ISOLATION OF CRYPTOCOCCUS NEOFORMANS FROM SOIL

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Sources of infection in the highly fatal human disease, cryptococcosis (torula meningitis), have not been determined. Like most other systemic mycoses, this disease is not contagious, is sporadic in appearance, and is widely distributed geographically (Benham, 1950; Cox and Tolhurst, 1946). Although a history of trauma preceding infection usually has not been obtained, in several cases presenting meningeal involvement small focal pulmonary lesions were found at necropsy. Cryptococcosis occurs also in animals, notably in the lungs and nasal granulomata of horses; and this literature has been recently reviewed by Saunders (1948). There has been no indication that the disease is transmitted directly to man from an animal reservoir. It therefore has been considered probable that *Cryptococcus neoformans* (*Torula histolytica*) is present in man's environment and that man is infected accidently or under unusual circumstances.

Some potential pathogens, because of the circumstances under which infection has occurred, are believed to occur in soil, although the fungi have never been isolated and recognized except from the human host. Others, such as *Aspergillus fumigatus*, are very abundant in soil and are frequently isolated by appropriate methods. The occurrence in soil or decaying vegetation has been demonstrated by actual isolation in culture of *Aspergillus* sp. (Raper and Thom, 1949), *Sporotrichum schenckii* (De Beurman and Gougerot, 1908; Emmons, 1951), *Nocardia asteroides* (Gordon and Hagan, 1936), *Phialophora verrucosa* (Conant, 1937), *Coccidioides immitis* (Stewart and Meyer, 1932; Davis *et al.*, 1942; Emmons, 1942), *Histoplasma capsulatum* (Emmons, 1949; Ajello and Zeidberg, 1951), and *Allescheria boydii* (Emmons, 1951; Ajello and Zeidberg, 1951).

It is interesting to note that the first strain of *Cryptococcus neoformans* to be named was isolated by Sanfelice (1894) from fruit juice. There are two later reports of its isolation from milk (Klein, 1901; Carter and Young, 1950), where it may have been present as a contaminant from the milk utensils since there was no evidence of illness in the cows from which the milk specimens were taken. Benham (1935) reported the isolation, from human skin and feces, of avirulent strains similar in other respects to *C. neoformans* from cryptococcosis. It has been isolated many times from man in cases of generalized or central nervous system cryptococcosis (torulosis).

The occurrence of many cases of cryptococcosis in many parts of the world without any recognized pattern of peculiar or significant occupational exposure suggests that *C. neoformans* may have a widespread distribution as a saprophyte in nature. The writer is not aware, however, of any reported direct evidence for this except for the isolations previously mentioned.

In the course of a study designed to reveal sources in nature of *Histoplasma capsulatum* an opportunity was provided to detect other pathogenic fungi which might be present as saprophytes in soil. In addition to the isolation of 9 strains of *H. capsulatum*, partially reported elsewhere (Emmons, 1949), *Aspergillus fumigatus*, *Allescheria boydii* (Emmons, 1951), *Nocardia asteroides*, and certain saprophytic fungi which seem to be potentially pathogenic were isolated. Some of this material will be made the basis of a future report.

It is the purpose of this paper to report the isolation of 4 strains of *C. neoformans* from these soil samples.

METHODS

During the past 4 years 716 samples of soil have been taken, mostly from farm premises in Loudoun County, Virginia (Emmons, 1949). Many of these were replicate samples taken at intervals from premises on which we had previously found rats or cats with histoplasmosis. The samples were collected in large (2 cm diameter) sterile culture tubes.

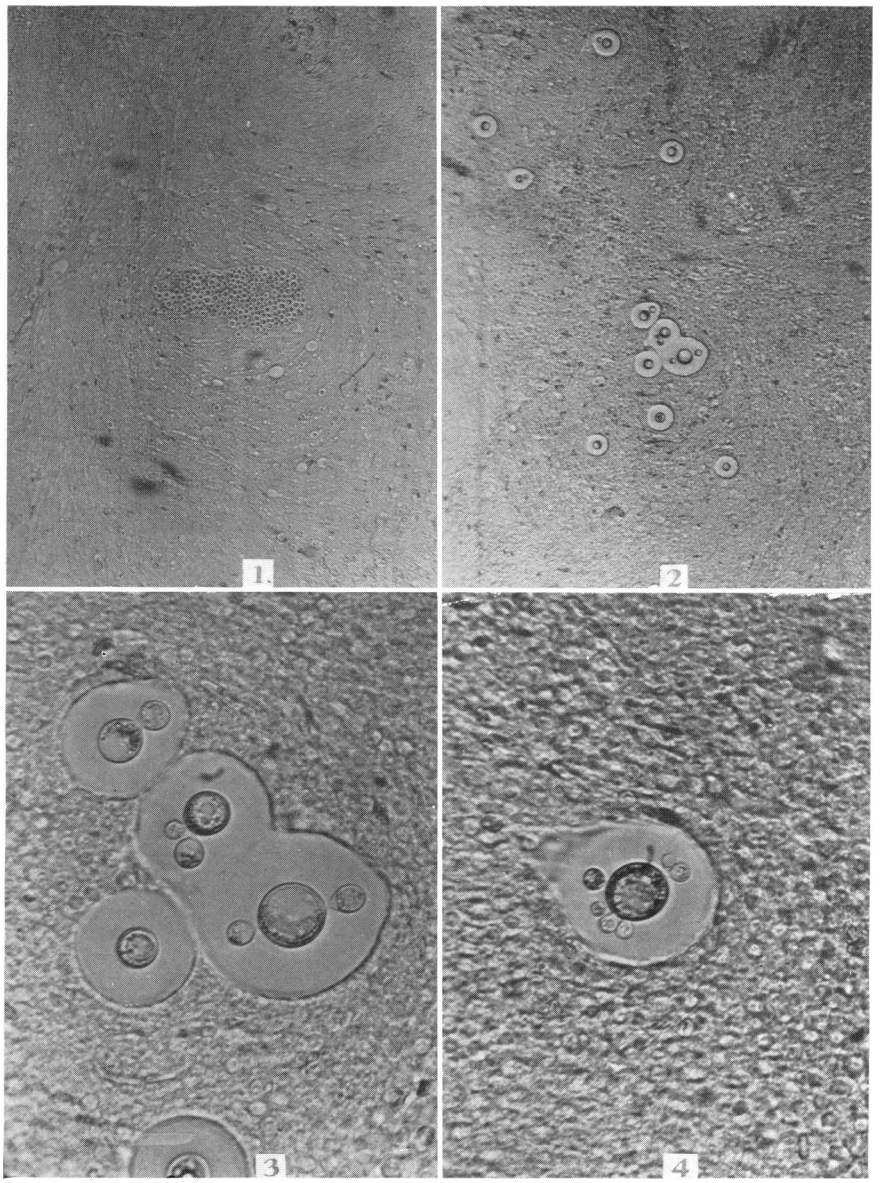
In the laboratory a portion of a soil specimen was suspended in physiological salt solution in a 100 ml graduate, stirred vigorously with a sterile wooden applicator, and left to settle at room temperature. After 2 to 3 hours a sample of 5 to 10 ml of suspension was pipetted from the upper portion of the column since it was believed that there was some concentration of the dry conidia of *Histoplasma* in the upper layers. However, there is no reason to suppose that there was any such concentration of *Cryptococcus*.

The suspension was then injected into the peritoneal cavity of each of 4 or 8 mice. A general purpose strain of white Swiss mouse was used and 1 ml of suspension per mouse was injected except in the case of specimens which appeared to have a high organic content when the dose was reduced to 0.5 ml. The mice were killed 4 to 6 weeks after inoculation, and cultures were made on modified Sabouraud's agar from the spleen and liver.

C. neoformans was isolated from 1 of 4, 2 of 4, 2 of 8, and 6 of 8 mice, respectively, receiving 4 of the soil samples. One of the 4 strains of *Cryptococcus* isolated gave rise in subsequent subculture to a mucoid mutant, which for convenience will be referred to as a fifth strain. On the basis of records and recollections of cultures it is believed that additional strains of this fungus were isolated early in the study, but they were lost before adequate examinations and positive identifications were made. In mice from which *C. neoformans* was isolated moderate enlargement of the spleen was observed. In one animal the spleen and liver were greatly enlarged and there was generalized lymphadenopathy. It seems probable that if the animals had been allowed to live for a longer period they would have shown lesions more characteristic of cryptococcosis.

RESULTS

Study of strains. This report is based upon a study of these 5 strains which have been compared with strains isolated from fatal cases of meningeal crypto-



Cryptococcus neoformans strain isolated from soil in experimentally infected mouse brain.

Figure 1. A group of small cells with relatively narrow capsules. × 100.

Figure 2. Large encapsulated cells. × 100.

Figure 3. Details of budding of large encapsulated cells. × 430.

Figure 4. Simultaneous budding at several points on the mother cell. × 430.

coccosis in man. They resemble strains of human origin in colony characteristics, morphology, fermentation reactions, and pathogenicity for mice.

In comparing strains of *C. neoformans* of saprophytic origin with strains from

fatal human cases, it is necessary to point out that the latter are not uniform (Benham, 1950; Cox and Tolhurst, 1946; Neill *et al.*, 1949). Strains vary in color, mucoid consistency of the colony, fermenting activity, and virulence for mice. Such differences were noted in the strains of human origin carried in our culture collection as well as in the strains isolated from soil.

The strains isolated from soil were moderately mucoid and were cream to light tan in color, becoming deep tan with age. One strain, as noted before, gave rise to a variant which was so highly mucoid that the colony flowed to the bottom of the tube when a slant culture was incubated in a vertical position. This mucoid variant did not differ from the other strains significantly in fermenting activity or virulence.

The 5 strains from soil (including the mutant) were compared with 3 strains of human origin and the original strain of Sanfelice. In testing for the production of acid all strains were transferred twice on sugar-free meat extract agar. Dunham tubes were then inoculated and incubated at 30 C for 3 weeks before final readings were made. No gas was formed in any sugar. Under these conditions all strains tested produced acid slowly in glucose, levulose, and mannose. Four of 5 soil strains, 1 of 3 human strains, and the Sanfelice strain produced acid slowly in maltose. Two of 5 soil strains, 1 of 3 human strains, and the Sanfelice strain produced acid slowly in starch. All human and soil strains produced acid slowly in sucrose. None produced acid in glycerol or lactose, and acid production was questionable in galactose.

All strains grew well at 37 C.

Intracerebral inoculation of all 9 strains killed white mice when the infecting dose was adjusted to an estimated 1,000 cells per mouse. However, a consistent pattern of deaths was not observed at this dose nor at 10^5 cells per mouse. Deaths occurred over an interval of 1 to 4 weeks, and it is believed that the erratic response was due to back leakage of the inoculating dose into the subcutaneous tissue and to differences in the exact loci of inoculations. Mice inoculated intracerebrally with much larger doses of human and soil strains died usually within 1 week.

Mice inoculated intraperitoneally developed a generalized infection which spread to the brain.

In the brains of mice dying of experimental cryptococcosis, the fungus could be found by crushing a piece of the brain under a cover slip (figures 1 to 4). In most cases there was a mixture of small cells with narrow capsules (figure 1), apparently representing young rapidly growing cells, and large cells with relatively wide capsules (figures 2 to 3), representing probably older cells. The cells show the characteristic spherical shape with buds arising at one or more points on the mother cell.

DISCUSSION

The possibility was considered that the experimental mice rather than the soil suspensions with which they were inoculated might be the source of the fungus. This possibility seems highly improbable since *Cryptococcus* has not

been isolated from stock mice in this laboratory, the pattern of isolations does not indicate naturally acquired disease in the stock mice, and the type of myxomatous lesion usually characterizing established or chronic infection was not observed in any of these mice.

This incidental finding in a search for another pathogenic fungus in soil is the first direct evidence that *C. neoformans* is present in soil. It offers an explanation for the source of infection in cases of meningeal cryptococcosis in which a primary pulmonary lesion has been demonstrated. The fungus has not yet been demonstrated in air, but it is probable that the type of thick encapsulated cell found in culture would be sufficiently resistant to drying to survive dissemination in air from a source in soil.

SUMMARY

Four strains of *Cryptococcus neoformans*, one of which gave rise to a highly mucoid variant, were isolated from soil by inoculation of mice with soil samples.

These strains were compared with 3 strains from human cryptococcosis and with the original Sanfelice strain. They are typical of the species, producing acid slowly in glucose, levulose, mannose, and sucrose and being virulent for mice.

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