

# Capsule Size of *Cryptococcus neoformans*: Control and Relationship to Virulence

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Capsule size of five isolates of *Cryptococcus neoformans* was controlled by cultivation in media containing varying amounts of sugar. High concentrations of sugar (e.g., 16%) suppressed encapsulation whereas low concentrations (e.g., 1%) allowed maximal encapsulation. Suppression of capsule size was attributed at least in part to the increased osmolarity of the medium because a medium with low sugar concentration but having high osmolarity (by virtue of added sodium chloride) also produced cells having small capsules. The extent of control was more marked with certain of the isolates than with others. Mice were intravenously inoculated with cells of a single isolate cultivated so as to have either small or large capsules, and virulence was measured by comparing death rates. Results indicate that virulence after such an inoculation is a constant characteristic of an isolate and is not affected by size of the capsule of the cells in the inoculum.

Cells of *Cryptococcus neoformans* in diseased tissue usually have large, distinctive capsules. When grown on ordinary media, however, such as Sabouraud glucose agar, different isolates may exhibit considerable variation in capsule size. Factors that may influence degree of encapsulation in culture are not clearly understood although there is evidence that control may be influenced by either environmental or nutritional conditions (5, 10). In 1970, Fahri, et al. (4) reported that encapsulation of cryptococci could be inhibited if any one of a variety of salts, or a high concentration of sugar, was incorporated into the medium. In the course of unpublished investigations carried out nearly two decades ago, we regularly suppressed encapsulation by increasing the amount of glucose in the medium. Herein are reported results of further investigations into suppression of encapsulation in vitro. As a corollary to these studies, the relationship between capsule size and virulence was reinvestigated, the first investigation in this laboratory having occurred in the earlier study referred to. Wherever relevant, results of some of these early experiments are cited.

## MATERIALS AND METHODS

**Organisms and animal inoculations.** *C. neoformans* 27, 98, 110, 145, and 153, from the collection at Tulane Medical Center, were used. Isolate 27 was extremely mucoid when grown on ordinary Sabouraud agar (2% glucose, 1% neopeptone, and 2% agar),

a characteristic which has been constant for nearly 30 years since primary isolation from spinal fluid of a patient who died of cryptococcosis. Isolate 98, originally from soil, was relatively dry in culture and had small capsules. The other isolates were intermediate in terms of mucosity and capsule size. One of these, 145, was used to infect mice. Seven-day-old cultures were harvested from agar and suspended in phosphate-buffered saline, and dilutions were prepared to contain varying concentrations of viable particles. Male CD-1 mice (Charles Rivers) were inoculated with 0.5 ml into a tail vein and, at the time of inoculation, the concentration of viable particles per inoculum was confirmed by triplicate pour plates.

**In vitro control of capsule size.** Sabouraud agar was prepared with glucose (1, 2, 4, 8, and 16%) and at a pH of 5.0 or 7.0. Plates containing the resulting 10 combinations of glucose and pH were conventionally streaked with the five isolates, incubated at both ambient temperature and 37°C, and observed daily for 14 days. Capsule size, as determined by microscopic examination of India ink preparations, and colony morphology were noted. Sabouraud broth was prepared, containing either 1 or 16% glucose and adjusted to pH 5.0 or 7.0. Inoculated broths were agitated at 150 gyrations per min during incubation at 37°C. Growth was observed microscopically each day for 4 days.

Growth was observed also on Sabouraud agar (pH 6) containing either (i) 1% glucose, (ii) 16% glucose, or (iii) 1% glucose with 2.9% NaCl, and incubated at the two aforementioned temperatures.

## RESULTS

**Effect of medium upon encapsulation.** Encapsulation was greatest in media that contained the lower amounts of glucose and was

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suppressed at the higher concentrations, in either broth or agar, and irrespective of temperature of incubation. When salt was substituted for the bulk of the glucose, the same degree of repression was observed, even though the salt-containing agar had the same low amount of glucose (i.e., 1%) that previously promoted large capsules (Fig. 1). Also as before, decrease in capsule size was accompanied by transformation of colonies from mucoid to dry and nonmucoid. Size of capsules was influenced also by pH—they were larger at pH 7 than at 5—but the effect was less pronounced than that of sugar concentration. The relationship between glucose concentration, pH, and capsule size was noted with all isolates, although not always to the same degree, and was most pronounced with strain 145. As can be seen in Fig. 2 and 3, growth of 145 on agar containing only 1% glucose and at a pH of 7.0 was mucoid—so much so that the colonies coalesced—and the cells were heavily encapsulated, whereas colonies on agar with 16% glucose and at pH 5.0 were discrete and dry, and the cells appeared almost devoid of capsules.

**Virulence experiments.** When groups of 10 mice were infected intravenously with  $10^2$ ,  $10^3$ ,  $10^4$ , or  $10^5$  viable particles of large- or small-capsuled *C. neoformans* 145, deaths began to occur first, as a rule, among those animals given cells having smaller capsules. Once dying started, however, there was no difference in the rate of dying with respect to size of the capsule, at any of the dosages except that of  $10^3$ . At this dosage cells of the mucoid culture appeared to be less virulent. Considering that the difference observed could have been due to the smallness of the group size, a second experiment was undertaken, repeating the dose of  $10^3$  but using 30 mice per group. With the larger number of animals, no disparity was seen; animals died at essentially the same rate, irrespective of whether cells in the inocula were thinly or thickly encapsulated (Fig. 4 and 5). In all experiments tissues of animals contained encapsulated cells, regardless of the condition of the inoculum.

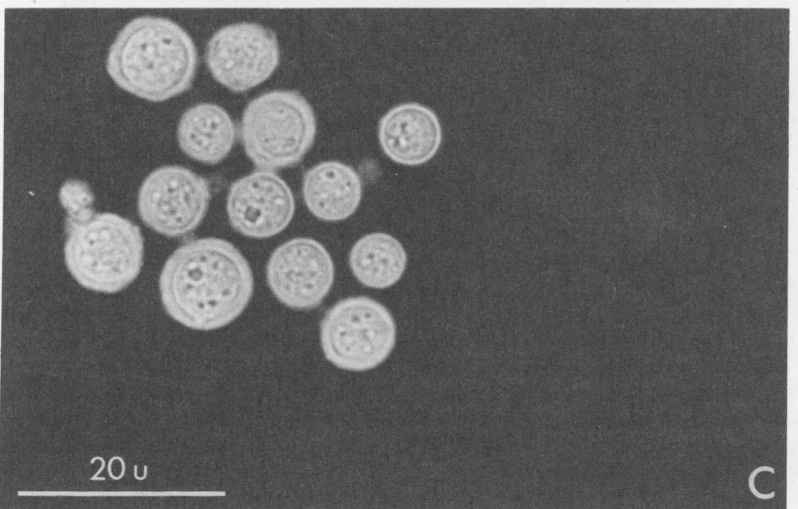
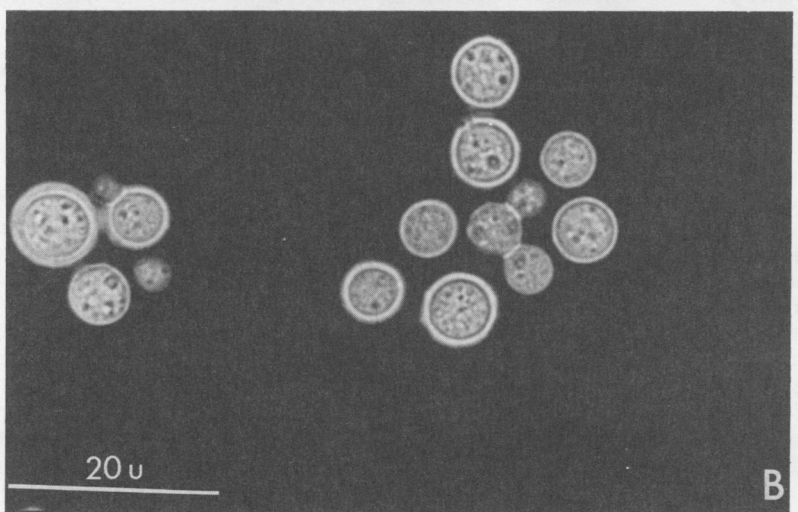
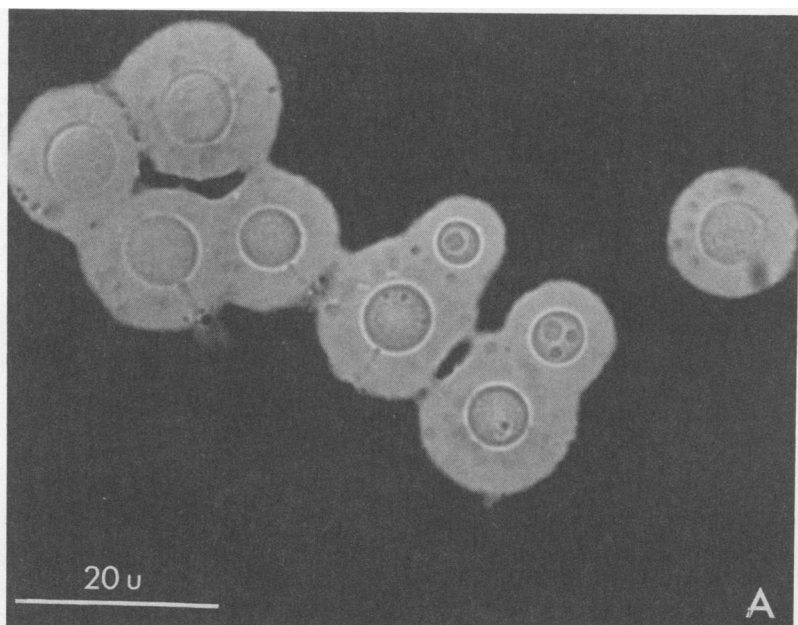
## DISCUSSION

To determine the effect of osmolarity upon capsule size, experiments were designed whereby the organism could be grown on an agar containing only 1% glucose (a concentration supportive of maximal encapsulation) but having a relatively high osmolarity, equal to that of the medium containing 16% glucose (suppressive to production of capsular material) and having the same pH. This was accom-

plished by addition of an appropriate amount of sodium chloride. When encapsulated cells were inoculated onto the low glucose-salt medium, growth proceeded without inhibition but encapsulation was suppressed, just as had occurred on the agar containing the high concentration of glucose. Capsule size was, therefore, at least in part, a function of the colligative properties of the medium. Still other factors must influence capsule size, however, for Littman (10) found that thiamine added to an agar medium would stimulate some nonmucoid isolates to become mucoid.

Whatever the possible mechanisms for reduced capsule size in vitro, it is tempting to postulate that capsular material shrinks in a medium of high osmolarity; that, in fact, cells may produce the same amount of capsular material, irrespective of osmolarity, but the volume occupied (and therefore the size of the capsule) in media of high osmolarity might be less because of shrinkage. Conversely, capsules formed in media of low osmolarity would expand, like a sponge. There is evidence, however, indicating that capsule size is not just a matter of shrinkage or swelling. For example, in experiments of Farhi et al. (4), although capsules appeared to become smaller after prolonged storage in dry soil, there was no enlargement after the stored cells were killed and transferred to water. In our own experience, capsules do not shrink during storage in brine, do not enlarge in water, and are not sloughed in water. In fact, the integrity of cryptococcal capsules is remarkable; we have found that pieces of mechanically broken cells carry their portions of capsule with them, even through vigorous homogenization by mechanical agitation—this despite the fact that solubilized capsular polysaccharide is readily detectable in body fluids and culture filtrates. That osmolarity is not the entire explanation is exemplified further by the isolated lesion seen occasionally in human material in which the organism appears devoid of capsule, despite residence in a milieu that presumably would be conducive to encapsulation.

Ability to control capsule size in vitro permitted an investigation of a possible relationship between capsule size and virulence, a relationship that has been debated for a number of years. For the most part, previous studies have been concerned with evaluating a series of isolates that varied in culture mucosity but which originated from different sources (6, 8, 13). In an extensive study in our own laboratory (unpublished data), virulence of 50 isolates, derived from a wide variety of sources, including



**FIG. 1.** India ink preparation of *C. neoformans* 145 grown on agar (pH 6) containing (A) 1% glucose, (B) 1% glucose and 2.9% NaCl, and (C) 16% glucose.

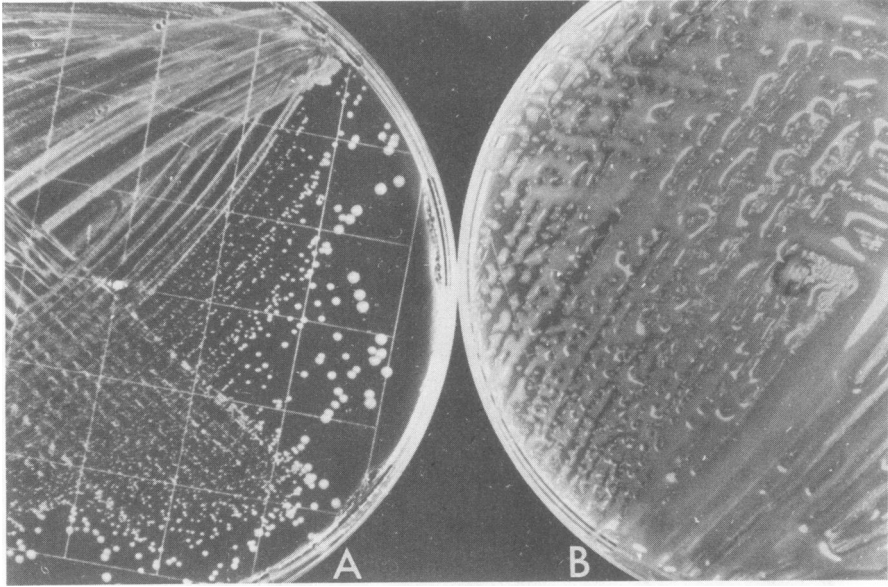


FIG. 2. *C. neoformans* 145 grown on agar plates containing (A) 16% glucose (pH 5) and (B) 1% glucose (pH 7).

soil, fatal human disease, and asymptomatic infection, varied greatly, and there was no correlation between virulence and size of capsules.

Another approach has been to compare virulence of mucoid or nonmucoid variants with that of a parent culture having opposite colony characteristics. Drouhet et al. (3) and Bulmer et al. (2) felt that they demonstrated a correlation between large capsules and virulence in this way. Kase and Metzgar (7), on the other hand, had a totally different experience with laboratory-derived variants. Their variant, which had a capsule of reduced size *in vivo* (personal communication) as well as *in vitro*, was more virulent than the mucoid parent culture. Failure of any isolate to form capsules *in vivo* has been the exception (14).

Perhaps the most convincing observation that size of capsule of cells in an inoculum seems unrelated to capacity to produce disease, at least in experimental animals, came from the work of Littman and Tsubura in 1959 (11). They controlled capsule size of a single isolate by varying the conditions of growth, and the two culture types had no difference in virulence. In a comparable experiment (unpublished data), we also were unable to demonstrate a relationship between virulence and capsule size in that there was no significant difference in the rate of dying between groups of 20 mice inoculated with  $10^8$  encapsulated cells (grown on a thiamine-enriched agar) or

the same dose of cells having virtually no capsule (grown on Sabouraud glucose agar). In fact, animals given the non-encapsulated cells actually died somewhat sooner. In the present study, a single isolate was again cultivated in such a way as to either suppress or permit maximal encapsulation. When the resultant large- or small-capsuled cells were compared for virulence in mice, there was no significant difference in the rate of dying. These consistent results convince us that a parenteral inoculation of cells having either large or small capsules may be capable of initiating fatal disease in laboratory animals, and this capacity is an inherent characteristic of the isolate, unrelated to capsular polysaccharide in the inoculum. This conclusion is reached despite observations that purified cryptococcal polysaccharide inhibits phagocytosis (1) and that phagocytosis correlates inversely with capsule size (e.g., 1, 12). Apparently the host is unable to dispose of the non-encapsulated inoculum before encapsulation resumes. In fact, even in the recent experiments, mice given cells having small capsules began to die before those inoculated with large-capsuled cells. Assuming this not to be an artifact, it is speculated that small-capsuled cells may be more extensively disseminated during the first few minutes of exposure, whereas large-capsuled cells, because of their large size, are more quickly trapped in the capillary bed of the lungs. It is further speculated

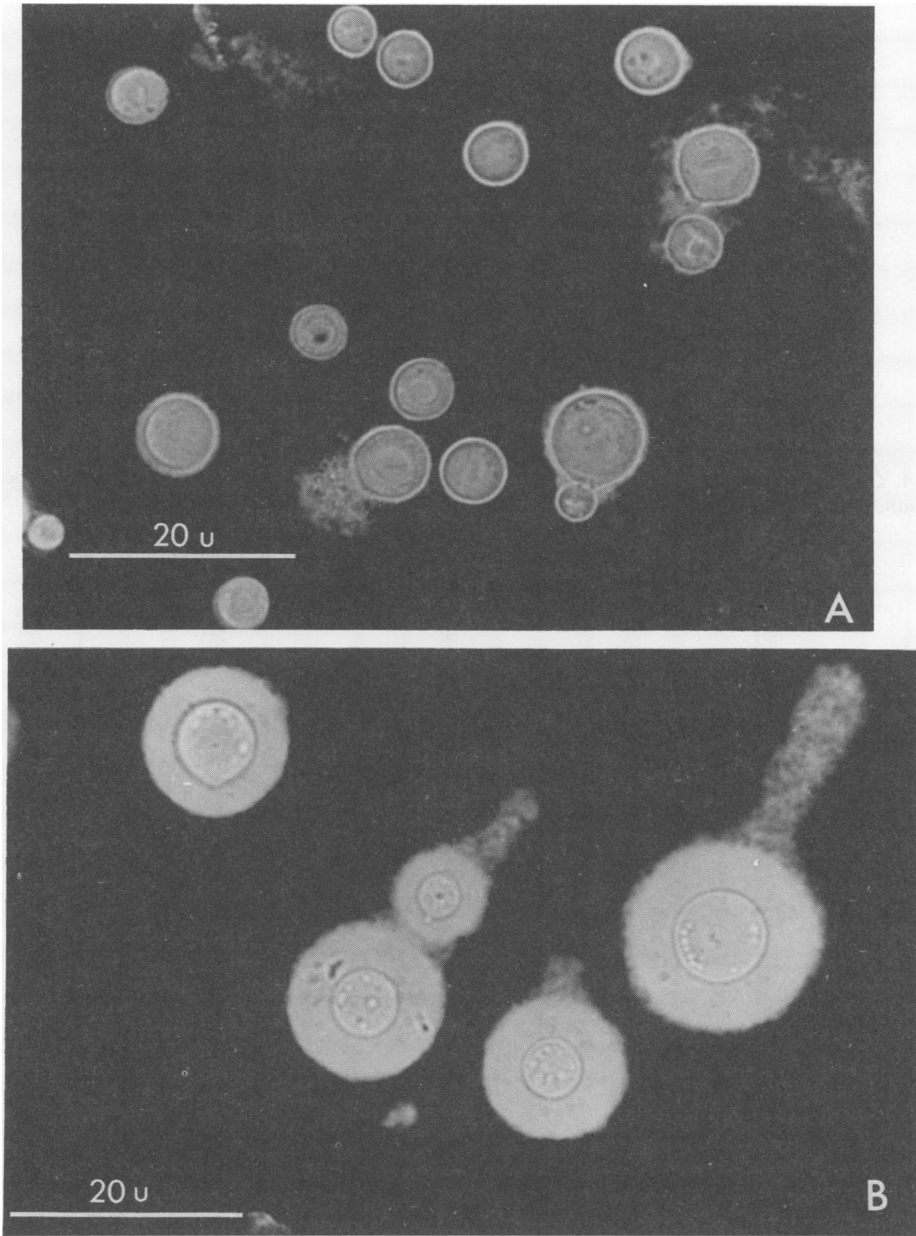


FIG. 3. India ink preparations of cultures depicted in Fig. 2. (A) 16% glucose (pH 5); (B) 1% glucose (pH 7).

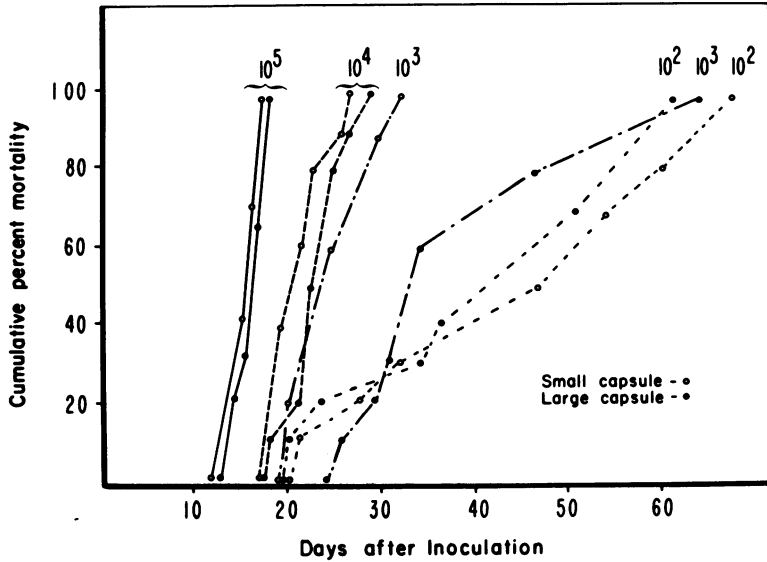


FIG. 4. Cumulative mortality of mice (groups of 10) infected intravenously with varying numbers of *C. neoformans* 145, cultivated under conditions conducive or repressive to encapsulation.

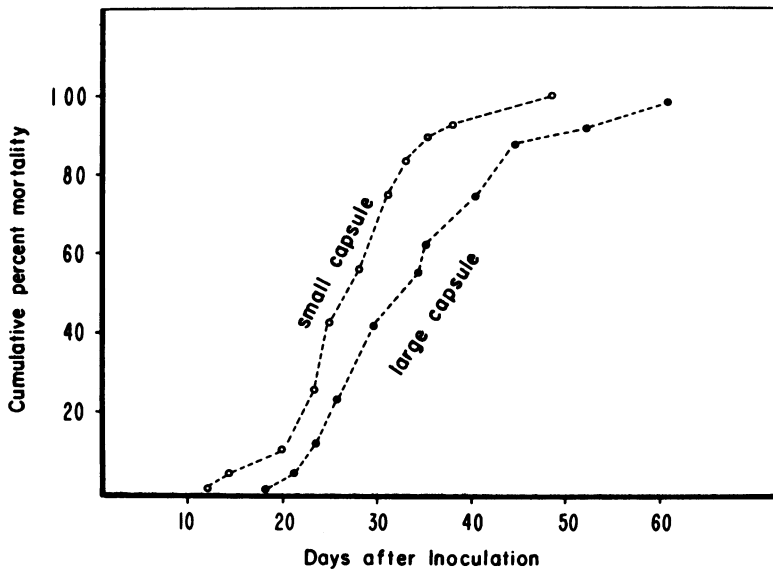


FIG. 5. Cumulative deaths of mice (groups of 30) infected intravenously with  $10^3$  viable units of *C. neoformans* 145, cultivated under conditions conducive or repressive to encapsulation.

that such an effect may play a role in the host-parasite interaction in these relatively artificial laboratory experiments. Not any of these experiments can be related, however, to how a normal human host would handle a naturally acquired inoculum of either small- or large-

capsuled cryptococci. Under the latter circumstances, it is probable that cells having a large capsule would be of a size such that they would not be able to traverse the respiratory tract and enter deep into the alveoli. This would be consistent with the hypothesis of Bulmer and his

associates (e.g., 4) that the infectious particle is a small-capsuled cell, but recent reports of perfect stages (e.g., 10) introduce the possibility of the even smaller basidiospore as an infectious unit.

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