3.

Spore treatments.—Specimens of *Calvatia cyathiformis* were collected in both the New Orleans, Louisiana area and the Tulsa, Oklahoma area. *Calvatia craniiformis* and *C. rubroflava* were from Tulsa, while *C. gigantea* was collected in Rochester, Minnesota. Voucher specimens of the Tulsa collections were deposited in the Barclay Herbarium of The University of Tulsa. Basidiospores were harvested from the mature gleba, passed through a 45 μm sieve and stored desiccated at room temperature in sealed containers with desiccant. Spores harvested this way were relatively pure (85% to 95%); the remainder of the material was primarily capillitium.

For electron microscopy, basidiospores were attached to sample stubs with Tempfix (Electron Microscopy Sciences, Ft. Washington, Pennsylvania), sputter-coated with gold, and examined with a Hitachi S2300 scanning electron microscope (SEM).

In preparing extracts, spores were defatted with ether (200 ml/g of spores) prior to extraction and allowed to air dry overnight. Spores were disrupted by vigorous shaking with glass beads in 0.125 M NH_4CO_3 (pH 8.1) using a Braun homogenizer (Melsungin, Germany) cooled with escaping liquid CO_2 for 2 min. Homogenate was recovered by washing the beads in a minimum volume (<100 ml) of cold buffer. The homogenized suspension was then centrifuged at 4 C for 30 min at 80,000 g, and the supernatant recovered and lyophilized. Lyophilized extracts were stored under desiccation until used for RAST, RAST inhibition, and isoelectric focusing.

Radioallergosorbent test (RAST).—Sera were obtained from six patients that were skin test positive to *C. cyathiformis* extracts and tested by RAST to determine levels of specific IgE (immunoglobulin class E) antibodies to *Calvatia* allergens as previously described (Horner et al., **1989**). Briefly, cyanogen bromide-activated paper disks (Whatman No. 1) were separately coated with *Calvatia* extracts (1 mg/disk) which had been resuspended in 0.1 M borate buffer, pH 8. Duplicate RAST disks were incubated overnight in 100 μl serum from subjects who skin tested positive to *C. cyathiformis* and then washed free of unbound serum with three changes of saline. During incubation serum antibodies specific for *Calvatia* antigens bound to *Calvatia* antigen on the disk. The disks were incubated with approximately 15,000 cpm affinity purified ^{125}I equine antihuman IgE (Kallested, Chaska, Minnesota) and unbound antibody removed by three saline washes. Residual counts were determined in a Beckman model 5500 gamma counter. RAST results were expressed as percent of total label added that bound to disks. Tests were run in duplicate and the mean used to calculate percent bound. RAST values >2% bound were considered a positive RAST reaction as previously described (Butcher et al., **1987**; Horner et al., **1989**).

RAST inhibition.—RAST disks, coated with the various *Calvatia* extracts were incubated with 50 μl pooled sera (containing equal volumes of five sera used for RAST) and 50 μl inhibiting antigen (10, 1, 0.1, 0.01, and 0.001 mg/ml of spore extracts in PBS). Inhibition of IgE binding to antigen coated disks is caused by competition between soluble and bound antigens with maximum inhibition occurring when the soluble and bound antigens are most similar. Results are presented as percent inhibition related to a PBS (negative control) solution. In addition, the amounts of antigen inhibiting RAST by 50% (ID_{50}) were calculated and the logarithm of these values plotted. Extracts which show significant inhibitory activity have relatively low ID_{50} values.

Isoelectric focusing.—*Calvatia* extracts were focused by electrophoresis in ampholine polyacrylamide gel plates, pH 3.5–9.5. Electrical settings were limited to 1500 V, 50 W and 30 mA with gels focused for approximately 1500 Vh. Following electrophoresis, gels were fixed in a solution of 3.5% sulfosalicylic acid and 11.5% trichloroacetic acid. Fixed gels were equilibrated in staining solvent (3:1:9, methanol : acetic acid : water) for 20 min and then stained in 0.1% Coomassie brilliant blue R 250 in staining solvent at 60 C (initially) for 20 min. Gels were destained with washes of staining solvent.

RESULTS

Aerobiology.—Basidiospores of *Calvatia cyathiformis* were identified on the Burkard slides and quantified during the fall seasons of 1988 and 1989. Spores of this puffball were present on 68% of the days during September and October in these years. The atmospheric concentration was generally low, below 100 spores/m^3, with the highest concentration of 409 spores/m^3 occurring on 10 Oct. 1989 (FIG. 1). Although the spores normally occurred singly on the air sampler slides,

occasional clusters containing up to 35 basidiospores were observed (FIG. 2). Basidiospores of other puffballs were also seen on these slides; however, no other *Calvatia* species were identified.

Spore morphology.—Under the light microscope, the basidiospores of *C. cyathiformis* appear light purplish-brown and globose with distinct spines. Spores of *C. rubroflava, C. craniiformis,* and *C. gigantea* are also globose, but appear almost colorless with indistinct spines or warts.

Electron microscopy showed that the four *Calvatia* species all have globose spores 3–5 μm in diam but each has distinctive spore ornamentation. Of the four species, *C. cyathiformis* has the largest spore (4–5 μm in diam) with large, irregular, and somewhat peltate spines (and occasional crests) that are up to 1 μm in height (FIG. 3). The spines are subtended by raised connectives that show signs of pitting or erosion. The number and density of the spines varies considerably even among spores from the same specimen. Spores from both the Oklahoma and Louisiana specimens share the same distinctive features.

Spores of C. *rubroflava* are globose, 3 to 3.5 μm in diam, with well spaced slightly curved spines that are up to 0.5 μm in height (FIG. 4). Raised connectives are visible but not as prominent as they are in *C. cyathiformis* spores.

Calvatia craniiformis has small globose spores approximately 3 μm in diam with verrucose ornamentation (FIG. 5). The verrucae are small (up to 0.2 μm in height) with rounded apices and are well spaced around the spore. The spores are similar to the *C. rubroflava* spores but the verrucae in *C. craniiformis* are small and not as prominent as the appendages in *C. rubroflava.*

Calvatia gigantea spores are globose to subglobose, 3 to 3.5 μm in diam, with scattered irregular warts that are up to 0.4 μm in height (FIG. 6). Although these spores show some similarity to *C. craniiformis* spores the ornamentation is larger and not as uniform.

RAST/RAST inhibition.—RAST was performed using sera from six patients who skin tested positive to *C. cyathiformis* extracts. The presence of IgE antibodies with specificity for *Calvatia* antigens was demonstrated by this technique. The percent binding of individual sera varied from 1.2% to 15.1% with the lowest average RAST binding occurring with *C. gigantea* extracts (FIG. 7).

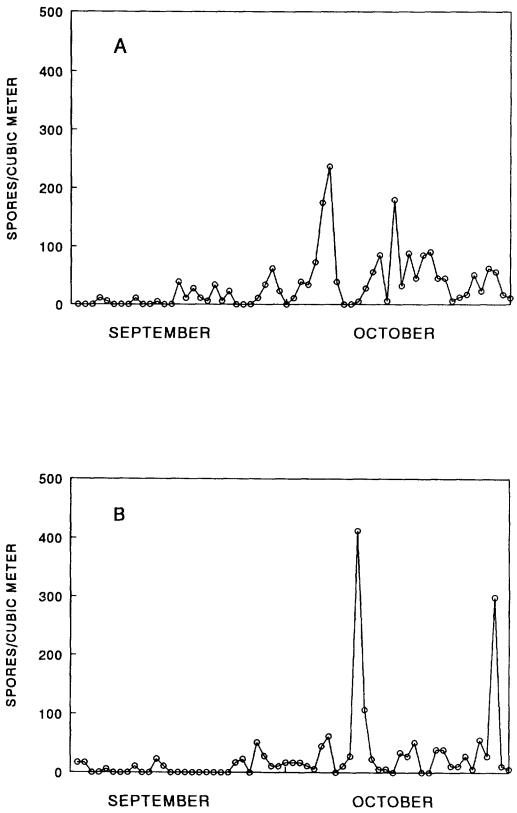
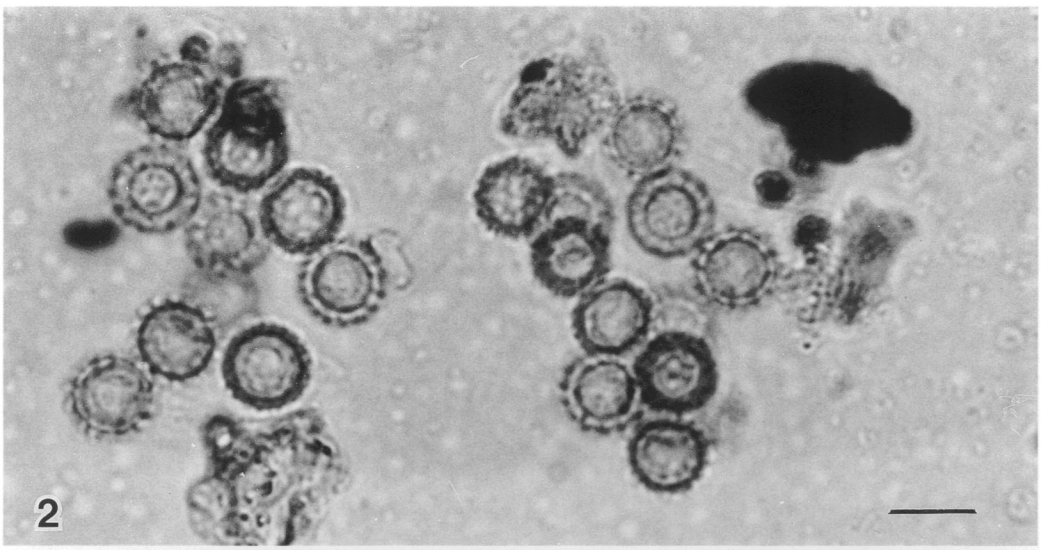
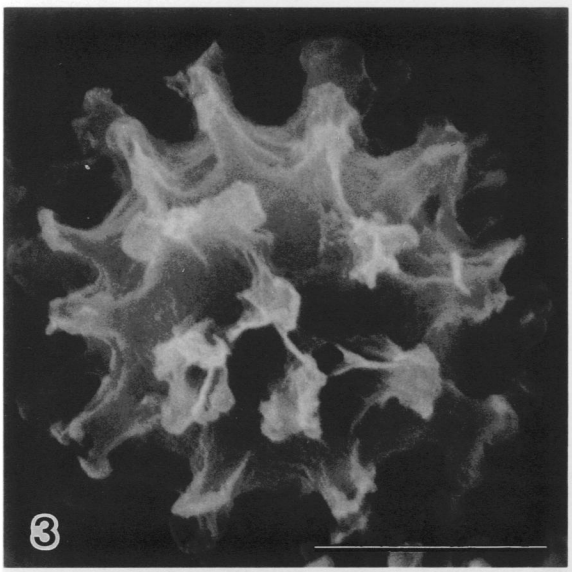


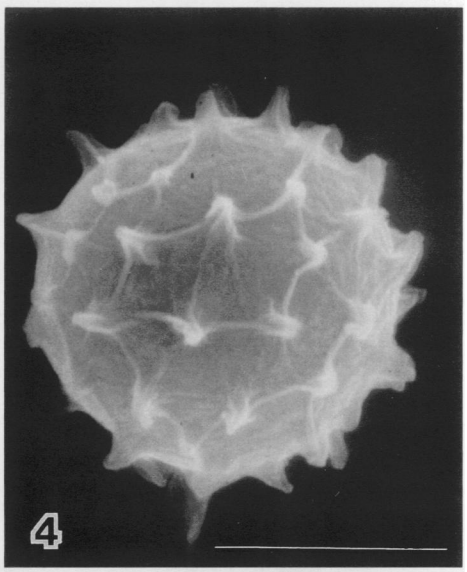
FIG. 1. Average daily concentration of atmospheric *Calvatia cyathiformis* spores in Tulsa, Oklahoma during 1988 (A) and 1989 (B).

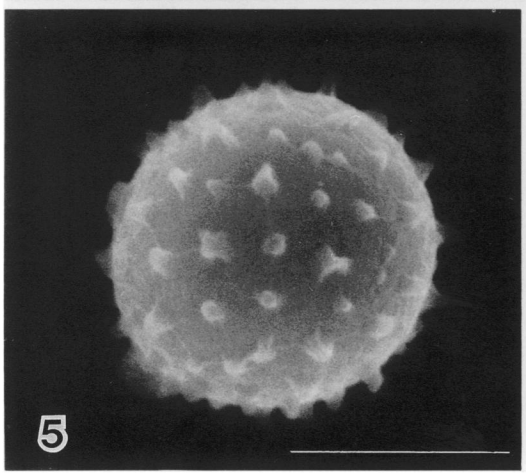
The ability of extracts of the various species to inhibit RAST of other species indicated common or cross-reacting allergens present in the spores of all species except *C. gigantea* (FIG. 8). The RAST values were generally low for *C. gigantea* extracts, and inhibition of *C. gigantea* RAST was also minimal. The homologous combination (*C. gigantea* inhibiting *C. gigantea*) only yielded 57% inhibition (data not shown). Inhibition dose 50 (ID_{50}) values (other than *C. gigantea*) were in the same range for all combinations except for *C. cyathiformis* (LA) inhibiting *C. cyathiformis* (OK) RAST (FIG. 9). For this combination the ID_{50} value was estimated by graphic interpolation.

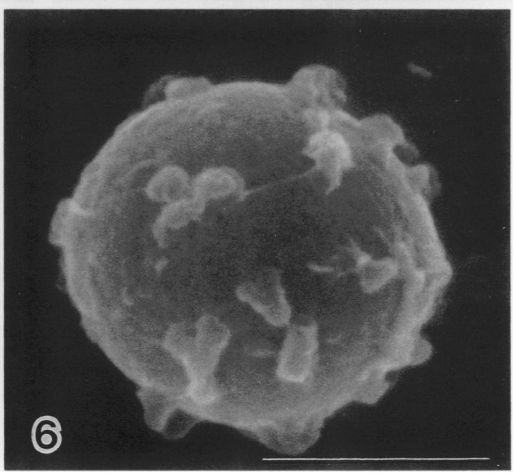
Isoelectric focusing.—The IEF protein banding showed unique bands for *C. craniiformis* and *C. rubroflava.* The patterns of the two *C. cyathiformis* extracts were essentially identical (FIG. 10).











Protein patterns were sufficiently intense with 400 μg extract for *C. craniiformis* and *C. rubroflava* but required 800 μg of *C. cyathiformis*; 800 μg gave no discernible pattern with *C. gigantea*. Two separate attempts with spore disruption failed to extract allergens. An additional extraction procedure from intact spores also failed to recover any activity (data not shown).

DISCUSSION

Spore morphology and ornamentation in the gasteromycetes has been extensively studied with SEM for the past 40 years; however, few studies have focused on the genus *Calvatia* (Burk et al., **1983**). This study confirms previous descriptions of *C. cyathiformis* (Heim and Perreau, **1971**) and *C. gigantea* (Perreau, **1971**) basidiospores as well as providing SEM descriptions of spore morphology in *C. rubroflava* and *C. craniiformis*.

Calvatia is a large genus with over 30 species in North America alone. Zeller and Smith (**1964**) divided the North American species into seven stirpes, or groups of closely related species, based on gross morphology and microscopic details of the gleba. The four species examined in the present study belong to three different stirpes with *C. craniiformis* and *C. rubroflava* closely related species in the same stirps. The present study supports this classification based on the details of spore ornamentation visible with the SEM and reinforces the view that *C. craniiformis* and *C. rubroflava* are closely related species. Although the spines of *C. rubroflava* are more prominent than the verrucae of *C. craniiformis,* they are clearly similar when compared to the peltate spines and crest of *C. cyathiformis* and the scattered warts of *C. gigantea*.

While the spores of these four species show unique ornamentation with the SEM, using light microscopy only *C. cyathiformis* is distinctive and was the only *Calvatia* species identifiable on air sampler slides. These basidiospores occurred singly or, occasionally, in clusters that resulted from the discharge of a clump of spores which were not fully dispersed by the wind. Although

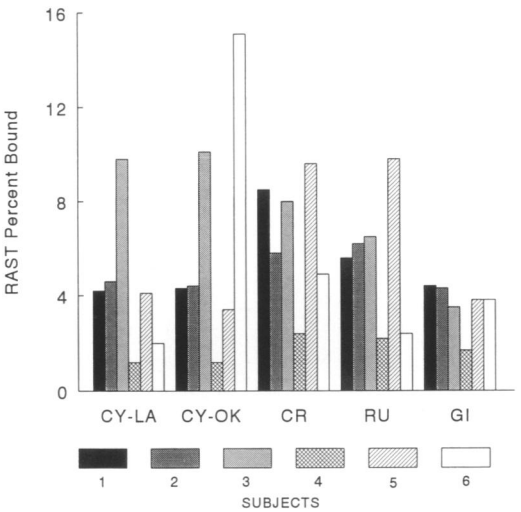


FIG. 7. RAST (Radio Allergosorbent Test) values for six subjects against five extracts of *Calvatia* spores. Abbreviations refer to *C. cyathiformis* from Louisiana (CY-LA) and Oklahoma (CY-OK), *C. craniiformis* (CR) and *C. rubroflava* (RU) and *C. gigantea* (GI).

gasteromycete spores have been previously identified from the atmosphere (Hasnain et al., **1985a**; Levetin, **1990**, **1991**), this is the first report to document atmospheric levels of *C. cyathiformis*. The spores occurred in the atmosphere on 68% of the days in September and October, but the concentrations recorded were generally low by comparison with other fungal aeroallergens (Lehrer et al., **1983**; Rubulis, **1984**; Hasnain et al., **1985a**; Burge, **1986**). There were few significant peaks in the *C. cyathiformis* spore concentrations during the two seasons under study. It should be noted, however, that most atmospheric concentrations are normally reported at the generic level including all species within that genus and not for an individual species. The lack of morphological uniformity of basidiospores within the genus *Calvatia* does not allow an accurate assessment of their atmospheric concentration. It is believed that spores of other *Calvatia* species are, in fact, present in the

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FIGS. 2–6. Spore morphology of *Calvatia* species by light microscopy (FIG. 2) and SEM (FIGS. 3–6). 2. Cluster of *Calvatia cyathiformis* spores on slide from Burkard Spore Trap. Bar = 5 μm. 3. *C. cyathiformis* spore, note large, irregular spines with raised connectives. Bar = 2 μm. 4. *C. rubroflava* spore with slightly curved spines. Bar = 2 μm. 5. *C. craniiformis* spore with small verrucae. Bar = 2 μm. 6. *C. gigantea* spore showing scattered irregular warts. Bar = 2 μm.

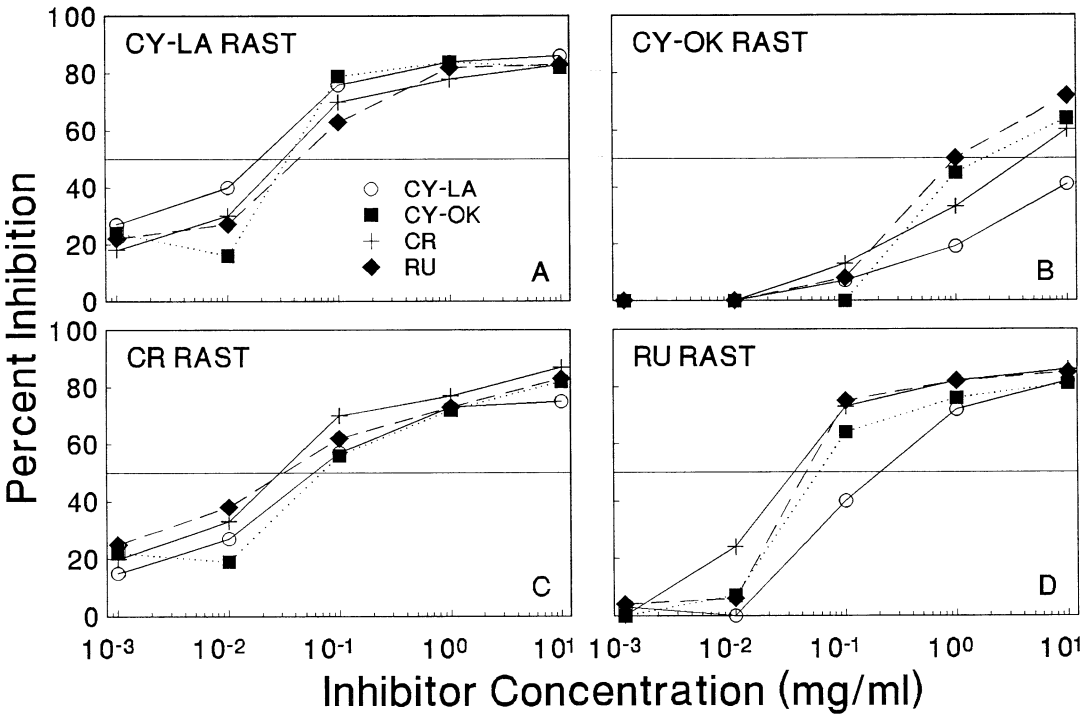
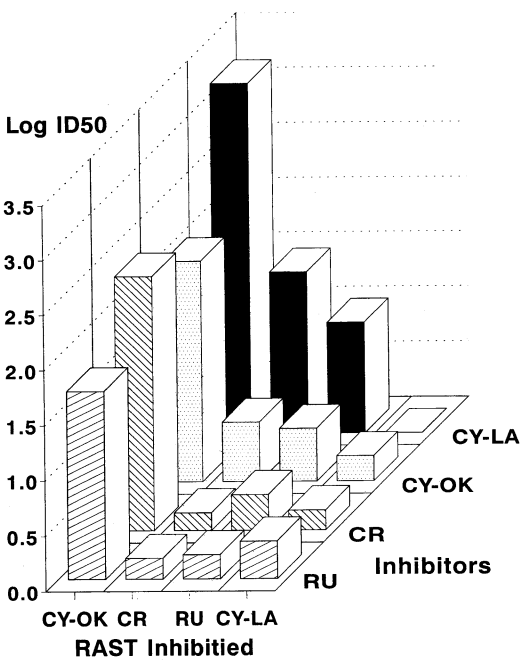


FIG. 8. Inhibition of *Calvatia* RASTs by different *Calvatia* extracts. *C. cyathiformis* from Louisiana (CY-LA) and Oklahoma (CY-OK), *C. craniiformis* (CR) and *C. rubroflava* (RU) were each coupled to activated disks and reacted with pooled RAST positive sera. Each RAST reaction was inhibited individually with increasing concentrations of each extract.



atmosphere and would significantly increase the total *Calvatia* basidiospore concentration.

It is widely accepted that there is a cause and effect relationship between aeroallergen exposure and allergic symptoms; however, threshold levels are virtually unknown. Atmospheric concentrations normally represent an average daily concentration for a large area. During a 24-h period, the atmospheric concentration for a particular aeroallergen may peak for only 1 or 2 h triggering symptoms in sensitive individuals; this peak may not be apparent in the daily average. In addition, atmospheric concentrations close to a source will

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FIG. 9. Log ID_{50} values for RAST inhibition. Inhibitor doses (μg) for 50% inhibition of each RAST/inhibitor combination were determined graphically and plotted as log values for comparison. Extracts with greater inhibitory activity have lower ID_{50} values. (CY-LA = *C. cyathiformis* from Louisiana; CY-OK = *C. cyathiformis* from Oklahoma; CR = *C. craniiformis*; RU = *C. rubroflava*).

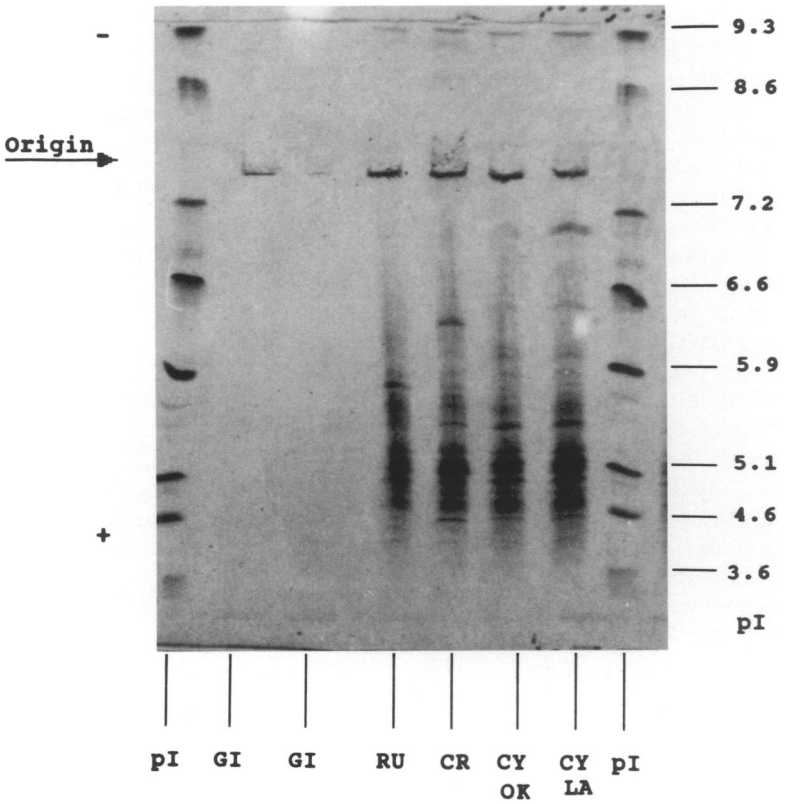

FIG. 10. Isoelectric focusing analysis of *Calvatia* extracts. Two extractions of *C. gigantea* (GI) failed to reveal protein bands. *C. rubroflava* (RU), *C. craniiformis* (CR) and *C. cyathiformis* from Oklahoma (CY-OK) and Louisiana (CY-LA) all revealed predominantly acidic protein bands with pI values between 4.6 and 6.6. Note that all except GI, have a basic protein of approximately pI 9.3.

be significantly higher than the daily average for a given area, and sensitive individuals nearby may experience symptoms.

Although these factors influence individual exposure levels, attempts have been made, nevertheless, to correlate atmospheric concentrations with symptoms. It has been reported (Solomon, **1980**) that grass pollen-sensitive patients normally develop symptoms when atmospheric levels reach 20 grains/m³. While comparable data for ragweed pollen are not available, Solomon (**1980**) suggested that ragweed pollen concentrations of 100 grains/m³ could elicit symptoms in ragweed pollen-sensitive individuals. Similarly there has been speculation upon the threshold concentrations for fungal spores. Published reports suggest that concentrations of 100 *Alternaria* conidia/m³ and 3000 *Cladosporium* conidia/m³ are reasonable estimates for these ubiquitous aeroallergens (Dhillon, **1991**). No estimates have been suggested for basidiospore concentrations; however, Salvaggio et al. (**1971**) have shown a positive correlation between increased hospital admissions due to asthma and high atmospheric levels of basidiospores. While there may be threshold concentrations below which no symptoms are experienced (Dhillon, **1991**), this is probably not an absolute value but a gradient based on individual sensitivities.

The present study showed that the Oklahoma specimens of *C. cyathiformis* contained allergens, as anticipated; however, one of the greatest differences in the present study was the contrast in allergenic activity between *C. cyathiformis* from Oklahoma and Louisiana. Although the protein patterns by IEF were identical, the spore extracts yielded different RAST and RAST inhibition values with the Oklahoma specimens showing greater allergenic activity. These variations between the *C. cyathiformis* collections may be as-

sociated with particular substrate or nutrient features and suggest that geographic differences in basidiospores should be considered as a possible variable related to allergenicity. Similar disparities were seen in RAST activity of *Pleurotus ostreatus* (Jacq. : Fr.) Kummer spore extracts obtained from specimens collected in different locations (Liengswangwong et al., **1987**).

RAST values indicate that spores of *C. craniiformis* and *C. rubroflava* also contain allergens. Protein patterns in these species differed from one another and from *C. cyathiformis* indicating that spores of each species may contain unique allergens. RAST inhibition studies, however, indicated that common or cross-reacting allergens were also present in the spores of all species except *C. gigantea.* Although not identifiable on air sampler slides, RAST and RAST inhibition studies suggest that both *C. craniiformis* and *C. rubroflava* are potentially important aeroallergens.

Calvatia gigantea gave indiscernible protein patterns. Low RAST values could explain the poor inhibition results with *C. gigantea.* Failure with this species may be due to the individual collection or could be a true species difference in protein content or extractability. These results could also be due to the selection of sera, since *C. cyathiformis* positive sera were used. Sera from *C. gigantea* skin-test-reactive subjects may yield better RAST results. Additional collections of *C. gigantea* basidiospores need to be tested before decisions on the allergenicity of this species are reached.

These studies indicate that although the four *Calvatia* species demonstrate a number of similarities, there are distinctive structural and allergenic properties as well. Further analyses of additional species are needed to fully assess the clinical importance of this group of fungi.

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