Medical Mycology August 2010, **48**, 775–779

informa
healthcare

Short Communications

Paramecium species ingest and kill the cells of the human pathogenic fungus *Cryptococcus neoformans*

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A fundamental question in the field of medical mycology is the origin of virulence in those fungal pathogens acquired directly from the environment. In recent years, it was proposed that the virulence of certain environmental animal-pathogenic microbes, such as *Cryptococcus neoformans*, originated from selection pressures caused by species-specific predation. In this study, we analyzed the interaction of *C. neoformans* with three *Paramecium* spp., all of which are ciliated mobile protists. In contrast to the interaction with amoebae, some *Paramecium* spp. rapidly ingested *C. neoformans* and killed the fungus. This study establishes yet another type of protist-fungal interaction supporting the notion that animal-pathogenic fungi in the environment are under constant selection by predation.

Keywords *Paramecium*, virulence factors, *Cryptoccocus neoformans*, environmental predators, evolution

Introduction

Cryptococcus neoformans is a free-living pathogenic fungus that primarily inhabits soil contaminated with decaying organic matter and bird excreta [1,2]. *C. neoformans* is unusual among the pathogenic fungi in that it has several well characterized virulence factors such as a polysaccharide capsule, degradative enzymes, and melanin pigment production [3]. The existence of well-defined mammalian virulence factors in *C. neoformans* is of particular interest since the organism normally inhabits soil and it is uncertain how those traits were selected through evolution. *C. neoformans* is a non-specific pathogen that has been reported to infect and/or cause disease in a myriad of taxonomically diverse organisms from mammals to birds to such invertebrates as insects and worms [4–8].

One of the enduring questions in the field of medical mycology is the origin and maintenance of virulence by microbes, such as *C. neoformans*, which have no requirement to inhabit vertebrate hosts for replication or survival. One theory to explain this phenomenon posits that selective pressures in the environment, possibly from protists, selected for the emergence of traits that could also function for mammalian virulence [9,10]. Supporting this view, the intracellular pathogenic strategy of *C. neoformans* for *Acanthomoeba castellanii* and *Dictyostelium discoideum* is strikingly similar to that observed with mammalian macrophages [11,12].

Thus far, the study of *C. neoformans* interactions with protista has been limited to amoeba species and social amoebae of slime molds [2,11,13,14]. In an effort to ascertain the nature of the interaction of *C. neoformans* with other unicellular protists, we explored the outcome of *C. neoformans* interactions with three species of paramecia. Our goal was to determine whether such interactions favored the paramecium or the fungus and to investigate the suitability of these hosts for the study of cryptococcal pathogenesis. Unlike *A. castellanii* and *D. discoideum*, which obtain nutritients by phagocytosis, *Paramecium* spp. are rapidly swimming grazers with a prodigious capacity for ingesting bacteria and fungi [15]. Here we show that in contrast to the interaction of *C. neoformans* with the

Received 5 May 2009; Received in final revised form 22 October 2009; Accepted 30 October 2009

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DOI: 10.3109/13693780903451810

amoeboid predators, *A. castellanii* and *D. discoideum*, where the fungus is rarely killed by the amoeba, certain species of paramecia can reduce fungal cell numbers.

Materials and methods

Organisms and culture conditions

Stocks of *Paramecium multimicronucleatum* and *Paramecium aurelia* (Carolina Biological Supply, Burlington, NC) were grown as per company instructions. *Paramecium tetraurelia* (stock 51) cultures were obtained from the American Type Culture Collection (ATTC, Manassas, VA) and grown in cereal grass media containing *Enterobacter aerogenes* (ATTC # 13048). Stocks of *Paramecium tetraurelia* were subcultured every two weeks in media containing *Enterobacter aerogenes*. A volume of 50 µl of a bacterial culture was supplemented weekly to sustain paramecial growth. *C. neoformans* strains H99 (serotype A), 24067 (serotype D), and NIH 112 (serotype B) were grown for 24 – 48 h in Difco Sabouraud Dextrose Broth (BD) at 30ºC and 150 rpm.

Ingestion assays

Light microscopy. *C. neoformans* cells were labeled with 1% Uvitex 2B solution in PBS (Polyscience, Inc Warrington, PA) for 10–30 min at room temperature (RT). Cells were then washed twice with spring water (Carolina Biological Supply, Burlington, NC) and approximately 30–35 cells of *P. multimicronucleatum* were added to 96-well tissue culture plates in cereal grass culture media. Paramecia were allowed to acclimate for 30 minat RT. Afterwards, 1×10^5 Uvitex 2B *C. neoformans*-labeled cells were added to the protist suspension. Plates were then incubated for 2 hat RT. Following incubation, the paramecia were fixed with 2.5% formaldehyde and visualized with a DAPI filter-equipped Zeiss Axiophot microscope.

C. neoformans *killing assays*. Approximately 1×10^5 *C. neoformans* cells were added to 100 cells of *P. tetraurelia* in a 96-well tissue culture plate for a 1:1000 effector to target ratio. *C. neoformans* cells in cereal grass media alone served as a control. Killing of *C. neoformans* H99 cells by *P. tetraurelia* cells was determined by counting colony-forming units (CFU) of *C. neoformans* and comparing colony counts at 0 and 24 hof co-incubation. At each time interval, the number of colonies associated with and without paramecia was determined. The protozoa were lysed by forcibly pulling the culture through a 30.5 gauge needle 10 times. This procedure lysed 86% of the paramecia cells and allowed intracellular *C. neoformans* to be counted. The procedure of pulling *C. neoformans* through a 30.5 gauge needle had no effect on *C. neoformans* viability (data not shown). The suspension containing the *C. neoformans* was then plated on Sabouraud Dextrose Agar (BD) plates containing 10 µg/ml of penicillin-streptomycin to inhibit bacterial growth. All plates were incubated at 30°C for 48 h All CFU counts were done in triplicate.

Transmission Electron Microscopy. *C. neoformans* was added to *P. tetraurelia* at a ratio of 1:1000 effector to target cells and was incubated at RT for 2 h The samples were then fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide followed by 2% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kv.

Statistical analysis

Statistical analysis was performed using two-way ANOVA (factors were time and presence/absence of *Paramecium* spp.). Comparison between the 24 hcontrol group (*C. neoformans* alone) and 24 hexperimental group (*C. neoformans* + *Paramecium* spp) was done by the Student's *t*-test. All statistical analyses were performed using Microsoft Excel 2003. A *P*-value of < 0.05 was considered statistically significant.

Results

Paramecium spp. ingest *C. neoformans*

To ascertain whether fungal cells were ingested by paramecia, ingestion assays were performed using *P. multimicronucleatum* and *C. neoformans* 24067. This assay involved labeling *C. neoformans* with the cell wall stain Uvitex 2B and observing the paramecium using fluorescent microscopy. The results showed that this *Paramecium* species engulfed *C. neoformans* cells during a 2 hincubation (Fig. 1A,C). Transmission electron microscopy experiments using *C. neoformans* H99 and *P. tetraurelia* confirmed this result by providing visual evidence of digested *C. neoformans* within *P. tetraurelia* food vacuoles (Fig. 1D,E). When viewed under the microscope in real time, ingestion of all *C. neoformans* by paramecia cells caused a transient decrease in the rate of protozoal swimming common to all *Paramecium* spp. during grazing (data not shown). When viewed at 24 h paramecia cells did not contain *C. neoformans* cells, further implying digestion of previously ingested cells (data not shown).

P. tetraurelia and *P. aurelia kill C. neoformans*

To ascertain the outcome of the interaction between different serotypes of *C. neoformans* and *Paramecium* spp., combinations of these species were co-incubated for 24 hand fungal colony counts were determined at t = 0 hand 24 h Two-way ANOVA revealed that CFU counts were affected by both time and the presence of both *P. tetraurelia* and *P. aurelia* ($P < 0.001$) but not by the presence of *P. multimicronucleatum*. After 24 h in the presence of *P. tetraurelia,* H99, 24067, and NIH 112 CFU counts decreased by 80%, 97%, and 81%, respectively, when compared to the 24 hcryptococcal CFU in the absence of paramecia (Fig. 2A). The CFU counts decreased by 82% for 24067 and by 51% for NIH 112 after 24 hincubation with *P. aurelia* when compared to the 24 hcontrol, as well (Fig. 2C). In the comparison amongst *C. neoformans* strains, both *P. tetraurelia* and *P. aurelia* killed strain 24067 cells while *P. multimicronucleatum* had no impact on the growth or survival of these cells (Fig. 2A–C). NIH 112 was killed by *P. aurelia*, however, the presence of *P. tetraurelia* merely reduced its growth relative to conditions without paramecia (Fig. 2A,C). The presence of *P. multimicronucleatum* did not affect the growth of NIH 112 when compared to conditions without the paramecia (Fig. 2B). The growth and survival of H99 was not affected by the presence of either *P. multimicronucleatum* or *P. aurelia*, but its growth was reduced in the presence of *P. tetraurelia* relative to conditions without paramecia (Fig. 2A–C).

C. neoformans presence is not lethal to *Paramecium spp.*

All three *Paramecium* spp. survived their interaction with *C. neoformans* with no evidence of protist killing after a 24-h incubation in the presence of *C. neoformans*.

Discussion

Incubation of *C. neoformans* with *Paramecium* spp. led to rapid ingestion of fungal cells by the protozoan. After the initial ingestion, there was a noticeable transient slowing of swimming motion by *Paramecium* spp. cells [15], but no visible damage to the protozoal cells occurred. Cells belonging to *C. neoformans* serotypes A, B, and D (with the exception of H99 incubated with *P. aurelia*) that were co-incubated with either *P. tetraurelia* or *P. aurelia,* either exhibited a reduction in growth, or were killed relative to conditions that contained no protozoal cells, evidenced by a reduction in colony counts. We interpret this result as

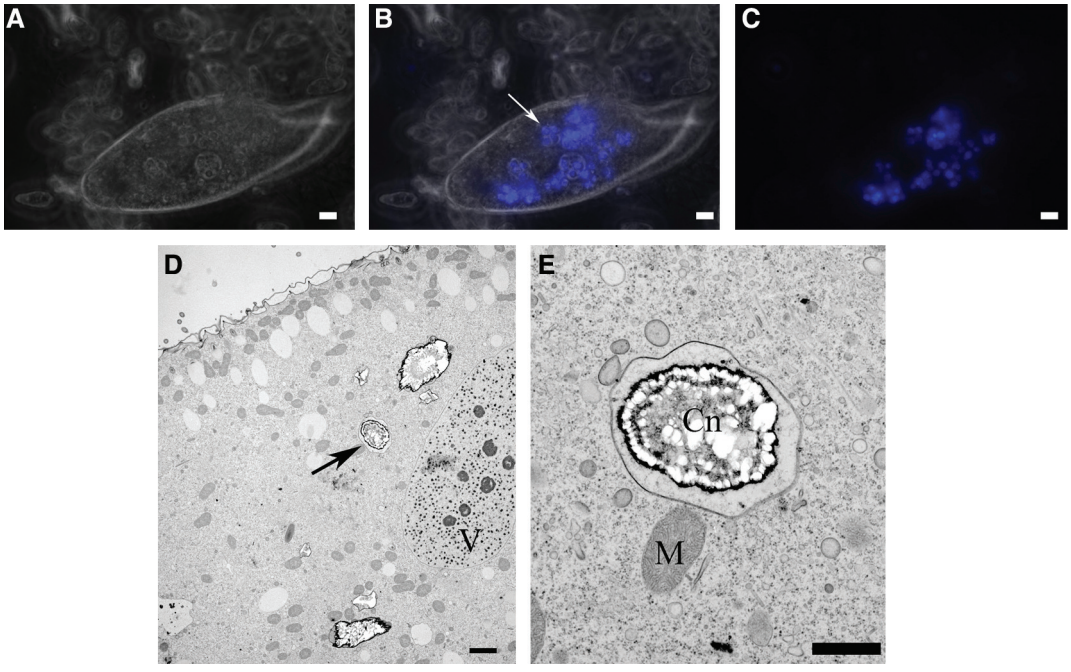

Fig. 1 Incubation of *Paramecium* spp. with *Cryptococcus neoformans* strains 24067 and H99 results in the ingestion of fungal cells. (A) Light microscopy, 400× magnification. (C) Corresponding fluorescence microscopy showing 1% Uvitex 2B labeled 24067. (B) Merged light and fluorescent microscopy images showing 24067 present within *P. mulitmicronucleatum* (arrow). (D) Transmission electron micrograph of H99 infected *Paramecium tetraurelia* (arrow). 2000× magnification, V = vacuole. (E) Magnified view of image (D). After a 2 h incubation, *P. tetraurelia* begins to digest *C. neoformans* in a membrane-bound food vacuole. 5000× magnification, Cn = *C. neoformans*, M = mitochondria. (A–C) Bar = 10 μm. (D) Bar = 2 μm. (E) Bar = 1 μm.

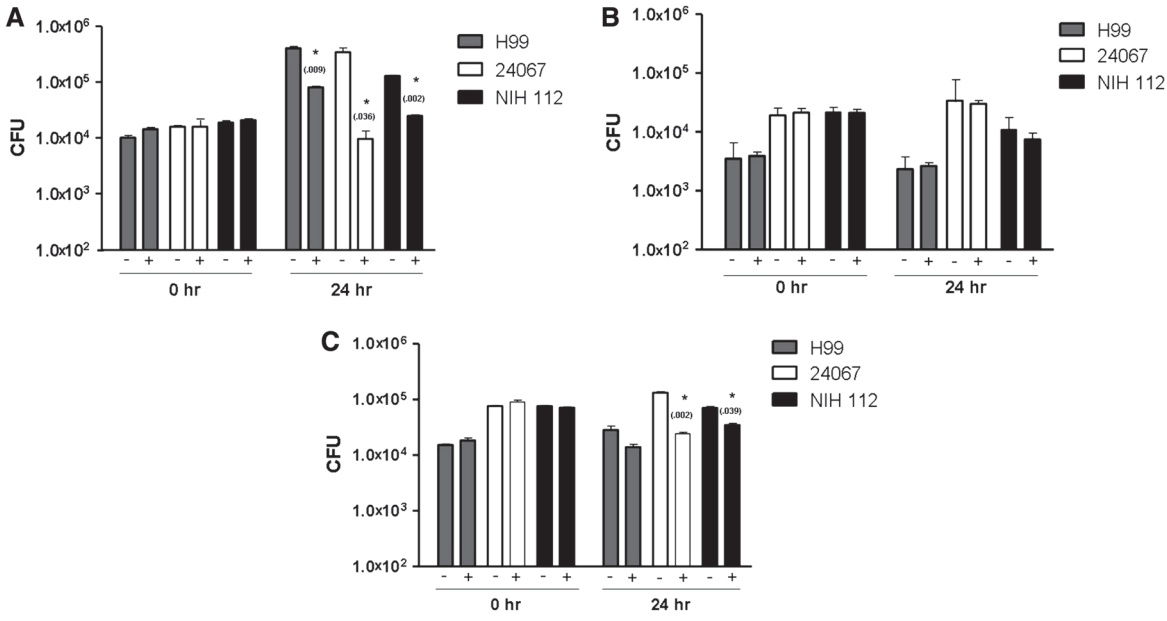

Fig. 2 Twenty-four hour incubation of *Cryptococcus neoformans* strains H99, 24067 and NIH 112 with *Paramecium tetraurelia*, *P. mulitmicronucleatum, and P. aurelia*. The histograms show average CFU+95% CI at time 0 and 24 h (A) Incubation of *C. neoformans* strains with *P. tetraurelia*. (B) Incubation of *C. neoformans* strains with *P. multimicronucleatum*. (C) Incubation of *C. neoformans* strains with *P. aurelia*. A plus (+) sign indicates the presence of a *Paramecium* species, whereas a minus (–) sign indicates the absence of a *Paramecium* sp.s. *indicates statistical significance ($P < 0.05$). Numbers in parenthesis denote the *P*-value when comparing the 24 h control group and 24 h experimental group. *P*-values were obtained using the Student's *t*-test.

indicating that some *Paramecium* spp. can prey on fungal cells and reduce fungal growth by killing cryptococcal cells. However, *P. multimicronucleatum* does not reduce CFU numbers for any of the *C. neoformans* strains studied (Fig. 2B). One possible explanation for this result is the fact that this species is the largest amongst all *Paramecium* spp. (~200–350 μm) [15]. The size of its cell body relative to *C. neoformans* may cause the paramecia to filter the fungus out of its feeding apparatus, as described [15]. Our data suggests that *Paramecium* spp. interact with *C. neoformans* differently than the interaction reported by Bunting *et al*. and Steenbergen *et al*. regarding *Acanthamoeba polyphaga* and *A. castellani*, respectively [11,16]. Unlike the interactions with *A. castellanii* and macrophages, interactions of *C. neoformans* with *Paramecium* spp. do not result in death of the protozoan after a 24-h incubation period [17]. In this regard, the interaction between cryptococci and paramecia also differs from the interactions of cryptococcal cells with *C. elegans* [18].

Based on our observations, the paramecium feeding apparatus and digestive system are not disabled by the virulence factors of *C. neoformans* serotypes A, B, and D. Consequently, the *C. neoformans-Paramecium* spp. interaction provides a fundamentally different system for studying fungal-host interactions than the amoeba, slime mold, and worm systems described previously. Additionally, our results describe another type of interaction that can be expected to constitute a major selective pressure for *C. neoformans* and other related yeasts in the environment.

Acknowledgements

We thank Andre Nicola for helping with the fluorescent microscopy work and for providing Uvitex 2B. We also thank Susana Frases-Carvajal and Nareen Abboud for their insight and advice regarding each of the experimental procedures.

This work was supported by National Institutes of Health grants AI33774, AI33142, and HL59842-01 to A.C. CJC was supported by Training Program in Cellular and Molecular Biology and Genetics, T32 GM007491. SZF is supported by the Dr Marshall Horwitz Fellowship of Yeshiva University.

Declaration of interest: None.

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This paper was first published online on Early Online on 15 March 2010.