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Melanin-Lacking Mutants of *Cryptococcus neoformans* and Their Virulence for Mice

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A double mutant of *Cryptococcus neoformans* which lacked the ability to produce melanin (Mel^-) on media containing diphenols and failed to grow at 37°C (temperature sensitive, Tem^-) was obtained by UV irradiation and subsequent cloning. The mutant showed two lesions in melanogenesis in that it lacked the active transport system for diphenolic compounds and also lacked phenoloxidase. Ultrastructures of the mutant and wild-type cells grown on a medium with or without L-dopa showed that only the wild-type cells grown on L-dopa medium formed a dark cell wall layer, presumably containing melanin. The mutant was crossed with a wild type, and the phenotypes of the progeny were analyzed. The analysis showed no linkage between the mating type and either Mel or Tem loci, but loose linkage was seen between Mel and Tem loci. The progeny, Mel^+ Tem^+, Mel^+ Tem^-, Mel^- Tem^+, and Mel^- Tem^-, were studied for their virulence in mice. Only Mel^+ Tem^+ types killed mice with an inoculum of 5×10^5 cells within 50 days.

Cryptococcus neoformans produces infection in both human and animals. Any part of the body may be involved, but the central nervous system is most commonly affected in humans (4, 8). Cryptococcosis has the highest case fatality among the mycoses (9), ranging from 18 to 37%.

The species *C. neoformans*, with two varieties (K. J. Kwon-Chung, J. E. Bennett, and J. C. Rhodes, Antonie Van Leeuwenhoeck J. Microbiol. Serol., in press), is the only true pathogen among nearly 20 species described in the genus *Cryptococcus* (19). Infections caused by cryptococci other than *C. neoformans* have been extremely rare and poorly documented. *C. neoformans* is unique among members of the genus in that it grows well at 37°C and produces a melanin-like pigment when grown on substrates containing di- or polyphenolic compounds (5, 20, 24, 28). The enzyme responsible for the pigment formation was determined to be phenoloxidase (20, 28) and was found to be membrane bound and constitutive (20). Some isolates of *C. laurentii* and *C. albidus* also grow at 37°C but do not produce the pigment when grown on media containing substrates for phenoloxidase (31, 32). These two species have only rarely been isolated from human cases of infection (6, 7, 17, 18), and either the identification of the species or their pathogenicity in these cases remains to be confirmed.

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Since the combination of growth at 37°C and ability to form melanin is unique to *C. neoformans*, we hypothesized that these two features may play an important role in its pathogenicity. One test of such a hypothesis would be to isolate mutants which lack one or both markers and to study their virulence in experimental animals. To achieve this goal, a mutant which failed to produce melanin (Mel) and failed to grow at 37°C was isolated. The mutant was crossed with a wild-type strain, and progeny with various combinations of the two markers were isolated to study their virulence in mice.

MATERIALS AND METHODS

Isolates, media, and growth conditions. The two tester strains, B-3501 (α type) and B-3502 (**a** type) (13), were used as the original parent strains. These cultures were maintained on malt extract slants at 25°C. To obtain a melanin-lacking (Mel^-), 37°C temperature-sensitive (Tem^-) mutant, B-3502 was subjected to UV irradiation. The cells to be irradiated were harvested from a 48-h-old slant culture and suspended in sterile physiological saline to obtain a concentration of 10^7/ml. The cell suspension was irradiated for 5 to 40 s as previously described (15). The irradiated cell suspension was periodically drawn and plated (0.2 ml/plate) on malt extract agar. The colonies formed on the malt extract agar plate were replica plated on asparagine minimal agar medium to test for prototrophy. The asparagine medium was prepared by dissolving the following in 1 liter of distilled water: 10 g of glucose; 3 g of KH_2PO_4; 0.5 g of $MgSO_4 \cdot 7H_2O$; 1 g of asparagine; and 50 μl of thiamine-vitamin solution (Bejectal;

Abbott Laboratories, North Chicago, Ill.). The isolates which grew on the minimal medium were streaked on L-dopa agar (5) and niger seed agar (29) to test their melanin-forming ability. In some cases, L-dopa was replaced by L-norepinephrine or dopamine. The concentrations of the diphenols and glucose were 0.5 mM and 0.1%, respectively (20). To test their temperature tolerance, cells grown on malt extract agar at 25°C for 48 h were streaked on the agar slants and incubated at 37°C for 3 days. The isolates which showed questionable growth were tested further by plating 100 to 200 cells/plate on malt extract agar and incubating the duplicate plates at 30 and 37°C for 3 days. The isolates which formed visible colonies at 30 but not 37°C were designated as the Tem^- isolates. To compare generation times between various isolates, inocula were harvested from 48-h cultures, washed three times with saline, suspended in yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.) broth with 2% glucose or asparagine minimal broth medium in Erlenmeyer flasks, and incubated for 48 h on a shaker at 25°C as well as at 37°C. The initial optical density of the broth culture was adjusted to 0.02 at 600 nm (Gilford spectrophotometer 250), and the absorbance was measured periodically.

Electron microscopy. The wild-type B-3501 and the melanine-less mutant (92t-1) were grown on asparagine agar with and without L-dopa for 4 days. The cells were harvested with a loop and transferred to a small tube containing 5 ml of 2.5% glutaraldehyde buffered in 0.2 M sodium cacodylate, pH 7.2, for 16 h (22, 27) at 4°C. The cells were centrifuged, and the pellets were washed in 0.2 M cacodylate buffer followed by a wash in 0.2 M sucrose buffered to pH 7.2 in 0.1 M sodium phosphate (26) for 16 h at 4°C. The cells were centrifuged, and the pellets were suspended in filtered 1% aqueous potassium permanganate for 3 h at 25°C. The cells were washed repeatedly with acetate-veronal buffer (calcium-free) and fixed in 1% OsO_4 for 16 h at 25°C. Subsequent washing, Noble agar embedment, and 0.5% uranyl acetate en bloc staining of cells were done according to the technique of Ryter and Kellenberger (26). The fixed, en bloc-stained cells were dehydrated through a graded series of ethanol and embedded in Spurr medium (30) as described previously (14, 22). Ultrathin sections were made on a LKB Ultratome III, using a Du Pont diamond knife. All sections were mounted on 300-mesh naked copper grids and were unstained. Micrographs were recorded on Kodak electron microscopy sheet film with a Hitachi HU11-C electron microscopy operating at 75 kV.

For the preparation of unstained sections, the cells were fixed in 2.5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) for 6 h at 25°C. The cells were centrifuged, and the pellets were washed in the cacodylate buffer embedded in agar cubes. Subsequent dehydration and epoxy infiltration and embedment were as described above.

Genetic analysis. Strain B-3501 (Mel^+ Tem^+) was crossed with B-3502 (Mel^+ Tem^+) and 92t-1 (Mel^- Tem^-) on hay infusion agar (25). Two progeny with phenotypes Mel^+ Tem^- and Mel^- Tem^+ obtained from B-3501 × 92t-1 were also crossed. After a 2-week incubation at 25°C, single basidiospores were randomly isolated by micromanipulation, and their phenotypes were analyzed. For the mating type analysis, progeny were crossed with B-3501 (α) and B-3502 (**a**). Their melanin-forming ability was scored by culturing them on niger seed agar. Their temperature tolerance was tested at 30 and 37°C.

Virulence study. Two parental isolates, 92t-1 and B-3501, and three isolates each of Mel^+ Tem^+, Mel^+ Tem^- , Mel^- Tem^-, and Mel^- Tem^+ were studied for their virulence for mice. The yeast cells from 48-h malt extract agar cultures were suspended in saline, and serial dilutions were prepared. The dilutions containing 10^6 and 10^7 cells/ml, as determined by both hemacytometer and plate count, were used as inocula. Female general-purpose (National Institutes of Health) white mice weighing 19 to 21 g were infected by injecting 0.5 ml of inocula into the lateral tail vein. Ten mice were injected with each inoculum of 5×10^6 and 5×10^5 cells for the observation of survival up to 60 days. Three other sets of 10 mice were used to test the in vivo growth rate of B-3501 (Mel^+ Tem^+), 92t-1 (Mel^- Tem^-), and a Mel^- Tem^+ progeny designated as Sb8. The mice were injected with 5×10^4 cells intravenously and were sacrificed periodically to culture the brain, spleen, and liver. The organs were weighed, mixed with an appropriate amount of saline, homogenized, and diluted before plating on malt extract agar plates. The colonies were counted after 4 to 5 days of incubation at 30°C.

Preparation of cell-free extracts. To obtain phenoloxidase from the two parent strains, B-3501 (Mel^+ Tem^+) and 92t-1 (Mel^- Tem^-), and three Mel^- Tem^+ progeny, Sb7, Sb8, and Sb31, the glucose-free medium treatment described by Polacheck et al. (20) was used. Briefly, cells were grown in YNB broth (800 ml) supplemented with 0.3% glucose for 18 h at 25°C. The optical density of the culture reached 0.6 to 0.7 (600 nm) at the end of the 18-h incubation. The cells were collected by centrifugation (12,000 × *g* for 15 min), washed with YNB broth (glucose-free), and then transferred to a flask with 800 ml of YNB broth on a shaker for 5 h. The cells were harvested by centrifugation and broken by blending with glass beads (21). After breakage, the supernatant was separated from the beads and centrifuged at 105,000 × *g* for 45 min in the cold to obtain cell-free extract.

Uptake of catecholamines and assay of phenoloxidase. Uptake of DL-3(3,4-dihydroxphenyl)[3-^{14}C]alanine, tartrate, DL-[*methylene*-^{14}C]noradrenaline DL-bitartrate, and [7-^{14}C]dopamine hydrochloride by B-3501, 92t-1, and their selected Mel^- Tem^+ progeny, Sb7, Sb8, and Sb31, was tested according to the method described by Polacheck et al. (20). The assays of phenoloxidase were carried out by both the colorimetric method according to Polacheck et al. (20) and the radiometric method described by Hearing et al. (10, 11).

RESULTS

Isolation and growth of the mutant. Five mutants which produced nearly white colonies on L-dopa or niger seed medium were isolated after a 15-s irradiation of B-3502 cells. These were all Tem^+ in that they grew at 37°C. Each mutant was cloned by diluting and plating the cells on malt extract agar. More than 200 clones were obtained from each isolate and were tested for

their temperature tolerance. One of the numerous clones tested was found to grow at 30°C but not at 37°C. This isolate produced only white colonies when cultured on niger seed, L-dopa, and norepinephrine agar and was designated as 92t-1 (Mel^- Tem^-). The growth rate of the mutant at 30°C on malt extract agar was the same as that of the wild types, B-3502 and B-3501. The mutant was a prototroph and grew well on asparagine minimal agar medium at both 25 and 30°C. The broth culture showed that the doubling time of 92t-1 was the same as for the wild types (2.5 h) in YNB with 2% glucose at 25°C with shaking. In the asparagine minimal broth, however, 92t-1 showed a generation time 2 h longer than that of the wild type. Under the microscope, most cells of 92t-1 grown on asparagine broth medium carried buds larger than those seen in B-3501 and B-3502. The separation of the bud appeared to be delayed in the mutant. The morphology of the cells was otherwise indistinguishable from that of the wild type. The degree of extracellular capsule formation was the same in 92t-1 and the wild type.

Electron microscopy revealed that the cells of B-3501 (Fig. 1D) and B-3502 grown on L-dopa agar and fixed with glutaraldehyde had an electron-dense layer in the cell wall, whereas the cells of 92t-1 grown on the same medium lacked this layer (Fig. 1C). The wild-type cells or the mutant grown on the medium without L-dopa also lacked the dark layer. Ultrathin sections of the cells fixed with glutaraldehyde, treated with potassium permanganate, and stained with uranyl acetate revealed a similar picture (Fig. 1A,B). The wild-type cells grown on L-dopa medium were the only ones that had an electron-dense layer in the cell wall (Fig. 1B). This layer appeared to be the melanin pigment. Under the light microscope, the dark cells of the wild type

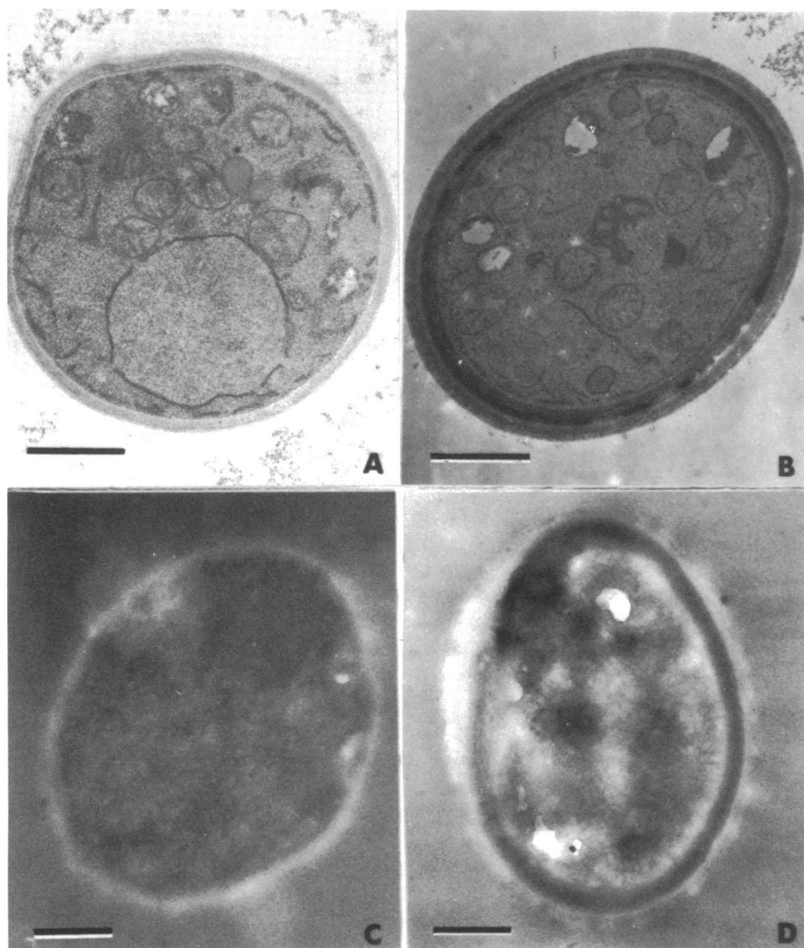

FIG. 1. Ultrathin sections of *C. neoformans* cells. Each bar represents 1 μm. (A) 92t-1 (Mel^- Tem^-) and (B) B-3501 (Mel^+ Tem^+) grown on L-dopa agar, fixed with glutaraldehyde, and treated with potassium permanganate and uranyl acetate. (C) 92t-1 and (D) B-3501 grown on L-dopa agar, fixed with glutaraldehyde, and not stained.

grown on L-dopa agar manifested the pigment only as part of the cell wall and not in the cytoplasm. The mutant produced white colonies on niger seed, L-dopa, L-norepinephrine, and L-dopamine agars at 25 and 30°C.

Genetic analysis. The mutant 92t-1 (**a** Mel^- Tem^-) was crossed with B-3501 (α Mel^+ Tem^+), and 160 single basidiospore cultures were obtained to analyze their mating type, temperature tolerance, and melanin-forming ability. Viability of the basidiospores from the cross was nearly 90%. The Mel^+ and Mel^- types were scored by culturing them on niger seed agar with 92t-1 and B-3501 as the controls (Fig. 2). Eight types of progeny were obtained: α Mel^+ Tem^+[30], **a** Mel^+ Tem^+[34], α Mel^+ Tem^-[11], **a** Mel^+ Tem^-[11], α Mel^- Tem^+[14], **a** Mel^- Tem^+[13], α Mel^- Tem^-[24], and **a** Mel^- Tem^-[23]. Table 1 presents the ratio of progeny with each marker and the relationship between any given two markers. All three markers appeared to be controlled by nuclear genes, and the mating type was not linked with either the Tem or the Mel locus. The Tem^- progeny were reduced in frequency, but the difference was not significant at the 5% level. A loose linkage was seen between the Tem and Mel loci. The ratio between the parental types and the recombinants was approximately 2:1. To confirm the loose linkage between these two loci, two progeny of opposite phenotypes, **a** Mel^+ Tem^- and α Mel^- Tem^+, were crossed, and 37 single basidiospore progeny were analyzed for their Mel and Tem markers. The number of progeny in the phenotypes Mel^+ Tem^+, Mel^+ Tem^-, Mel^- Tem^+, and Mel^- Tem^- were 7, 14, 12, and 4, respectively. The loose linkage between Mel and Tem loci was confirmed, since the ratio between the parental types and the recombinants was 2:1 among the progeny obtained from the cross Mel^+ Tem^- $\times$ Mel^- Tem^+. To confirm the lack of spontaneous mutation among the progeny produced by the pair of two wild types, Mel^+ Tem^+, B-3501, and B-3502 were crossed. Ninety-nine single basidiospore cultures were analyzed for their Mel and Tem phenotypes. The cross between two wild-type isolates produced only the Mel^+ Tem^+ type, indicating the absence of spontaneous Mel^- or Tem^- progeny.

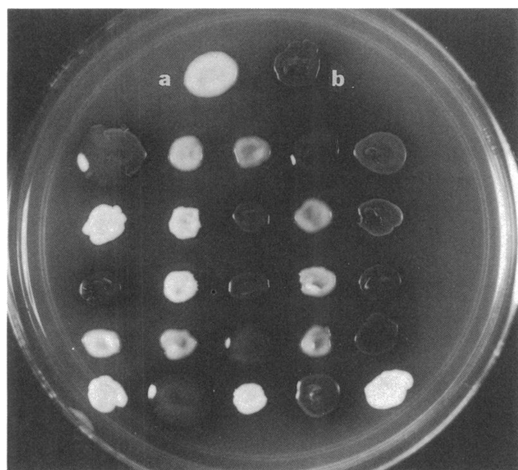

FIG. 2. Segregation of Mel^- and Mel^+ among the progeny of 92t-1 (a) $\times$ B-3501 (b).

Uptake of diphenols and assay of phenoloxidase. The radiolabeled L-dopa, L-norepinephrine, and dopamine were not taken up by 92t-1 (Fig. 3) nor by the three Mel^- Tem^+ progeny which were used for the virulence test. The wild type, B-3501, showed active transport and incorporation of all three diphenols. The change in the uptake rate of the wild type after 10 min was significant. It was assumed that after 10 min the internal pools were saturated, and further accumulation was due to the phenoloxidase-catalyzed incorporation of dopa into the insoluble melanin rather than true, active transport. Two pieces of data supported this view: the internal pool of dopa measured by washing the cells with 10% trichloroacetic acid was in steady-state concentration of 0.1 nmol/ml (dry weight) after 10 min, and when a mutant lacking phenoloxidase was used (Rhodes, Polacheck, and Kwon-Chung, unpublished data), a plateau was reached at that time. Table 2 shows the results of phenoloxidase assays in various isolates. Both radiometric and colorimetric assays showed that 92t-1 and the three Mel^- Tem^+ progeny used for the virulence study lacked the enzyme. The isolate 92t-1 showed a negligible amount of the intermediate, 5,6-hydroxyindole, which had an absorbance maximum at 300 nm. The wild-type isolate, B-3501, showed (Table 2) a specific activity similar to that of the enzyme, as reported by Polacheck et al. (20).

Virulence. The temperature-sensitive isolates of Mel^+ Tem^- and Mel^- Tem^- types were completely avirulent under the conditions used here. All mice which received 5×10^5 or 5×10^6 cells remained healthy beyond 60 days. It was evident that the temperature-sensitive isolates did not survive in mouse organs since the culture of 92t-1 could not be recovered from the animals after 48 h. The temperature-tolerant isolates were of two kinds (Table 3). All Mel^+ Tem^+ types killed 100% of the mice inoculated with 5×10^6 cells within 30 days. With the inoculum of 5×10^5 cells, 80 to 100% of the mice were killed within 50 days. The Mel^- Tem^+ isolates, on the other hand, were avirulent, and all mice survived beyond 60 days. The surviving mice were all sacrificed on day 70. Figure 4 shows the

TABLE 1. Segregation pattern of mating type, melanin formation, and temperature sensitivity in F_1 progeny from a 92t-1 (**a** Mel^- Tem^-) × B-3501 (α Mel^+ Tem^+) cross

Marker	Ratio		P (χ^2)
	Observed	Expected	
α:**a**	79:81	80:80	>0.9
Mel^+:Mel^-	86:74	80:80	>0.4
Tem^+:Tem^-	91:69	80:80	0.08
α Mel^+:α Mel^-:**a** Mel^+:**a** Mel^-	41:38:45:36	40:40:40:40	>0.7
α Tem^+:α Tem^-:**a** Tem^+:**a** Tem^-	44:35:47:34	40:40:40:40	>0.5
Tem^+ Mel^+:Tem^+ Mel^-:Tem^- Mel^+:Tem^- Mel^-	64:27:22:47	40:40:40:40	<0.0001

results of the in vivo growth study using one each of the Mel^+ Tem^+ and Mel^- Tem^+ types.

The Mel^+ Tem^+ isolate, B-3501, showed exponential growth in the brain, whereas only moderate growth was seen in the spleen and liver (Fig. 4A). The Mel^- Tem^+ isolate, Sb8, showed no sign of multiplication in any organ (Fig. 4B). The cells of Sb8 disappeared from the brain within 4 days, although they survived in the liver and spleen for at least 2 to 3 weeks.

DISCUSSION

This study confirms the findings of Shaw and Kapica (28) that cryptococcal cells deposit melanin in the cell wall when grown on media containing diphenols. Our Mel^- isolates grown on L-dopa medium showed a hyaline wall in the unstained section, whereas the wild type had a dark layer in the cell wall. The darkness of the layer was accentuated in the sections stained with uranyl acetate.

Melanogenesis in *C. neoformans* is initiated by phenoloxidase (28). Unlike mushroom or human phenoloxidase (tyrosinase), the enzyme from *C. neoformans* oxidizes a wide range of *o*-diphenols but not tyrosine (20, 28). Polacheck and Kwon-Chung (unpublished data) found two relatively stable intermediates in the melanin synthesis by *C. neoformans*, and they were identified as dopachrome and 5,6-hydroxyindoquinone. These findings show that the pigment synthesized by *C. neoformans* growing on diphenolic media is a melanin of a sort commonly found in various plants and animals (16).

The Mel^- Tem^+ isolates studied here showed at least two lesions in melanogenesis in that they lacked the active transport system for diphenolic compounds as well as for phenoloxidase. These isolates were avirulent for mice, whereas their siblings with Mel^+ Tem^+ phenotypes retained virulence equivalent to that of the wild-type parent. There is no question that both Mel^+ and Tem^+ phenotypes are associated with virulence in *C. neoformans*, since Mel^- Tem^+ or Mel^+ Tem^- were equally avirulent for mice. It was clear that UV irradiation caused the Mel^- phe-

TABLE 2. Specific activity of phenoloxidase in the wild-type and mutants

Substrate	Isolate	Colorimetric assay[a]		Radiometric assay
		300 nm	480 nm	
L-Dopa				
	B-3501	13,100	3,640	651
	92t-1	11	<1	1.4
	Sb7	<1	<1	0.2
	Sb8	<1	<1	0.6
	Sb31	<1	<1	0.1
Norepinephrine				
	B-3501	26,620	8,220	634
	92t-1	123	<1	1.0
	Sb7	<1	<1	0.4
	Sb8	<1	<1	0.6
	Sb31	<1	<1	0.3

[a] Specific activity (enzyme units per milligram of protein).

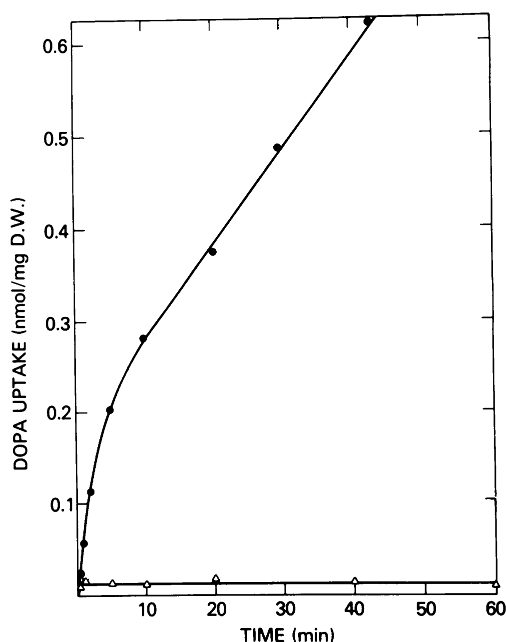

FIG. 3. Uptake of radiolabeled L-dopa by B-3501 (●) and 92t-1 (△).

TABLE 3. Virulence of the parental isolates and their temperature-resistant progeny

Cells	Cells/ mouse	% Dead[a]	Survival[b]
Parental isolate			
B-3501 (Mel^+ Tem^+)	5×10^6	100	17
	5×10^5	100	26
92t-1 (Mel^- Tem^-)	5×10^6	0	
	5×10^5	0	
Progeny			
Mel^+ Tem^+			
Sb2	5×10^6	100	12
	5×10^5	100	23
Sb58	5×10^6	100	27
	5×10^5	80	46
Sb45	5×10^6	100	14
	5×10^5	90	29
Mel^- Tem^+			
Sb7	5×10^6	0	
	5×10^5	0	
Sb8	5×10^6	0	
	5×10^5	0	
Sb31	5×10^6	0	
	5×10^5	0	

[a] Within 50 days after injection.
[b] Average day of death after injection.

notype but not the Tem^- phenotype in 92t-1, since the original five Mel^- mutants obtained by 15-s irradiation all grew at 37°C. Although the exact frequency of Tem^- cells was not determined, it appeared to be less than 1 in 10^3 Mel^- cells obtained by UV irradiation. Isolation of revertant Mel^+ Tem^+ from Mel^- Tem^- was attempted but failed, possibly because the Mel^- Tem^+ isolates carried multiple lesions in melanin synthesis. In our laboratory, Rhodes et al. (unpublished data) isolated a spontaneous Mel^- Tem^+ mutant which lacked phenoloxidase but had an active transport system for the diphenols. These mutants were also avirulent for mice. However, the isolation of revertants was readily obtained after repeated subculture on malt extract agar. The revertants recovered the wild-type virulence. These studies indicate that, regardless of whether the Mel^- phenotype is due to the deficiency in uptake of diphenols or phenoloxidase, the isolates lose virulence for mice.

Isolation of Mel^- strains from clinical specimens is extremely rare. We have not encountered any naturally occurring Mel^- types among the 1,200 *C. neoformans* clinical isolates thus far accumulated in our laboratory. Some isolates which were received as Mel^- from other laboratories were in fact all Mel^+ when the glucose level in niger seed medium was reduced to less than 0.5%. The lack of Mel^- types among clinical isolates provides indirect evidence that Mel^+ is associated with the pathogenicity of *C. neoformans*.

Strain 92t-1 and the three Mel^- Tem^+ progeny had a doubling time similar to that of the wild-type parent in YNB medium (2% glucose). In asparagine minimal medium, however, the growth rate was slower in the Mel^- type, and the doubling time was 2 h longer than that of the wild type. We have not determined the nutritional factors affecting the growth rate. It is unlikely for two reasons that the loss of virulence in these isolates is due to the slower growth rate. First,

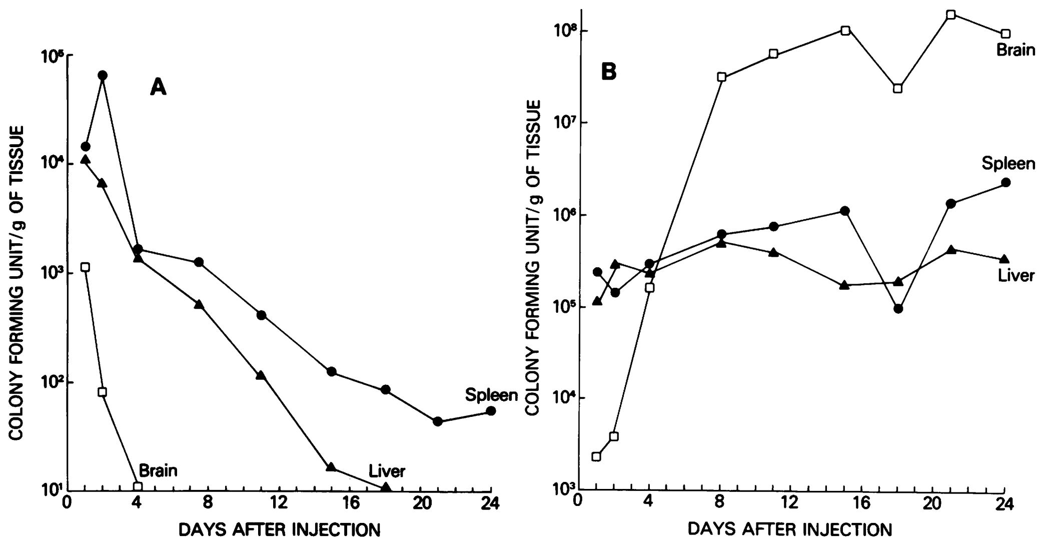

FIG. 4. Colony-forming units of (A) B-3501 (Mel^+ Tem^+) and (B) Sb8 (Mel^- Tem^+) in mouse organs. Original inoculum, 10^4 cells.

the host tissue is a complex medium almost certainly more complete than YNB medium in its nutritional make-up. Second, the in vivo growth curve definitely showed that the Mel^- types neither multiplied nor survived in the animal organs. The host defense system appeared to remove the mutant cells effectively while allowing the wild-type cells to grow profusely in the brain.

The genes responsible for Mel, Tem, and mating types segregated independently. Although 92t-1 appeared deficient in both diphenol uptake and phenoloxidase activity, the ratio of Mel^+ and Mel^- among 160 progeny obtained from B-3501 × 92t-1 was 1:1. One simple explanation for this phenomenon is that the locus for Mel^- may be due to the loss in a gene controlling both uptake of diphenols and synthesis of phenoloxidase. Alternatively, the loci for the uptake of diphenols and the synthesis of phenoloxidase may be tightly linked, or lack of active transport may not be sufficient for the Mel^- phenotype. Bowman and Ahearn (3) noticed that melanin production in **a** mating type isolates of *Filobasidiella neoformans (C. neoformans)* was delayed or weak compared with α isolates. We observed that B-3502 (**a** type) produced less intense pigment and a much larger extracellular capsule than B-3501 (α type). The color was usually more intense in the isolates which produced small capsules. Capsule formation and mating type have been shown in a previous study (13) to be unrelated.

Although the role of phenoloxidase or melanin in virulence is unknown, several hypotheses may be suggested. The melanin deposited in the cell wall may act as a shield against the host defense mechanisms. There is no known enzyme which hydrolyzes melanin or any antibody which recognizes this melanin (2). It has been shown that fungi with melanin on the wall survive microbial or enzymatic lysis better than those lacking melanin (2, 12, 23). *C. neoformans* grows preferentially in the brain, and the brain is rich in the catecholamines which serve as substrates for the phenoloxidase (1). Although *C. neoformans* cells grown in brain tissue are never black or dark, they may contain some faint pigment which is not easily detectable. In the rat brain, the concentration of catecholamines is reported to be 1.04 μg/g of tissue (1). When we grew *C. neoformans* on a medium containing 1.04 μg of dopamine or norepinephrine per ml, the colonies became light tan, whereas the colonies on the control medium were white after 3 to 5 days of incubation. One other explanation may be that the substrates for phenoloxidase enhance the growth of *C. neoformans* or the formation of extracellular capsule in the brain. In vitro, the addition of L-dopa or norepinephrine to a culture medium promotes neither the growth nor the formation of capsule. However, in vivo growth conditions are entirely different, and the situation cannot be directly compared with in vitro conditions.

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