

Basidiospore allergen release: Elution from intact spores

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Background: We sought to test the hypothesis that allergens are readily released by intact basidiospores in vitro and that different species release allergens in different patterns that are related to spore wall structure.

Methods: To assess basidiospore allergen releasability, basidiospore allergens were extracted from disrupted spores and eluted from intact spores, and the allergenic potency of the extracts and eluates was compared in *Calvatia cyathiformis*, *Psilocybe cubensis*, *Lentinus edodes*, and *Pleurotus ostreatus*.

Results: There was a significant species difference; on the basis of dry weight, the yield of extract and eluates from *Pl. ostreatus* greatly exceeded the yield from *C. cyathiformis* extract and eluates. As measured by RAST inhibition, the allergenic potency of *Pl. ostreatus* and *L. edodes* spore eluates reached the potency of disrupted spore extract in less than 4 hours. Allergen potency of *Ps. cubensis* and *C. cyathiformis* eluate approached the potency of disrupted spore extract only after 8 and 24 hours. Allergen staining intensity in sodium dodecyl sulfate polyacrylamide gel electrophoresis IgE immunoblot correlated with RAST activity. *C. cyathiformis* and *Ps. cubensis* spores have thick walls, whereas *Pl. ostreatus* and *L. edodes* spore walls are thin and hyaline (nonpigmented). Thus spore allergen release may relate to spore wall characteristics.

Conclusions: These studies indicate that intact basidiospores of all four species release allergens, but the release patterns differ by species, and these differences may be clinically significant. (*J ALLERGY CLIN IMMUNOL* 1993;92:306-12.)

Key words: Fungal allergens, fungi, basidiomycete, spore wall, allergen extraction

Basidiospore allergens analyzed to date have been primarily from extracts of disrupted spores.¹⁻⁷ Such extraction favors the release of all soluble proteins. However, intact spores are probably the mode of exposure, and thus intact spores are a more clinically relevant source of allergen. Also, spore disruption may release components that are not normally released on spore deposi-

Abbreviations used

PBS: Phosphate-buffered saline
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

tion. Such compounds could complicate allergen purification and characterization/standardization and are also undesirable in a diagnostic or therapeutic extract because they might cause unwanted side effects.

Spore disruption could also release proteases that degrade or alter native allergens. The alteration of native proteins could affect the IgE binding sites of particular antigens. Releasability of antigens might also affect the intensity of exposure and influence IgE responses. These hypotheses are speculative, but their exploration would aid understanding of the immunopathogenesis of asthma.

We sought to test the hypotheses that allergens are readily released by intact basidiospores in

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Presented in part at the Forty-seventh Meeting of the American Academy of Allergy and Immunology, San Francisco, Calif., March 1-6, 1991.

Supported by NIH Grant No. AI 20331, an American Lung Association Research grant to Dr. Horner, and by University of Tulsa Faculty Research Grant No. 3107-21.

Received for publication July 28, 1992; revised Jan. 27, 1993; accepted for publication Feb. 8, 1993.

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0091-6749/93 \$1.00 + .10 1/1/46355

vitro and that species release allergens in different patterns that are related to spore wall structure. To determine the amount of allergen activity and the qualitative nature of proteins released from intact spores, we compared allergen extracts from mechanically disrupted spores and from intact spores at several time intervals.

MATERIAL AND METHODS

Spore collection

Pleurotus ostreatus (Jacq. ex Fr.) Kummer spores (greater than 95% pure) were obtained from spore prints of field-collected caps. *Calvatia cyathiformis* (Bosc.) Morg. spores (about 80% pure) were removed from the interior of dry, mature puffballs collected in the Gulf South region. The nonspore material (about 20%) was capillitium, which is sterile hyphae from the interior of the fruit body. *Lentinus edodes* (Berk.) Sing. (greater than 95% pure) spores were harvested from spore prints of commercially obtained caps. *Psilocybe cubensis* (Earle) Sing. spores were purchased as a pure preparation (Florida Mycology Institute, Pensacola). The RAST potency was determined for eluates of intact spores from all four species. Additional quantities of *Calvatia* and *Pleurotus* spores were available and these were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) IgE immunoblot and assessed for total extractable yield. Spore purity was checked by light microscopy.

Spore extraction

Spore disruption. Spores were disrupted by vigorous shaking with glass beads in 0.125 mol/L NH_4HCO_3 with a Braun (Melsungen, Germany) homogenizer. This treatment was previously demonstrated to disrupt more than 95% of spores. The suspension was centrifuged (27,000 g); the supernatant was recovered and lyophilized. Lyophilized extract was stored desiccated until used.

Wetting intact spores. Spores were wetted in NH_4HCO_3 buffer by shaking them in the Braun homogenizer without glass beads. This treatment produced a uniform suspension of intact spores, yet had no discernible effect on spore morphology when the spores were viewed microscopically. Spores of *Pl. ostreatus*, *L. edodes*, and *Ps. cubensis* were all easily wet and uniform suspensions could be prepared with mild shaking. However, to standardize the extraction procedure, all spores were wetted by shaking them in the homogenizer without glass beads to disperse them in extraction buffer. This yielded a suspension of wetted, intact spores, which was stirred at 4° C until sampled.

Spore elution. Spores were suspended and then samples of the suspension were taken after 0, 2, 4, 8, and 24 hours of incubation and centrifuged; the supernatants were collected. Ultraviolet absorbance (280 nm) of samples (appropriately diluted) and pH were recorded. Supernatants were frozen and lyophilized. Pelleted spores were mounted in lactophenol/cotton blue and

examined by light microscopy; no changes were apparent in gross physical condition.

RAST inhibition

RAST discs were prepared by incubating cyanogen-bromide activated cellulose discs overnight in a solution of spore extract (from disrupted spores) in borate buffer at a concentration of 1 mg (dry weight) per disc, as described previously.⁸ Coated discs were washed the next day with ethanolamine and then stabilized in a solution of phosphate-buffered saline (PBS)/Tween 20 (Sigma Chemical Co., St. Louis, Mo.)/bovine serum albumin. Sera for RAST inhibitions were obtained after informed consent was obtained from atopic subjects who had positive results to a skin test to the basidiomycete being tested. Pools of equal volumes of sera from five subjects were used for each RAST. For RAST inhibition the discs were incubated with 50 μl pooled sera and 50 μl inhibiting antigen (10, 1.0, 0.1, 0.01, or 0.001 mg/ml of spore extracts in PBS). Results were presented as percent inhibition of a PBS control solution according to the formula $[1 - (\text{test solution}/\text{PBS})] \times 100$ as previously described.⁸

SDS-PAGE IgE immunoblot

Samples were analyzed by discontinuous SDS-PAGE (15% T/2.5% C) in a PROT RESOLV (Phase, Lubeck, Germany) minigel apparatus.⁹ Lyophilized samples were reconstituted in 1 ml PBS and diluted in SDS-PAGE sample buffer (1:4 for *Pleurotus* and 2:3 for *Calvatia*). Five microliters of each diluted sample were applied per lane. Semidry blots were prepared from the gels onto activated nitrocellulose.^{10, 11} Blots were overlaid with pooled patient sera (diluted 1:8 in PBS/Tween) overnight; bound IgE was labeled with ¹²⁵I anti-IgE (Sanofi, Chaska, Minn.) and visualized by autoradiography.

Electron microscopy

Spore samples of *C. cyathiformis*, *P. ostreatus*, *L. edodes*, and *P. cubensis* were separately embedded in molten 2% agar cooled to 45° C. Blocks (approximately 2 mm on a side) were cut from the solidified agar/spore mixture and fixed overnight in 2% glutaraldehyde in 0.2 mol/L (pH 7.2) cacodylate buffer.¹² Spores were post-fixed with 1% OsO_4 in cacodylate buffer for 1 hour, dehydrated in an ethanol series, and embedded in Spurr's resin.¹² Thin sections were cut with a diamond knife on an LKB Ultratome V (LKB, Piscataway, N.J.) and stained with 2.5% aqueous uranyl acetate followed by lead citrate.¹³ Specimens were viewed with a Hitachi H-700 transmission electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Spore extraction

It was difficult to wet and produce a uniform spore suspension with *Calvatia* spores. A uniform

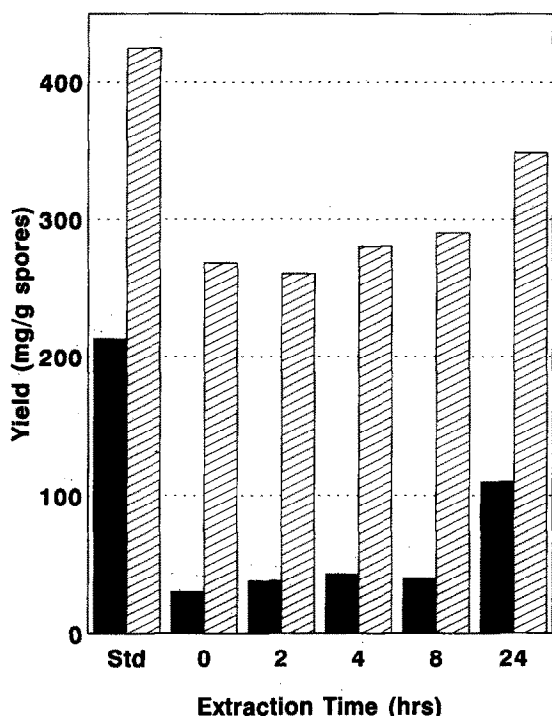


FIG. 1. Yield of allergen extracts from basidiospores. Standard (Std) extract was prepared from disrupted spores. Eluates were prepared by incubating wetted spores in buffer for 0, 2, 4, 8, and 24 hours.

spore suspension was not attained either by vigorous manual shaking, continuous stirring, or with wetting agents. Unwetted spores either remained clumped in dry pockets in the liquid or floated at the surface and thus could not be evenly dispersed through the extraction fluid. This problem was overcome by wetting all spores in the Braun homogenizer. Glass beads were omitted from the spores that were eluted intact. This method produced a uniformly wetted spore suspension and examination by light microscopy indicated that spores were intact and not discernibly changed.

The standard extraction process (shaking with glass beads) completely disrupted spores as determined by light microscopy. Release of material from intact spores varied. For example, intact *Pl. ostreatus* spores released from two to nine times more eluate (dry weight) than spores of *C. cyathiformis* did. Eluate yield (dry weight) from intact spores of both species increased to maximum values at 24 hours (Fig. 1). Intact spores of *Pl. ostreatus* and *C. cyathiformis*, respectively, yielded 63% and 14% of the yield of extract at 0 hours and 82% and 52% at 24 hours.

The pH values of the elution buffer for *Calvatia* changed from 8.02 to 8.15 and those for *Pleurotus* from 7.70 to 7.94. Release of ultraviolet (280 nm) absorbing material from *C. cyathiformis* increased

steadily over time. *Pleurotus* released 5- to 10-fold more ultraviolet absorbing material than *Calvatia*. The absorbance values for *Pleurotus* decreased by 34% at 2 hours, relative to the initial value, and then increased steadily to 38% higher than the initial value at 24 hours. The absorbance value for *Calvatia* initially was very low, but steadily increased over 24 hours, reaching 414% of the original value.

RAST inhibition

Allergen activity was released in two general patterns. Significant levels of RAST inhibitory activity were present in eluates of *C. cyathiformis* and *Ps. cubensis* spores only after 8 hours (Fig. 2). Even after 24 hours, the eluates from these two species did not have as much inhibitory activity as the extract from disrupted spores (Fig. 2). Conversely, *Pl. ostreatus* and *L. edodes* eluates from short elution periods had substantial RAST inhibitory activity comparable to the inhibitory activity in disrupted spore extract. Thus one pattern was rapid and complete, whereas the second pattern required 8 hours or more for partial allergen release.

Immunoblots

Allergens were present, but faint, in the eluate at 0 hours of *C. cyathiformis*. Allergen staining intensity in eluates from intact spores remained relatively weak until 24 hours (Fig. 3). Band intensity in the eluate at 24 hours appeared similar to that for extract from disrupted spores. Allergen staining intensity from eluates of intact spores of *Pl. ostreatus* at 0 hours was comparable to that of the extract from disrupted spores (Fig. 4). As suggested by the RAST inhibition activity of these eluates, allergen bands were intense even after short elution periods.

Electron microscopy

The spore wall structures of these species were compared by examination of ultrathin sections by transmission electron microscopy (Fig. 5). This examination revealed that *C. cyathiformis* and *Ps. cubensis* spores had thick electron-dense wall structures. By contrast, the spore walls of *Pl. ostreatus* and *L. edodes* were very thin, unornamented, and not electron-dense. The spore wall of *Ps. cubensis* was about 22 to 24 times thicker than the walls of *Pl. ostreatus* and *L. edodes*. The intracellular structure of the thin-walled species, *Pl. ostreatus* and *L. edodes*, was also much better resolved than was the intracellular structure of the thick-walled species.

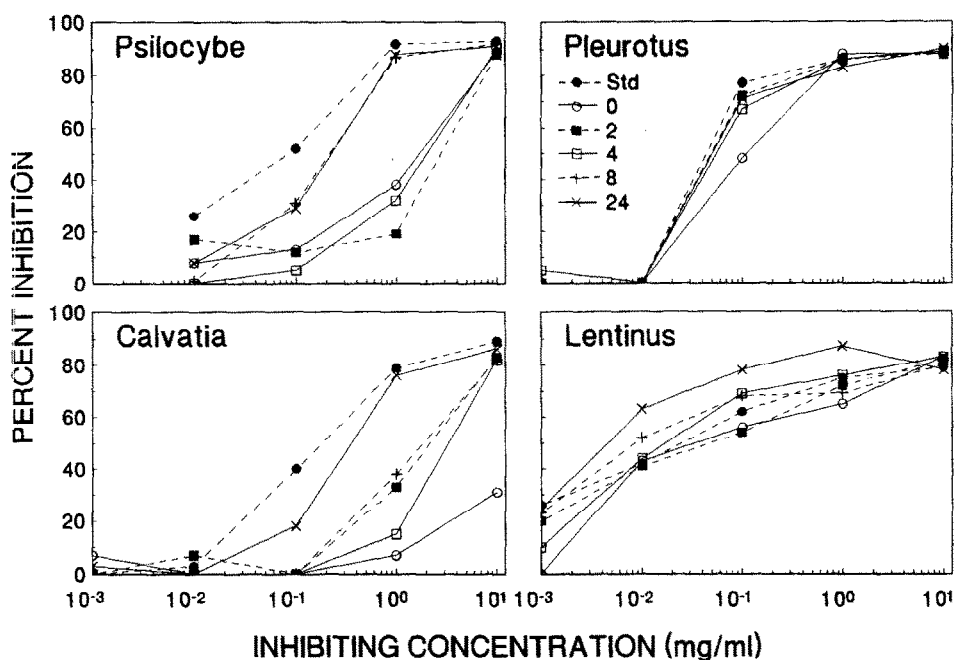


FIG. 2. RAST inhibition of spore extracts from *Ps. cubensis*, *Pl. ostreatus*, *C. cyathiformis*, and *L. edodes*. RAST discs were coupled with standard extract (disrupted spores) for each species. RAST was then inhibited with standard (Std) extract and with eluates harvested from incubations of 0, 2, 4, 8, and 24 hours from that species.

DISCUSSION

Allergen activity was eluted from intact spores of all species tested. These species are from three families and hence this release appears to be a general feature of basidiospores. Extracts of disrupted spores had greater yields, on a dry weight basis, than extracts of intact spores.

Two patterns of allergen release were seen. As compared with extractable activity from disrupted spores, one pattern was a full, rapid release of activity and one was a slow, partial release of activity. These patterns appeared to correlate with spore wall structure. The present study included *Ps. cubensis* and *C. cyathiformis*, which have thick walls, and *Pl. ostreatus* and *L. edodes*, which have very thin walls. Allergens were eluted from thin-walled spores more quickly and in greater quantity than from the thick-walled species. Different patterns have also been reported for allergen release from conidia of *Cladosporium* and *Alternaria*.^{14, 15} Optimal incubation times were 15 minutes for *Alternaria*, but maximal allergen release required 4 hours for *Cladosporium*. Thus species-related differences in allergen release may be common to all fungal groups.

There are other important differences in the wall structure of the spores in these species. *Ps. cubensis* has melanized spores and *C. cyathiformis* has prominently ornamented spores. Each

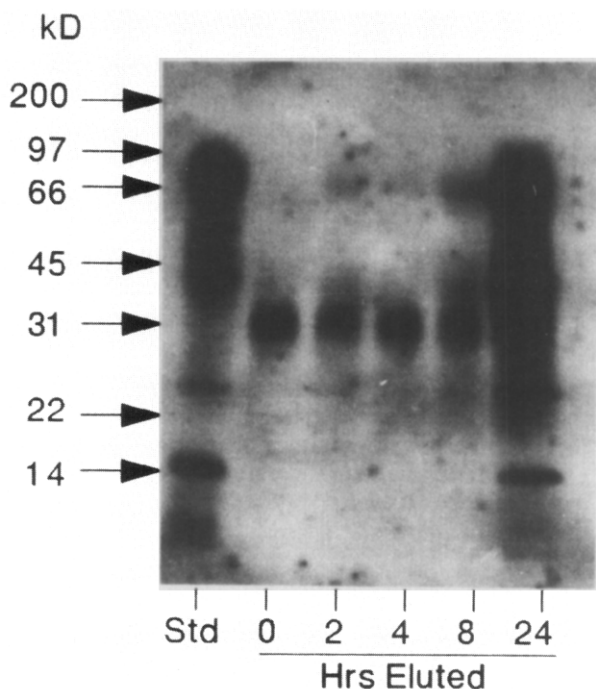


FIG. 3. SDS-PAGE IgE immunoblot analysis of *C. cyathiformis* extractions from disrupted (Std) spores and eluates from intact spores incubated for 0, 2, 4, 8, and 24 hours.

of these features is a potentially confounding factor of the correlation between thin spore walls and rapid allergen release. A determination of the

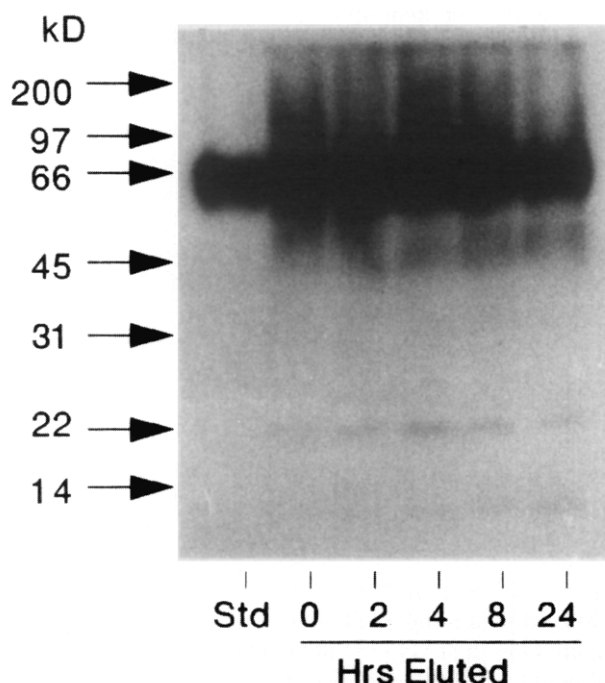


FIG. 4. SDS-PAGE IgE immunoblot analysis of *Pl. ostreatus* extractions from disrupted (*Std*) spores and eluates from intact spores incubated for 0, 2, 4, 8, and 24 hours.

importance of these other factors will require assessment of additional species. Basidiospore walls also are impregnated with lipids. All spores used in the present study were defatted with ethyl ether before use; hence, lipids apparently do not determine the allergen release pattern.

The relative protein content of the spores in our study is another variable. Wall material is a greater portion of the thick-walled spores, as seen in the micrographs in Fig. 5. Because wall material contains relatively low amounts of protein (2% to 10%) compared with the amount of cytoplasm,^{16, 17} thin-walled spores contain more protein per spore than comparably sized thick-walled spores. Differences in spore protein content might account for the different total yields from thin- and thick-walled spores, but would not explain the different release rate of allergen activity.

Basidiomycete and ascomycete cell walls consist of layers of chitin microfibrils embedded in a β -glucan-protein matrix.^{16, 18} The absence and different thicknesses of the layers account for the spore wall differences. The episporium, the thick middle layer, may be up to 1 μ m thick in *Psilocybe* but is greatly reduced in spores of *L. edodes* and *Pl. ostreatus*; these also are devoid of the outermost layer.^{19, 20} Thicker spore walls could retard

and reduce allergen release simply by adsorbing allergens. Alternatively, the thicker wall may lend additional structural integrity to prevent membrane damage and allergen leakage. Support for either possibility will require study of additional species.

Pl. ostreatus and *L. edodes* are in one family (Tricholomataceae), *Ps. cubensis* is in a different family, and *C. cyathiformis* is in a separate order (puffballs).²¹ Thus rapid allergen release might occur only in the Tricholomataceae. However, the allergens in all three families cross-react allergenically.^{4, 22} Thus it is unlikely that a general feature such as allergen release would be completely unique to any one family if the allergens being released share epitopes among families.

The location of allergens before release is of some interest. Presumably basidiospore allergens originate in the cytoplasm because basidiospore production does not occur inside another cell. It is feasible, however, that allergens are deposited in the wall before release, inasmuch as fungal walls do contain protein (up to 10%).

Allergen secretion in the lung by *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis is very different from allergen deposition caused by inhaled basidiospores. However, a recent study used sera from patients with allergic bronchopulmonary aspergillosis to immunolabel and detect the location of allergens, which were found in the cytoplasm, cell membrane, and cell walls of hyphae and spores of *A. fumigatus*.²³ Thus allergens occur in *Aspergillus* fungal walls, even if only transiently as they are released. The greatest concentration of allergen was in the plasma membrane at the hyphal tip. The hyphal tip is the area with the greatest metabolic rate in the mycelium, thus presumably is the site of synthesis. Indirect evidence from *Cladosporium* also suggests that allergens may be released from the outer wall layers and also from internal (inner wall or cytoplasm) compartments of the conidia.¹⁴

Because exposure to liquid is important, the permeability of the spore wall could be an important factor that determines allergen release. Ultrastructural studies of rust teliospore development noted that as spores matured, the wall thickened and fixation difficulties increased; these occurrences were attributed to decreased permeability of the thicker wall of more mature spores.²⁴ This is only indirect evidence, but it suggests that wall permeability and solute flow through the walls decrease in thicker spore walls.

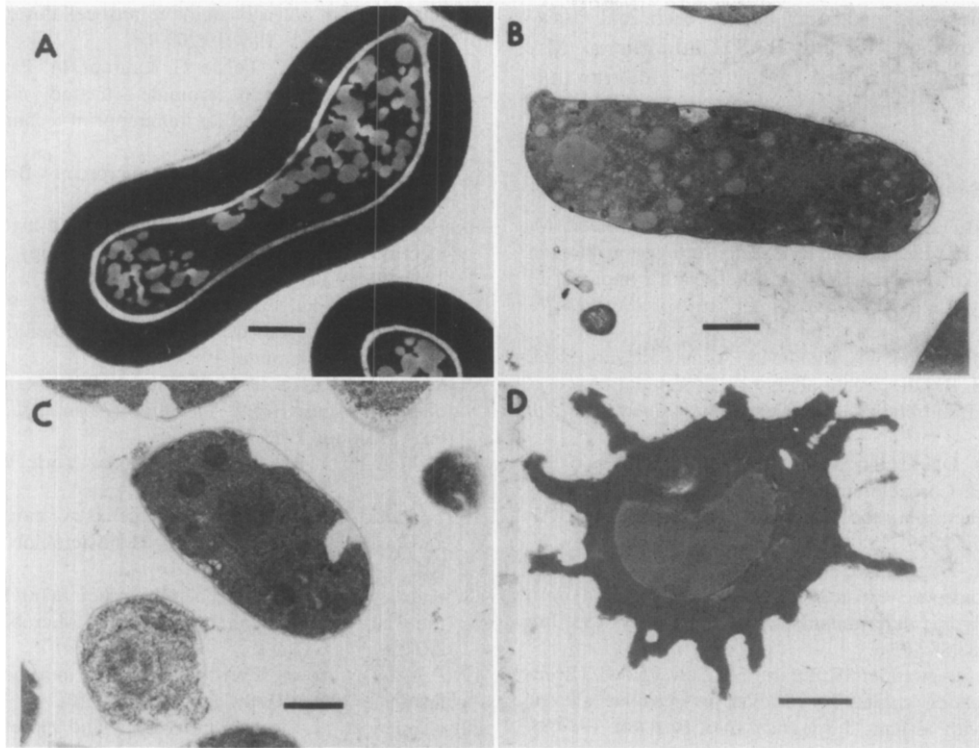


FIG. 5. Transmission electron micrographs of ultrathin sections of basidiospores. Scale bars indicate 1 μm . General dimensions (length $\times$ width) and wall thickness follow species names. **A**, *Ps. cubensis* (9 to 14 $\times$ 5 to 9 μm , about 1000 nm). Note thick spore wall. **B**, *Pl. ostreatus* (7 to 11 $\times$ 2.5 to 4 μm , about 50 nm). Note thin spore wall and excellent preservation of organelle structure. **C**, *L. edodes* (6 to 7 $\times$ 2.5 to 3.5 μm , about 50 nm). Note thin spore wall. **D**, *C. cyathiformis* (4 to 6 μm diameter, about 900 nm). Note thick spore wall and echinulate surface.

Differences in releasability of antigens might affect IgE production or even determine the allergenicity of antigens. Rapid versus slow release of allergens could determine the proportion of subjects that respond to an allergen, that is, speed of release could determine which are the major (or minor) allergens. Such concepts remain speculative, but a clear indication either way would contribute to our understanding of the immunopathogenesis of asthma.

The greater and more rapid release of allergen from thin-walled spores should be clinically significant. Spores that impact on surfaces within the bronchial tree are cleared by muco-ciliary flow within 6 hours.²⁵ Thus slow release of allergenic activity from thick-walled spores should spread allergen along a swath of bronchial epithelium. Conversely, the large, rapid release of allergen from the thin-walled spores should concentrate allergen in a small area over a short time. This variable allergen deposition might be a clinically

significant difference between thin-walled and thick-walled spores.

The present study demonstrated significant differences between the allergen-release pattern of thin-walled and thick-walled basidiospores. If this difference is a general feature in basidiomycetes, then significant clinical differences may exist among types of basidiospores. This feature requires confirmation with a larger number of species. However, if valid, this feature would aid in the identification of relevant allergenic fungi. Conversely, such a distinction would allow clinically less important spore types to be disregarded. Concentrating on more important spores would increase the value of aeroallergen monitoring efforts. The recognition of such differences would also be important for studies with intact spores, such as bronchoprovocation challenges. Finally, in the laboratory, disruption of thin-walled spores for extract preparation appears unnecessary.

Margie McCants provided expert technical assistance with extractions and RAST inhibitions. Mr. Richard Portman assisted with the electron microscopy.

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