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Tumor Induction by *Cryptococcus neoformans*

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A human isolate of *Cryptococcus neoformans* strain CIA, which was originally obtained in 1963, produced fatal disease in mice. Postmortem examinations showed extensive central nervous system disease. After using this yeast for research for 7 years, we found that it began to produce large tumors after intraperitoneal inoculation in mice. The present study showed that the tumors consisted of large agglomerates of yeast cells, capillaries, and reticular stroma. In concomitant experiments, the same *C. neoformans* strain, introduced into the lungs of mice in an aerosol, produced asymptomatic infections. A definition for the term "cryptococcoma" is proposed.

Sanfelice (21), credited with the first isolation of *Cryptococcus neoformans* in 1894, referred to this yeast as *Saccharomyces neoformans* because it appeared to induce tumor-like masses in laboratory animals. His original report was followed by a series of six papers (22), in which he implicated yeast as an etiological agent of cancer. In the same decade, other investigators (13, 20) published evidence in support of Sanfelice's hypothesis. However, Buschke (3) and Busse (4), in their accounts of the first recorded case of human cryptococcosis, disagreed with the parasitic theory of cancer. In 1896, Curtis (6) described a patient with cryptococcosis associated with meningitis. Because the organism he isolated produced "tumors" in rats (similar to those described by Buschke), he named it *Saccharomyces tumefaciens*. Foulerton (9) was the first American investigator to describe tumor-like lesions produced by a yeast. He named the organism *S. tumefaciens* albus. Around the turn of the century, controversy over yeasts as etiological agents of cancer subsided with the reports of Plimmer (19), who believed that the tumors apparently produced by yeasts were not true neoplasms, and with those of Greenough (11) and Nichols (18), who concluded that the identification of inclusion bodies seen in malignant cells as yeasts was not justified.

In 1916, Stoddard and Cutler (23) published the first monograph on cryptococcosis, recognizing it as a distinct disease entity. Many of their conclusions on host tissue response, route of dissemination, and pathogenesis remain valid today. However, they so emphasized meningitis as a clinical feature of the disease that even until recently few other forms were accepted.

Pulmonary cryptococcosis as a distinct form of the disease was brought to the attention of other workers by Freeman (1) in 1931. He classified the lung lesions into two types and reported that only 20 pulmonary cases existed in the world literature to that date. Currently, meningitis is the most frequently reported disease form, although many investigators believe that pulmonary cryptococcosis is very common but is seldom diagnosed (7). In fact, Littman and Schnerson (16) speculated that 5,000 to 15,000 clinical or subclinical cases of pulmonary cryptococcosis may occur annually in New York City alone.

Since the late 1800's, little attention has been paid to the tumor-like masses caused by *C. neoformans*. Notable exceptions to this are the recent reports of a case of mammary cryptococcosis grossly simulating carcinoma in a human (5) and tumor-like masses in the organs of *Profelis temmincki* (25).

In 1963, we began studies on cryptococcosis using a human isolate of *C. neoformans* in mice. After intraperitoneal or intracerebral inoculation, death inevitably ensued with associated central nervous system disease. By 1969, however, equal doses of this organism were beginning to have an entirely different effect on the same strains of mice. Survival time was greatly extended, and intraperitoneal inoculation was followed by tumor induction. The nature of these tumors is the subject of the present report.

MATERIALS AND METHODS

Organism. The human isolate of *C. neoformans* strain CIA used in these studies was originally obtained from the Cryptococcus Research Laboratory, Veterans Administration Hospital, Oklahoma City.

Since 1963, the culture has been maintained by subculturing once or twice weekly on Sabouraud dextrose agar (Difco) at room temperature. Encapsulated cells were harvested directly from this medium and suspended in saline after a 72-hr incubation period at 25 C. Nonencapsulated cells were obtained by culturing on a low *p*H (L*p*H) medium (8) for 72 hr at 25 C.

Animals. CBA/J and CE/J mice were obtained from Jackson Laboratories (Bar Harbor, Me.). Strain WM white mice were obtained from a colony maintained at this institution.

Interaperitoneal infection. An initial study was designed to study the pathogenesis of cryptococcosis in CBA/J mice using cells of *C. neoformans* (strain CIA.)

Encapsulated cells were harvested from Sabouraud dextrose agar, suspended in 0.85% saline solution, and injected intraperitoneally into three groups of mice (each consisting of four animals). The first group received 10^6 cells in 0.1 ml of saline per animal; the second, 10^7 cells; and the third, 10^8 cells. Three additional groups of mice (four animals each) were similarly inoculated with 10^4, 10^5, and 10^6 nonencapsulated cells of *C. neoformans*. Cell counts were made with a hemacytometer and adjusted with saline.

Four control animals received intraperitoneal injections of heat-killed *C. neoformans* (60 C for 60 min) suspended in saline, and four received saline alone.

All animals were observed daily for up to 200 days. Necropsy was performed on the day of death for any mice which failed to survive or those which were sacrificed early.

Airborne infection. *C. neoformans* cells were cultured on Sabouraud dextrose agar medium for 3 days at 25 C, transferred to yeast nitrogen base medium (Difco), and incubated for 10 days at 25 C. The suspension was then dispensed into the nebulizer of an Airborne Infection Apparatus (Tri R Instrument Co., Jamaica, N.Y.). The inhalation of yeast cells was verified by removing the lungs and trachea of mice immediately after aerosol exposure and by washing the lungs with saline. The saline was then plated onto Sabouraud dextrose agar to assay for *C. neoformans* cells. The objective of this study was to have CBA/J mice inhale yeast cells, which would lead to infection by the pulmonary route.

Over a 12-month period before the experiments described below, the airborne infection apparatus was tested several times using similar instrument settings and a standard suspension of yeast cells. The number of organisms recovered from the aerosol-exposed chamber air varied from 0 cells/liter to 1,000 cells/liter of chamber air. All three sampling methods yielded similar variability. The methods were: (i) exposure of a petri dish (placed in the chamber), containing Sabouraud dextrose agar, with an aluminum-covered lid which could be mechanically uncovered during a release of aerosol and covered again before decontamination, (ii) removal of chamber air during aerosol exposure through a water impinger, with 1:10 and 1:100 dilutions of the resulting fluid being plated onto Sabouraud dextrose agar, and (iii) withdrawal of aerosol-exposed chamber air, by means of a diaphragm pump, through a membrane filter (0.22 μm; Millipore Corp.), which was then removed and pressed onto the surface of a petri dish containing Sabouraud dextrose agar. Despite the failure of these sampling methods as reliable indicators, one factor in the operation of the apparatus did prove to correlate with the recovery of *C. neoformans* from the lungs of aerosol-exposed mice. This factor was the appearance of a visible aerosol suspension in the secondary air from the nebulizer (described below).

In four separate experiments, 45 CBA/J mice were placed in the chamber (in accordance with the manufacturer's directions) and exposed for 120 min to a *C. neoformans* aerosol produced by a primary air pressure of 15 psi. The secondary air (i.e., the air carrying the nebulized cells) was adjusted for greatest visible cloud density (usually about 5 liters/min). Aerosol exposure was followed by a 30-min decontamination cycle, which included 15 min of ultraviolet radiation.

Age and strain differences in susceptibility to C. neoformans. A final set of experiments was designed to assess the possible influence of age or strain upon susceptibility to *C. neoformans* infection. The classes of mice selected for IP inoculation with 10^6 encapsulated cells of *C. neoformans* were: (i) CBA/J mice, 200 days of age or older, (ii) CBA/J mice, 35 days of age, (iii) CE/J mice, 35 days of age, and (iv) WM mice, 35 days of age. An additional group of CBA/J mice over 200 days of age received injections of heat-killed cells.

Postmortem examination. At necropsy, the liver, spleen, and kidneys were removed, examined grossly, and sectioned at the greatest dimension. Cultures were made from these organs on Sabouraud dextrose agar medium. After 3 days of incubation at 25 C, these were examined for growth of *C. neoformans*. The thoracic cavity was also exposed during necropsy and the heart and lungs were examined, as were the abdominal organs. The skullcap was removed and the brain was inspected in situ. After gross examination, all organs were preserved in 10% neutral buffered Formalin.

Microscopic examination. Postmortem tissue specimens were prepared either by sectioning or by making impressions from fresh unfixed tissues onto microscope slides. After the organs had been fixed in 10% neutral buffered Formalin for 48 hr at room temperature, the fixed tissues were dehydrated, cleared in xylene, and infiltrated and mounted in Tissuemat (Fisher Scientific Co.); 5-μm sections were then cut on a microtome (International Rotary). Sections were stained either with hematoxylin and eosin or by Lillie's allochrome method (14).

Because many of the delicate tissues of the brain may be destroyed by removal of this organ in the fresh state, the head was severed from the body, the external soft tissues and mandible were carefully dissected away, and the brain was fixed in situ. The skull, with brain intact, was then decalcified by treatment with 10% hydrochloric acid until all calcium salts were removed. After decalcification, the skull was washed in tap water for 30 min and cut into

sections 5 to 7 mm thick. These sections were examined for lesions with a dissecting microscope. Selected thick sections were infiltrated with paraffin, microtome-sectioned, and stained by the methods described above.

Culturing from organs. Because *C. neoformans* cells were often undetectable upon examination of serial-sectioned organs with a microscope, it was sometimes desirable to assay for small numbers of *C. neoformans* cells by another method. An entire organ was homogenized for 3 min in 5 ml of saline in a blendor (Omnimixer Corp.). The homogenate was then dilution-plated onto Sabouraud dextrose agar medium.

RESULTS

Intraperitoneal infection. Within 80 days after IP inoculation with either 10^6, 10^7, or 10^8 encapsulated *C. neoformans* cells, approximately 90% of the surviving CBA/J mice developed subcutaneous masses (cryptococcomas) at or near the site of injection (Table 1). The three groups of CBA/J mice similarly inoculated with 10^4, 10^5, or 10^6 nonencapsulated cells developed similar cryptococcomas at a similar rate (Table 2). None of the animals receiving killed cells of *C. neoformans* in saline, or saline alone, developed these lesions.

When two mice which had been inoculated with encapsulated cells were sacrificed on day 100 after inoculation, external examination revealed firm, lobulated, subcutaneous masses in the right lower quadrant of the abdomen (Fig. 1 and 2). These lesions were fixed, immobile, and adherent to the skin. The lesions resembled those seen in the surviving inoculated mice. One mouse had a lesion 9 mm in diameter and the other a lesion 25 mm in diameter. Both were located subcutaneously, were invasive, and penetrated the abdominal wall. The masses were white, translucent, firm, nonencapsulated, and lobulated. In one animal, the invasive growth surrounded part of the small intestine. Wet impressions were made from these tissues and large (25- to 40-μm), encapsulated cells of *C. neoformans* were observed. When these masses were removed and weighed, the tumor from the mouse receiving 10^8 cells weighed 1.2 g (19% of its body weight), and that from the mouse receiving 10^7 cells weighed 0.7 g (4.3% of its total body weight.) The tumors were embedded in paraffin, sectioned, stained, and examined with a microscope. They consisted primarily of yeast cells and a few macrophages, fibroblasts, collagen fibers, and a capillary bed (Fig. 3). The integrity of the mass might be attributed to the collagen network which provided a stroma. Normal tissue was compressed by the enlarging spherical masses of *C. neoformans*. In the cryptococcomas, most of the yeast cells were large and distorted, but in some areas there were small (1.5 to 3.0 μm) yeast cells that had not been observed in the preparations made from fresh tissues. Examinations of sections of other abdominal organs (kidney, liver, and spleen)

TABLE 1. *Survival and tumor production in mice inoculated intraperitoneally with cells of C. neoformans (strain CIA) grown on Sabouraud dextrose agar*

Dose (no. of cells)	No. of animals	No. of animals surviving/no. with tumors, at day:										
		0	20	40	60	80	100	120	140	160	180	200
10^8	4	4/0	4/0	2/0	2/0	2/2	1/1[a]	1/1	0/0	0/0	0/0	0/0
10^7	4	4/0	4/0	4/0	4/0	3/3	2/2	1/1	0/0	0/0	0/0	0/0
10^6	4	4/0	4/0	4/0	4/0	4/3	2/2	2/2	1/1	1/1	1/1	1/1
Saline control	4	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0
Killed cells control	4	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0

[a] One animal in this group was sacrificed to observe extent of lesion.

TABLE 2. *Survival and tumor production in mice inoculated intraperitoneally with cells of C. neoformans (strain CIA) grown on low pH medium*

Dose (no. of cells)	No. of animals	No. of animals surviving/ no. with tumors, at day:										
		0	20	40	60	80	100	120	140	160	180	200
10^6	4	4/0	3/0	2/0	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1
10^5	4	4/0	4/0	4/0	4/0	4/3	2/2	2/2	1/1	0/0	0/0	0/0
10^4	4	4/0	4/0	4/0	4/0	4/4	3/3	3/3	1/1	1/1	0/0	0/0

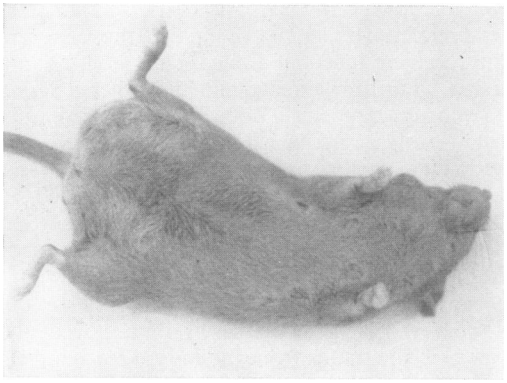

FIG. 1. *CBA/J mouse with large subcutaneous cryptococcoma 100 days after intraperitoneal inoculation with encapsulated cells of C. neoformans.*

FIG. 2. *CBA/J mouse dissected to demonstrate invasive mass in the abdomen and abdominal musculature, 100 days after intraperitoneal inoculation with encapsulated cells of C. neoformans.*

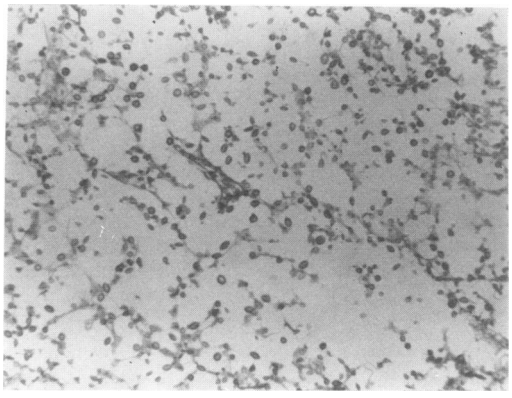

FIG. 3. *Abdominal cryptococcoma induced by intraperitoneal inoculation of C. neoformans, macrophages, capillaries, and collagen fibers. Lillie's allochrome stain.* ×*400.*

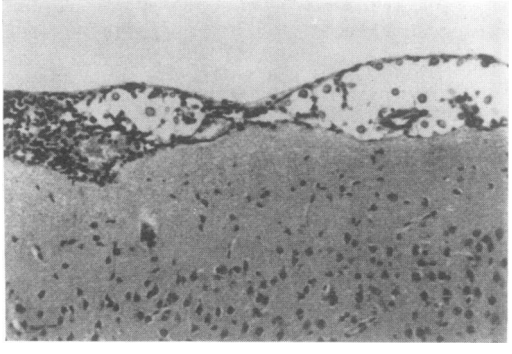

FIG. 4. *Subpia-arachnoid lesion with lymphocytic infiltrate from mouse infected by intraperitoneal inoculation with C. neoformans. Hematoxylin and eosin stain.* ×*100.*

revealed yeast cells and the reticular stroma (described above) outside the capsule of the organs. The lungs of these two mice were removed and examined grossly and with a microscope. No lesions or yeast cells were observed. The cerebellum and cerebrum were removed and subpia-arachnoid lesions were seen upon examination using a microscope (Fig. 4 and 5). Some yeast cells were also seen in perivascular lesions deep within the cerebrum (Fig. 6).

Airborne infection. As mentioned previously only one factor in the operation of the apparatus was observed consistently to correlate with the recovery of *C. neoformans* from the lungs of aerosol-exposed mice, namely, the appearance of a visible aerosol suspension in the secondary air from the nebulizer (although the intensity of aerosol appeared to be unrelated to the number of organisms recovered). Conversly, an invisible aerosol was always associated with the absence of *C. neoformans* in the lungs of the exposed mice.

From each of the four groups of CBA/J mice exposed to the aerosol for 120 min, one or more animals were selected immediately after exposure, and lung washings were examined for *C. neoformans*. In one group of eight mice, *C. neoformans* cells were found in the lungs of six mice thus examined, but no *C. neoformans* cells were detected in serial sections of the lungs of the remaining two animals.

In another group of 10 mice, three were sacrificed immediately after exposure to determine the number of viable cells in the lung washings. The remaining seven mice were again exposed to *C. neoformans* aerosol 15 days later and were observed for an additional 180 days.

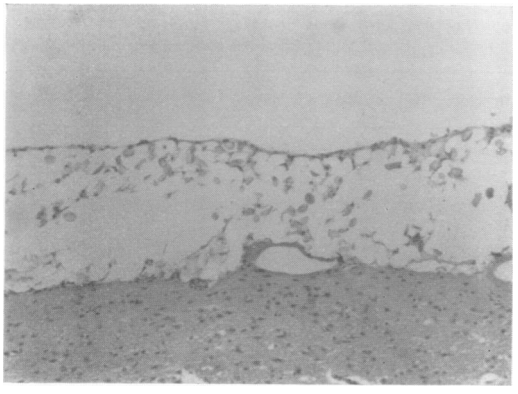

FIG. 5. *Subpia-arachnoid lesion from mouse infected by intraperitoneal inoculation with C. neoformans. Lillie's allochrome stain.* ×*400.*

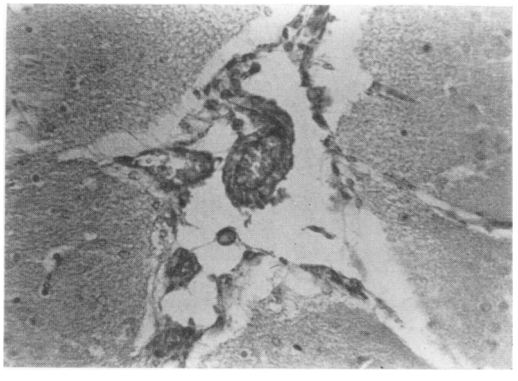

FIG. 6. *Deep perivascular lesion in cerebrum of mouse infected by intraperitoneal inoculation with C. neoformans. Lillie's allochrome stain.* ×*400.*

During the observation period, four animals died and necropsy and examination of tissues by microscope revealed no *C. neoformans* cells. The three animals which remained alive on day 180 after the second aerosol exposure were sacrificed. Serial sections of the lungs, liver, and brain of one of these contained no observable *C. neoformans* cells. However, approximately 50 *C. neoformans* cells were detected in the brain of one mouse by whole-brain homogenization and by dilution plating, and 200 *C. neoformans* cells were found in the brain and 15 in the liver of the second animal. No yeast cells could be cultured from lungs or kidneys of either animal or from the liver of the first mouse.

Age and strain differences in susceptibility to C. neoformans. When mice of different ages and strains were inoculated intraperitoneally with viable, encapsulated *C. neoformans* cells, 200-day-old CBA/J mice died at a greater rate (only 17% surviving at day 70) than the 35-day-old CBA/J mice (50% surviving at day 70; Table 3). In contrast to the younger mice, none of the older animals developed cryptococcomas during this experiment. They were found to have large spherical lesions (3 to 5 mm) of the central nervous system (Fig. 7). These lesions were translucent and, in one animal, herniation of the part of the cerebrum medial to the lesion was observed.

DISCUSSION

Most workers (16) believe the respiratory route is the normal site of entry for *C. neoformans*. With some exceptions, investigators seem to agree that particles with a diameter of 2.0 to 6.0 μm are capable of penetrating the lower respiratory tract. Ishaq et al. (12) and Farhi et al. (8) demonstrated that many *C. neoformans* cells lie in this size range. Our study demonstrates that cells of *C. neoformans* can enter mouse lungs in an aerosol.

The Airborne Infection Apparatus used in our experiments was designed by Middlebrook (17), who used it to infect mice with tubercle bacilli. He operated it at a secondary air flow rate of 20 liters/min and reported that 50% of the nebulized organisms were found in the chamber air. In our study, *C. neoformans* could not be recovered this easily. In addition to certain physical problems inherent in the apparatus, the characteristics of *C. neoformans* make operation of the chamber somewhat inefficient. The secondary air rapidly extracts moisture from nebulized particles, causing dry organisms to be carried in the secondary air. If the secondary air flow is reduced to 5.0 liters/min, an aerosol cloud is formed that becomes visible when it enters the chamber. Only under this condition, i.e., exposure to a visible aerosol cloud, could yeast cells be recovered from the lungs of mice in our study. Even after optimum aerosol exposure, the recovery of *C. neoformans* from the mouse lung was below that reported by Middlebrook for the tubercle bacillus. Since plates of Sabouraud dextrose agar exposed in the bottom of the aerosol chamber had a heavy growth of *C. neoformans*, many of the yeast cells in the chamber must be heavy particles with a rapid settling rate. Thus, the larger cells would probably never reach the lungs.

When CBA/J mice inhaled nebulized *C. neoformans* cells, infection without signs of disease was induced and maintained over a long period of time. The fact that *C. neoformans* cells are found in soil and commonly in pigeon

TABLE 3. *Effect of strain and age of mice on survival after intraperitoneal injection of 10^6 cells of C. neoformans*

Mouse strain	Age of mice (days)	No. surviving/percentage surviving, at day:							
		0	10	20	30	40	50	60	70
CBA/J	200	30/100	28/93	27/90	23/77	19/63	14/47	11/37	5/17
CBA/J	35	18/100	16/89	15/83	15/83	14/78	13/72	11/61	9/50
WS	35	10/100	9/90	9/90	9/90	9/90	8/80	7/70	7/70
CE/J	35	8/100	8/100	8/100	8/100	8/100	8/100	7/88	7/88
CBA/J[a]	200	10/100	9/90	8/80	9/80	8/80	8/80	8/80	7/70

[a] Noninfected control group.

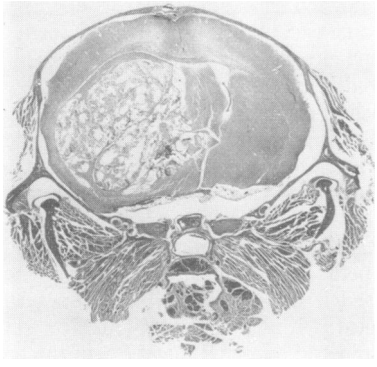

FIG. 7. *Large central nervous system lesion typical of those found in mice more than 200 days old receiving intraperitoneal inoculations of C. neoformans. Specimens were obtained by fixing the whole head in 10% Formalin and by decalcifying. Lillie's allochrome stain.* ×20.

droppings (1) suggests that man is continuously exposed to air containing this organism. The finding that mice can inhale cells of *C. neoformans* in an aerosol and harbor them in their central nervous system for the greater part of their life span further suggests that man, too, may be subject to *C. neoformans* infection. Although little evidence has been provided in support of endogenous infections in man, one must remember that most human patients with cryptococcosis at the present time have a predisposing disease, and that little evidence generally exists to establish an association between the patient and soil or pigeon excreta known to contain *C. neoformans*. Possibly, many individuals may be infected with or harbor *C. neoformans* and yet not develop the disease due to an equilibrium between host and microorganism. Should a situation develop which favors the proliferation of yeast cells (i.e., "predisposing factors," or a more subtle decrease in the host defense mechanisms), the infection may become a disease. Indeed, some of the mice in our study could be infected with rather small numbers of cells without apparent disease. This contrasts to the results of a study by Vanbreuseghem (24), who reported little variation in virulence with the quantity of cells inoculated.

Age appears to play a role in the response of mice to experimental cryptococcosis. CBA/J mice 35 days of age, when injected intraperitoneally with *C. neoformans* cells, developed large masses near the injection site. These consisted of encapsulated yeast cells, capillaries, macrophages, fine strands of collagen, and connective tissue cells. Examined with a microscope they were identical to tumors described by previous workers (1, 9). CBA/J mice over 200 days of age, on the other hand, failed to develop cryptococcomas, but died with central nervous system lesions containing *C. neoformans* cells. These lesions were similar in all mice and included small perivascular lesions deep in the brain. Meningeal lesions were typically below the pia-arachnoid, causing the meninges to be elevated above the surface of the cerebrum while depressing the cerebral surface. Occasionally, these areas were infiltrated by lymphocyte-type cells. When this infiltration occurred, it apparently was selective, since a lesion lying beside the one infiltrated frequently showed no such response (Fig. 4). Some mice had large lesions that produced herniation of the central nervous system (Fig. 7). These large lesions were not as firm as those in the peritoneum, and they had none of the connective tissue elements. As in the peritoneal lesions, most of the cryptococcal capsular material was removed in the preparation of the tissue, accounting for the large clear areas in the lesions.

Strain differences apparently are also a factor in the course of murine cryptococcosis. For example, 35-day-old white mice survived inoculation with *C. neoformans* longer than CBA/J or CE/J mice. However, survival time could not be correlated with any tissue changes.

An important feature in our research on *C.*

neoformans has been the use of strain CIA. We obtained our original culture in 1963. After subculturing this organism for 6 to 7 years, we observed that, after intraperitoneal inoculation, an occasional mouse developed abdominal masses. Over the next 2 years the organism began to produce a higher percentage of these masses. Thus, in some unknown manner, the virulence of the strain was altered. This is inconsistent with the findings of others (24). Since the tumors failed to develop in aged mice, these masses may represent some form of host defense mechanism, although the lack of tissue response is puzzling. Perhaps strains of *C. neoformans* differ in their mode of infection and the host response which they elicit.

Since we have been unable to find any record of a descriptive term for these tumors in the literature, we propose that they be called cryptococcomas. We suggest that this term be applied to tumor-like masses in animal tissues which are composed of yeast cells of the genus *Cryptococcus*, along with macrophages, connective tissue, and vascular elements.

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