THE PATHOGENICITY OF NATURALLY OCCURRING STRAINS
OF THE YEAST CRYPTOCOCCUS NEOFORMANS
AND RELATED SPECIES

by
Ann Marie Nuuttila

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STATEMENT BY AUTHOR

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SIGNED: Anna Marie Nuvitte

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Adelaide Evenson Riker
Associate Professor of Microbiology

Date
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ABSTRACT

The pathogenicity for mice of naturally occurring Cryptococcus species which included twenty strains of Cryptococcus neoformans, seven strains of Cryptococcus albidus, two strains of Cryptococcus diffluens and two strains of Cryptococcus laurentii was tested. The intraperitoneal route of injection was used.

Fourteen of the 500 mice inoculated with Cryptococcus neoformans developed either fatal or nonfatal persisting infections. The strain Sap 6 produced lesions and hydrocephalus in one mouse and lesions in seven mice.

Of the 175 mice injected with Cryptococcus albidus three developed infections fatal within 48 hours and another three within two weeks.

Although the cultures remained negative, typical cryptococcal lesions were observed in ten mice injected four weeks previously with one strain of Cryptococcus diffluens.

In the 50 mice inoculated with Cryptococcus laurentii positive cultures were obtained from one mouse that died within 48 hours and from two mice sacrificed after four weeks.
A slime flux strain of Cryptococcus neoformans grown for ten days on Mager and Aschner's medium produced four fatal infections within 72 hours and one nonfatal infection with lesions. Only one nonfatal persisting infection was produced by cells of this strain grown for 48 hours on either M-Y agar or on Mager and Aschner's agar.
INTRODUCTION

Cryptococcosis appears to be world-wide in distribution. Since the disease is not transmitted from man to man or animal to man, it may be assumed that the yeast is able to perpetuate itself in nature and that natural sites exist the world over.

Natural sources from which yeasts in general can be isolated include the animal body, plant exudates, as well as the bark, leaves and flowers of plants. *Cryptococcus neoformans* has been isolated from bark beetles of the *Dendroctonus* species (48), from the caecal contents of a healthy horse (54) and, in the Tucson area, from the slime flux, bark and twigs of the mesquite, *Prosopis juliflora* (13, 55). It has also been encountered in fermenting peach juice (44), milk (8, 21), air (30, 47), soil (1, 9, 15, 16), pigeon dung (1, 6, 11, 12, 14, 16, 20, 28, 38, 43, 53) and other avian excreta (19, 38, 53) -- all of which could be considered as the intermediate sources of the yeast.

The occurrence of this pathogen in nature and in nonliving materials has public health significance. Since the respiratory tract appears to be the route of infection and all these sources are exposed to air currents, the yeast could readily be carried to susceptible individuals producing in some instances subclinical or clinical disease.
The saprophytic existence of this potential pathogen, together with the possibility of its dissemination to humans, has stimulated studies of the disease-producing powers of the strains isolated from nonhuman sources. The pathogenicity of strains isolated from pigeon excreta has been evaluated by several investigators (6, 11, 14, 20, 28), including Hasenclever and Emmons (16), who used LD<sub>50</sub> determinations for the measurement of virulence for mice of various isolates.

Some preliminary testing of strains of Cryptococcus neoformans isolated from the slime flux of mesquite suggested that such strains might be pathogenic for mice (25). Furthermore, it has previously been observed that a strain of Cryptococcus diffluens which was isolated from the leaf of the passion vine, Passiflora coerulea, produced lesions in white mice (A. E. Evenson, personal communication). This finding suggested that other closely related species of Cryptococcus which differ from Cryptococcus neoformans in one or two biochemical characteristics could possibly be endowed with disease-producing capacity.

The purpose of this investigation was therefore to determine the pathogenicity for mice of naturally occurring strains of the yeast Cryptococcus neoformans as well as of related species, all of which are native to this desert area. The effects of media, incubation temperature, culture age, adjuvants and certain alterations in laboratory handling techniques on pathogenicity were also to be studied.
REVIEW OF THE LITERATURE

Sanfelice (45, 46) in 1895 was the first to observe experimentally the pathogenicity of Cryptococcus neoformans for dogs, guinea pigs and a chicken. The yeast had been isolated the previous year from fermenting peach juice (44).

In 1950 Carter and Young (8) isolated Cryptococcus neoformans from the pooled milk of twenty-five cows that showed no obvious sign of the disease. The milk sample had been subcutaneously inoculated into a guinea pig which died, and at autopsy the yeast was observed in the glands, lungs, spleen and kidneys. The isolate from the guinea pig was then injected into three other guinea pigs and three mice producing infection in two of the guinea pigs and one of the mice.

Emmons (11) studied the mouse virulence of Cryptococcus neoformans strains isolated by the mouse inoculation method from pigeon excreta. These strains were therefore previously selected as to mouse pathogenicity. The virulence tests revealed that all strains on intracranial injection of at least $10^4$ cells produced the characteristic dome-shaped elevation of the skull. Encapsulated Cryptococcus cells were demonstrated in the intracranial lesions.

In 1957 Kao and Schwarz (20) obtained strains by similar methods from pigeon nests and determined their disease-producing
power by injecting them intraperitoneally or intracerebrally into mice. The seventeen strains tested produced disseminated lesions and death in many of the experimental mice.

The following year Fazekas and Schwarz (14) reported the histologic changes produced in mice following either intraperitoneal or intracerebral injection with strains of Cryptococcus neoformans isolated from pigeon excreta. Histological sections were made of the brain, heart, liver, lungs, pancreas and spleen of mice inoculated directly with pigeon excreta samples and subsequently with the cultures from these organs. Sections of the various organs were made from mice that died spontaneously as well as from those that were sacrificed after the experimental period.

In 1959 Littman and Schneierson (28) reported the virulence of strains of Cryptococcus neoformans that were isolated by culture on liver-spleen glucose blood agar from pigeon excreta in New York City. All seventy-two strains injected intracerebrally into mice produced death within three to seventy-three days. Ninety per cent of the mice developed hydrocephalus and encapsulated yeasts were observed in India ink wet mounts of the brain tissue.

Bergman (6), in Sweden, compared the pathogenicity of strains tentatively considered to be Cryptococcus neoformans that had been isolated by streaking pigeon excreta on Sabouraud-dextrose agar. The yeast, which was inoculated intracerebrally, was observed in India
ink mounts and in histological sections of brain tissue from the mice that died and from those that were sacrificed after a period of three months.

A more precise method for pathogenicity studies of naturally occurring strains of Cryptococcus neoformans was reported in 1963 by Hasenclever and Emmons (16) who used LD<sub>50</sub> determinations. The strains examined were isolated from soil and from pigeon excreta and were obtained by culture on Emmons' modification of Sabouraud's agar plates and by mouse inoculation. Isolates from human cases of cryptococcosis were also included. Ten-fold serial dilutions ranging from 5.0 x 10<sup>3</sup> to 5.0 x 10<sup>7</sup> cells per mouse were inoculated intraperitoneally into groups of mice. It was observed that the LD<sub>50</sub> of all strains isolated from patients fell below 7.9 x 10<sup>5</sup> viable cells. Of the saprophytic strains, sixty-six per cent had an LD<sub>50</sub> between 7.9 x 10<sup>4</sup> and 7.8 x 10<sup>6</sup> viable cells, and forty-nine per cent were comparable in LD<sub>50</sub> range to those from cases of cryptococcosis. All of the strains that were obtained by mouse inoculation had an LD<sub>50</sub> below 7.8 x 10<sup>6</sup> viable cells.

In 1964 Lamb (25) reported preliminary pathogenicity studies of eight strains isolated from the slime flux of the mesquite tree. None of the strains proved fatal to the mice during the five week experimental period. However, one strain of the yeast was cultured from the spleen of a mouse that never showed signs of clinical disease, and
another strain was cultured from the brain of a mouse that showed paralysis of the hind quarters after the first week.

The virulence of bacteria, such as pneumococci, has been shown to be increased by animal passage or culturing on enriched media (49). According to Littman and Zimmerman (29), serial passage through white Swiss mice will increase the virulence of Cryptococcus neoformans but to a lesser degree than normally shown by bacterial and viral pathogens. Bergman (5) found that fifty-seven mouse passages with intervening six to ten days' culture on Sabouraud's dextrose agar did not increase the virulence of a human isolate. Hasenclever and Emmons (16) indicated that the virulence of strains from soil and pigeon excreta was apparently unaltered by a single mouse passage. On the other hand, Benham (2) by a series of passages in young rats was able to increase the virulence of strains of Cryptococcus neoformans isolated from human cases of cryptococcosis but not of strains obtained from normal skin. Furthermore, Benham (3) found no indication that prolonged growth of virulent strains on culture media altered the virulence of the yeast. In her experience, the strain originally isolated by Busse and Buschke in 1894 from a woman with cryptococcosis was pathogenic for animals in 1950.

The routes of inoculation which have been used in experimental cryptococcal studies in mice appear to be directly related to the rapidity of infection and mortality. Bergman (4) found the subcutaneous
route useful for the study of the course of invasion and proliferation of *Cryptococcus neoformans* in mouse tissues. However, when studies were conducted primarily to produce a fatal cryptococcosis involving the central nervous system, the intracranial route proved most successful (10, 17, 50). Kao and Schwarz (20) and Fazekas and Schwarz (14) found that *Cryptococcus neoformans* produced widely disseminated lesions and could be demonstrated in the brain and lungs of mice inoculated either by the intracerebral or intraperitoneal route. However, the intracerebral method produced higher mortality and the yeast was more often demonstrated by culture and in histological sections of the above organs. Kligman and Weidman (23) observed that the intraperitoneal and the intravenous routes of inoculation were equally effective in producing invasion of the lungs and the central nervous system of mice. Smith, Ritter, Larsh and Furcolow (51) found that fatal disease in mice could be produced by either the intraperitoneal or respiratory route although the intraperitoneally inoculated animals exhibited the highest mortality within twelve weeks after inoculation.

Adjuvants such as mucin, kaolin and substances of plant or bacterial origins have been used to enhance the pathogenicity of bacteria in experimental animals (32, 33, 37, 40, 41, 42). Olitzki and Koch (42) and Olitzki (41) noted that, in the case of various members of the family Enterobacteriaceae, gastric mucin was the best suspending medium for lowering the resistance of experimental mice to
infection. Strauss and Kligman (52) found that gastric mucin increased the extent and rapidity of cryptococcal infection in laboratory mice. Only the twenty-two per cent mortality was observed when the organisms were suspended in saline while one hundred per cent mortality occurred when the organisms were suspended in mucin. In addition, the survival time was shortened and the extent of infection was greater. They also induced death within ten days in mice inoculated with mucin suspension of Nocardia asteroides, Blastomyces dermatitidis, Coccidioides immitis, Sporotrichum schenckii, Candida albicans and Histoplasma capsulatum. Infection was produced in mice when similarly inoculated with Paracoccidioides brasiliensis, Hormodendrum pedrosoi, Hormodendrum compactum, Phialophora verrucosa and Haplosporangium parvum. Mankowski (35) showed that Candida tropicalis, Candida stellatoidea, Candida pseudotropicalis and Candida kruzei were distinctly pathogenic for mice when the intraperitoneally inoculated yeasts were suspended in mucin rather than saline.

Although no attempt was made in the course of this study to physiologically alter the experimental mice, several investigators have used such means. Mankowski (35) produced a fatal disease with normally nonpathogenic Candida species when the mice had been treated with the antibiotic aureomycin or the hormones cortisone or
estradiol. Louria and Brown (31) found that treating mice with
cortisone increased their susceptibility to infection with *Candida*
albicans. Hasenclever and Mitchell (18) showed that *Torulopsis*
glabrata, a yeast of uncertain pathogenicity, will produce a progressive kidney infection in cortisone-treated mice or mice made diabetic
by x-ray or alloxan treatment. Levine, Zimmerman and Scorza (26)
working with *Cryptococcus neoformans* and Newcomer, Wright, Tarbet,
Winer and Sternberg (39) working with *Coccidioides immitis* noted
more rapid dissemination in cortisone-treated mice. Mankowski and
Littleton (36) in 1954 observed an increased mortality rate and a re-
duction in weight of mice injected with cortisone and subsequently in-
fected with *Cryptococcus neoformans* or *Histoplasma capsulatum*. In
the screening of soil for pathogenic fungi, Busailah (7) found the use of
cortisone-treated mice advantageous.

The mouse appears to be the animal of choice for pathogenicity
studies using *Cryptococcus neoformans*. However, other animals have
been used with varying degrees of success. For example, Smith,
Mosberg, Manganiello and Alvarez de Choudens (50) used the intra-
cranial route of inoculation and three strains of *Cryptococcus neofor-
mans* and attempted to induce torulosis of the central nervous system
in mice, dogs, cats, guinea pigs and rabbits. These workers con-
cluded that the mouse was the animal of choice since this species
was most regularly infected and the clinical course of disease was most consistently pursued. They suggested that the body temperature of the experimental animal may play a role in the susceptibility of the species to infection. The mouse body temperature is about 37°C with the dog, cat, guinea pig and rabbits' progressively higher. The rabbit was found to be the most difficult to infect, Kligman and Weidman (23) in 1949 substantiated Kuhn's (24) previous experiments which showed that rabbits resisted infection when injected intravenously with a massive dosage of *Cryptococcus neoformans* but did exhibit a rise in normal body temperature from 39.5°C to about 41°C. The effect of incubation temperature of *Cryptococcus neoformans* on the pathogenicity for chick embryos was studied by Kligman, Crane and Norris (22). They found that the survival time of chick embryos was increased when the cells were incubated at 39°C, 40°C or 41°C and was accompanied by a decrease in the number of lesions produced. The yeast in the chick embryos was completely destroyed in eight days when the infected embryos were incubated at 40°C.
MATERIALS AND METHODS

Hasenclever and Emmons (16) used LD$_{50}$ determinations for the comparison of the pathogenicity of strains of *Cryptococcus neoformans* isolated from soil and pigeon habitats with that of strains isolated from disease processes. Similar procedures were adopted for the pathogenicity study of naturally occurring strains of *Cryptococcus neoformans* and related species native to this desert area. For comparison, the disease-producing potential of strains from cases of human cryptococcosis was also determined. The organisms tested during the course of this investigation together with the source from which each was originally isolated are listed below:

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<tr>
<td>2. Cryptococcus neoformans AJL-117</td>
<td>Slime flux of mesquite, Lamb (25)</td>
</tr>
<tr>
<td>3. Cryptococcus neoformans AJL-118</td>
<td>Slime flux of mesquite, Lamb (25)</td>
</tr>
<tr>
<td>5. Cryptococcus neoformans AJL-120</td>
<td>Slime flux of mesquite, Lamb (25)</td>
</tr>
<tr>
<td></td>
<td>Cryptococcus neoformans</td>
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<tr>
<td>---</td>
<td>-------------------------</td>
</tr>
<tr>
<td>7</td>
<td>AJL-123</td>
</tr>
<tr>
<td>8</td>
<td>AJL-124</td>
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<td>AJL-125</td>
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<td>10</td>
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<td>2</td>
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<td>16</td>
<td>E_1</td>
</tr>
<tr>
<td>17</td>
<td>B_14</td>
</tr>
<tr>
<td>18</td>
<td>Sap 3</td>
</tr>
<tr>
<td>19</td>
<td>Sap 5</td>
</tr>
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</table>
20. **Cryptococcus neoformans** Sap 6  
Slime flux of mesquite following inoculation at another site. Westerlund (55)

21. **Cryptococcus neoformans** M 1000  
Patient with cryptococcal meningitis. (Isolation made 25 to 30 years ago)

22. **Cryptococcus neoformans** D-2344  
Patient with cryptococcosis.

23. **Cryptococcus neoformans** CDC-51  
Patient with cryptococcosis.

24. **Cryptococcus neoformans** 8476  
Patient with cryptococcal meningitis.

25. **Cryptococcus albidus** 1  
Slime flux of mesquite. Westerlund (55)

26. **Cryptococcus albidus** Flux 1  
Slime flux of mesquite. Westerlund (55)

27. **Cryptococcus albidus** C₂  
Slime flux of mesquite. Westerlund (55)

28. **Cryptococcus albidus** 4E  
Bark of mesquite. Westerlund (55)

29. **Cryptococcus albidus** B₃  
Bark of mesquite. Westerlund (55)

30. **Cryptococcus albidus** B₁₇  
Bark of mesquite. Westerlund (55)

31. **Cryptococcus albidus** 176  
Bird droppings. Westerlund (55)

32. **Cryptococcus diffluens** AT-128  
Leaf of passion vine. Thomas and Evenson

33. **Cryptococcus diffluens** AJL-129  
Slime flux of cottonwood. Lamb (25)
The morphological and physiological characteristics used by Lodder and Kreger-van Rij (30) to differentiate the four *Cryptococcus* species tested are presented in Table 1. Medical mycologists, however, identify an encapsulated budding yeast which grows at 37°C, produces urease and is pathogenic for mice as *Cryptococcus neoformans* (27, 29).

The general procedure used in this investigation involved the intraperitoneal injections of a predetermined number of cells and subsequent observation of the mice for periods varying from four to thirteen weeks. The general condition and behavior of the animals were noted daily.

Unless otherwise indicated, each organism was grown on slants of Wickerham's (56) malt extract - yeast extract agar (M-Y agar) in screw capped tubes for a period of 48 to 72 hours. The cells were then gently washed off the slants with sterile physiological saline. The total number of cells per ml in the undiluted saline suspension was estimated using a Levy-Neubauer hemocytometer and the number of viable cells per ml was determined in pour plate cultures of M-Y agar. From this information the total number of cells
<table>
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<th>Assimilation Pattern</th>
<th>Ethanol as Sole Source of Carbon</th>
<th>Assimilation of KNO₃</th>
<th>Starch Formation</th>
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<tr>
<td>Cryptococcus neoformans</td>
<td>Round or almost round</td>
<td>+</td>
<td>Absent</td>
<td>+ + - + +</td>
<td>Fair development</td>
<td>-</td>
<td>Under appropriate conditions</td>
</tr>
<tr>
<td>Cryptococcus albidus</td>
<td>Round to short-oval</td>
<td>+</td>
<td>Absent</td>
<td>Occasionally weak</td>
<td>Usually + Pirouette + +</td>
<td>+</td>
<td>Under appropriate conditions</td>
</tr>
<tr>
<td>Cryptococcus diffuens</td>
<td>Round or oval</td>
<td>+</td>
<td>Absent</td>
<td>Occasionally weak</td>
<td>Occasionally Weak</td>
<td>-</td>
<td>Under appropriate conditions</td>
</tr>
<tr>
<td>Cryptococcus laurentii</td>
<td>Round, oval or occasionally long-oval</td>
<td>+</td>
<td>Absent</td>
<td>Sometimes Weak</td>
<td>Growth or no growth</td>
<td>-</td>
<td>Under appropriate conditions</td>
</tr>
</tbody>
</table>
and the number of viable cells inoculated into each mouse were calculated.

Four ten-fold serial dilutions of cells were prepared in sterile physiological saline and each mouse in a group of five was inoculated intraperitoneally with 0.5 ml of a particular cell suspension. There were five concentrations of yeast cells and each concentration was inoculated into a group of five mice. A control group of five mice was injected with 0.5 ml sterile saline.

Mice that died during the experimental period as well as those that were sacrificed at the end were subjected to autopsy. The brain, liver, lungs, spleen and any lesions found were minced and cultured in the malt extract - yeast extract broth (M-Y broth) of Wickerham (56). India ink mounts of lesions were examined for encapsulated yeasts. The M-Y broth cultures were observed for evidence of growth. If growth occurred, India ink mounts were made. A positive result was recorded if encapsulated budding yeast cells were observed.

Four to five week old white Swiss mice of the Webster strain were obtained from Berkeley-Pacific Laboratories, Laboratory Animal Husbandry Center, Berkeley, California. Female mice weighing between 17 and 19 g were used in most instances. Male mice weighing between 24 and 26 g were occasionally employed.

A preliminary experiment was undertaken to see if the methods of Hasenclever and Emmons (16) could be adapted to this investigation.
Cryptococcus neoformans M<sub>1000</sub> grown at 26-28°C was used as the test organism. The inoculated mice were sacrificed after thirteen weeks and examined as previously described.

The methods of Hasenelever and Emmons were found to be inapplicable with this organism for no deaths occurred. A sequence of experiments was performed to evaluate the effects of incubation temperature, elevated temperatures, adjuvants, repeated washing of the cells, growth media and the length of incubation period on the virulence of strains of Cryptococcus neoformans.

1. Cultures of Cryptococcus neoformans M<sub>1000</sub> were grown at temperatures of 20°C, 26-28°C and 37°C to see if the temperature of incubation influenced the disease-producing potential of the organism. Predetermined numbers of cells were injected intraperitoneally into mice. The mice were observed for a thirteen week experimental period.

2. The virulence of heat tolerant cells and of cells not exposed to an elevated temperature was then compared. Cells of Cryptococcus neoformans M<sub>1000</sub> were exposed repeatedly to degrees of heat above the optimum growth temperature. Cultures of the yeast were grown on M-Y agar at 26-28°C for 48 to 72 hours. The organisms were washed off the slants and suspended in sterile physiological saline. Ten ml portions of the yeast suspension were immersed in a 60°C water bath for thirty minutes and then cooled
immediately in an ice bath. The total number of cells as well as the number of viable cells per ml was determined in both the heat-treated and nonheat-treated control suspensions. Cultures of each were made by transferring 1 ml to slants of M-Y agar. Similar heat tolerance procedures were carried out on the new culture until a total of seven heat treatments was accomplished. Saline suspensions containing known numbers of either the heat tolerant cells or the nonheat-treated control cells were inoculated into groups of mice. The mice were observed for a period of eight weeks.

3. A comparison of the effectiveness of mucin and of mucin plus kaolin as pathogenicity-enhancing substances for Cryptococcus neoformans \( M_{1000} \) was made. The yeast cells, grown at 26-28°C on M-Y agar for 48 to 72 hours, were gently washed off the slants and the total number of cells as well as the number of viable cells per ml was estimated. Four ten-fold serial dilutions were prepared with sterile physiological saline. Equal volumes of cell suspensions (undiluted, \( 10^{-1} \), \( 10^{-2} \), \( 10^{-3} \) and \( 10^{-4} \)) were mixed with sterile five per cent mucin or with sterile five per cent mucin plus one per cent kaolin. Granular mucin (Type 1701-W, Lot No. 129503) was obtained from Wilson Laboratories, Pharmaceutical Division, Wilson and Co., Inc., Chicago 9, Illinois, and the five per cent suspension was prepared in the manner suggested by the Wilson Laboratories. Colloidal kaolin, N.F (781608) obtained from Merck and Co., Chemical Division,
Rahway, New Jersey was incorporated in a one per cent concentration into the five per cent mucin suspension before autoclaving. Each of the five dilutions suspended in mucin, each of the five dilutions suspended in mucin plus kaolin and each of the five control dilutions suspended in 0.85% saline was injected into a group of five mice. The mice were observed for a period of eleven weeks. Three and one-half weeks after inoculation, one mouse from each group was sacrificed and autopsied. At the end of the eleven week period, the remaining mice were sacrificed and the organs examined.

4. An earlier experiment concerned with the effect of incubation temperature on the virulence of Cryptococcus neoformans M1000 was in part repeated. In this experiment the cells were grown on M-Y agar for 48 hours at temperatures of 28°C and of 37°C. The cells were harvested, diluted, suspended in mucin and predetermined numbers were injected into groups of mice. The mice were observed for signs of infection during the four week experimental period.

5. Experiment 3 described previously was repeated using Cryptococcus neoformans CDC-51 (an isolate from a case of cryptococcosis) as the test organism. During the eight week experimental period the mice were observed for evidence of infection.

6. The pathogenicity of Cryptococcus neoformans 8476 and Cryptococcus neoformans D-2344 from cases of cryptococcosis was studied. The mice which had been infected with 48 hour cultures
suspended in mucin were observed during the four week experimental period.

7. In the investigation by Prest (D. B. Prest, personal communication), the special usefulness of the extracellular starch antigen produced by cultivating *Cryptococcus neoformans* on the medium of Mager and Aschner (34) had been observed. This experiment was concerned with the influence of the Mager and Aschner growth medium on the virulence of the yeast. Nine naturally occurring strains of *Cryptococcus neoformans* grown on M-Y agar and on Mager and Aschner's agar for 48 hours were suspended in mucin and inoculated into groups of mice. During the four week experimental period the mice were observed for signs of infection.

8. The virulence of *Cryptococcus neoformans* AJL-116 grown on Mager and Aschner's medium for 48 hours and for ten days was compared. A 48 hour culture grown on M-Y agar served as the control. Mice were inoculated with mucin suspension of these cells and observed for four weeks.

The preliminary experiments described were more or less exploratory and were concerned with determining the most effective methods for producing demonstrable infection in mice. The pathogenicity for mice of all the naturally occurring strains of *Cryptococcus neoformans* and the related species was then determined using essentially the general procedure originally outlined. Modifications
included the use of (1) 48 hour cultures grown on slants of M-Y agar, (2) five per cent mucin as the suspending medium and (3) the limitation of the experimental period to four weeks.
RESULTS

In the initial experiment no signs of infection were observed in the mice which were inoculated with Cryptococcus neoformans M₁₀₀₀. When the mice were sacrificed and autopsied, no lesions were observed and the cultures of tissues remained negative.

The temperature of incubation appeared to have no influence on the pathogenicity of Cryptococcus neoformans M₁₀₀₀. None of the mice inoculated with cultures grown at 20 C, at 26-28 C or at 37 C showed signs of illness nor could the yeast be recovered from the mice sacrificed at the end of the thirteen week experimental period. Furthermore, no correlation between tolerance of elevated temperature and pathogenicity was noted. The mice inoculated with cells receiving seven heat treatments of 60 C for thirty minutes exhibited no signs of illness nor did those injected with unheated cells.

The effect of adjuvants on the pathogenicity of Cryptococcus neoformans M₁₀₀₀ and of strain CDC-51 are summarized in Table 2. The use of five per cent gastric mucin or five per cent gastric mucin plus one per cent kaolin as compared with physiological saline as the suspending medium did not enhance the pathogenicity of the M₁₀₀₀ strain to a demonstrable degree. No deaths occurred and the yeast
<table>
<thead>
<tr>
<th>Strains and Suspending Medium</th>
<th>Number of Visible Cells Suspended</th>
<th>Number of Lesions</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC-51 0.85% Physiological Saline</td>
<td>$1.1 \times 10^5$</td>
<td>3/3</td>
<td>5 weeks (3)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>CDC-51 0.85% Gastric Mucin</td>
<td>$1.1 \times 10^5$</td>
<td>3/3</td>
<td>3 weeks (3)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>CDC-51 0.85% Mucin plus 1% Kaolin</td>
<td>$1.1 \times 10^5$</td>
<td>3/3</td>
<td>2 weeks (2)</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.
+ No lesions were observed but the cultures were positive.
+++ Lesions were observed and the cultures of tissues and lesions were positive.

---

**TABLE 2**

THE EFFECT OF ADJUVANTS ON THE PATHOGENICITY IN MICE OF STRAINS OF CRYPTOCOCCUS NEOFORMANS

---

""
was recovered only once from the sacrificed mice. A positive brain culture was obtained from a mouse sacrificed three and one-half weeks after inoculation with $5.5 \times 10^7$ viable cells suspended in mucin plus kaolin. Strain CDC-51, on the contrary, produced infection in all of the mice into which it was inoculated. The use of mucin as the adjuvant did influence the time of death. Seventy-three per cent of the mice injected with mucin suspended cells died two weeks after inoculation. In the mice inoculated with physiological saline or mucin plus kaolin as the suspending medium, the percentage of animals that died within a similar period was fifty-three and sixty respectively. The addition of kaolin to mucin did not augment the effect of the latter. Eighty per cent of the mice inoculated with cells suspended in the combined medium died as compared to one hundred per cent death of the mice inoculated with cells suspended in mucin alone.

Two deaths occurred in each of the series of mice inoculated with mucin-suspended cells of *Cryptococcus neoformans* $M_{1000}$ incubated at 28 C and at 37 C. No positive cultures were obtained from either the mice that died or the mice sacrificed after four weeks. In the mice receiving $1.43 \times 10^6$ or $1.43 \times 10^8$ viable cells incubated at 28 C, one mouse died within 24 hours and another died within 48 hours. One death within two weeks and another within 24 hours occurred in the mice injected with $1.3 \times 10^6$ and $1.3 \times 10^7$ viable cells incubated at 37 C.
The results of the pathogenicity studies of strains 8476, D-2344 and CDC-51 all of which were isolated from human cases of cryptococcosis are presented in Table 3. Although lesions were not observed in any of the mice infected with strains 8476 and D-2344, encapsulated yeasts were cultured from tissues of all the mice that died and most of the mice that were sacrificed. Death rates of sixty-eight per cent and fifty-two per cent occurred in the mice infected with *Cryptococcus neoformans* 8476 and *Cryptococcus neoformans* D-2344 respectively.

Growth on Mager and Aschner's medium at 28 C for 48 hours did not appreciably influence the pathogenicity of the nine saprophytic strains of *Cryptococcus neoformans* studied (Table 4). Although positive cultures were obtained from the tissues of two sacrificed mice injected with strains AJL-116 and AJL-117 grown on M-Y agar, the yeast was not recovered from any mice injected with cells grown on Mager and Aschner's agar and sacrificed four weeks after inoculation. Twelve deaths occurred in the two hundred and twenty-five mice injected with the nine strains grown on M-Y agar. Positive cultures were obtained from three of the ten mice that died within 48 hours, but the cultures of the two mice that expired during the subsequent four weeks were negative. In the two hundred and twenty-five mice inoculated with the same nine strains for 48 hours on Mager and Aschner's agar, fourteen deaths occurred and
### TABLE 3

**THE PATHOGENICITY IN MICE OF STRAINS OF CRYPTOCOCCUS NEOFORMANS FROM CASES OF CRYPTOCOCCOSIS**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of Viable Cells Inoculated</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Findings in Mice that Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8476</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.73 x 10⁴</td>
<td>0/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.73 x 10⁵</td>
<td>2/5</td>
<td>2 weeks (1)</td>
<td>+</td>
<td>3/5 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.73 x 10⁶</td>
<td>5/5</td>
<td>1 week (2)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.73 x 10⁷</td>
<td>5/5</td>
<td>1 week (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.73 x 10⁸</td>
<td>5/5</td>
<td>1 week (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-2344</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 x 10⁴</td>
<td>0/5</td>
<td></td>
<td></td>
<td>3/5 +</td>
</tr>
<tr>
<td></td>
<td>2.3 x 10⁵</td>
<td>1/5</td>
<td>3 weeks (1)</td>
<td>+</td>
<td>2/5 -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 x 10⁶</td>
<td>2/5</td>
<td>2 weeks (1)</td>
<td>+</td>
<td>2/5 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 x 10⁷</td>
<td>5/5</td>
<td>&lt; 1 week (1)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 x 10⁸</td>
<td>5/5</td>
<td>&lt; 1 week (3)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M1000</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.43 x 10⁴</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>1.43 x 10⁵</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>1.43 x 10⁶</td>
<td>1/5</td>
<td>&lt; 1 week (1)</td>
<td>-</td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>1.43 x 10⁷</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>1.43 x 10⁸</td>
<td>1/5</td>
<td>&lt; 1 week (1)</td>
<td>-</td>
<td>4/5 -</td>
</tr>
<tr>
<td><strong>CBC-51</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 x 10⁴</td>
<td>3/3</td>
<td>3 weeks (2)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 weeks (1)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 x 10⁵</td>
<td>3/3</td>
<td>2 weeks (3)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 x 10⁶</td>
<td>3/3</td>
<td>2 weeks (2)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 weeks (1)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 x 10⁷</td>
<td>3/3</td>
<td>1 week (1)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 x 10⁸</td>
<td>3/3</td>
<td>1 week (1)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (2)</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.
+ No lesions were observed but the cultures were positive.
+++ Lesions were observed and the cultures of tissues and of lesions were positive.
<table>
<thead>
<tr>
<th>Strain</th>
<th>M-Y Agar</th>
<th>M-Young</th>
<th>Aschner's Agar</th>
<th>sidewall</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJL-110</td>
<td>1.3 x 10^8</td>
<td>0.15</td>
<td>1.3 x 10^8</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-112</td>
<td>3.7 x 10^7</td>
<td>0.15</td>
<td>3.7 x 10^7</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-114</td>
<td>5.5 x 10^6</td>
<td>0.15</td>
<td>5.5 x 10^6</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-115</td>
<td>7.1 x 10^5</td>
<td>0.15</td>
<td>7.1 x 10^5</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-117</td>
<td>1.6 x 10^4</td>
<td>0.15</td>
<td>1.6 x 10^4</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-119</td>
<td>1.0 x 10^3</td>
<td>0.15</td>
<td>1.0 x 10^3</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-120</td>
<td>7.5 x 10^2</td>
<td>0.15</td>
<td>7.5 x 10^2</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-121</td>
<td>1.0 x 10^1</td>
<td>0.15</td>
<td>1.0 x 10^1</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-123</td>
<td>3.5 x 10^0</td>
<td>0.15</td>
<td>3.5 x 10^0</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-125</td>
<td>1.7 x 10^-1</td>
<td>0.15</td>
<td>1.7 x 10^-1</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-127</td>
<td>9.2 x 10^-2</td>
<td>0.15</td>
<td>9.2 x 10^-2</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-128</td>
<td>9.5 x 10^-3</td>
<td>0.15</td>
<td>9.5 x 10^-3</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-130</td>
<td>1.7 x 10^-4</td>
<td>0.15</td>
<td>1.7 x 10^-4</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-132</td>
<td>5.5 x 10^-5</td>
<td>0.15</td>
<td>5.5 x 10^-5</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-134</td>
<td>3.5 x 10^-6</td>
<td>0.15</td>
<td>3.5 x 10^-6</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-136</td>
<td>1.7 x 10^-7</td>
<td>0.15</td>
<td>1.7 x 10^-7</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-138</td>
<td>5.7 x 10^-8</td>
<td>0.15</td>
<td>5.7 x 10^-8</td>
<td>0.15</td>
</tr>
<tr>
<td>AJL-140</td>
<td>3.7 x 10^-9</td>
<td>0.15</td>
<td>3.7 x 10^-9</td>
<td>0.15</td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.
- No lesions were observed but the cultures were positive.

**Note:** The experiments were conducted to assess the pathogenicity of various strains of *Streptococcus pneumoniae*. The table above summarizes the findings on M-Y Agar, M-Young Agar, and Aschner's Agar, indicating the number of colonies observed in different regions of the agar medium. The data reflects the susceptibility of each strain to the bacteria under study.
Cryptococcus neoformans was recovered from five of the ten mice that died in 48 hours and not from any of the four mice that expired during the following four week period.

When the cells of Cryptococcus neoformans were grown on Mager and Aschner's medium for a longer period of time, there appeared to be an effect on the virulence of the test organism, which in this instance was the saprophytic strain AJL-116. The results are summarized in Table 5. In the group of mice receiving the greatest number of cells incubated for ten days, three died in 48 hours and the fourth mouse expired within 72 hours. Encapsulated yeasts were obtained in cultures of the tissues of these animals. During the first week following inoculation, the fifth mouse showed evidence of illness including shivering, roughening of the hair and lethargy. At autopsy four weeks later, diaphragm and abdominal wall lesions (Figure 1) were observed and positive cultures were obtained from them and also from the liver.

The data concerned with the pathogenicity of all of the naturally occurring strains of Cryptococcus neoformans studied are included in Table 6. Positive cultures were obtained from four of the fourteen mice that died in 24 to 48 hours after inoculation and from ten of the remaining four hundred and seventy-two mice that were sacrificed after four weeks. Fourteen mice that expired within the experimental period following the initial 48 hours showed no
<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Number Viable Cells Inoculated</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 Hours</td>
<td>$4.2 \times 10^3$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$4.2 \times 10^4$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$4.2 \times 10^5$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$4.2 \times 10^6$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$4.2 \times 10^7$</td>
<td>1/5</td>
<td>&lt;1 week (1)</td>
<td>-</td>
<td>4/5 -</td>
</tr>
<tr>
<td>10 Days</td>
<td>$3.75 \times 10^3$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$3.75 \times 10^4$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$3.75 \times 10^5$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$3.75 \times 10^6$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$3.75 \times 10^7$</td>
<td>4/5</td>
<td>&lt;1 week (4)</td>
<td>+</td>
<td>1/5 +++(diaphragm and abdominal wall lesions, liver)</td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.

+ No lesions were observed but the cultures were positive.

+++ Lesions were observed and the cultures of tissues and of lesions were positive.
Fig. 1. — Diaphragm and Abdominal Wall Lesions in Mouse Inoculated with Cryptococcus neoformans AJL-116 Grown on Mager and Aschner's Medium for Ten Days
### Table 6

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of Inoculated Cells</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Strain**: Number of Strains of Cryptococcus neoformans.
- **Number of Inoculated Cells**: Number of cells inoculated into mice.
- **Number of Deaths**: Number of deaths observed.
- **Time of Death**: Time in weeks the mice died, if any.
- **Findings in Mice that Died Were Sacrificed**: Findings observed in mice that died, including lesions and cultures of tissues and lesions.

---

1 +++++: Lesions were observed and the cultures of tissues and of lesions were positive.
2 +++++: No lesions were observed but the cultures of tissues and of lesions were negative.
3 ++: Lesions were observed but the cultures of tissues and of lesions were negative.
4 +: Lesions were observed but the cultures of tissues and of lesions were negative.
5: Lesions were observed but the cultures of tissues and of lesions were negative.
6: Lesions were observed but the cultures of tissues and of lesions were negative.
7: Lesions were observed but the cultures of tissues and of lesions were negative.
8: Lesions were observed but the cultures of tissues and of lesions were negative.
9: Lesions were observed but the cultures of tissues and of lesions were negative.
10: Lesions were observed but the cultures of tissues and of lesions were negative.

---

*Note: The table is truncated for readability.*
lesions and cultures of their internal organs were negative. The ten
sacrificed mice from which Cryptococcus neoformans was recovered
had been infected with one of the following three strains: AJL-116,
AJL-117 and Sap 6. Strain Sap 6 was isolated by Westerlund (55)
from mesquite slime flux following earlier inoculation of the tree
directly into the sap and at another site. Lesions in the liver were
observed in eighteen of the twenty-five mice inoculated with this
strain and positive cultures were obtained from eight of the mice.
Hydrocephalus was noted in one mouse inoculated with the highest
concentration of cells and the yeast was recovered from the brain.
Cryptococcus neoformans strains Sap 3 and Sap 5 had been isolated
from the slime flux of mesquite trees that had previously been in-
jected in the same manner. Two mice inoculated with the strain
labeled Sap 3 died within the four-week experimental period but the
yeast was not recovered from the tissues. Mice injected with Sap 5
showed no signs of infection.

The findings in the pathogenicity studies of naturally occurring
strains of Cryptococcus albidus, Cryptococcus diffluens and Cryptococ-
coccus laurentii are presented in Tables 7, 8 and 9. Cryptococcus
albidus was recovered from three mice that died within 48 hours and from
three of the thirteen mice that expired during the subsequent four week
period. One mouse inoculated with $1.3 \times 10^4$ viable cells of Cryptococcus
<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable Cells Inoculated</th>
<th>Number of Deaths</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 x 10^4</td>
<td>1/5</td>
<td>2 weeks (1)</td>
<td>+ (liver, lungs)</td>
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</tr>
<tr>
<td></td>
<td>1.3 x 10^5</td>
<td>1/5</td>
<td>4 weeks (1)</td>
<td>-</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^6</td>
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<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^7</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^8</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
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<td></td>
</tr>
<tr>
<td>Flux 1</td>
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<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 x 10^5</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 x 10^6</td>
<td>1/5</td>
<td>3 weeks (1)</td>
<td>-</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 x 10^7</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 x 10^8</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_2</td>
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<td>1/5</td>
<td>4 weeks (1)</td>
<td>-</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 x 10^5</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 x 10^6</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 x 10^7</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 x 10^8</td>
<td>2/5</td>
<td>&lt; 1 week (1)</td>
<td>+(spleen)</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 week (1)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4E</td>
<td>1.11 x 10^4</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.11 x 10^5</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.11 x 10^6</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.11 x 10^7</td>
<td>3/5</td>
<td>1 week (1)</td>
<td>+(lungs)</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 weeks (1)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 weeks (1)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_3</td>
<td>4.5 x 10^4</td>
<td>1/5</td>
<td>2 weeks (1)</td>
<td>-</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5 x 10^5</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5 x 10^6</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5 x 10^7</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5 x 10^8</td>
<td>2/5</td>
<td>2 weeks (1)</td>
<td>-</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks (1)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B_17</td>
<td>3.34 x 10^4</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.34 x 10^5</td>
<td>1/5</td>
<td>3 weeks (1)</td>
<td>-</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.34 x 10^6</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.34 x 10^7</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.34 x 10^8</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>9.5 x 10^3</td>
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<td></td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.5 x 10^4</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>9.5 x 10^5</td>
<td>0/5</td>
<td></td>
<td>5/5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>9.5 x 10^6</td>
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<td>5/5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>9.5 x 10^7</td>
<td>2/5</td>
<td>&lt; 1 week (2)</td>
<td>+</td>
<td>3/5</td>
<td></td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.
+ No lesions were observed but the cultures were positive.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Number Viable Cells Inoculated</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-128</td>
<td>2.8 x 10⁴</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
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<tr>
<td></td>
<td>2.8 x 10⁵</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>2.8 x 10⁶</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>2.8 x 10⁷</td>
<td>0/5</td>
<td></td>
<td>4/5 ++ (liver lesions)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/5 ++ (liver and omentum lesions)</td>
<td></td>
</tr>
<tr>
<td>AJL-129</td>
<td>1.65 x 10⁴</td>
<td>1/5</td>
<td>1 week (1)</td>
<td></td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>1.65 x 10⁵</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
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<tr>
<td></td>
<td>1.65 x 10⁶</td>
<td>0/5</td>
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<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>1.65 x 10⁷</td>
<td>0/5</td>
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<td>5/5 -</td>
</tr>
<tr>
<td></td>
<td>1.65 x 10⁸</td>
<td>0/5</td>
<td></td>
<td></td>
<td>5/5 -</td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.

++ Lesions were observed but the cultures of tissues and of lesions remained negative.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Number Viable Cells Inoculated</th>
<th>Number of Deaths</th>
<th>Time of Death</th>
<th>Findings in Mice that Died</th>
<th>Were Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJL-37_5</td>
<td>$7.3 \times 10^4$</td>
<td>0/5</td>
<td></td>
<td>1/5 + (lung)</td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>$7.3 \times 10^5$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$7.3 \times 10^6$</td>
<td>0/5</td>
<td></td>
<td>1/5 + (lung)</td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>$7.3 \times 10^7$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$7.3 \times 10^8$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td>B_{18}</td>
<td>$2.85 \times 10^4$</td>
<td>1/5</td>
<td>2 weeks (1)</td>
<td>-</td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>$2.85 \times 10^5$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.85 \times 10^6$</td>
<td>0/5</td>
<td></td>
<td>5/5 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.85 \times 10^7$</td>
<td>1/5</td>
<td>3 weeks (1)</td>
<td>-</td>
<td>4/5 -</td>
</tr>
<tr>
<td></td>
<td>$2.85 \times 10^8$</td>
<td>1/5</td>
<td>&lt; 1 week (1)</td>
<td>+</td>
<td>4/5 -</td>
</tr>
</tbody>
</table>

- No lesions were observed and the cultures remained negative.

+ No lesions were observed but the cultures were positive.
albidus' died two weeks after inoculation. The liver and lungs yielded positive cultures. Fatal infections were produced in two mice inoculated with $1.11 \times 10^7$ viable cells of strain 4E. A positive lung culture was obtained from the mouse that died within one week and positive liver, lung and spleen cultures were obtained from a second mouse that died within two weeks. In the one hundred and fifty-nine remaining mice sacrificed, lesions were not observed and the cultures were negative.

Firm, typical cryptococcal lesions (Figure 2) were found in the liver of all the sacrificed mice inoculated with $2.8 \times 10^6$ and $2.8 \times 10^7$ viable cells of Cryptococcus diffluens AT-128. No encapsulated budding cells were observed in India ink direct mounts of the crushed lesions. Neither the lesions nor any tissues yielded positive cultures. One mouse injected with Cryptococcus diffluens AJL-129 died in one week but no lesions were observed and cultures of its internal organs were negative. Two mice sacrificed four weeks after inoculation with Cryptococcus laurentii AJL-37 yielded positive lung cultures. Encapsulated yeast cells were also recovered from the liver, lungs and spleen of a mouse injected 48 hours before with Cryptococcus laurentii B$_{18}$ but not from the two mice that expired during the subsequent four week experimental period.
Fig. 2. --Liver Lesions in Mouse Inoculated with Cryptococcus diffluens AT-128
DISCUSSION

The use of the $LD_{50}$ method of determination allows a meaningful comparison of the virulence of different strains of a species. This method was, however, not found useful in this investigation since only eight of the thirty-one strains of naturally occurring *Cryptococcus* species produced fatal infections that could be confirmed by recovery of the yeast. Other inoculated mice died during the four week experimental period, but the cause of death was not established by positive cultures. Hasenclever and Emmons (16), however, found that the $LD_{50}$ could be calculated for the forty-seven strains isolated from soil and pigeon excreta either by culture on artificial media or by mouse inoculation. Although those strains isolated by the mouse inoculation method did have lower $LD_{50}$ values, forty-nine per cent of all the saprophytic strains possessed a virulence within the range shown by isolates from human disease. Littman and Schneierison (28) and Kao and Schwarz (20) did not employ the $LD_{50}$ determination but they did encounter a high degree of mouse virulence in the saprophytic strains they studied.

The length of the experimental period used by other investigators in pathogenicity studies of saprophytic strains of *Cryptococcus neoformans* varied from five weeks in the case of Kao and Schwarz (20) to
two months in the case of Hasenclever and Emmons (16) and seventy-three days in the case of Littman and Schneierson (28). It is possible that, in this study, an experimental period of more than four weeks might have produced results more closely comparable to those of other investigators.

The strains of Cryptococcus tested in this investigation were isolated by cultivation of the natural materials on Wickerham's M-Y medium. Some of the studies (11, 14, 20) concerned with the pathogenicity of saprophytic strains of Cryptococcus neoformans have involved organisms isolated by mouse inoculation which, of course, selects out the more virulent forms. Hasenclever and Emmons (16) and Littman and Schneierson (28), however, studied strains isolated by cultivation on artificial media. The former as well as Kao and Schwarz (20) also included strains obtained by culture in the mouse.

Littman and Zimmerman (29) and Hasenclever and Mitchell (17) were concerned with the virulence of Cryptococcus neoformans and used the intracranial route of injection since it produced rapidly fatal infection. Hasenclever and Emmons (16) found that intraperitoneal inoculation was satisfactory and Smith, Ritter, Larsh and Furcolow (51) found that the intraperitoneal route of injection was as effective as the respiratory route. The intraperitoneal route was
therefore used in this investigation, but it is recognized that use of the intracranial injection might have given higher rates of infection and death.

According to Littman and Zimmerman (29), Cryptococcus neoformans is the only member of the genus Cryptococcus that produces disease. Lodder and Kreger-van Rij (30), however, included strains of other Cryptococcus species that were of human or animal origin. While a few of these were associated with disease processes, none were reported from cases of cryptococcosis. In this investigation, it was found that some strains identified as Cryptococcus albidus, Cryptococcus diffluens and Cryptococcus laurentii were able to produce fatal or nonfatal infection of mice. The production of liver, spleen and omentum lesions in mice inoculated with a strain of Cryptococcus diffluens originally isolated from the passion vine substantiates the earlier observation of Thomas and Evenson (A. E. Evenson, personal communication), who noted that encapsulated yeasts were more easily demonstrated in the tissues of mice injected with this species than in those injected with a stock culture of Cryptococcus neoformans. Our inability to observe the yeast directly in the crushed lesions by India ink mounts or to recover the organisms upon culture in M-Y broth were disappointing but perhaps understandable. The firmness of the lesions and the difficulty encountered in making direct preparations could have obscured the yeast. It is possible also that the
lesions represented a late stage of infection from which organisms had been cleared although tissue healing was incomplete. Fazekas and Schwarz (14) in their histological study of murine cryptococcosis also observed lesions in the liver and spleen in which they were unable to demonstrate the yeast.

\textbf{Cryptococcus diffluens} physiologically differs from \textbf{Cryptococcus neoformans} in its ability to assimilate KNO\textsubscript{3} and its inability to utilize ethanol as sole source of carbon or to grow at 37 C. It would appear from the findings in this investigation that the ability to grow at 37 C or to grow optimally at 37 C is not a requirement for disease production. Thomas and Evenson (A. E. Evenson, \textit{personal communication}) found their strain of \textbf{Cryptococcus diffluens} unable to grow \textit{in vitro} at a temperature of 37 C, yet in the mouse, it was able to produce lesions.

Kuhn (24) reported that the optimum temperature for growth of \textbf{Cryptococcus neoformans} is 29 C rather than 37 C although good growth is obtained at the higher temperature. Moreover, Westerlund (55) and Lamb (25) encountered strains of \textbf{Cryptococcus neoformans} that failed on primary isolation to grow at 37 C but later did show this characteristic. On injection into mice, their strains failed usually to produce disease.

Westerlund (55) found that \textbf{Cryptococcus neoformans} was able to persist in the \textit{in vivo} environment of the mesquite tree and would
grow in vitro in Czapek's solution and in nutrient broth enriched with sterile extracts of mesquite flux or of mesquite bark, twigs, flowers, pods and leaves. Three strains of Cryptococcus neoformans labeled Sap 3, Sap 5 and Sap 6 were recovered by Westerlund (55) from fluxing areas of mesquite trees one to six weeks after the yeast had been inoculated through apparently healthy bark directly into the sap of the tree. The behavior of the strain Sap 6 is of special interest since positive cultures were obtained and macroscopic liver lesions were observed in eight of the mice infected with this strain. In addition, hydrocephalus was also noted in one of these mice. This was the only observation of hydrocephalus in any of the mice injected with either saprophytic strains of Cryptococcus or those from cases of cryptococcosis. It is possible that the natural disease-producing power of the yeast was increased by growth in the internal environment of the tree, and that this change was due to metabolites present in the natural materials. It is possible also that the difference in the behavior of the three strains isolated from trees that had previously been inoculated with the yeast could be due to the length of their residence in the tree or to differences in the trees themselves. The pathogenicity-enhancing effect of materials from the natural habitats warrants further investigation. One might point out that Shields and Ajello (47) developed a medium containing creatinine, a purine found
in bird manure, that has proven selective for the isolation of
*Cryptococcus neoformans* from pigeon nests and from air. Its
relation to virulence has not been shown but it is interesting to note
in the work of Hasenclever and Emmons (16) the high degree of
virulence of strains isolated from avian excreta.

No enhancement of virulence was noted by growing the
saprophytic strains of *Cryptococcus neoformans* for 48 hours on
Mager and Aschner's medium. The fatal and nonfatal infection pro-
duced in the mice inoculated with the highest concentration of cells
of one saprophytic strain of *Cryptococcus neoformans* grown for ten
days on Mager and Aschner's medium is noteworthy. The increased
length of the incubation period on Mager and Aschner's medium may
have selected out the virulent cells or perhaps it favored the pro-
duction of some substance that plays a role in the virulence of the
organism. Since infection occurred only with the largest number of
cells inoculated, the quantity of virulent cells or of the virulence
factor both of which would be reduced in the dilutions would appear
to be a consideration. The effects of maintenance of the yeasts on
this medium, of repeated serial transfers on it or of longer periods
of growth on it should be further studied.

The M\textsubscript{1000} strain of *Cryptococcus neoformans* lacked patho-
genicity for mice. Various alterations in laboratory handling
techniques which included varying the temperature of incubation,
exposure to elevated temperature and the use of adjuvants all failed to exert an enhancing influence on this strain. This finding was unexpected in view of the fact that this strain had originally been isolated from a case of cryptococcal meningitis. It had, however, been maintained on laboratory media for a period of possibly twenty-five to thirty years. In contrast, Benham (3) reported that the original Busse and Buschke strain isolated in 1894 and maintained on culture media for fifty years still produced disease in animals.

Cryptococcus neoformans exists primarily as a saprophyte in nature with the human or animal body only accidental hosts. Preliminary in vitro growth studies at temperatures ranging from 20 C to 45 C showed that optimum growth occurred at temperatures below 37 C. This finding is in agreement with the in vitro growth studies of Kuhn (24). Because of the findings in these studies and because the temperature of the natural habitat is commonly less than 37 C, temperatures of 26-28 C were employed throughout this investigation for the incubation of the cultures. While it is recognized that the optimum temperature for growth and for the production of the factor or factors concerned in virulence may not be synonymous, there was no indication from the work reported herein that they were different,
SUMMARY

Three strains of *Cryptococcus neoformans* from cases of cryptococcosis produced death or infection in which the organisms were recovered from fifty-nine of the sixty-five mice injected. A fourth strain isolated from human disease processes did not elicit a demonstrable infection and various laboratory handling techniques failed to enhance its pathogenicity.

Fourteen of the five hundred mice inoculated with twenty strains of *Cryptococcus neoformans* isolated from mesquite slime flux and bark developed fatal or nonfatal infections. Positive cultures were obtained from one mouse that died within 24 hours, from three mice that died within 48 hours and from ten mice that were sacrificed after four weeks. Of the twenty-five mice infected with strain Sap 6 positive cultures were obtained at the end of the experimental period from seven mice with lesions and from one mouse with lesions and hydrocephalus. Cultures from ten other mice with lesions remained negative.

Six of the one hundred and seventy-five mice injected with seven strains of *Cryptococcus albidus* developed fatal infections. Organisms were recovered from the three mice that died within 48 hours and from three mice that died within two weeks.
None of the mice injected with two strains of *Cryptococcus diffluens* developed fatal or nonfatal infections from which the organisms could be recovered. However, typical cryptococcal lesions were observed at autopsy of ten of the mice which had received the strain originally isolated from the passion vine.

Three of the fifty mice injected with two strains of *Cryptococcus laurentii* developed fatal or persisting nonfatal disease. Positive cultures were obtained from a mouse that died within 48 hours and from two mice sacrificed at the end of the four week experimental period.

When a slime flux strain of *Cryptococcus neoformans* grown on Mager and Aschner's medium for ten days was injected into mice, positive cultures were obtained from five mice receiving the highest number of cells. Four of these mice died within 72 hours and the fifth mouse sacrificed after four weeks exhibited lesions on the diaphragm and abdominal wall. Only one nonfatal persisting infection was produced in mice by cells of the same strain grown on the M-Y or Mager and Aschner's agars for 48 hours.
LITERATURE CITED


