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Relationship of Virulence Factor Expression to Evolved Virulence in Mouse-Passaged *Cryptococcus neoformans* Lines

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Serial passage of *Cryptococcus neoformans* in mice increases virulence relative to the nonpassaged line. Postpassaged lines showed no difference in the expression of most known virulence factors, with the exception that the more virulent lines had smaller capsules in vitro. These data imply that other mechanisms of virulence remain to be discovered.

One approach to study the evolution of virulence experimentally is to serially passage a microbe in a host (8) and then compare the pre- and postpassaged lines to identify traits that affect changes in virulence. The yeast *Cryptococcus neoformans* is commonly found in the environment and causes disease primarily in immunocompromised humans. Its best-recognized virulence factors are the capsule (1, 14), melanin production (15, 25), laccase (17, 22), phospholipase (5), urease (6), and growth rate (18). Passage of *C. neoformans* in B10 major histocompatibility complex-congenic mice and BALB/c mice increased virulence relative to the nonpassaged line, using time to death as the measure of virulence (19). These observations prompted this study to investigate the mechanism responsible for the virulence increase.

The *C. neoformans* lines used in this study were derived by serial passage in mice and are described in detail in reference 19. Capsule size in vitro was measured as described in reference 26. Capsule size in vivo was measured from frozen brain and liver homogenates scraped into a microcentrifuge tube, washed once with 100 μl phosphate-buffered saline, resuspended in 10 μl phosphate-buffered saline, and measured as described in reference 26. To determine if the lines differed in their ability to release capsular glucuronoxylomannan (GXM) into the medium, capsules were induced in DME (as for measuring capsule size) and the concentration of GXM in the supernatant was measured the next day by capture enzyme-linked immunosorbent assay as described previously (2).

Melanization was assessed qualitatively by colony color on L-dopa plates after incubation at 30°C for 3, 5, and 7 days. The amount of color produced was scored based on colony photographs using a 0 to 5 scale, with 0 being white and 5 corresponding to black. Laccase activity was measured as described previously (13). Extracellular phospholipase activity was determined as described previously (9). Urease activity was determined as described previously (16).

All lines were grown in yeast-peptone-dextrose medium at 37°C overnight and then diluted in 10 ml yeast-peptone-dextrose medium and the growth rate measured as described previously (10) from CFU and turbidity. CFU were measured three times (every 4 h). The doubling time was calculated using the following formula: time × {0.693/[ln(final OD/initial OD)]}, in which OD is the optical density.

The phagocytic efficacy of the macrophage-like cell line J774.16 was measured by the method used in reference 24, with minor modifications. Macrophage killing was measured by the method used in reference 12, with modifications. Briefly, J774.16 cells were opsonized with either 10% guinea pig serum or 10 μg/ml of the monoclonal antibody 18B7. Postpassaged lines and macrophages were incubated in a 1:1 ratio for 4- and 18-h intervals, and viability was assessed by trypan blue exclusion.

A logistic correlation was used to test if virulence factors were correlated with time to death (virulence) during the last passage. A standard least-squares test with simple contrasts was used to test for significant differences between nonpassaged and postpassaged *C. neoformans* macrophage killing.

Surprisingly, there was no correlation between time to death and the following line characteristics: capsule GXM release in vitro, secreted extracellular laccase, extracellular phospholipase secretion, urease activity, growth rates, and in vitro phagocytosis with mouse intraperitoneal macrophages (Table 1). There was also no significant difference in macrophage killing between the nonpassaged H99 and any postpassaged line (Table 1). Furthermore, there was no correlation between the amount of melanization and the time to death (Table 1). Eight of the 14 *C. neoformans* passaged lines produced some melanin by day 7, but only 5 of those 8 produced a significant amount of melanin by day 7 (Table 1). There was no correlation between capsule size in the brain or liver of moribund mice and passage time to death (Table 1). However, a negative correlation between capsule size in vitro and passage time to death (virulence) was found ($P = 0.0347$, $R^2 = 0.28$) (Table 1). All but one (F_1) of the passaged *C. neoformans* lines produced a capsule smaller than the capsule of the nonpassaged H99 (Fig. 1) in vitro.

There was a statistically significant negative correlation between increased virulence (decreased time to death) and smaller capsule size in vitro ($P = 0.0347$) that is opposite that of conventional views on capsule size and virulence. This observation, combined with the finding that mice infected with

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TABLE 1. Correlation analysis for virulence factors and *C. neoformans* strains

C. neoformans strain or parameter[a]	Time to death (days)	Ordinal melanin[b] (day 7)	Laccase (OD, 420 nm)	Capsule size (μm^3)			Doubling time (h)	Phospholipase index[c]	Urease (OD, 560 nm)	Phagocytosis index in J774.16 cells[d]	Macrophage killing[e]	GXM released in vitro (μg/ml)
				In vitro	Liver	Brain						
D1	41	4	0.2	870	NA	NA	2.6	0.5	0.1	1.1	2.3	5.1
H99	39	0	0.2	1,200	NA	NA	3.0	0.6	0.2	0.9	9.0	1.9
F1	39	4	0.2	1,900	390	360	3.2	0.6	0.1	0.7	2.8	8.1
B1	32	2	0.2	270	760	1,200	3.7	0.6	0.1	1.5	13	26
K2	31	2	0.3	520	1,600	660	3.1	0.6	0.1	1.1	1.0	11
F2	26	0	0.2	630	320	320	3.2	0.5	0.2	0.8	5.3	15
B2	25	0	0.2	680	620	780	3.2	0.6	0.1	1.3	18	10
K1	24	3	0.2	850	1,300	2,200	2.8	0.7	0.2	1.0	1.0	11
Q2	22	0	0.2	380	440	560	3.0	0.5	0.0	1.4	2.0	0.1
Balb1	15	3	0.3	240	630	640	3.2	0.6	0.0	1.1	1.8	1.7
Alt d/q	14	0	0.3	350	360	460	3.6	0.6	0.2	0.6	2.8	46
Q1	13	2	0.2	660	1,300	1,200	3.4	0.6	0.1	1.1	1.5	6.0
D2	7	0	0.2	750	1,100	1,400	2.6	0.6	0.1	1.3	3.5	16
Balb2	7	2	0.2	250	220	730	3.3	0.6	0.2	0.8	4.3	3.8
Alt het	6	0	0.2	430	970	840	3.3	0.6	0.1	0.7	2.5	12
Control strains												
H99D	14	0	0.2	1,000	1,600	980	2.9	0.5	0.1	1.2	11	26
H99E	NA[f]	2	0.3	2,700	NA	NA	2.4	0.6	0.1	2.9	19	2.0
24067	NA	3	0.2	1,300	NA	NA	2.7	1.0	0.0	3.1	19	4.1
R^2 with time to death		0.17	0.01	**0.28**[g]	0.01	0.03	0.03	0.03	0.04	0.01	0.02	0.04
P value		0.11	0.80	**0.03**	0.81	0.58	0.52	0.53	0.48	0.67	0.57	0.45

[a] Strain names reflect the major histocompatibility complex genotypes or strains of mice in which *C. neoformans* was passaged (19). Strains H99Duke (H99D), H99Einstein (H99E), and 24067 are controls. AH d/q indicates *C. neoformans* passaged in alternating d/d and g/g MHC genotypes. Alt het indicates *C. neoformans* passaged in alternating heterozygote MHC genotypes. H99 is the nonpassaged *C. neoformans* strain.

[b] 0, no color; 1, absence or presence of color; 2, some color; 3, moderate amount of color; 4, a lot of color.

[c] Ratio of the colony diameter to the diameter of the colony plus the precipitation zone.

[d] Ratio of macrophages associated with *C. neoformans* to the total number of macrophages counted.

[e] Number of dead macrophages at 18 h.

[f] NA, not available.

[g] Boldface indicates significant correlation.

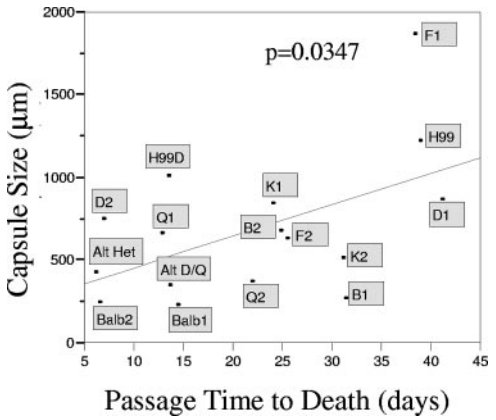

FIG. 1. Correlation between capsule size and passage time to death. Points are the average in vitro capsule size of each line plotted against passage time to death. Strain names indicate the MHC genotype or strain of mice in which *C. neoformans* was passaged (19).

passaged lines had higher GXM serum levels (data not shown), suggests that increased virulence was due either to more in vivo capsule shedding or to an increased growth rate in vivo. Since soluble capsular polysaccharide can mediate many deleterious effects on the immune system, including alteration in cytokine regulation (21, 23), interference with leukocyte migration (7), and apoptosis (4), the finding of smaller capsules and increased serum polysaccharide suggests a potential explanation in addition to increased growth rate in vivo, for the increased virulence of the highly virulent mouse-passaged lines.

Growth rate/doubling time was tested in vitro in different media, but no correlation with time to death was found. We did not measure growth rate in vivo because that would require separating differences in replication rate from changes in tissue burden as a result of clearance by immune cells. Since increased growth rate in vivo may still be a possible mechanism for the increased virulence seen in these mouse-passaged lines of *C. neoformans*, we cannot exclude this mechanism.

From a microbe-centric perspective, there are several potential explanations for these observations. First, the virulence factors that remained unchanged in postpassage lines may function in a qualitative manner such that only their presence is required for virulence. The fact that quantitative differences in virulence factor expression have not been associated with virulence in *C. neoformans* supports this view. Second, mouse passage may not affect essential fungal characteristics that arose in the environment and function as virulence factors in mammals. In this regard, we note that the capsule of *C. neoformans* seldom elicits high-titer or protective antibody responses in natural infections (3, 20), and consequently, this trait may not be under strong immune selection pressure. Similarly, murine infection does not elicit antibodies to laccase (11). Third, line adaptation to survival in mice may involve selection for other virulence factors.

In conclusion, mouse passage can increase the virulence of *C. neoformans* without selecting for significant differences in many well-characterized virulence factors. The increased virulence seen in the highly virulent lines may be due to a combination of increased growth rate in vivo, increased serum capsular polysaccharide, or changes in undiscovered virulence factors.

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