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Phenoloxidase Activity and Virulence in Isogenic Strains of *Cryptococcus neoformans*

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A naturally occurring Mel^- variant of *Cryptococcus neoformans* was isolated from the wild type. The effect of phenoloxidase activity on virulence was analyzed on genetically constructed Mel^+ and Mel^- isolates. The traits Mel^+ and virulence in mice, as measured by cumulative mortality and replication potential in brain tissue, cosegregated among the progeny of a $Mel^+ \times Mel^-$ cross. Revertants (Mel^R) isolated during the course of the cumulative mortality experiment were used to compare virulence in isogenic sets of Mel^- and Mel^R. In two separate sets of such isolates, Mel^+ phenotype and virulence coreverted. Measurement of substrate uptake and phenoloxidase activity showed that loss of detectable phenoloxidase was the basis for the Mel^- phenotype and that enzyme activity reappeared in the Mel^R isolates. An intermediate phenotype, Mel^{bg}, was also described. Cosegregation and coreversion of the melanin phenotype and virulence suggest that phenoloxidase is a virulence factor in *C. neoformans*.

Cryptococcus neoformans is an encapsulated yeast-like fungus of worldwide distribution. It can cause, in an appropriate host, life-threatening meningitis. Although *C. neoformans* shares a number of physiological and morphological characteristics with other members of the genus, e.g., production of urease, utilization of inositol, and possession of a polysaccharide capsule (7), it is the only species that grows well at 37°C and is a primary pathogen. In addition to its ability to grow at host body temperature, an obvious requisite for a pathogen (14; K. J. Kwon-Chung, J. E. Bennett, and J. C. Rhodes, Antonie van Leeuwenhoek J. Microbiol. Serol., in press), *C. neoformans* is the only member of the genus with demonstrable phenoloxidase activity (4, 17, 20, 22). This latter property is used extensively as the basis for a presumptive identification test in which colonies of *C. neoformans*, when grown on a medium containing a suitable substrate for phenoloxidase, turn brown or black.

Previous studies to determine virulence factors in *C. neoformans* have focused on the contribution of the polysaccharide capsule (6). Its antiphagocytic properties as well documented (1, 3, 10, 12, 16), and acapsular mutants appear to be avirulent in mice (2, 11). Coupling these data with the fact that most of the clinical isolates are encapsulated suggests that the capsule may be a contributing factor for virulence. However, capsule alone seems to be insufficient, since correlation between the level of virulence and degree of capsulation does not exist (6) and all other members of the genus are also encapsulated. The coincidence of pathogenic potential and possession of phenoloxidase, combined with preliminary evidence for a casual relationship between the two (K. J. Kwon-Chung, I. Polacheck, V. Hearing, and T. Popkin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F16, 322), led us to pursue phenoloxidase as a possible virulence factor. Building on work presented in companion papers (14, 18), we examined the relationship between melanin phenotype and virulence for mice in isolates of *C. neoformans* derived from a naturally occurring variant with weak melanogenic ability and in isogenic sets differing only in phenoloxidase activity. By showing cosegregation and coreversion of melanin phenotypes and virulence, we strengthened the hypothesis that phenoloxidase activity is one of the important virulence factors in *C. neoformans*.

MATERIALS AND METHODS

Isolates. Wild-type strains B-3501 and B-3502 (13) of *C. neoformans* were used. B-3502 B^-, a spontaneous variant characterized by weak melanin production (Mel^{wk}), was isolated from B-3502 and was used to construct the strains listed in Table 1. All isolates used in the study were derived from single basidiospores and had capsule sizes equivalent to that of B-3501. Appropriate crosses were made on V-8 juice agar with neutral pH (Kwon-Chung et al., in press), and single basidiospores were isolated after 2 to 3 weeks by micromanipulation. Derivation of the isogenic sets is described in the Results section. All cultures were maintained with monthly transfers on malt extract agar (7).

TABLE 1. Parental isolates and progeny used in the study

Parental crosses[a]	Progeny
B-3501 (α, Mel$^+$) × B-3502B$^-$ (**a**, Mel$^-$)[a]	sb26 (**a**, Mel$^-$)
B-3501 (α, Mel$^+$) × sb26 (**a**, Mel$^-$)[a]	Br-1 (α, Mel$^+$) Br-2 (α, Mel$^+$) Br-4 (α, Mel$^+$) w-2 (**a**, Mel$^-$) w-4 (**a**, Mel$^-$) w-5 (**a**, Mel$^-$)

[a] Mating type and phenotype on *Guizotia* seed agar are given in parentheses for each isolate.

Growth rates. Growth rates of the isolates were compared in glucose-yeast extract broth (20 g of glucose and 10 g of yeast extract per liter; Difco Laboratories, Detroit, Mich.), using a shaking water bath (Precision Scientific Group, Chicago, Ill.) set at 37°C and 100 oscillations per min. Cultures were grown under test conditions for two 24-h periods before beginning the measurements. Increase in optical density was monitored at 600 nm on a model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Virulence studies. Female all-purpose albino mice (18 to 20 g) from the National Institutes of Health were used. The animals were housed 10 to a cage and given food and water ad libitum. Isolates were grown in glucose-yeast extract broth for 48 h at 25°C, 100 oscillations per min, in a shaking water bath. Cells were harvested by centrifugation and washed twice with sterile physiological saline. Appropriate numbers of cells, as determined by hemacytometer count, in 0.25 ml of saline were injected into mice intravenously (i.v.) via lateral tail vein. The viability of the inoculum was confirmed by plating. For determination of viable *C. neoformans* cells in brain tissue, mice were sacrificed by carbon dioxide asphyxiation. Their brains were removed aseptically, weighed, and homogenized in saline with a mortar and pestle. Appropriate dilutions were plated in duplicate on malt extract agar plates. After a 48-h incubation at 30°C, colonies were counted. In determining the geometric means, animals with negative cultures were assigned a value of 1.0, consistent with the calculated sensitivity of the test procedure. All inocula and brain isolates were screened for maintenance of phenotype by testing 10 to 60 colonies on *Guizotia* seed agar (21) modified to contain only water, seed extract, agar, and 1 g of glucose per liter.

Tissue samples for histology were fixed in acetate-buffered 10% Formalin. Embedding, sectioning, and staining were performed by Baker Histology, Alexandria, Va.

Substrate uptake and enzyme assay. Uptake of DL-3(3,4-dihydroxyphenyl)[3-^{14}C]alanine tartrate ([^{14}C]DOPA, 58 mCi/mmol; Research Products International Corp., Mt. Prospect, Ill.) was performed as described by Polacheck et al. (18).

Phenoloxidase activity was assayed by the colorimetric procedure of Polacheck et al. (18), using L-DOPA and L-norepinephrine (Sigma Chemical Co., St. Louis, Mo.) as substrates. The incubation period before the 5-h starvation step was extended to 18 h, resulting in a final optical density of ca. 1.0 at 600 nm. Before testing, the phenotype of the inoculum was verified on *Guizotia* seed agar.

Statistical tests. Cumulative mortality data were analyzed with the Wilcoxon test. The two-tailed Student *t* test was used for the colony count data. A generalized form of the *t* test was employed for analysis of the brain weight data.

RESULTS

Derivation of B-3502 B$^-$ and Mel$^-$ isolates. *C. neoformans* B-3502 was streaked on malt extract agar to obtain isolated colonies. Numerous colonies were then transferred as point inocula onto *Guizotia* seed agar. Whereas most resultant colonies produced the deep-brown pigment characteristic of *C. neoformans*, rare clones or sectors showing poor pigment production were selected to repeat the process. B-3502 B$^-$, which is light tan after a 7-day incubation, was isolated after four such cycles. Repeated recloning of this Mel^{wk} isolate resulted in no further diminution of pigment intensity. To obtain the white Mel$^-$ isolates used in this study, the crosses shown in Table 1 were performed and the melanin phenotype of the progeny was tested.

Growth rate. The growth rate in vitro at 37°C was determined for the Mel$^+$ isolates, B-3501, Br-1, Br-2, and Br-4, and for the Mel$^-$ isolates, sb26, w-2, w-4, and w-5. In the Mel$^+$ group, the doubling times ranged from 2.4 to 3.0 h, with a mean of 2.7 h. The mean doubling time for the Mel$^-$ isolates was 2.8 h, ranging from 2.4 to 3.1 h. The difference in growth rate between the two groups was not significant.

Virulence studies. To examine the relative virulence of Mel$^+$ and Mel$^-$ isolates, 10 mice were injected i.v. with a mean dose of 8×10^5 viable cells of each isolate. Deaths among members of the groups were recorded for 28 days, when all mice which had received Mel$^+$ strains had died. The cumulative mortality for mice injected with Mel$^+$ isolates was much greater ($P < 0.001$) than that for mice receiving Mel$^-$ strains (Fig. 1). Indeed, over 50% of the mice in the Mel$^-$ groups survived for 8 weeks. When dead animals from the Mel$^-$ groups were examined, Mel$^+$ revertants (Mel^R) were isolated from the brains of five of six mice in which cultures were performed (Fig. 2A). Although the proportion of Mel^R colonies in these cultures varied, revertants averaged 50% of the colonies screened.

Because studies from this laboratory (14) had shown that UV-induced Mel$^-$ mutants do not exhibit the exponential growth in brain tissue that characterizes wild-type *C. neoformans*, experiments were performed to examine this possibility in these spontaneous Mel$^-$ mutants. At

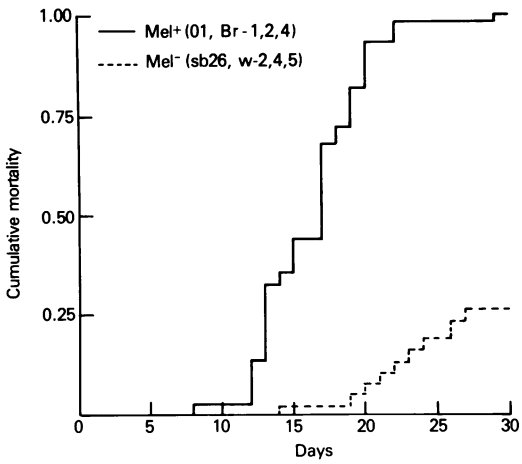

FIG. 1. Cumulative mortality of mice injected i.v. with 8×10^5 cells of Mel^+ or Mel^- isolates.

specified intervals after i.v. injection of ca. 10^4 viable cells of strains B-3501, Br-1, sb26, and w-2, three mice from each group were sacrificed and the mean colony-forming units per gram of brain tissue were determined (Fig. 3). The numbers of both Mel^+ isolates B-3501 and Br-1 increased steadily over the first 12 days and then reached a plateau. The recovery of viable sb26 cells from mouse brains was consistently near zero. Although they were always much lower than those seen with Mel^+ strains, numbers of viable w-2 cells in mouse brains fluctuated more than did those of the parent Mel^- isolate. No frank Mel^+ revertants were isolated from the Mel^- groups during the course of this experiment.

The data on colony-forming units were pooled over time to obtain the mean $\log_{10}$ viable organisms per gram of brain (Table 2). Although there were differences between isolates of the same phenotype, the most striking difference ($P < 0.001$) was found between the Mel^+ and Mel^- groups. An almost 10^4-fold higher titer was recovered from mice receiving Mel^+ inocula as compared with that from mice receiving Mel^- strains.

Derivation of isogenic sets. Revertants of sb26 and w-5 (Fig. 2A) were isolated from the brains of mice that died during the cumulative mortality studies. These two isolates, sb26 Mel^R and w-5 Mel^R, and two Mel^- isolates obtained from mouse brains, sb26 Mel^- (m) and w-5 Mel^- (m), were studied along with the original Mel^- mutants (Fig. 2B). The Mel^- (m) isolates were included in these isogenic sets because previous work with *C. neoformans* (19) had shown that mutant phenotype and virulence do not always corevert after mouse passage.

Virulence studies on the isogenic sets. To determine whether there was a difference in virulence among the members of these sets, groups of 12 mice each were injected with ca. 2×10^6 viable cells. Cumulative mortality was recorded for 21 days, the time when all of the mice which had received Mel^R isolates were dead. The mortality in the Mel^R group was significantly greater ($P < 0.001$) than that of either the Mel^- or the Mel^- (m) group (Fig. 4A and B). This relationship was true whether the origin of the strains was sb26 or w-5. Even if the observation period were extended to 5 weeks, the cumulative mortality in the Mel^- groups did not exceed 30%.

Histological examination of two mice from each group at 6 days after inoculation revealed

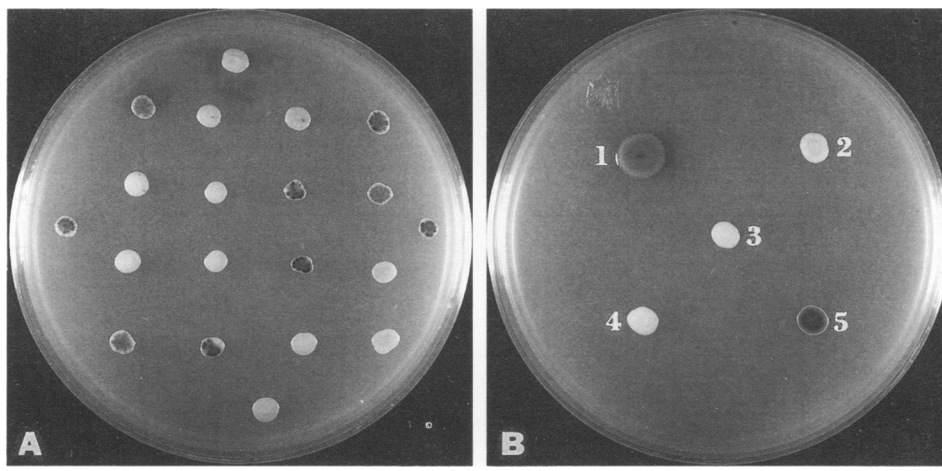

FIG. 2. Colonies after 5 days of incubation on *Guizotia* seed agar. (A) Random screening of colonies recovered from a mouse that died of w-5 infection showing almost 50% Mel^R phenotype. (B) The w-5 isogenic set and progenitor isolates: 1, B-3501; 2, sb26; 3, w-5 Mel^-; 4, w-5 Mel^- (m); 5, w-5 Mel^R.

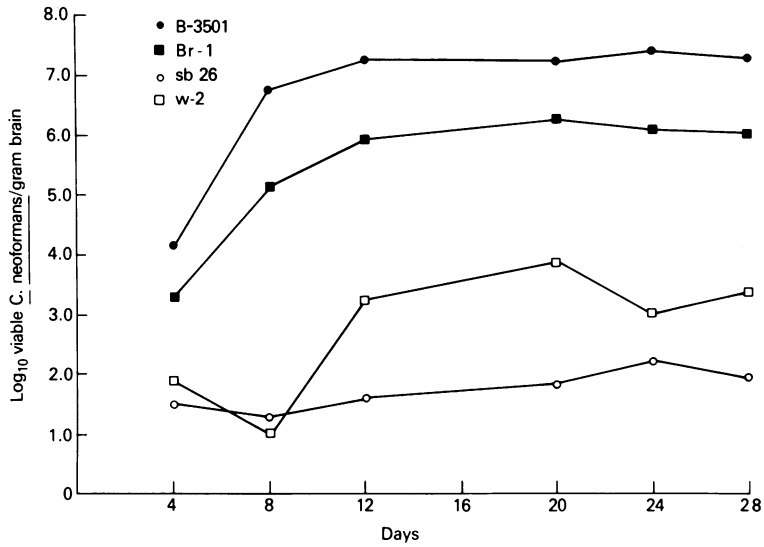

FIG. 3. Colony-forming units of Mel$^+$ and Mel$^-$ isolates recovered from mice that received 10^4 cells i.v.

TABLE 2. Mean $\log_{10}$ viable *C. neoformans* cells per gram in brains of mice infected with Mel$^+$ and Mel$^-$ isolates

Organism	Mean $\log_{10}$ titer[a]	P[b]
Mel$^+$		
B-3501	6.69	<0.01
Br-1	5.48	
Mel$^-$		
sb26	1.75	<0.02
w-2	2.77	
Mel$^+$ (B-3501 + Br-1)	6.08	<0.001
Mel$^-$ (sb26 + w-2)	2.26	

[a] The data were pooled over time to determine the overall mean titer.

[b] The level of significance obtained when the data were compared with that from the two-tailed Student *t* test.

that, whereas the Mel^R isolates proliferated in brain tissue (Fig. 5B), the Mel$^-$ strains had virtually disappeared. Interestingly, sb26 Mel$^-$ (m) behaved like sb26 Mel$^-$ and was undetectable in these sections; w-5 Mel$^-$ (m), however, appeared to be proliferating to almost the same extent as was w-5 Mel^R (compare Fig. 5A and B).

To quantitate this replication in brain tissue, mice were injected i.v. with ca. 7×10^3 viable cells and the number of colony-forming units per gram of brain was determined for all six isolates. These results (Fig. 6A and B) confirmed the phenomena observed in the histological sections of brain. Comparison of geometric mean values (Table 3) shows that both sb26 Mel$^-$ and Mel$^-$ (m) were found in significantly lower numbers than was the Mel^R isolate. Although w-5 Mel$^-$ (m) grew significantly better than did w-5 Mel$^-$, its titer of colony-forming units was still lower than that obtained with w-5 Mel^R. In addition, when the ability of members of the isogenic sets to cause cerebral swelling was examined (Fig. 7A and B), only in the mice infected with Mel^R isolates were the brains significantly ($P < 0.001$) increased in weight.

Determination of the biochemical basis of the Mel$^-$ phenotype. Defects in substrate uptake and phenoloxidase activity were examined as possible bases for the Mel$^-$ phenotype. The uptake rate of [^{14}C]DOPA by sb26 Mel$^-$ was calculated from data at 1, 2, and 5 min to be 0.17 nmol/mg of cells (dry weight). This represents approximately 35% of the value obtained for the wild-type isolate B-3501. Because both progenitors, B-3501 and sb26, demonstrated substrate uptake, the strain w-5 Mel$^-$ was not tested.

Finally, phenoloxidase activity was determined for selected isolates which had been characterized in animals. Values shown in Table 4 illustrate the loss of enzymatic activity by Mel$^-$ isolates and the reappearance of this activity in Mel^R strains. The values shown were obtained with L-DOPA as the substrate, although results were essentially the same when L-norepinephrine was used (data not shown).

When enzymatic activity was examined on a constant background, i.e., w-5, relative levels of detectable phenoloxidase activity correlated with relative virulence in mice. The level of activity seen in w-5 Mel$^-$ (m) was intermediate

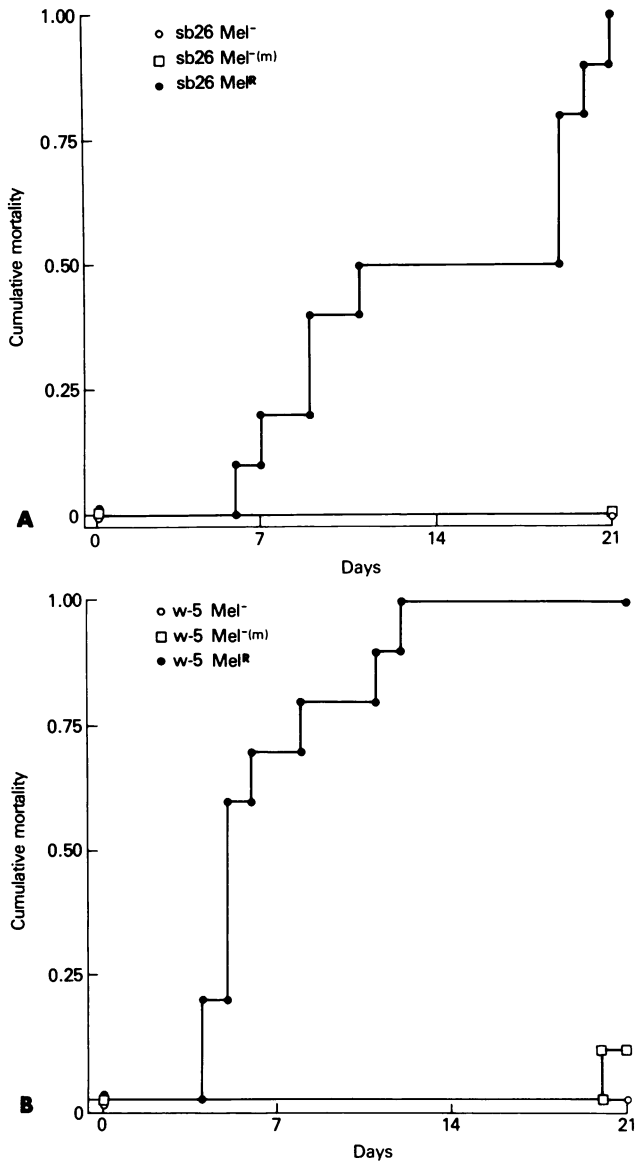

FIG. 4. Cumulative mortality of mice injected i.v. with 2×10^6 cells. (A) sb26 isogenic set; (B) w-5 isogenic set.

between those of its Mel^- and Mel^R counterparts. This intermediate level was expressed as the development of light-tan colonies on *Guizotia* seed agar after ≥7 days of incubation, in contrast to the white colonies of w-5 Mel^-.

DISCUSSION

In this paper, we have examined the relative virulence in mice of *C. neoformans* isolates differing in their melanin-forming ability. By using a naturally occurring variant isolated from the wild type to genetically construct Mel^+ and Mel^- isolates for study, we hoped to avoid the possible introduction of covert lesions by mutagenic agents (14, 19). It is not known whether this variant represented natural, low-frequency heterogeneity in the population or resulted from a spontaneous mutation.

In the first set of experiments, the cumulative mortality and replication rate in brain tissue was compared among genetically heterogeneous isolates with either the Mel^+ or the Mel^- phenotype. Despite the essentially identical in vitro doubling times at 37°C and capsule sizes of the organisms, infection of mice with Mel^+ isolates produced 100% mortality in 30 days (Fig. 1). In

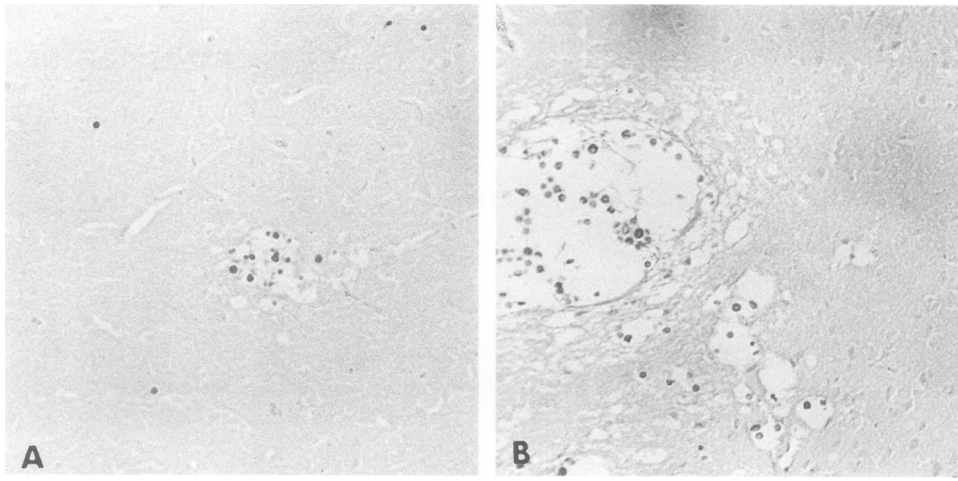

FIG. 5. Gomori methenamine silver-stained brain sections taken 6 days after a 2×10^6 i.v. inoculum. (A) w-5 Mel^- (m); (B) w-5 Mel^R.

contrast, Mel^- isolates killed only 30% of infected animals in 30 days; extending the observation period to 8 weeks increased this figure only to 43%. Further support for the association between Mel^+ and virulence for mice came from the recovery of Mel^R isolates from five of six of the Mel^- animals which died during the course of the experiment. Determination of growth potential of Mel^+ and Mel^- isolates in the brains of infected mice provided data which correlated with the difference in virulence displayed by the two phenotypes in the cumulative mortality study. These two experiments provided strong evidence for cosegregation of Mel^+ and virulence among progeny from a $Mel^+ \times Mel^-$ cross. The cosegregation of these two traits agrees with the findings of Kwon-Chung et al. (14), who also showed loss of virulence in Mel^- progeny.

Relatively minor, although still significant, differences seen between geometric mean titers of organisms per brain of parent and progeny isolates (Table 2) were not wholly unexpected. Previous studies have shown that numerous factors, e.g., the ability to grow at 37°C or to form the polysaccharide capsule, as well as phenoloxidase activity are important for the expression of virulence in *C. neoformans* (2, 11, 14; Kwon-Chung et al., in press). Certainly, in isolates with disparate genetic backgrounds, there is no reason to expect these or other, yet undefined virulence determinants to be equivalent.

In an attempt to isolate differences in melanogenesis for study, we used Mel^R strains obtained in the cumulative mortality experiment to establish isogenic sets differing only in melanin phenotype (Fig. 2B). In addition to the original Mel^- isolate and its revertant, Mel^R, these isogenic sets, derived from sb26 and w-5, included a Mel^- strain isolated from a mouse brain and designated Mel^- (m). These isolates were included because, in work with *C. neoformans* auxotrophic mutants, Rhodes and Howard (19) recovered from mice an Arg^- strain with possessed virulence equivalent to that of the parental wild type. Unexpectedly, the w-5 Mel^- (m) isolate defined an intermediate phenotype, which we have called beige, described below.

Behavior of sb26 isogenic strains conformed with that predicted from previous results. Both Mel^- isolates were essentially avirulent for mice, failing to produce lethal infections in mice receiving a relatively high i.v. inoculum (Fig. 4A), to proliferate to any significant extent in brain tissue (Fig. 6A), or to cause cerebral swelling in mice injected i.v. with viable cells (Fig. 7A). In contrast, sb26 Mel^R possessed all of the qualities associated with virulence in *C. neoformans* which were not found in the Mel^- strains. The coreversion to Mel^+ and virulent in sb26 Mel^R strengthens the association between these two phenotypic characters that was suggested by the data on their cosegregation.

Previously, this laboratory has reported UV-induced Mel^- mutants of *C. neoformans* (14) which have neither demonstrable substrate uptake nor phenoloxidase activity. The Mel^- isolates described here lack only phenoloxidase activity, further implicating this lesion as the basis for their lack of virulence. Also, the rate of [^{14}C]DOPA uptake reported for isolate sb26 Mel^-, which was lower than that of the wild type, may have resulted from rapid saturation of intracellular pools in the absence of phenoloxidase.

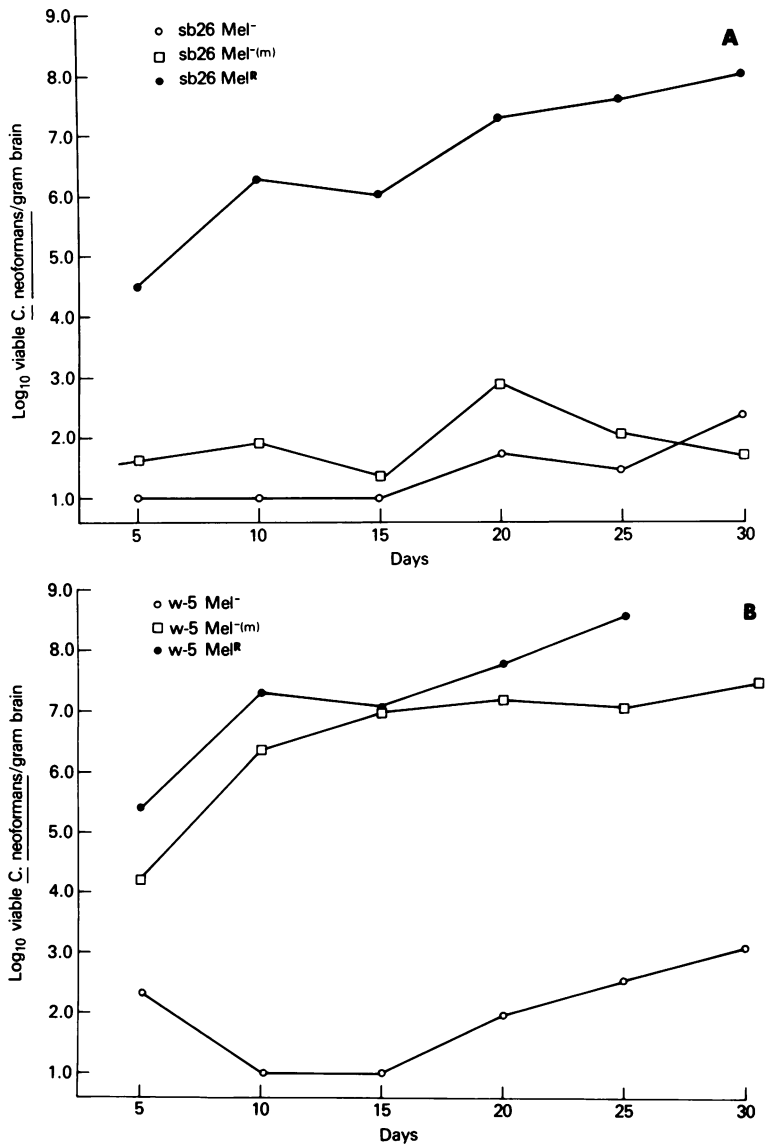

FIG. 6. Colony-forming units of isogenic strains recovered from mice that received 7×10^3 cells i.v. (A) sb26 isogenic set; (B) w-5 isogenic set.

Data obtained with the w-5 isogenic strains supported the hypothesis that phenoloxidase and virulence in mice are linked in *C. neoformans*. The introduction of an intermediate phenotype, however, may initially obscure this relationship. The results of the cumulative mortality experiment paralleled those obtained with the sb26 set; both Mel^- isolates did not produce lethal infections in mice, whereas the Mel^R isolate did (Fig. 4B). Surprisingly, when the ability of these isolates to multiply in brain tissue was measured, the titers of w-5 Mel^- (m) were only slightly lower than those of w-5 Mel^R (Table 3).

This finding posed an apparent dilemma, i.e., the failure of w-5 Mel^- (m) to kill mice during the time studied despite its ability to proliferate in brain tissue. We then reexamined the data collected in the viable count experiment. Hydrocephalus and swelling of the brain itself are major pathological processes in human cryptococcal meningitis (5) that also develop in infected mice where they are measurable as increased brain weight. Only the Mel^R isolates studied

TABLE 3. Mean $\log_{10}$ viable *C. neoformans* cells per gram in brains of mice infected with isogenic strains differing in melanin phenotype

Strain designation	Mean $\log_{10}$ titer of following isolate[a]:	
	sb26	w-5
Mel^-	1.42	1.77[b]
Mel^- (m)	1.92[c]	6.38
Mel^R	6.64[d]	7.27[e]

[a] The data were pooled over time to determine overall mean titers.
[b] $P < 0.001$ when compared with w-5 Mel^- (m) or Mel^R.
[c] $P > 0.20$ when compared with sb26 Mel^-.
[d] $P < 0.001$ when compared with sb26 Mel^- or Mel^- (m).
[e] $P < 0.01$ when compared with w-5 Mel^- (m).

TABLE 4. Phenoloxidase activity in selected isolates of *C. neoformans*

Isolate	Phenoloxidase activity[a]
B-3501[b]	1,091
sb26 Mel^-	0 (0)
sb26 Mel^R	87 (8.0)
w-5 Mel^-	61 (5.6)
w-5 Mel^- (m)	126 (11.5)
w-5 Mel^R	698 (64.0)

[a] Specific activity based on Δ absorbancy at 480 nm/mg of protein, using L-DOPA as the substrate. Protein determinations were performed by the method of Lowry et al. (15). Numbers in parentheses represent specific activity (percentage of wild-type activity).
[b] Wild-type Mel^+ isolate.

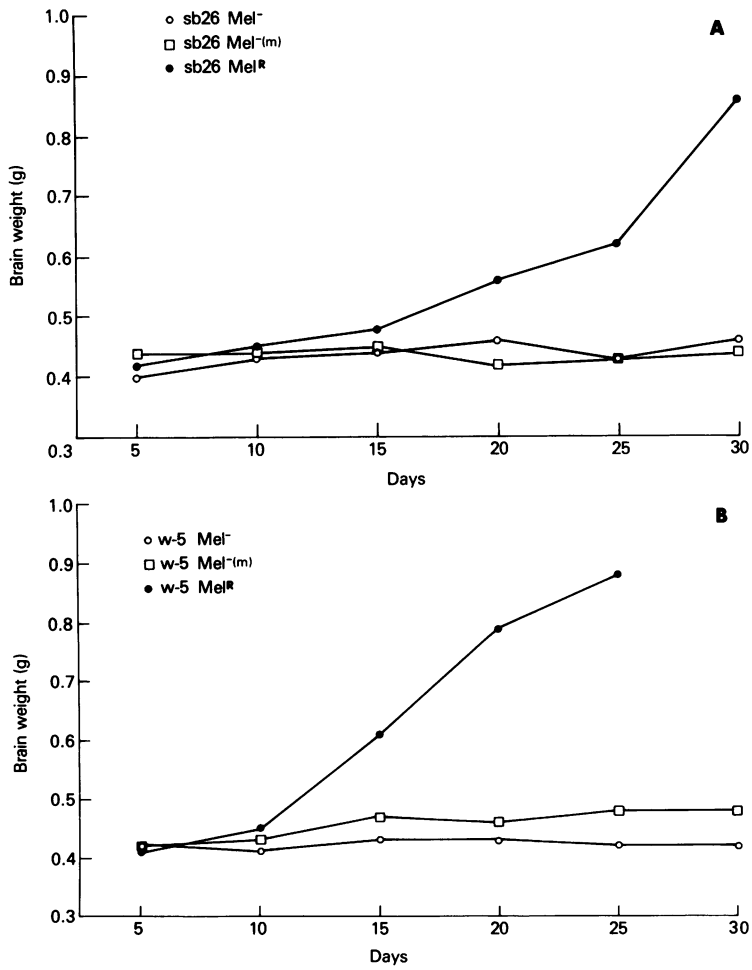

FIG. 7. Mean brain weight versus time for mice receiving 7×10^3 cells of the isogenic strains. (A) sb26 isogenic set; (B) w-5 isogenic set.

here caused a significant ($P < 0.001$) increase in brain weight during the course of the experiment (Fig. 7A and B). Although the exact cause of this enlargement is not known, the direct implantation of cryptococcal polysaccharide into the brains of rats has been shown to cause cerebral edema (8, 9). Attempts to demonstrate differences in capsule size between w-5 Mel^- (m) and w-5 Mel^R in vitro in India ink preparations of cells grown under identical conditions or in vivo in mucicarmine-stained histological sections of brain were not successful. Differences in shedding of soluble polysaccharide could not be ruled out, although this possibility seemed unlikely owing to the presumed identical genetic backgrounds of the isolates. Maximizing the value of χ^2 generated from a contingency table of low and high brain weights versus low and high viable counts suggested that a titer of 6.8 to 6.9 $\log_{10}$ viable cells per brain was the critical mass of *C. neoformans* required for developing brain swelling in mice. This value would agree with viable counts obtained in animals infected with w-5 Mel^- (m) and w-5 Mel^R (Fig. 6B) and the resultant brain enlargement. For this reason, we favor the explanation that a specific burden of yeast cells is required to cause morbid cerebral edema or hydrocephalus for the results obtained with the w-5 isogenic set. However, we are unable to prove this explanation or to disprove ones involving shedding of soluble polysaccharide or elaboration of undefined edema-causing substances.

Levels of phenoloxidase were assayed on all three w-5 isolates (Table 4), and the relative enzymatic activities, high, intermediate, and low, for Mel^R, Mel^- (m), and Mel^-, respectively, correlated with their relative virulence for mice discussed above. The combination in w-5 Mel^- (m) of intermediate virulence in mice; production of tan, rather than brown or white, colonies on *Guizotia* seed agar; and intermediate phenoloxidase activity seems sufficient to define a new phenotype that we have called beige and designated Mel^{bg}.

Comparison of the amounts of phenoloxidase detected in the sb26 and w-5 isogenic sets suggests several conclusions. Perhaps the most important is that, within an isogenic set, relative phenoloxidase levels may reflect relative virulence, but between isolates of disparate genetic backgrounds, this correlation may not apply. This finding is in keeping with the idea that virulence is a polygenic phenomenon in *C. neoformans* in which numerous factors may make contributions to the virulent phenotype (2, 6, 11, 14, 16; Kwon-Chung et al., in press). Second, the amount of phenoloxidase required to produce the observed phenotypes may not be absolute but may differ among isolates, based on the genetic background on which the trait is expressed. Finally, differences in detectable levels of phenoloxidase related to growth conditions or to comparative difficulty in breaking the yeast cells to obtain the membrane-bound enzyme (18) may make direct comparisons of activity between disparate isolates impractical. We feel that the possibility of these last points substantively influencing the data herein is slight because repeated assays on these isolates grown under various conditions and broken at different times gave the same hierarchy of phenoloxidase levels as that shown in Table 4. These conclusions require that other isolates of *C. neoformans* be tested before we can form accurate conclusions on the prediction value with respect to virulance of phenoloxidase determinations. However, the cosegregation of Mel^+ (phenoloxidase$^+$) and mouse virulence in progeny from a Mel^+ (phenoloxidase$^+$) × Mel^- (phenoloxidase$^-$) cross and the coreversion of the two traits from avirulent Mel^- isolates in two separate isogenic sets provide compelling evidence that phenoloxidase is a virulence factor in *C. neoformans*.

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