Spores as infectious propagules of *Cryptococcus neoformans*

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Abstract

_Cryptococcus neoformans_ and _Cryptococcus gattii_ are closely related pathogenic fungi that cause pneumonia and meningitis in both immunocompromised and immunocompetent hosts and are a significant global infectious disease risk. Both species are found in the environment, and acquired via inhalation leading to an initial pulmonary infection. The infectious propagule is unknown, but hypothesized to be small desiccated yeast cells or spores produced by sexual reproduction (opposite or same sex mating). Here we characterize the morphology, germination properties, and virulence of spores. Comparative morphological analysis of hyphae and spores produced by opposite sex mating, same sex mating, and self-fertile diploid strains was conducted by scanning electron microscopy, yielding insight into hyphal/basidial morphology and spore size, structure, and surface properties. Spores isolated by microdissection were found to readily germinate, even on water agarose medium. Thus, nutritional signals do not appear to be required to stimulate spore germination, and as yet unknown environmental factors may normally constrain germination in nature. As few as 500 colony forming units of a spore-enriched infectious inocula (~95% spores) of serotype A _C. neoformans_ var. _grubii_ were fully virulent (100% lethal infection) in both a murine inhalation virulence model and the invertebrate model host _Galleria mellonella_. In contrast to a previous report on _C. neoformans_ var. _neoformans_, spores of _C. neoformans_ var. _grubii_ were not more infectious than yeast cells. Molecular analysis of isolates recovered from tissues of infected mice (lung, spleen, brain) provides evidence for infection and dissemination by recombinant spore products. These studies provide a detailed
morphological and physiological analysis of the spore, and document that spores can serve as infectious propagules.
Introduction

Humans are exposed to infectious agents via inhalation, cutaneous exposure, and from the microbiota. Transmission can involve direct human-human, intermediate animal or insect vectors, or exposure to environmental sources. Animals are exposed to pathogenic fungi via direct/fomite transmission of dermatophytes that infect skin/nails, animal-animal transmission by inhalation of the obligate pathogen *Pneumocystis*, and bloodstream penetration by *Candida* species from the gastrointestinal microbiota (46).

Many pathogenic fungi (dimorphic fungal pathogens, molds such as *Aspergillus fumigatus*, and the basidiomycete *Cryptococcus*) are environmental and exposure is by inhalation of conidia/spores, hyphal fragments, or yeast cells. Particles >5 microns are subject to efficient mucociliary airway clearance and smaller infectious propagules more readily deposit deep in the lungs/alveoli (18). As such, spores/conidia represent known or suspected infectious propagules for many pathogenic fungi. Moreover, as spores are often stress-resistant, abundant, and readily aerially dispersed, animals may more often encounter spores than other infectious forms. Studies of *Schizophyllum commune* reveal abundant spores present in air above the ocean at distances up to a mile from shore (24).

*Cryptococcus neoformans* is a common, opportunistic human fungal pathogen that causes meningoencephalitis in immunocompromised individuals and, if untreated, is uniformly fatal (6, 40). Cryptococcosis is caused by three varieties or sibling species that diverged 10–40 mya, and exhibit different environmental distributions and virulence properties (32, 43). *C. neoformans* var. *grubii* (serotype A) is the major cause (95%) of infections worldwide and >99% of infections in AIDS patients (6). *C. neoformans* var. *neoformans* (serotype D) strains account for <5% of infections worldwide but serotype
AD hybrids cause up to 20% of clinical infections in Europe (2, 9, 31). The serotype A and D lineages are globally distributed, associated with pigeon guano, and typically infect immunocompromised hosts.

The closely related species *Cryptococcus gattii* (serotypes B and C) is more geographically restricted to tropical/subtropical regions, associated with trees, and commonly infects immunocompetent hosts and less frequently AIDS patients and other immunocompromised hosts (6, 29). A *C. gattii* outbreak has been occurring on Vancouver Island since 1999, and recently expanded to the Canadian mainland and the United States (5, 12, 25, 38, 52).

The infectious propagules for many human fungal pathogens are thought to be spores. Humans are exposed to *Cryptococcus* by inhalation leading to an initial pulmonary infection that can be asymptomatic or limited, or can disseminate. In many individuals, the initial pulmonary infection is cleared; in others, the organism establishes a dormant latent granulomatous form in the hilar lymph nodes (10, 17). Individuals can die harboring granulomas without overt disease (1, 19, 48). In response to immunosuppression, either primary infection or recrudescence of latent infection results in dissemination via the bloodstream. The organism can infect many organs and tissues, but exhibits a predilection to infect the central nervous system (45). The suspected infectious propagules for Cryptococcus are spores or small, desiccated, less encapsulated yeast cells that are an ideal size for alveolar deposition. *C. neoformans* var. *neoformans* serotype D spores have been reported to be up to 100-times more infectious than yeast cells in an immunocompromised murine inhalation model (50). Studies to purify spores and analyze their pathogenicity in animal models using different infection methods have
been previously reported, documenting that spores can serve as infectious propagules under certain conditions, including when directly inoculated intracerebrally, intravenously, or by inhalation (7, 50, 59).

*Cryptococcus neoformans* and *C. gattii* have defined sexual cycles that produce abundant basidiospores, which could represent infectious propagules (13, 23, 27, 28, 39, 42). However, if spores are the infectious propagule, and spores are produced by mating between α and α cells, how and where would this occur in the largely α unisexual population? A potential resolution was the discovery of that α isolates can produce hyphae, basidia, and basidiospores via monokaryotic fruiting (11, 54), a modified sexual cycle that only requires one of the two mating types (4, 12, 21, 33-36, 49). This might be a route by which infectious propagules are produced in nature; however, serotype A var. *grubii* and *C. gattii* strains do not reproducibly undergo monokaryotic fruiting under laboratory conditions. Population genetics studies provide evidence they do so in nature (4, 21, 36, 49), but this remains to be established under defined laboratory conditions.

As most previous studies on spore infectivity focused on serotype D, we focused investigations on serotype A spores and virulence properties. Our analysis examined spore size, shape, and surface properties, and conditions supporting spore germination. Importantly, we demonstrate infectious inocula highly enriched for spores serve as efficient infectious propagules in both a murine inhalation model and an invertebrate host. Analyses of isolates recovered from infected animals provide evidence recombinant spore progeny are infectious. Spores were not more virulent than yeast cells in either virulence assay. Taken together, our studies provide electron microscopic views of the spore and demonstrate that both spores and yeast can serve as infectious propagules.
Materials and methods

Strains. Serotype A strains H99 (αA) (44), KN99a (αA) (42), Bt63 (αA) (37), diploid KN2B5#19 (αAAα) (42), serotype D strains JEC20 (αD) and JEC21 (αD) (20, 30), XL280 (αD) (34), and C. gattii strains WM276α (αB, VGI), R265α (αB, VGII), B4546a (αC, VGIII), NIH312α (αC, VGIII), and MMRL2651α (αC, VGIV) (12) were used in mating assays and scanning electron microscopy (SEM) analysis. For matings, equal amounts of a and α cells were mixed together with a flat end toothpick on MS medium (57) and incubated at room temperature in the dark for 2 to 4 weeks until spore chains were visible under the microscope at the culture edges.

Scanning Electron Microscopy. Mating cultures were excised from the agar plate around the colony edge and fixed in 3% glutaraldehyde in 0.1 M Na cacodylate buffer, pH=6.8 for several days at 4°C. Samples were then rinsed in three 1-hour changes of cold 0.1 M Na cacodylate buffer, pH=6.8 followed by a graded dehydration series of 1-hour changes in cold 30% and 50% EtOH and held overnight in 70% EtOH. Dehydration was completed with 1-hour changes of cold 95% and 100% EtOH at 4°C warming to room temperature in 100% EtOH. Two additional 1-hour changes of room temperature 100% EtOH completed the dehydration series. The samples were then critical point dried in liquid CO₂ (Samdri-795, Tousimis Research Corp., Rockville MD) for 15 minutes at the critical point. The agar pieces were mounted on stubs with double stick tape, pressed down completely around the edge, and then sealed with silver paint to ensure good conductivity. The samples were sputter coated with 50Å of Au/Pd (Hummer 6.2, Anatech
U.S.A., Hayward CA). Samples were held in the vacuum desiccator until viewed with a scanning electron microscope (JEOL JSM 5900LV) at 15 kV. Spore sizes were calculated by measuring at least fifty individual spores from each cross for all the samples. Statistical significance was determined by calculating P-value by using the t-test provided by QuickCalcs (GrapPad online software).

**Basidiospore isolation.** To isolate basidiospores, strains from opposite mating type H99α and KN99a (serotype A) or JEC20α and JEC21a (serotype D), were mixed in equal proportions on MS medium plates with flat end toothpicks. Matings were conducted at room temperature in the dark for 2 to 4 weeks until robust sporulation was observed by microscopy. Basidiospores on the edges of a mating colony were removed with a glass Pasteur pipet and transferred to a YPD plate, and individual basidiospores were transferred to fresh YPD medium by microdissection, as described previously (22). Isolated basidiospores were incubated at 30°C for 2 to 3 days to allow the spores to germinate, and the resulting yeast colonies were subcultured in fresh YPD medium. To determine the mating type, isolates were co-cultured with the reference tester strains, JEC20a and JEC21α grown on V8 (pH=7) medium in the dark at room temperature (30). An isolate and tester strains were cultured alone on the same plate as controls. The mating reaction plates were examined for the formation of mating hyphae, which signaled the initiation of sexual reproduction. Genomic DNA extractions followed a procedure previously reported (51). Mating and mitochondrial DNA inheritance were determined by PCR analysis using specific primers for STE20a (5'-
ATCAGAGACAGAGGAGCAAGAC, 5'-CTAACTCTACTACCTCACGG); 
STE20α (5'-AGCTGATGCTGTGGATTGAATAC, 5'-TGCAATCACAGCACCTTAC 
ATAG)(35) and mitochondrial DNA inheritance by using Da3/Da20 (5'- 
GCAATAGCATATACCATCCCG, 5'-GACACTACACAAGATGCCTC) and the PCR 
conditions were as described previously (35, 51).

Germination studies. Spores were dissected by micromanipulation from mating cultures 
as described previously (22). To determine the signals that trigger spore germination, 
spores were dissected onto different media with limiting nutrients, including carbon and 
nitrogen sources. Plates were observed by microscopy at regular 6 to 8 hr. intervals for 
microcolonies to determine the germination efficiency. Ten to twenty individual spores 
from the cross between H99α and KN99α were dissected onto 2% water agar medium 
with no added nutrient, carbon, or nitrogen sources. Plates with dissected spores were 
incubated at 30°C and observed under the microscope at regular time intervals. In order 
to completely eliminate nutrients, 2% agarose water medium was prepared and the 
germination efficiency of spores observed. To determine the conditions favoring spore 
germination, agarose medium plates were incubated in inverted position at 30°C, and 
control plates with dissected spores were incubated in the standard upright position. In 
another set of experiments, spores containing agarose plates were parafilm wrapped to 
limit CO2 escape and to determine the effect of CO2 accumulation on germination, and 
control plates were incubated without parafilm. To determine the effects of carbon and 
nitrogen source on spore germination, spores were dissected onto 2% agarose media 
supplemented with 2% glucose, and 10 mM cAMP separately in individual plates. Also
to analyze an effect between fungal and bacterial cultures, which might influence fungal development by releasing quorum sensing molecules, we used bacterial cultures of *E. coli* strain OP50 or *Salmonella enterica* strain SL1344 grown in FB medium overnight. Agarose plates were prepared by mixing 2% agarose in culture supernatant and spores were dissected onto these plates directly by micromanipulation. Plates were incubated at 30°C and microcolonies were observed at the place of spore deposition. We further compared the germination rate between the serotype A and serotype D spores by dissecting the spores onto 2% water agarose medium from the mating crosses between H99α and KN99a (serotype A) and JEC21α and JEC20a (serotype D), incubating at 30°C until the colonies were formed, and observation by microscopy at regular intervals.

**Murine virulence studies.** Basidiospores were isolated carefully from the edges of mating colonies between H99α and KN99a on MS medium with a glass Pasteur pipet tip without touching the surrounding yeast colonies, and suspended into Eppendorf tubes containing PBS. Yeast cells (H99α and KN99a) were grown overnight on MS medium for the same time as mating cultures and harvested and washed with PBS. The inocula size was confirmed by counting cells in a hemocytometer and by CFU determination.

Animals were infected as previously described (8). Groups of 4- to 6-week-old female A/JCr mice were anesthetized by intraperitoneal injection of phenobarbital (~0.035 mg/g). Five or ten animals for each sample of spores, and H99α and H99α/KN99a co-infections (1:1 ratio, both from medium plates and overnight cultures) were infected by carefully instilling an inocula of 500 colony forming units each in 50 µl of PBS by directly dropping into the nares using a pipette man to imitate an inhalation
mode of infection (42). After inoculation of fungal cells, animals were monitored twice daily, and the animals showing signs of severe morbidity (weight loss, extension of the cerebral portion of the cranium, abnormal gait, paralysis, seizures, convulsions, or coma) were sacrificed by CO\textsubscript{2} inhalation. Survival was plotted against time, and P-values to determine significance of survival curves were calculated by plotting Kaplan-Meier survival curves and performing the log-rank test using Prism software (Version 4.0a, Prism Computational Sciences, Incorporated, Madison, Wisconsin, USA).

To determine the number of recombinants inhaled by the animals and their dissemination to cause infection, the lungs, spleen, and brain from two animals from each group were harvested, weighed, and homogenized in 5 ml of sterile PBS. Serial dilutions of the organ samples were plated on SD agar plates containing 100 \(\mu\text{g/ml}\) chloramphenicol and incubated at 37°C overnight. Colonies were tested for mating types (\(\alpha\) and \(\alpha\)) both by PCR using \(\text{STE20}_\alpha\) and \(\text{STE20}_\alpha\) specific primers (51), and by mating the isolates with tester strains (JEC20\(\alpha\) and JEC21\(\alpha\)) on V8 (pH=7) medium incubated in dark at room temperature. Mitochondrial DNA inheritance was determined by PCR with primer pair \(\text{Da3}/\text{Da20}\) (51).

\textit{Galleria mellonella} infections. The greater wax moth virulence assays followed a protocol previously reported (41). Greater wax moth larvae were purchased from Vanderhorst, Inc. (St. Marys, Ohio). Larvae from a single shipment were used for each experiment. An inoculum of 500 yeast cells (1:1 \(\alpha\) and \(\alpha\)) or a spore-enriched preparation (500 CFU’s, ~93% spores) were injected into the larvae in the posterior pseudopod and
the larvae were incubated at 37°C and at room temperature/dark post inoculation. Larvae were examined daily, and those not responding to touch were scored as inviable.

**Macrophage uptake of *C. neoformans* cells.** J774A.1 cells were removed from T75 flasks by scraping, centrifuged at 1000x g for 8 min., and resuspended in complete medium. Live cells were counted with a hemocytometer using Trypan Blue exclusion to identify dead cells. Cells were then adjusted to a concentration of 1 x 10⁵ cells/ml, and 300 µl of this suspension was plated in 8-well coverslip chambers at a concentration of 1 x 10⁵ cells/ml, allowed to adhere, and activated with 300 µl fresh complete medium containing 100 ng/ml LPS (serotype 0111:B4) and 200 U/ml mouse interferon-gamma (R&D Systems) overnight. *C. neoformans* cells of strain H99α or spores (H99α and KN99a mating) were washed three times and resuspended in sterile PBS at a concentration of 2 x 10⁶ yeast/ml. Yeast cells were then incubated with 2 μg/ml of 18B7 anti-capsular antibody or alone for 1 hr at 37°C shaking in an Eppendorf Thermomixer 5436 (Eppendorf, Westbury NY). For macrophage infection, 10 µl of pre-treated yeast cells (20,000 yeast cells) were inoculated into wells containing activated macrophages at time = 0. The number of yeast cells or spores was chosen to represent a Multiplicity of Infection (M.O.I.) of 1:1, with the assumption that the macrophages doubled overnight (53). Data represent experiments performed at least three times with similar results.

**Time lapse microscopy.** Time lapse microscopy was performed using a Zeiss Axiovert Time Lapse station (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped with a Hamamatsu Orca AG camera (Hamamatsu, Bridgewater, NJ). An exposure of 240 was
used along with an LD acroplan 40X objective for optimal brightfield cell imaging. Images were obtained for five independent fields per well at 1-minute time intervals for a total of 120 min. Movies were compiled using Metamorph software.
Results

Morphology of *C. neoformans* and *C. gattii* spores produced by mating and fruiting

A detailed analysis of hyphal morphology and spores produced during *C. neoformans* and *C. gattii* sexual reproduction was conducted by scanning electron microscopy (SEM) (Figure 1, see also Supplemental Figure 1). Hyphae, basidia, and spores produced by serotype A x A, D x D, B x C and C x C strain crosses were analyzed and compared. These matings involve an initial cell-cell fusion, and the resulting zygote then forms a filamentous dikaryon that produces hyphae decorated with fused clamp connections, basidial fruiting structures, and long chains of basidiospores emanating from the basidial surface.

Hyphae terminated in basidia that were globular at the apex and cylindrical at the base. Four independent basidiospore chains were seen arising from the basidial surfaces, which were otherwise smooth (Figure 1). Sterigmata were not observed on the basidial surface for any of the crosses examined. The spores have rough surfaces, are elongated, and form four long, intact chains unless disrupted. Serotype A and D spores were 1 µm wide but differed modestly in length. Spores from serotype A strains (H99α x KN99α (VNI x VNI) or (H99α and Bt63α (VNI x VNB)) were rough and 2 to 3 µm long (Figures 1 and 3). Spores produced by a self-fertile aAAα diploid strain (KN2B5#19) were smoother, slightly curved at the tip, and shorter (2 to 2.5 µm long). Spores from crosses of congenic serotype D strains (JEC21α x JEC20α) were 2 to 2.5 µm long (Figures 1 and 3). Around fifty individual spores from each cross were measured to determine the spore size from all the samples. Statistical significance was determined by calculating the P values where the two-tailed P-value equals to 0.0012. Both serotype A and D spores
produced by mating had a roughened, prominent surface, with numerous spike-like surface protrusions, which may represent novel cell surface proteins or carbohydrates (cell wall/capsule). The spores produced by monokaryotic fruiting of the serotype D strain XL280α were roughened but modestly longer than those produced by serotype D a-α mating, ranging from 2 to 3 µm in length and 1 to 1.25 µm in width. As shown in Figure 3 and Supplemental Figure 1, the spore shape was asymmetric, and rounded on the basidial distal end and flattened on the basidial proximal end. Spores in chains were always arranged in a head to tail fashion. These differences in the spore ends likely reflect the process of budding and the action of specific hydrolytic enzymes that lead to spore-spore, and spore-basidial, separation. In summary, spores produced by mating and monokaryotic fruiting of serotype A and D strains are morphologically similar with a rough surface, and have similar, albeit not identical sizes and shapes.

Interestingly, no capsular staining was observed in our studies using anti-GXM antibody in spores from serotype A mating cultures, (see Supplement Figure 3). Whereas, control yeast cells showed affinity towards anti-GXM antibody, suggesting the presence of polysaccharide surrounding the yeast cell surface (see Supplemental Figure 3).

Interestingly, spores from Cryptococcus gattii mating are considerably more elongated compared to C. neoformans var. grubii (serotype A) and C. neoformans var. neoformans (serotype D) (Figures 1 and 2). Spores from C. gattii matings were dramatically elongated, in some cases slightly curved, and with a smooth surface (Figure 2). In C. gattii mating cultures, four basidiospores arise from the basidia and elongate during their development. Long spore chains were rarely seen in this species, possibly because the elongated spores are more readily discharged from the chains. No sterigmata
on the basidia were observed in these crosses, similar to the findings with *C. neoformans*. Different *C. gattii* molecular types were compared to spores produced by serotype A and D strains (see Supplemental Figure 1). In all cases, *C. gattii* spores were much longer in size, ranging from 3 to 5 µm in length and 1 µm in width. Control yeast cells differed in size and structure to spores produced by both mating and fruiting. Yeast cells in all of the backgrounds measured ~5 µm or larger, and were round and encapsulated. Taken together, our findings are in accord with previous and recent studies (3, 47) and reveal additional insights into spore morphology.

**Spore germination signals**

We next addressed signals that trigger spore germination. Typically, microdissected spores are germinated on rich YPD medium for genotypic and phenotypic characterization. To determine signals influencing spore germination, different media were tested with limited nutrients, such as reduced carbon and nitrogen sources. Germination efficiency was analyzed with spores isolated from H99α and KN99α matings by micromanipulation. On distilled water 2% agar medium plates with no added nutrients, spores were isolated by micro dissection and incubated at 30°C. Spores were observed by microscopy at 6 to 8 hr intervals and the production of 2 to 3 cell microcolonies was scored as indicative of spore germination and yeast cell division. Under these conditions, spore germination was modestly delayed (<48 hours) compared to YPD medium (<24 hours), but there was no difference in the frequency of spore germination (~70-80%) and within one week of incubation microcolonies visible to the naked eye were produced (see Supplemental Figure 2). To test whether spore
germination might result from trace contaminating nutrients in agar, similar studies were conducted on distilled water medium prepared with 2% agarose. Spores microdissected onto 2% agarose medium with no added nutrients or carbon and nitrogen sources still germinated (within 48-72 hrs) to form microcolonies with similar efficiency. Addition of 10 mM cAMP to this medium had no effect. Thus, it appears that there is little to no obligatory nutritional signal required to trigger spore germination under these experimental conditions.

We next tested if conditions in nature or the laboratory normally constrain spore germination. We tested spores on agarose medium plates incubated under different conditions, including parafilm-sealing plates to restrict air flow and increase CO₂ levels, incubation upright vs. inverted to alter humidification and dessication, and using conditioned medium from bacterial cultures. None increased or decreased spore germination. We conclude spores are self-sufficient for nutrients required for germination and germinate under a variety of environmental conditions. On the other hand, intact spore chains produced from mating remain dormant on mating medium for months. Thus, other signals, such as contact sensing, may inhibit germination in spore chains, or stimulate germination when an aerial spore lands on a solid surface in nature.

**Spores are aerosolized by wind but not by agitation**

In considering if spores serve as infectious propagules, a key question is whether spores become airborne. Previous studies provide evidence that particles small enough to be spores are present in air, but whether these are spores, or small desiccated yeast cells, or both, is unknown (14, 25). Serotype A mating mixtures and control yeast cells on the
surface of V8 solid medium were exposed to different conditions to test if spores are released from mating reactions. No CFU were observed when mating reactions or control yeast cells were gently agitated overnight or vigorously vortexed for 2 minutes above fresh YPD medium, indicating spores are not readily released.

We next tested if exposing plates to an airstream mimicking wind would trigger spore release. Mating and yeast cultures on MS media were exposed to a standard laboratory airstream and the resulting cells were trapped onto a closely attached fresh YPD agar plate. Both mating and yeast culture plates exposed to an airstream led to release and capture of CFU. Seven hundred forty two CFU were captured from the mating plate while only 24 CFU were captured from the control yeast plate. Thirty two CFU derived from the mating plate were randomly selected and analyzed for genomic and mitochondrial markers. Among the 32 isolates analyzed, cells that were \( \text{MAT} \alpha \) (12 isolates) all exhibited the \( \text{MAT}a^- \) type mitochondria. These results suggest that although both spores and yeast cells are readily aerosolized by wind, spores can be discharged at a higher efficiency (~20 fold). These findings provide evidence as to how spores might become airborne infectious propagules in nature.

**Spore purification attempts and successful approach**

For further studies, we investigated a variety of different approaches to separate spores and yeast cells of serotype A. We focused on serotype A spores, as these are the cause of >95% of cryptococcal infections globally. We first tested filtration approaches based on size. Mating mixtures with spores, hyphae, and yeast cells were passed through a filter with a 3 µm exclusion size. We hypothesized that the larger yeast cells would be
retained on the filter because of their greater size (>5 µm) and spores would pass through the filter yielding a spore-enriched preparation. Spores were detected as recombinant products of mating using serotype A mating parents with dominant drug resistance markers, such that spores could be distinguished from parental yeast cell genotypes. Using this approach, we observed a high proportion of contamination attributable to yeast cells of the parental genotypes (data not shown), and attribute this to the presence in mating cultures of a high proportion of smaller yeast cells of a size similar to that of spores, which may result from desiccation promoted mating.

Second, in attempt to separate serotype A spores and yeast cells based on size and buoyancy, step and/or discontinuous gradients were used under a variety of conditions. Commercially available gradients (Percol, Renografin) and lab prepared sucrose gradients were used at concentrations from 40-90% gradients. None yielded sufficiently enriched spores to eliminate contamination by yeast cells (not shown).

Third, selective killing of yeast cells at different temperatures for varying incubation periods was tested. Treatment of a mixture of spores and yeast cells at higher temperature (i.e. 55°C for 10 minutes), resulted at best in ~80% spore preparations, but this level of enrichment varied (data not shown).

As a final approach to obtain purified spores, we took a novel tact. A standard micromanipulation procedure, similar to that used to microdissect individual spores from mating cultures, was adapted to obtain sufficient quantities of spores to analyze virulence. Spore chains from mating culture edges were carefully dissected using the tip of a capillary glass tube and then re-suspended in water or PBS for further analysis. By
microscopic inspection, these samples contained a large proportion of spores (data not shown).

The proportion of spores in these preparations was estimated by germinating CFU, isolating genomic DNA from isolates, and subjecting this to PCR analyses for mitochondrial DNA inheritance ($Da_3/Da_20$), mating type ($STE20\alpha$ and $STE20\alpha$), and genotyping using sequence specific primers. The parental strains H99$\alpha$ and KN99$\alpha$ are congeneric, but differ at the mating type locus, harbor unique mitochondrial genomes (51), and differ in one other genomic region. KN99$\alpha$ (and KN99$\alpha$) harbor a recombinant mitochondrial genome that was generated during the backcrosses used to derive this congenic strain pair from strains H99$\alpha$ and 125.91$\alpha$ as their progenitors (51). Previous studies have documented that the mitochondria are uniparentally inherited from the $\alpha$ parent following mating (55, 58).

By scoring segregation of the mitochondrial genome and mating type, we can ascertain that the $\alpha$ progeny that inherit the $\alpha$ parental mitochondrial genome are products of mating, and we can infer that an equivalent number of $\alpha$ progeny are similarly derived. In other words, H99$\alpha$ crossed with KN99$\alpha$ yields F1 progeny with four theoretically possible genotypes: two parental ($\alpha$ mito 1 and $\alpha$ mito 2) and two recombinant ($\alpha$ mito 2 and $\alpha$ mito 1). But because of uniparental mitochondrial inheritance, the vast majority of progeny inherit mitochondrial genotype 2 and there are in practice two types of progeny: ($\alpha$ mito 2 and $\alpha$ mito 2). The frequency of recombinant F1 progeny can therefore be calculated as twice the number of the $\alpha$ mito 2 recombinants. This method of analysis might underestimate the number of spores analyzed, given that mitochondrial recombination and rare mitochondrial transmission from the $\alpha$ parent also occur. We
also analyzed the segregation of one genomic region that perdured in the KN99a/α genome from the original parental strain 8-1, and which differs from H99α. This genomic region segregated in a mendelian fashion (1:1) and independently of the MAT locus, based on RFLP analysis, providing direct genomic evidence that these represent products of meiosis. The purity of the spore preparations/inocula were calculated to be at least ~93% spores/7% yeast cells by molecular and mating type analysis, and these spore preparations were used for further analysis.

**Spores are infectious in a murine inhalation model**

Previous studies indicated that spores from serotype D are up to 100 times more infectious than yeast cells in a murine inhalation model (50). Here we addressed the pathogenicity of serotype A spores. Spores produced by mating of the serotype A strains H99α and KN99α were isolated by micromanipulation, and 500 colony forming units (~93% spores, see Figure 4a) were resuspended in saline as the infectious inocula. The spore enriched inoculum was compared with three different types of yeast cell inocula: 1) H99α alone, 2) a 1:1 mixture of H99α and KN99α yeast cells grown in liquid culture, and 3) a 1:1 mixture of H99α and KN99α yeast cells (grown individually on V8 mating medium and mixed just prior to infection) to control for desiccation. Two experiments were conducted in which groups of 5 (experiment 1) or 10 (experiment 2) mice per sample were infected via intranasal inoculation and monitored daily. Because spores germinate within 48 to 72 hours, even on water agarose medium, spore preparations were prepared in PBS and used for animal infections within 4 hours of preparation.
The spore-enriched inocula used for these infections was analyzed for mitochondrial DNA inheritance and mating types by PCR using gene specific primers and by mating on V8 (pH=7) plates with tester strains JEC20a and JEC21α (Figure 4A, Table 1). Based on this analysis, we calculate that the spore preparation used for infections contained ~93% spores and ~7% contaminating parental yeast cells. For the infectious inocula of 500 CFU, which was validated by counting spores/cells with a hemocytometer and plating assays, this corresponds to 465 spores and 35 yeast cells.

All infectious inocula, including the spore-enriched preparation and the yeast cell controls, were found to be highly infectious in the murine inhalation model. In both independent experiments, all inocula resulted in 100% lethal infection by 34 days post-infection (Figure 4). While the spore-enriched preparation was highly infectious, progression to lethal infection was modestly delayed compared to yeast cells in both independent experiments, with 100% mortality by day 25 for H99 yeast cells and day 30 for the spore-enriched inoculum in experiment 1, and day 23 for H99 compared to day 34 for the spore inocula in experiment 2. This difference in the virulence of yeast cells and spores was statistically significant ($P < 0.0027$ for spores vs H99 yeast cells in experiment 1 and $P < 0.0018$ for experiment 2). Notably, there was no statistically significant difference in the relative virulence between the three different yeast inocula (for experiment 1, H99 vs. H99/KN99 liquid culture, $P < 0.2799$ and H99 vs. H99/KN99 plate cultured, $P < 0.1606$; for experiment 2, H99 vs. H99/KN99 liquid culture, $P < 0.9237$ and H99 vs. H99/KN99 plate cultured, $P < 0.0816$). These findings support the conclusion that both yeast cells and a highly spore-enriched preparation are infectious in animals, but that the virulence of spores is modestly delayed compared to yeast cells.
Given that the spore-enriched infectious inoculum contained a majority of spores, but also a small fraction of contaminating yeast, further studies were conducted to document that animals had been infected with spores. Lung, spleen, and brain tissues were recovered from sacrificed animals that had been infected with the spore-enriched preparation. Colony forming units were recovered and analyzed for mating type, mitochondrial markers, and the genomic marker by PCR using gene-specific primers, mating on plates with tester strains (JEC21α and JEC20a), and RFLP analysis (Figure 5, Tables 2-5). As shown in Figure 5A, B, and C, of the isolates recovered from lung, spleen, and brain, the vast majority had inherited the mitochondrial genome from the α mating parent, whereas roughly equal proportions of a and α isolates were recovered. As discussed above, the recovery of α isolates with the a mitochondrial genome provides evidence these isolates derive from spores produced by sexual reproduction. This conclusion is supported by segregation analysis of the genomic region that differs between H99α and KN99a (Supplemental Table 5). Based on this analysis, the vast majority of the recovered isolates were recombinant. Thus, following inhalation, spores deposited in the lungs, germinate and disseminate to both the spleen and the brain of infected animals.

To determine the possibility of murine infection caused by contaminating yeast cells (~7%) in pure spore preparation, we further conducted an experiment with low infection inocula (5, 25, 100 and 300 CFU per mice) of H99α yeast cells (see Supplemental Figure 4). Mice infected with 300 CFU succumbed to lethal infection by 31-34 days post-infection and with 5 CFU the survival of mice was prolonged until day 70 post-infection. Compared to mice infected with the spore-enriched preparation (465
spores/35 yeast cells), which showed severe infection by day 33 to 35 post infection, with the lower yeast cell inocula alone (25 yeast cells), survival plots demonstrate initiation of infection symptoms 50 days post-infection with 100% mortality delayed until day 61 (see Supplemental Figure 4). Thus, the potent virulence of the spore-enriched inocula cannot be ascribed to the small percent of contaminating yeast cells.

**Spores are infectious in an invertebrate host model**

*C. neoformans* spores were also virulent in the invertebrate host model *Galleria mellonella*. Similar to the murine virulence studies, larvae were infected with 500 CFU of spores produced by H99α and KN99α mating, H99α yeast cells, or a 1:1 mix of H99α and KN99α cells from either an overnight liquid culture or from plate conditions. Infections were performed by injecting 5 µl of the infectious inocula in PBS into the terminal pseudopod, and infected larvae were incubated at room temperature (24°C) or 37°C. Saline-injected larvae served as controls, and in all cases survived for the duration of the experiment (8 to 14 days).

As shown in Figure 6, all four infectious inocula were highly virulent in *G. mellonella* larvae, resulting in 100% lethal infection by day 7 or day 13 at 37°C compared to 24°C respectively. In the invertebrate host at 24°C, spores and yeast cells were of equivalent virulence at 24°C (*P* < 0.4161), and there was little or no difference in virulence between the three infectious yeast inocula (H99 vs. H99/KN99 liquid culture *P* < 0.2751, H99 vs. H99/KN99 plate culture *P* < 0.0417). This experiment was repeated with similar results (see Supplemental Figure 5). Similar to the murine model, spores were modestly less virulent than yeast cells in the invertebrate host at 37°C in one
virulence test (H99 vs. spores, \( P < 0.0027 \), Figure 6A), whereas spores and yeast were equally virulent in the second independent experiment (H99 vs. spores, \( P < 0.1149 \), Supplemental Figure 5). These findings in an invertebrate host provide additional evidence that both yeast cells and spores are infectious.

**Spore and yeast cell interactions with alveolar macrophages**

The capacity of murine macrophages to phagocytose *C. neoformans* spores and yeast cells was evaluated by time lapse microscopy. As positive and negative controls, unopsonized yeast cells and yeast cells opsonized with 18B7 monoclonal antibody, respectively, were assessed in parallel. Giles and Hull have recently found that spores do not require opsonization for phagocytosis (15). Our findings confirm their conclusions, and phagocytosis of unopsonized *C. neoformans* spores was clearly observed under our experimental conditions (Supplemental movie 1), with the first phagocytosis events detected by 55 minutes post-incubation. Efficient phagocytosis of antibody-opsonized yeast cells by macrophages was evident by 30 minutes post-incubation (Supplemental movie 3). As expected, no phagocytosis of unopsonized control yeast cells was observed (Supplemental movie 2). Control acapsular mutant strain *cap59* was also tested and found to be readily phagocytosed by macrophages, in accord with previous studies that the capsule can serve to inhibit phagocytosis (Supplemental movie 4) (26). In summary, phagocytosis of yeast cells required opsonization, as expected, whereas phagocytosis of spores did not.

As previously described, our spore preparations are highly enriched for spores (~93%), but some yeast cells are still present in the spore sample serving to mimic what
might normally transpire during infections acquired in nature. Intriguingly, some yeast cells present in the spore sample were observed to be phagocytosed by macrophages (Supplemental movie 1); this result was unexpected since no phagocytosis of unopsonized encapsulated yeast cells alone was observed. These results suggest that an unknown factor(s) present in the spore sample may potentiate the capacity of mouse macrophages to phagocytose encapsulated yeast cells, a phenomenon that typically requires the presence of an opsonin such as complement or antibody. Alternatively, activation of phagocytosis by a spore may lead to concomitant uptake of a yeast cell by macrophages. Taken together, our results confirm recent findings of others (15) that macrophages can readily phagocytose C. neoformans spores in the absence of opsonin, an event that may be critical for further disease progression or the establishment of latency.
Discussion

The infectious propagules in several predominant human fungal pathogens are thought to be spores. Fungal spores are readily aerosolized and function as effective dispersal agents because of their shape, size, and ability to survive under harsh environmental conditions. Like many other fungal infections such as those caused by Aspergillus, Histoplasma, and Coccidioides, Cryptococcal infection is initiated when airborne infectious particles are inhaled into the human respiratory tract. Infectious particles larger than 5 microns are cleared by mucociliary action, whereas particles smaller than 2 microns penetrate and lodge in the alveoli, and subsequently disseminate and cross the blood brain barrier to penetrate the central nervous system (18, 50).

In previous studies, attempts were made to isolate pure spores from mating cultures and studies were performed in murine inhalation models with spores to analyze their pathogenicity (50, 59). Sukroongreung et al. performed studies using spores from \textit{C. neoformans} var. \textit{neoformans} (serotype D) and reported that spores from this lineage are up to 100-times more infectious than yeast cells (50). Spores from Cryptococcus measure ~3 microns, which readily lodge in the alveoli, compared to yeast cells that are typically >5 microns. Desiccated yeast cells, which are similar in size to spores, may also efficiently enter the alveoli and disseminate. Our studies focused on spores to understand their morphology, germination signals, and host response studies with controlled infectious inocula. We focused on the clinically most significant serotype, \textit{C. neoformans} var. \textit{grubii} (serotype A), which is the major cause (95%) of infections worldwide and greater than 99% of infections in AIDS patients (6).

Comparative studies on spore morphology from different \textit{C. neoformans} strains
and varieties provide a detailed view of surface properties and size and shape differences between spores from different serotypes and control yeast cells. Spores from serotype A measured between 2 to 3 µM; compared to serotype D spores measuring 2 to 2.5 µM. Spore sizes were determined by calculating around 50 individual spores from each cross and statistical analysis using t-test results in the two-tailed P-value equals to 0.0012, which is considered to be statistically significant. Spike-like protrusions on the surface of spores from serotype A and D were commonly observed. We hypothesize that the rough surfaces of the spores may represent novel cell surface proteins or carbohydrates. In a self-fertile diploid strain (αAAα) the spores were smooth, and measured 2 to 2.5 µM. Control yeast cells are round, >5 µM, and highly encapsulated. No visible capsule was observed on the spore surface (Figures 1 and 3). Furthermore, in studies with anti-GXM antibody, no signal for polysaccharide capsule was observed on spore surface, resulting in exposure of glucans (α-1,3 and β-1,3) on the cell surface, as detected by staining with specific antibodies (see Supplemental Figure 3). Control yeast cell capsule stained with anti-GXM antibody whereas no staining was seen with anti-glucan antibodies indicating the capsule overlays glucan structures (see Supplemental Figure 3). Interestingly, spores from C. gattii are more elongated compared to C. neoformans var. grubii and C. neoformans var. neoformans. C. gattii spores are elongated with smooth surfaces, measuring 3 to 5 microns in length (Figure 2). Sterigmata were not observed on the basidial surfaces for any of the crosses studied. This study on comparative morphology between different serotypes of Cryptococcus revealed distinguishable features between spores of C. neoformans, C. grubii, and C. gattii and both confirms and extends previous findings (47).
To examine the role of nutrients in triggering germination, we tested different media with reduced or no nutrients. When spores from serotype A (H99α and KN99a) mating were microdissected on 2% water agarose medium plates, microcolonies were observed (Supplemental Figure 2). Our findings indicate that spores germinate readily, even under severe nutrient limiting conditions. We also tested the ability of spores and yeast cells to aerosolize under laboratory conditions by exposing mating plates to an airstream mimicking natural wind currents. These studies indicate that spores are aerosolized and disperse more rapidly than yeast cells.

To test spores as infectious propagules, we enriched spores (~93%) from mating cultures (H99α and KN99a) and used these as infectious inocula in both murine and invertebrate host models. All of the infection experiments were conducted using spores from serotype A (H99α and KN99a) matings. In these studies, *C. neoformans* var. *grubii* spores were fully virulent (100% lethal infection) both in the murine inhalation virulence model and in the invertebrate host model *Galleria mellonella*. In two independent murine experiments, 500 CFU were used as the infectious inocula. Infectious inocula used for infection studies were typed for molecular and mating markers, confirming that ~93% of CFUs were recombinant. Dissemination of spores in infected mice was analyzed by typing CFUs isolated from lung, spleen and brain of infected animals. These CFUs were also typed for molecular and mating type markers, confirming that the infections were caused by recombinant mating products. Our results support the conclusion that spores are able to enter the lungs, germinate, and cause infections in the murine model.

Animals or larvae infected with yeast (mono or co-inoculated) progressed more rapidly to lethal infection compared to spores, and this result is statistically significant
In contrast, previous studies had shown that spores were up to 100 times more infectious than yeast cells for serotype D (50). We hypothesize that there could be serotype differences in the virulence pattern between spores from serotype A and D. Sukroongreung et al. conducted experiments with serotype D spores, whereas, all of our virulence studies were conducted with serotype A spores. These are now recognized as distinct species that diverged 18.5 mya (32, 56), so they may differ in virulence. There could also be host species-specific differences, such that spores are more infectious than yeast cells in humans but not in mice. This might be influenced by species-specific details of the airway anatomy, or innate or adaptive immune differences. Spores might remain in a dormant phase before causing infection, which could explain the delayed mean time to mortality we observed for mice instilled with spores compared to those instilled with yeast cells. Spores may evade host immune cell recognition and subsequent responses via efficient uptake by macrophages and delayed germination. To test this and mimic human immune responses during latency, a rat model (16) may be more appropriate compared to mice in which disease progresses more rapidly.

In nature, infectious spores are likely produced by α-α same sex mating in the largely unisexual population. Spores produced by unisexual mating could differ from those produced by opposite sex mating. It could also be that the course of infection differs when animals are infected with a mixture of mating types compared to spores from unisexual mating of only one mating type. Previous studies reveal the potential for cell-cell interactions during co-infection with a and α yeast cells (42); similar interactions might occur or be magnified during infection with a mixture of spores of opposite mating types. Given that spores are readily phagocytosed without opsonin, alveolar macrophages...
may contain both \( a \) and \( \alpha \) spores, and pheromone cell-cell communication in this setting could be more robust and influence the course of infection. This would not occur during infection with just \( \alpha \) yeast cells or spores. Finally, our studies employed a nasal instillation model, and a frank inhalation model might be required to appreciate differences between spores and yeast cells.

In our \textit{in vitro} analysis with alveolar mouse macrophages, spores were readily phagocytosed without opsonin, whereas control encapsulated yeast cells require opsonization to be phagocytosed by macrophages. We observed that yeast cells mixed with spores could be phagocytosed by macrophages when spores were also present. These observations confirm and extend the findings of Giles and Hull, who discovered that spores do not require opsonin to be phagocytosed by macrophages (15). We postulate that \( \beta-1,3 \) glucans on spores and acapsular yeast cells are readily exposed to dectin 1, leading to rapid phagocytosis by macrophages. Whether the phagocytosis of encapsulated yeast cells in the presence of spores involves factors released by spores, or concomitant uptake triggered by the spore surface, remains to be investigated.

\textbf{Acknowledgments}

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References


Figure legends

**Figure 1.** Scanning electron microscopy (SEM) analysis of *C. neoformans* spores.
Basidia bearing basidiospores from the mating cultures of *C. neoformans var. grubii* a) H99α and KN99a, b) H99α and Bt63a, c) *C. neoformans var. neoformans* spores from the cross between JEC21α and JEC20a, d) monokaryotic fruiting structures from the serotype D α strain XL280, e) spores from the self-filamentous diploid (aAAα) strain KN2B5#19, and f) control yeast cells from strain H99α were observed by scanning electron microscopy (SEM) and photographed. Sexual spores from *C. neoformans var. grubii* are elliptical with a rough surface that is apparent from the early stages of spore development. Four long intact spore chains on the basidia were commonly seen in the mating cultures from serotypes A and D.

**Figure 2.** *C. neoformans var. gattii* sexual spores are elongated. Spores from mating cultures of *C. gattii* strains are elongated with a smooth surface compared to spores from serotypes A and D. Basidia bearing basidiospores that are rod shaped and slightly curved were observed in most of the *C. gattii* crosses. Different VG types were analyzed by incubating crosses on MS medium, with incubation for 3-4 weeks until the spore chains appeared at the edges of the mating colonies. Developing spores at the early stages were round, as shown in a), WM276α mating with B4546a, and fully developed, elongated basidiospores were seen in crosses between strains b) R265α and B4546a, c) NIH312α and B4546a, d) MMRL2651α and B4546a by scanning electron microscopy (SEM).
Figure 3. **Comparison of morphology and spore size.** Spores produced by mating, fruiting, or diploid self-filamentous *C. neoformans* strains were analyzed by scanning electron microscopy (SEM). A comparative profiling of the spores from a) H99α and KN99α serotype A mating, b) JEC21α and JEC20α serotype D mating, c) monokaryotic fruiting of the serotype Dα strain XL280 and d) spores produced by the self-filamentous diploid (aAaα) strain KN2B5#19 is shown.

Figure 4. **Molecular and mating types analysis of inoculum used for virulence studies.** A) A proportion of the spore inoculum used in the virulence studies was scored for recombination by marker analysis. Mitochondrial, *MATa*, and *MATα* markers were analyzed by PCR using gene specific primers (see methods), and RFLP analysis of one genomic region that perdured in the KN99α/α genome from the original parental strain 8-1, and which differs from H99α. A representative sample of the recombination and mating type analysis conducted is shown in panel A. Mating was performed on V8 (pH=7) medium with tester strains JEC20α and JEC21α and scored for mating type, as shown in Table 1. B and C) Animal studies were performed using five (B) or ten (C) mice, each infected by inhalation with either spores from crosses between strains H99α and KN99α or corresponding yeast cells of H99α and KN99α in an equal (1:1) ratio and/or H99α alone as a control.

Figure 5. **Recombinant spore products infect mice and disseminate.** Colony forming units from infected mouse tissues (lung, spleen, and brain) were recovered by plating on
Sabouraud dextrose agar medium supplemented with 100 µg/ml chloramphenicol. Molecular and mating type analyses were performed as described in the legend to Figure 4. A representative agarose gel of PCR products is shown. Mitochondrial DNA inheritance and mating types were typed by PCR using gene specific primers. Mating types were also determined by mating the isolates with the tester strains (JEC20α and JEC21α) and are shown below the respective panels. The molecular and mating type analysis of isolates recovered from the infected mice lung tissues (A), with the corresponding parental strains to the right. Recombinants recovered from the infected mice spleen (B), and recombinants recovered from infected mice brain (C). A large proportion of recombinants were recovered from the infected animal tissues indicating that spores used as inocula caused infections in mice and disseminated to distant organs. For a complete listing of typing please refer to the supplementary Tables 1-3.

**Figure 6. Spores cause lethal infection in *Galleria mellonella*.** Larvae were infected with spores from a cross between strains H99α and KN99a and a 1:1 mixture of H99α and KN99a yeast cells or H99α alone by injection and incubated at 37°C (panel A) or room temperature (panel B). Viability was assessed by responsiveness to touch. Survival curves were plotted, and the statistical significance of the survival curves between spores and yeast cells was analyzed (see results).
Figure 1

Aα x Aa
H99 x KN99a

Aα x Aa
H99 x Bt63

Dα x Dα
JEC21 x JEC20

Dα
XL280

αAAa
Diploid

Aα
H99
Figure 2
Figure 3
Figure 4

A

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- Mito
- SNP8
- STE20a

B

![Graph showing percent survival over days post infection](image)

C

![Graph showing percent survival over days post infection](image)
Figure 5

A. Isolates recovered from lung

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B. Isolates recovered from spleen

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C. Isolates recovered from brain

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Figure 6

37°C

Days Post Infection

Percent survival

PBS
Spores
H99 yeast
H99+KN99-o/n yeast
H99+KN99-plate yeast

24°C

Days Post Infection

Percent survival

PBS
Spores
H99 yeast
H99+KN99-o/n yeast
H99+KN99-plate yeast