INFECTION AND IMMUNITY, Apr. 1978, p. 262-266
0019-9567/78/0020-0262$02.00/0

Vol. 20, No. 1
Printed in U.S.A.

Cryptococcus neoformans: Pseudohyphal Forms Surviving Culture with *Acanthamoeba polyphaga*

J. B. NEILSON, M. H. IVEY, AND G. S. BULMER*

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Received for publication 21 November 1977

During experiments on the gastrointestinal tract as a possible portal of entry for *Cryptococcus neoformans*, we occasionally observed the free-living amoeba, *Acanthamoeba polyphaga*, growing in the presence of *C. neoformans* cultured from mouse feces. Examination of the amoebic trophozoites revealed that they were engorged with yeast cells. Over a period of 2 to 3 weeks of incubation, the amoebae apparently killed most of the yeast cells. Some of the surviving *C. neoformans* cells formed atypical colonies which contained pseudohyphae. Seven other strains have since been cultured with this amoeba. Pseudohyphal forms were found among the surviving colonies in all strains tested. Virulence studies were performed on one randomly selected pseudohyphal isolate from each of the eight strains of *C. neoformans*. Pseudohyphal isolates from seven of the eight strains failed to kill mice 30 days after intracranial inoculation. The potential role of soil amoebae in the control of *C. neoformans* in nature is discussed.

Cryptococcus neoformans, a pathogenic encapsulated yeast, occurs worldwide in soils, especially those which contain pigeon excreta (26). Although *C. neoformans* is usually described as an encapsulated yeastlike cell 2.5 to 14 μm in diameter (8, 20), there are numerous reports of unusual cell shapes, e.g., pseudohyphal and hyphal forms (6, 9, 12, 13, 20, 21, 25, 27) and exceptionally large (7) and small (22) cell sizes. The recently discovered sexual stages of *C. neoformans* contain hyphae with clamp connections, dolipore septal complexes, and basidia which bear haploid basidiospores (17-19). Farhi et al. (10) reported that cells of *C. neoformans* inoculated into sterile soil persisted as a relatively small, non-encapsulated yeast. Neilson et al. (22) recently reported isolating cells of *C. neoformans* less than 1.1 μm in diameter from soil seeded with *C. neoformans* weeks earlier. These small yeast cells were capable of causing cryptococcosis in mice.

Acanthamoeba polyphaga is a free-living amoeba found widely distributed in soil and water (23). *A. polyphaga* is not considered to be a pathogen (23), although it has been implicated in a few cases of corneal ulceration (16) and primary amoebic meningoencephalitis (14).

In a series of notes published in 1930, Castellani (2-5) reported the isolation of an amoeba from cultures of *C. pararoseus*. He observed that the trophozoites often contained yeast cells in their protoplasm and proposed that the amoeba was "parasitic on the yeast" (2, 3). Further studies revealed that the isolated amoebae were capable of using several species of yeast and bacteria as food sources (3, 4, 6).

Previous studies in our laboratory involved the recovery of *C. neoformans* from feces of mice which were inoculated intragastrically with the yeast (J. R. Green, M.S. thesis, University of Oklahoma Health Sciences Center, Oklahoma City, 1977). In certain experiments, we observed, in a few instances, colonies of amoebae growing among the colonies of *C. neoformans* on the trypan blue selective medium (28). These amoebae were identified as *A. polyphaga* (F. C. Page, personal communication). Microscopic examination of the *A. polyphaga* showed that the trophozoites had ingested yeast cells. Since *Acanthamoeba* species, as well as *C. neoformans*, are free-living soil organisms found throughout the world, we have initiated a series of investigations to determine if this amoeba might exert any biological control over the pathogenic yeast *C. neoformans*. This study reports on changes in growth characteristics and alteration in virulence of *C. neoformans* when cultured with this amoeba. In future papers we will report on the killing of *C. neoformans* by *A. polyphaga* and the interaction of these two organisms in soil.

MATERIALS AND METHODS

Preparation of sterile amoebic cysts. Cysts of *A. polyphaga* were obtained by growing the amoebae (isolated from mouse feces) in culture at 25°C for 2 weeks with *C. neoformans* on trypan blue agar me-

dium (28). The cysts were washed from the medium with sterile distilled water and centrifuged at 500 × *g* for 10 min. Using the procedure described by Singh et al. (24), the cysts were treated with 1.5 to 2.0% (wt/vol) hydrochloric acid for 24 h to kill yeast cells, amoebic trophozoites, and partially formed cysts. The remaining mature viable cysts were washed with distilled water and suspended in 2% (wt/vol) sterile sodium deoxycholate solution for 1 h at 25°C and then further washed several times with distilled water to remove small particles and water-soluble products released by the dead cells of *C. neoformans*. Finally, cysts were suspended in sterile distilled water and stored at 4°C. The viability of the cysts was determined by rapid eosin staining, using 0.125% (wt/vol) eosin solution in distilled water. Eosin stained the dead cysts within a few seconds, whereas the living cysts remained unstained for 10 to 15 min. The above procedure effectively killed yeast cells without reducing the percent viability of *A. polyphaga* cysts.

Yeast cultures. Eight strains of *C. neoformans* were obtained from human cases either at the University of Oklahoma Health Sciences Center or the University of Saigon School of Medicine. All cultures were maintained on Sabouraud dextrose agar at 25°C.

Isolation of pseudohyphal yeasts. The eight strains of *C. neoformans* were streaked for heavy growth in a large cross on trypan blue agar medium in petri plates. A loop of sterile cysts of *A. polyphaga* was placed at the intersect of each cross. After 2 to 3 weeks of incubation at 25°C, most of the cells of *C. neoformans* in the streak had been replaced by amoebic cysts; however, at various places along the streak, a few surviving colonies of *C. neoformans* were observed. In India ink preparations, 3 to 20 (average, 14) colonies per plate were studied microscopically. Percentages of colonies which contained predominately pseudohyphal forms rather than yeastlike cells were calculated. From each strain, a colony composed of predominately pseudohyphal cells was streaked for isolation. Biochemical characteristics of both yeast and pseudohyphal forms of *C. neoformans* were determined, using Uni-Yeast-Tek plates (Corning Medical Diagnostic, Roslyn, N.Y.). Cultures were maintained on Sabouraud dextrose agar medium at 25°C.

Virulence experiments. Virulence of the different morphological forms of *C. neoformans* for 4-week-old Swiss white mice from the Charles River Laboratory (Wilmington, Mass.) was determined. Initially, sixteen groups of four mice each were inoculated with 4-day-old cultures of both the original yeast and the pseudophyphal isolates of eight different strains of *C. neoformans*. Each animal was inoculated intracranially with 10^5 cells suspended in 0.05 ml of sterile saline. The inocula were quantitated with a hemacytometer and confirmed by viable dilution plating. For pseudohyphal forms, each branching unit was counted as one cell on the hemacytometer. No more than a 2% error was recorded by dilution plating methods. Tests were repeated with each pseudohyphal isolate, using an inoculum of 10^6 cells per mouse. The brains of these mice were studied microscopically after death and cultured on trypan blue agar. Animals still alive 30 days after inoculation were sacrificed. Brains were removed and studied as above.

RESULTS

Pseudohyphal isolates. When *C. neoformans* was inoculated on medium containing cysts of *A. polyphaga*, excystment occurred within 24 h. The emerging trophozoites began to multiply and migrate out from the center of the petri plate along and through the yeast cell growth. During this process, amoebae were seen engorged with cells of *C. neoformans*. The amoebae subsequently encysted. After 2 to 3 weeks of incubation it was evident, visually and microscopically, that the yeast in the original streaks had been consumed and replaced with cysts of the amoeba, except for the occasional surviving yeast colonies along the lines of the streaks. These colonies apparently developed from cells which were not killed by the amoebae (Fig. 1). Microscopic examination revealed that from 8 to 64% of these colonies were composed of pseudohyphal forms of *C. neoformans* (Table 1).

Figure 2 is a photomicrograph of pseudohyphal cells from strain CIA suspended in lactophenol cotton blue. The morphology of the cells is typical of this particular pseudohyphal isolate. There was, however, considerable variation among the pseudohyphal isolates of the eight strains studied; e.g., pseudohyphae of various strains were curled or straight, ranged in length from 15 to 150 μm, and showed various amounts and degrees of branching. In four of the pseudohyphal strains (HB4C-S, CIA-S, G-S, Saigon 11-S), occasional clamp connections were observed. In all cases, the pseudohyphal isolates were remarkably different from the encapsulated yeast cells in the original clinical isolates. Pseudohyphal strains Saigon 11-S, Saigon 15-S, and CIA-S had little or no capsule, whereas the other five pseudohyphal isolates were encapsulated.

In describing the surviving colonies, the term "predominantly pseudohyphal" is used because occasional yeastlike cells were observed. We feel that these cells then elongate into pseudohyphae. The pseudohyphal isolates remained stable for several months; i.e., none of them, with the exception of 184-S (see below), reverted to the typical yeast form.

Biochemical characteristics of all pseudohyphal isolates conformed with accepted parameters for the identification of *C. neoformans*.

Virulence. All eight clinical isolates of *C. neoformans* killed mice when a 10^5 inoculum was used. India ink preparations revealed the presence of encapsulated yeast cells in the brains of these animals. Viable cells of *C. neoformans* were cultured from smears of brain tissues.

Seven of the eight pseudohyphal isolates did not kill mice when a 10^5 inoculum was used

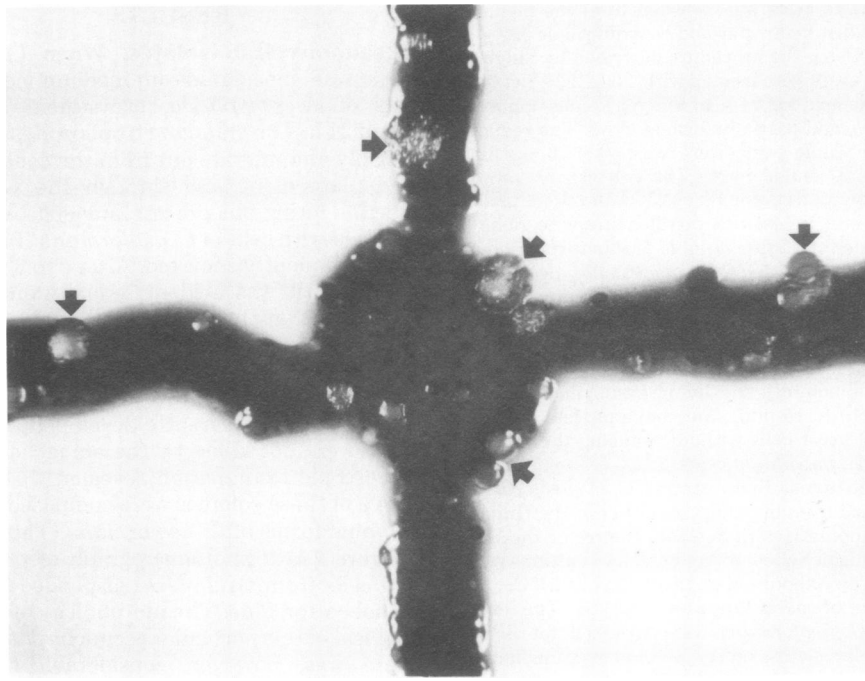

FIG. 1. *Surviving colonies of C. neoformans (arrows) from strain CIA appearing through original growth of yeast and A. polyphaga.*

TABLE 1. *C. neoformans co-cultured with A. polyphaga: surviving yeast colonies which formed pseudohyphae*

Strain of *C. neoformans*	Colonies composed of pseudohyphae (%)
G	58
Throng	33
Saigon 15	8
CP110	55
Saigon 11	20
184	50
HB4C	64
CIA	15

(Table 2). The one exception, strain 184-S, killed mice; however, upon re-examination, it was discovered that a high percentage of the cells of the inoculum of this isolate had reverted to the encapsulated yeast form.

As a further check for virulence, additional animals were inoculated with 10^6 cells of the eight pseudohyphal isolates. With the exception of strain 184-S, few of the animals died, indicating again that seven of the eight pseudohyphal forms were relatively avirulent for mice (Table 2). Again, the one exception to this was strain 184-S, which killed four out of four animals; however, it was noted again that most of the cells of the inoculum reverted to the encapsulated-yeast parent type.

Thirty days postinoculation, surviving animals which were inoculated with the pseudohyphal isolates were sacrificed, and the brains were removed. In India ink preparations, occasional fungal elements were observed and, in most instances, viable cells of *C. neoformans* were cultured from brain portions smeared on trypan blue agar medium. The resulting colonies were similar to the original pseudohyphal isolate both macroscopically and microscopically.

DISCUSSION

During experiments on the isolation of *C. neoformans* from mouse feces, we occasionally observed the development of colonies of *C. neoformans* which were surrounded by a halo. Microscopic examination of the halo area revealed amoebic cysts. Amoebae also were present and appeared to be ingesting cells of *C. neoformans*. This chance observation led us to wonder if an amoeba such as the widely distributed free-living soil amoeba *A. polyphaga* (Puschkarew 1913) might exert some biological control over *C. neoformans* in nature.

The effect of the amoeba on eight strains of *C. neoformans* was tested. In all instances, this mixed culture resulted in the death of the majority of yeast cells. However, after 2 to 3 weeks of incubation of the *C. neoformans* with the amoebae, occasional colonies of *C. neoformans* grew up among the layer of encysted amoebae. Many of these colonies were composed of pseu-

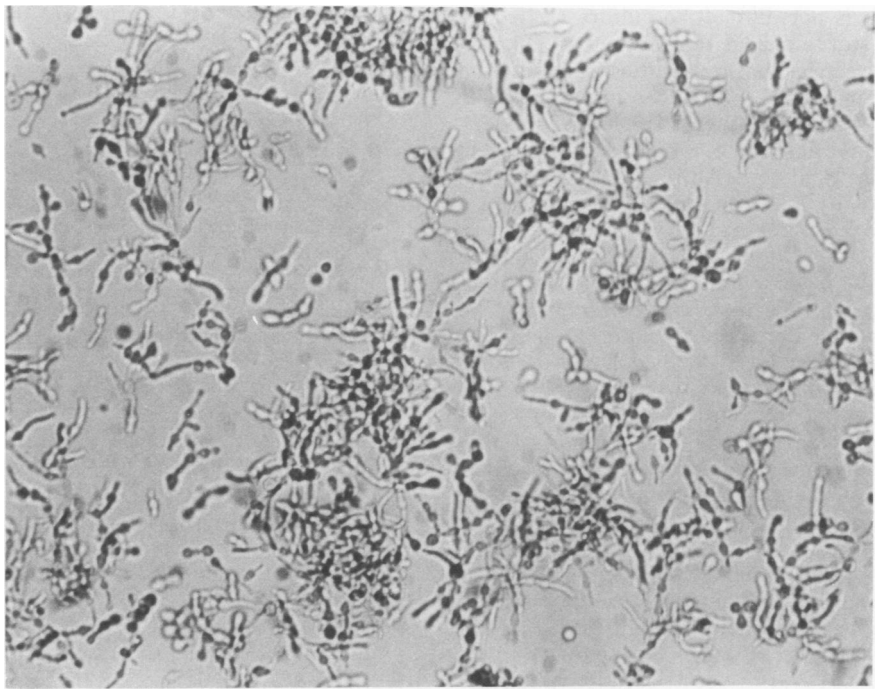

FIG. 2. *Pseudohyphal cells from C. neoformans strain CIA suspended in lactophenol cotton blue.*

TABLE 2. *Virulence of C. neoformans cells inoculated intracranially into mice*

C. neoformans strain	No. of animals inoculated/no. of animals deceased[a]	
	10^5 [b]	10^6 [b]
G	4/4	ND[c]
G-S[d]	0/4	1/4
HB4C	4/4	ND
HB4C-S	0/4	0/4
Saigon 15	4/4	ND
Saigon 15-S	0/4	0/4
CP110	4/4	ND
CP110-S	0/4	0/4
Saigon 11	4/4	ND
Saigon 11-S	0/4	1/4
184	4/4	ND
184-S[e]	4/4	4/4
Throng	4/4	ND
Throng-S	0/4	0/4
CIA	4/4	ND
CIA-S	0/4	0/4

[a] 30 days postinoculation.
[b] Yeast cells per mouse.
[c] ND, Not done.
[d] S indicates pseudohyphal forms.
[e] This strain reverted to the yeastlike form with occasional pseudohyphal cells before inoculation into mice.

dohyphal forms. Although the amoebae may, in some manner, have induced the formation of pseudohyphal forms, another explanation is also possible. During 14 years of research on cryptococcosis, we have occasionally seen pseudohyphal cells in numerous strains of *C. neoformans* both in vivo and in vitro (unpublished). Others have made similar observations (6, 12, 13, 20, 21, 25, 27). Perhaps pseudohyphal forms of *C. neoformans* are always present, and the amoeba *A. polyphaga* exerts a selective environmental pressure which favors their survival by ingesting the yeast cells but not the pseudohyphae. By demonstrating that pseudohyphal forms survived in eight different strains of *C. neoformans,* it appears that our observations do not represent an isolated, unusual event. Perhaps the ability of this yeast to form occasional pseudohyphae affords a biological "escape hatch" which insures survival of the species.

In our experiments we found that pseudohyphal isolates of *C. neoformans* were avirulent in mice. In contrast, other investigators (12, 21, 25, 27) have reported the isolation of virulent hyphal forms. Although avirulent hyphal (or pseudohyphal) forms of *C. neoformans* may not be of direct interest clinically, they may be significant in the epidemiology of *C. neoformans.* Possibly they represent forms in nature which have a selective advantage under certain conditions; under other conditions the yeast form may be advantageous. Perhaps virulence can be regained, such as in the case of strain 184-S, especially if selective pressures begin to favor the yeast form.

Since it is not difficult to culture *C. neoformans* in sterile soil in the laboratory (10, 15), one might expect that it would be isolated often from soil in nature. Such does not seem to be the case. Felton et al. (11) isolated *C. neoformans* from 4 out of 538 randomly selected soil samples in Oklahoma. Although environmental factors greatly affect the maintenance and proliferation of this organism in soil (15), there may be other reasons for its infrequent isolation from nature. The ingestion and killing of large numbers of yeast cells of *C. neoformans* and the survival of pseudohyphal forms in the presence of *A. polyphaga* (as described here) or other unknown organisms may account for its infrequent isolation. Perhaps pseudohyphal forms might not be identified as *C. neoformans* due to its atypical appearance and avirulence for mice. All of these factors need to be considered in further elucidation of the epidemiology of *C. neoformans*.

It is now known that *C. neoformans* may exist either encapsulated or non-encapsulated (10), in sizes ranging from giant forms over 50 μm in diameter (7) to small forms less than 1.1 μm in diameter (22), and in yeast, pseudohyphal, hyphal, or sexual forms with basidia and basidiospores. This organism should no longer be considered as existing solely as an encapsulated yeast, 2.5 to 14 μm in diameter.

The mode of killing and the interaction of soil amoebae and *C. neoformans* in nature are the subjects of continuing investigations in this laboratory.

ACKNOWLEDGMENTS

We thank R. A. and A. M. Fromtling for helpful discussion in preparation of this manuscript.

This investigation was supported by Public Health Service grant AI-05022 from the National Institute of Allergy and Infectious Diseases.

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