Cryptococcus neoformans: Size Range of Infectious Particles from Aerosolized Soil

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Although cryptococcosis is characterized as a chronic central nervous system disease, it is generally accepted that the lungs are the primary portal of entry for the etiologic agent. Despite this, there is a distinct lack of evidence that viable airborne particles of Cryptococcus neoformans are small enough to reach the alveoli. Two encapsulated strains and one nonencapsulated strain of C. neoformans were inoculated into 250-g quantities of sterile soil. Throughout the 0 to 12 weeks of incubation, this soil was aerosolized in a sealed chamber with a Waring blender. Samples of the resultant dust cloud were taken with an Anderson air sampler from which the numbers and sizes of viable airborne particles were determined. Of the viable organisms aspirated into the air sampler, 15% were 0.65 to 2 μm in diameter. As incubation time in soil increased, the size of the particles decreased, and increased numbers of C. neoformans cells 0.65 to 2 μm in diameter were isolated. The presence of viable cells <2 μm in soil aerosols indicated that, under certain conditions in nature, C. neoformans cells exist in sizes that are capable of deep lung deposition.

Since the first isolation of Cryptococcus neoformans by Sanfelice (13) in 1894, researchers have speculated upon the primary route of infection by this pathogen. In general, fungi are particularly suited for airborne dissemination; thus, the respiratory tract has been suggested as an important portal of entry for this organism, as well as the etiologic agents for other important systemic mycoses (3, 8, 9, 11, 12; R. M. Karauoi, Diss. Abstr. Int. B 36B:1583-1584).

It is difficult to conceptualize the lung as a portal of entry for particles of C. neoformans, which are usually described as being heavily encapsulated yeast cells, 4 to 20 μm in diameter (3). Wright (15) and Hatch (6) have shown that airborne particles entering through the larynx are generally smaller than 10 μm. It is considered that particles larger than 5 μm are swept out of the lungs by ciliary action. However, particles that are minimally affected by the cleansing ciliary action and succeed in penetrating the respiratory system to the alveoli are smaller than 2 μm (6).

In 1968, Ishaq et al. (7) reported that the capsule size of C. neoformans appeared to decrease with increased incubation of the organism in soil. Farhi et al. (4) reported in 1970 that particles of C. neoformans appeared to exist in soil as relatively small, nonencapsulated cells. They detected viable and infectious 3-μm cells of C. neoformans and suggested that the organism, under certain conditions, may exist naturally in the soil in a nonencapsulated state. These experiments led us to speculate that if the lung is the primary portal of entry, then the infectious particle of C. neoformans must be small enough to enter the lung and, in particular, must be deposited within the alveolus-bearing structures, which most investigators of lung physiology place at less than 2 μm. The purpose of this investigation was to determine the sizes and numbers of viable airborne particles of C. neoformans in laboratory-simulated dust clouds, with particular attention to their ability to grow in soil as particles <2 μm.

MATERIALS AND METHODS

Organism. C. neoformans CIA is a moderately encapsulated strain (10) isolated from a patient with cryptococcal meningitis at the Veterans Administration Hospital, Oklahoma City, Okla. Strain G is a heavily encapsulated strain isolated from a patient in Saigon, Vietnam. Strain M7 is an ultraviolet radiation-induced mutant of CIA that is nonencapsulated and avirulent for mice (2).

Air sampler. An air sampler (Anderson Air Samplers, Inc., Salt Lake City, Utah) was used to aspirate air from test chambers in which soil bearing yeast cells was aerosolized. The air sampler is a sievelike, six-stage unit in which each succeeding stage has holes of smaller diameter. The sampler is designed to draw air successively through each stage at an increasing velocity. This regulated velocity causes deposition of larger, denser particles on the upper stage.
and smaller, less-dense particles on the lower stages (1). Each stage contains a petri dish containing culture agar; thus, the sizes and numbers of viable airborne particles can be counted after appropriate incubation. The sampler was calibrated to aspirate air at a rate of 19.5 liters/min.

Experiments with the pathogenic strains CIA and G were performed within the confines of an Airborne Infection Apparatus (Tri-R Instrument Co., Jamaica, N.Y.). The nonpathogenic strain M7 was aerosolized in a modified 2.8-liter Erlenmeyer flask that was connected by a short latex sleeve to a Waring blender. This modified flask, referred to as an "octopus," was designed for future studies on aerosol infections of mice.

Preparation and seeding of soil samples. Yeast cells, used as the soil inoculum, were harvested from a 72-h (room temperature) culture grown on trypan blue mycological agar medium, which is selective and differential for C. neoformans (14). The cells were suspended in sterile, distilled water, and 1 ml containing 10^9 hemacytometer-counted yeast cells was pipetted into 500-ml mason jars containing 250 g of soil that had been autoclaved at 15 lb/in^2 for 1 h on 3 consecutive days. The jars were lightly capped and stored at room temperature until used.

Aerosolization and sampling of inoculated soil. After 0 to 32 weeks of incubation at room temperature, soil containing cells of either strain CIA or G was poured into a Waring blender located in the Airborne Infection Apparatus. The Airborne Infection Apparatus was sealed, and the soil was blended for 1 min. The resulting thick dust cloud was permitted to settle for 15 min, and then a 1-min air sample was aspirated.

At weekly intervals during 1 to 6 weeks of incubation of strain M7 in soil, the contents of a mason jar was poured into a Waring blender to which the "octopus" chamber was attached. The air sampler was connected to an outlet on the "octopus." This method allowed direct sampling of the air within the chamber. Inoculated soil samples were blended for 1 min, and then the ensuing dust cloud was drawn from the blender jar into the "octopus" chamber by a vacuum line. This produced a homogeneous dust cloud within the 2.8-liter chamber. The vacuum was turned off after 15 s, then the dust cloud was aspirated for 30 s through an air sampler.

After each chamber sampling, the plates containing aspirated dust and yeast cells were removed from the various air sampler stages and were incubated at room temperature. Colonies were counted after 96 h of incubation. The positive hole count method developed by Anderson (1) was used. India ink preparations, made from randomly selected colonies, were examined microscopically for encapsulated (CIA or G) or nonencapsulated (M7) yeast cells.

Virulence experiments. Cells of encapsulated strains CIA and G that were isolated from colonies growing on air sampler stages 1, 5, and 6 (7 to 11, 1 to 2, and 0.65 to 1-μm particle sizes, respectively) were subcultured to trypan blue agar medium and incubated for 3 days at room temperature. Cells were suspended in 0.85% saline and adjusted to a concentration of 10^9/ml (ascertained by hemacytometer counts), and 0.05 ml, containing 5 × 10^6 cells, was inoculated intracranially into 3-week-old Swiss white mice. Six groups of animals (each with four animals) were inoculated. Stage-1 cells (7 to 11 μm) served as controls. Death of the animals due to cryptococcosis was recorded up to 10 days postinoculation.

RESULTS

C. neoformans particle size and distribution from aerosolized soil. During preliminary experiments, we noticed that very few C. neoformans cells were isolated on the air sampler plates when freshly inoculated soil was aerosolized and sampled. These low numbers of cells at zero time may be attributed to the moist conditions of the sample, which inhibited formation of dust clouds and might have promoted clumping of the yeast cells in the soil. Optimal incubation time for this study appeared to range from 1 to 12 weeks.

Table 1 shows the size distribution of viable C. neoformans cells in aerosolized soil in relation to particle size. The distributions were based on 12 aerosol experiments per strain with optimally incubated soil samples.

The viable aerosolized cells from the moderately encapsulated strain CIA ranged from <1 to 11 μm in diameter. It is important that 22.4% of the aerosolized C. neoformans CIA cells were <2 μm; 9.1% were <1 μm; and 13.3% were 1 to 2 μm. Of the remaining viable organisms aspirated onto the air sampler plates, 10.3% were 2 to 3 μm; 19.0% were 3 to 5 μm; 11.1% were 5 to 7 μm; and 37.2% were 7 to 11 μm. When samples of colonies cultured from the air sampler plates were examined microscopically in India ink preparations, encapsulated cells ranging from 4 to 20 μm were seen.

Cells of the heavily encapsulated G strain were also isolated on all six stages of the air sampler. Unexpectedly, large numbers (15.1%) of the airborne particles were <2 μm in diameter; 6.2% were <1 μm; and 8.9% were 1 to 2 μm. Of the remaining isolated cells, 15.8% were 2 to 3 μm; 20.2% were 3 to 5 μm; 16.0% were 5 to 7 μm; and 31.9% were 7 to 11 μm.

Table 1. Size distribution of viable C. neoformans cells in relation to particle size from aerosolized soil

<table>
<thead>
<tr>
<th>Air sampler stage no.</th>
<th>C. neoformans cell size (μm)</th>
<th>Viable cells cultured from aerosolized soil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CIA</td>
<td>G</td>
</tr>
<tr>
<td>1</td>
<td>7.0-11.0</td>
<td>37.2</td>
</tr>
<tr>
<td>2</td>
<td>4.7-7.0</td>
<td>11.1</td>
</tr>
<tr>
<td>3</td>
<td>3.3-4.7</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>2.1-3.3</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
<td>1.1-2.1</td>
<td>13.3</td>
</tr>
<tr>
<td>6</td>
<td>0.65-1.1</td>
<td>9.1</td>
</tr>
</tbody>
</table>
Nonencapsulated cells of strain M7 aerosolized with soil also showed a wide range in particle size. A small percentage (7.8%) of the viable M7 cells isolated were <2 \mu m in diameter; 2.3% were <1 \mu m; and 5.5% were 1 to 2 \mu m. Of the remaining viable cells, 13.6% were 2 to 3 \mu m; 27.0% were 3 to 5 \mu m; 14.7% were 5 to 7 \mu m; and 36.9% were 7 to 11 \mu m.

Figure 1 illustrates the percentage of viable C. neoformans cells isolated from the laboratory-induced dust clouds on the basis of size. The percentages illustrated are composite values for the three C. neoformans strains studied and are expressed as the mean plus or minus the standard error of the mean. Of particular note is the first column, which indicates that 5.9 \pm 0.87% of the viable C. neoformans cells isolated from aerosolized soils was 0.65 to 1 \mu m. C. neoformans cells have not been previously reported to be this small.

**Virulence tests.** Within 10 days post-intracranial inoculation with either C. neoformans CIA or G cells, 100% of the mice died of cryptococcosis (Table 2). All of the G-strain-inoculated mice died from 4 to 10 days postinoculation. M7 virulence studies were not performed, since this strain has previously been shown to be avirulent for mice (2). These results indicate that all viable airborne particles of C. neoformans, regardless of their size, are virulent for mice.

**DISCUSSION**

Figure 2 illustrates the approximate level of penetration of most airborne particles within the lung (1, 6). The depth and location of deposition are largely dependent on the size of the particles. Inhaled particles larger than 3 \mu m in diameter generally do not penetrate further than the trachea and primary bronchi. However, particles smaller than 3 \mu m can be deposited within the secondary and terminal bronchi, and those smaller than 1 \mu m are capable of alveolar deposition.

Our studies indicate that viable airborne cells of C. neoformans do exist as very small particles and that the particle sizes are definitely compatible with alveolar deposition. In our studies of three C. neoformans strains, we repeatedly isolated yeast cells <1 \mu m in diameter. The percentages of cells <1 \mu m were 9.1, 6.2, and 2.3% for strains CIA, G, and M7, respectively. An earlier report by other investigators (9) stated that a very small percentage of the C. neoformans cells isolated from a natural habitat by Anderson air samplers were <5.5 \mu m. They did not report isolating any C. neoformans cells that were <1 \mu m (9). Although strains of C. neoformans may vary in their cell sizes, we believe that this is not a major reason for the apparent

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**Table 2. Virulence of C. neoformans cells isolated from air sampler stages 1, 5, and 6**

<table>
<thead>
<tr>
<th>C. neoformans strain</th>
<th>Stage no.</th>
<th>Cell size (\mu m)</th>
<th>No. of animals inoculated/no. of animals deceased</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td>1</td>
<td>7.0–11.0</td>
<td>4/4</td>
</tr>
<tr>
<td>CIA</td>
<td>5</td>
<td>1.1–2.1</td>
<td>4/4</td>
</tr>
<tr>
<td>CIA</td>
<td>6</td>
<td>0.65–1.1</td>
<td>4/4</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>7.0–11.0</td>
<td>4/4</td>
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<td>G</td>
<td>6</td>
<td>0.65–1.1</td>
<td>4/4</td>
</tr>
</tbody>
</table>

**Fig. 1.** Mean ± standard error of the mean size distribution of viable C. neoformans cells in relation to particle size from aerosolized soil.

**Fig. 2.** Potential depth of deposition for particles inhaled into the human respiratory tract.
discrepancies between our data and those of Powell et al. (9). In all instances, we sampled dust clouds for 1 min or less with the air sampler. In contrast, Powell et al. (9) sampled dustladen air for 4 h. It was our experience that lengthy exposure periods resulted in plugging the holes of the air sampler. This could explain why they detected few particles less than 5 μm in diameter.

The percentages of isolated airborne particles reported in this study reflect only the actual number of culturable cells that were isolated from the soil aerosols. Microscopic examination of the sampler plates revealed the presence of numerous yeast cells that did not develop into colonies. It is unknown whether these cells were dead or were simply in a latent state. Continued incubation of the sampler plates for 2 weeks failed to resolve this question of viability.

It is generally accepted that upper respiratory removal of airborne particles (e.g., by ciliary action or exhalation) is essentially 100% for particles 10 or more μm and ca. 80% for particles 5 to 10 μm. Clearance efficiency by these mechanisms decreases with the inhaled particle size, so that the clearance efficiency for particles 1 to 2 μm approaches zero. Particles <1 μm are generally unaffected by pulmonary clearing methods and thus remain in the lung (6).

Several studies have shown that cryptococcosis can be induced when experimental animals are inoculated by the airborne route (10, 12; R. M. Kataouli, Diss. Abstr. Int. B 36B:1583–1584). Environmental studies have revealed that C. neoformans cells exist in soil in a relatively small and nonencapsulated state (4, 7). In our work, as incubation time in soil increased, the size of the C. neoformans particles decreased, with increased numbers of 0.65- to 2-μm yeast cells being isolated from aerosols. This is probably a reflection of our previous finding that C. neoformans cells become smaller and tend to lose their capsules with increased incubation time in soil. However, this is not to infer that C. neoformans cells are always small and nonencapsulated in soil in nature. Since capsule production seems to be intimately linked with the amount of water present (4, 7), it seems logical that capsule production may be an "on-off" affair depending upon the environmental conditions. For example, after a rain, the amount of moisture and transported nutrients in the soil would increase dramatically. Perhaps, under such conditions, capsule production would be "turned on," with a subsequent increase in capsule size, whereas during long, dry periods, capsule production would be "turned off," with a subsequent decrease in capsule size. Since lung deposition is linked to particle size, the infectious capability of C. neoformans cells might also vary. Although speculative, this concept is supported by the findings of Farhi et al. (4) which show that the number of encapsulated C. neoformans cells in soil increased from 13 to 63% 18 h after the addition of water.

Although the thrust of these investigations was to show that viable particles of C. neoformans can be small enough to be deposited deep into the human lung, it may be equally as important to speculate about the C. neoformans cells that are larger than 5 μm in diameter. Since most particles larger than 5 μm are cleared through the gastrointestinal tract, we have concurrently undertaken an investigation into the possibility that the gastrointestinal tract might be an additional portal of entry for C. neoformans. Findings from this study, currently in preparation, indicate that C. neoformans can replace the normal cecal flora in a small percentage of mice. The possibility that such a situation might exist in man is suggested by the report by Felsenfeld (5) in which Cryptococcus spp. were isolated from the feces of 39.2% of hospital "ward patients" and from the feces of 24% of "new admissions" patients.

In summary, these studies indicate that soil-grown C. neoformans cells can be small enough to be deposited within the human lung. These studies also support our previously published speculation that, under certain environmental conditions, the infectious particle of this pathogen may reside in the soil in a relatively small, nonencapsulated state.

ACKNOWLEDGMENTS

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