Most Environmental Isolates of *Cryptococcus neoformans* var. *grubii* (Serotype A) Are Not Lethal for Mice[▽]

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Most cases of cryptococcosis are caused by *Cryptococcus neoformans* var. *grubii* (serotype A), which is widespread in the environment, where it is primarily associated with pigeon excreta. A number of molecular epidemiological studies indicate that many environmental and clinical isolates of serotype A are indistinguishable. However, the murine virulence of environmental strains of *C. neoformans* has not been thoroughly evaluated. We used the murine intranasal model of cryptococcosis to compare the lethality of clinical and environmental strains of serotype A that possessed identical genotypes as determined by amplified fragment length polymorphisms (AFLP) and multilocus sequence typing (MLST). Eleven environmental strains were tested, and only one caused disease within 60 days postinfection, at which time the experiments were terminated. Conversely, 7 of 10 clinical isolates were lethal for mice at median times of 19 to 40 days. Passing environmental isolates in mice (up to three times) did not significantly increase their lethality. In follow-up studies, we developed a new genotyping technique based on hybridization with TCN2 and TCN4 retrotransposon-specific probes. Although the retrotransposon banding patterns were unstable after prolonged incubation in the laboratory, this method was able to differentiate clinical and environmental strains that had the same AFLP/MLST genotypes.

Cryptococcus neoformans var. *grubii* (serotype A) is a ubiquitous saprobic yeast. In nature, it can be routinely isolated from avian, especially pigeon, habitats, soil contaminated with avian excreta, and decayed wood (10, 18, 20, 25, 35, 36). Mammalian infection with *C. neoformans* is acquired by the inhalation of airborne yeasts or spores. The ensuing pulmonary infection is often asymptomatic. Alternatively, this exposure may lead to subacute, invasive disease. The yeast cells may disseminate to any part of the body, but *C. neoformans* is neurotropic, and the typical, life-threatening clinical manifestation of cryptococcosis is meningoencephalitis. Cryptococcal disease occurs predominantly in people with impaired immunity, but individuals with apparently normal host defenses may also develop grave infections (5). With rare exceptions, cryptococcosis is not transmissible among humans or other animals (5).

Although cryptococcosis can be caused by other species of *Cryptococcus* or any of the three serotypes of *C. neoformans* (A, D, or AD), most clinical and veterinary cases worldwide are caused by isolates of serotype A, which is also the most prevalent serotype among environmental samples. Genotypic analyses of isolates of serotype A have identified three genetically isolated subpopulations, designated VNI, VNII, and VNB (26–28). Representative isolates of all three populations have been cultured from patients and the environment. However, VNI strains of serotype A are globally dominant in patients, veterinary cases, and the environment. For example, using amplified fragment length polymorphisms (AFLP) and multilocus sequence typing (MLST), we genotyped numerous environmental VNI isolates from North America and found

identical genotypes in clinical and environmental samples (25, 28). In contradistinction, strains of VNII are much less common in patients, and they are exceptionally rare in the environment (3, 28, 31). Regarding the VNB population, both clinical and environmental isolates appear to be confined to southern Africa (28).

Since VNI strains with identical genotypes can be isolated from clinical specimens and environmental samples, it is widely accepted that fully virulent strains are present in the environment (5, 6, 21). However, few studies have experimentally tested the pathogenicity of environmental isolates of *C. neoformans*, and none have compared the virulence of clinical and environmental isolates with the same genotypes. In 2006, Silva et al. compared the virulence of 62 strains of serotype A that were isolated from pigeon excreta in Brazil (39). Using an intravenous mouse model of experimental cryptococcosis, they observed that the environmental strains varied in their abilities to cause disease; some mice developed symptoms of cryptococcosis, but others remained asymptomatic (39). In 1989, Fromtling et al. evaluated the murine virulence of environmental and clinical isolates of *C. neoformans* from Puerto Rico (14). They also used an intravenous model of infection, and they determined that the mean lethal dose resulting in the death of 50% of the infected mice was significantly higher for environmental strains than clinical strains (14). In the next-most-recent report of the virulence of environmental strains, published in 1963, the authors compared 21 clinical isolates of *C. neoformans* with 47 isolates from soil contaminated with pigeon feces (19). The isolates were tested for virulence in a murine intracerebral model of cryptococcosis, in which suspensions of the yeast cells were injected directly into the cerebra of the mice. They concluded that “the strains isolated from [human] cases of cryptococcosis were more virulent than the soil strains; however, almost half [$<50\%$] of the soil strains dem-

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TABLE 1. Strains of *C. neoformans* used in this study^a

Strain	Origin	Molecular type	Genotype	Underlying condition	Median time of death ^b	CFU/brain	CFU/lung
A1-35-8	NC, pigeon excreta	VNI	A1/M1		>60	4	2.5 × 10 ³
A1-38-2	NC, pigeon excreta	VNI	A1/M1		>60	1	1.3 × 10 ³
A1-84-14	CA, pigeon excreta	VNI	A1/M1		34	6.9 × 10 ⁴	3.2 × 10 ⁵
A1-19-11	NC, pigeon excreta	VNI	A1/M1		>60	NP	NP
A1-46-3	NC, pigeon excreta	VNI	A1/M1		>60	NP	NP
A7-35-23	NC, pigeon excreta	VNII	VNII ^c		>60	15	1.1 × 10 ⁴
A2-38-23	NC, pigeon excreta	VNI	A2/M2		>60	1	2.8 × 10 ³
A2-19-13	NC, pigeon excreta	VNI	A2/M2		>60	0.5	84
A2-102-5	NC, pigeon excreta	VNI	A2/M2		>60	NP	NP
A4-34-6	NC, pigeon excreta	VNI	A4/M4		>60	7	3.1 × 10 ³
A5-35-17	NC, pigeon excreta	VNI	A5/M5		>60	1.2 × 10 ^{3d}	1.0 × 10 ⁵
C23	BAL	VNI	A1/M1	Lung transplant	23	2.7 × 10 ⁵	3.6 × 10 ⁵
C11	BAL	VNI	A1/M1	Sarcoma	30	NP	NP
C22	Lung biopsy	VNI	A1/M1	Empyema	>60	NP	NP
C54	CSF	VNI	A1/M1	HIV positive	>60	NP	NP
C36	CSF	VNI	A1/M1	Lymphoma	28	NP	NP
C27	CSF	VNI	A4/M4	Leukemia	40	5.7 × 10 ⁵	5.6 × 10 ⁶
C8	CSF	VNI	A5/M5	Leukemia	24	8.3 × 10 ⁵	1.0 × 10 ⁷
C12	Lung biopsy	VNII	VNII	Renal transplant	>60	1.3 × 10 ³	2.3 × 10 ⁵
C44	CSF	VNII	VNII	Renal transplant	19	1.4 × 10 ⁵	3.0 × 10 ⁸
C45	CSF	VNII	VNII	Asthma	21	NP	NP
H99	CSF	VNI	A1/M1	HIV positive	23	1.4 × 10 ⁴	5.6 × 10 ⁶

^a NC, North Carolina; CA, California; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage fluid; HIV, human immunodeficiency virus; NP, not performed. All strains have the α mating type allele.

^b Number of days after which 50% of animals exhibited $\geq 20\%$ weight loss and were sacrificed. Bold denotes lethal strains.

^c VNII group contains strains with several closely related AFLP/MLST genotypes; the nomenclature has not been fully resolved.

^d Unusually high tissue burden in a single mouse that developed cryptococcosis on day 42 resulted in the skewed results for the A5-35-17 strain. When dissected, this mouse had 6,083 CFU/brain and 1.6×10^6 CFU/lung, whereas the remaining animals had an average of <2 CFU/brain and 4.3×10^4 CFU/lung (Fig. 3).

onstrated virulence within the range shown by the isolates from human disease..." (19). The murine virulence of *C. neoformans* was also studied in the 1950s (11, 12, 23). However, at that time, selective media for the in vitro isolation of *C. neoformans* from the environment were not available, and in these reports, mice were used for the primary isolation of environmental strains by injecting suspensions of pigeon guano into mice and, after several weeks, culturing their livers and spleens for *C. neoformans*. Obviously, the environmental strains they recovered were preselected for their ability to cause infections. Here, we selected clinical and environmental strains of serotype A with identical AFLP and MLST genotypes and compared their pathogenicities in mice to determine whether there was any correlation between genotype and virulence for mice.

MATERIALS AND METHODS

Strains and media. The 22 MAT α strains of *C. neoformans* serotype A subpopulation VNI or VNII used are listed in Table 1. Ten strains were isolated from the environment in North Carolina and one from California (25), 10 clinical strains were isolated from patients at Duke Hospital between 2000 and 2002 (25), and the virulent strain H99 was included as a control (9). Environmental strains were isolated from dried pigeon excreta as described previously (25). Briefly, samples of pigeon excreta were suspended in sterile water; mixed; serially diluted; plated on Staib agar (40) supplemented with 0.2 g/liter chloramphenicol (Sigma-Aldrich, St. Louis, MO), 0.025 g/liter gentamicin (EM Science, Gibbstown, NJ), and 0.1 g/liter (0.1 g/10 ml 95% ethanol) biphenyl (Alfa Aesar, Ward Hill, MA); and incubated at either 30° or 35°C for 3 to 5 days. Brown yeast colonies were selected, grown in pure culture on Staib plates without antibiotics, and confirmed to be *C. neoformans* by standard morphological criteria, serotyping with commercial monoclonal antibodies (Iatron, Tokyo, Japan), and molecular genotyping (28). All strains were maintained at -80°C . For the experimental infections, strains were cultured on yeast extract-peptone-dextrose (YPD) agar medium, colony purified, and grown at 37°C overnight in yeast nitrogen base broth supplemented with 1% (wt/vol) glucose. Under sterile conditions, yeast

cells were washed three times with phosphate-buffered saline (PBS), enumerated by hemocytometer counts, and resuspended to the appropriate concentrations in PBS.

AFLP and MLST strain typing. An analysis of AFLP markers was performed as previously reported (26). An MLST analysis was also performed as described, and the following 12 unlinked genetic loci were analyzed: *CAP10*, *CAP59*, *IGS1*, *GPD1*, *LAC1*, *MPD1*, *MP88*, *SOD1*, *PLB1*, *TEF1*, *TOP1*, and *URE1* (28). The following criteria were used to select these MLST loci: (i) the primer-binding sites were designed to be situated within protein-coding sequences to maximize the number of strains for which a particular locus can be PCR amplified; (ii) each MLST locus contained variable noncoding DNA regions, such as introns or intergenic regions, to ensure the likelihood of finding substantial polymorphisms among strains; and (iii) the MLST loci are physically unlinked, either on different chromosomes or separated by at least 100 kb (28).

Murine virulence studies. Mice were infected intranasally as described previously (9, 33). Briefly, groups of eight-week-old inbred male BALB/c mice (5 or 10 mice per group) were individually anesthetized by an intraperitoneal injection of pentobarbital. The unconscious mice were suspended on a silk thread by their maxillary incisors to ensure that their necks were fully extended. The mice were then infected intranasally with 5×10^5 yeast cells in 50 μl PBS, which was pipetted slowly into the nares and inhaled by the mice. The viable census of yeast cells in the inoculum was confirmed by plating serial dilutions of the inoculum. Mice were monitored and weighed daily, and those that exhibited $\geq 20\%$ weight loss were sacrificed by CO₂ inhalation and necropsied. The statistical significance of the survival data was assessed by the Mantel-Cox log rank test. Murine experiments were approved by the Duke University Institutional Animal Care and Use Committee protocol A082-07-03.

Quantitative analysis of tissue census of *C. neoformans*. Quantitative assessments of the tissue burdens were performed for the representative strains of each genotype with lethal and nonlethal phenotypes (Table 1). Mice that developed severe signs of cryptococcosis ($\geq 20\%$ weight loss) were sacrificed and dissected. Mice that did not develop any signs of infection were kept for 60 days and then sacrificed and dissected. The brain and lungs of each mouse were removed and homogenized in a biosafety cabinet under sterile conditions using a tissue homogenizer; serial dilutions were prepared in sterile PBS and plated on YPD agar medium supplemented with 100 $\mu\text{g/ml}$ chloramphenicol and incubated at 30°C for several days. Colonies of *C. neoformans* were counted, and the total number

FIG. 1. Genetic relationships among strains of serotype A based on 12 MLST markers (6,835 bp) (28) visualized by the neighbor-joining dendrogram. Three molecular types (VNI, VNI I, and VNB) and four AFLP/MLST genotypes (A1, A2, A4, and A5) are indicated. Vertical lines represent strains with identical genotypes. Strains used in the virulence experiments are colored: blue represents clinical strains and red environmental strains. The topology of the VNB clade has been truncated.

of CFU per organ was calculated. Wilcoxon rank sum analysis was used to determine the significance of the CFU per organ data.

Repeated passage in mice. Groups of five mice each were infected with strains A1-38-2 and A7-35-23 as described above. These two environmental strains represent the two major molecular types, VNI and VNI I; in addition, the A1/M1 genotype of strain A1-38-2 is the most common genotype and frequently isolated from the environment and patients. At 60 days postinfection, the mice, which were completely healthy in appearance and behavior, were sacrificed by CO₂ inhalation. The lungs were removed aseptically, homogenized with a tissue homogenizer, and cultured on YPD plates at 37°C. Single colonies were selected and frozen at -80°C. For subsequent infections, frozen cells were thawed, grown in YPD broth, washed, and enumerated as described. Groups of five mice each were inoculated with the passaged strains and monitored for 60 days. At day 60, lungs were dissected, single colonies of *C. neoformans* were purified and propagated in YPD medium, and new groups of animals were infected. Experimental infections with passaged strains were performed three consecutive times.

Southern hybridization with probes specific for retrotransposons TCN2 and TCN4. Approximately 400-bp fragments of the TCN2 and TCN4 elements containing putative reverse transcriptase were amplified by PCR and labeled using a PCR DIG probe synthesis kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The following PCR primers were used: TCN2-f, 5'-TGTTTCTCATGCCGCGTTGT; TCN2-r, 5'-TACGACTGGACCC GCCTGAT; TCN4-f, 5'-CAGCCTTCGGCGATAACCAC; and TCN4-r, 5'-AC GAGACGGTGTGGCCATT. Samples of approximately 10 ng genomic DNA were digested with two restriction endonucleases, HindIII and PstI, and electrophoresed in 0.8% agarose in 0.5× Tris-borate-EDTA buffer at 30 V for 48 h. Using routine methods, DNA fragments were transferred by capillary action from the gel to a positively charged nylon membrane (Roche Diagnostics). Southern blot hybridizations were performed according to the conditions rec-

ommended by the probe manufacturer (Roche). The blots were hybridized and washed under high-stringency conditions (65°C).

RESULTS

Environmental strains are less virulent than clinical strains in mice. We compared the murine pathogenicity of 11 environmental and 10 clinical strains of serotype A. As indicated in Table 1 and Fig. 1, these strains represent two of the three major subpopulations of serotype A, VNI and VNI I, and they are designated according to their source ("A" for environmental and "C" for clinical) as well as their AFLP and MLST genotypes (abbreviated A1/M1, A2/M2, etc.). The selected strains represent four phylogenetic clades of VNI (A1/M1, A2/M2, A4/M4, and A5/M5) and two closely related VNI I genotypes. Table 1 also compares the lethality of each strain or the median times to death or sacrifice of the mice, as well as the CFU per brain or lungs at the time of death. These data have demonstrated that clinical and environmental isolates of serotype A vary greatly in their ability to cause disease in mice that were challenged by a route that simulates the natural history of cryptococcosis. Unlike clinical isolates, most environmental strains are nonpathogenic and nonlethal over 60 days. Eleven environmental strains were tested, and only one,

FIG. 2. Survival of mice infected with environmental and clinical strains of *C. neoformans*. Blue lines represent clinical strains and red lines environmental strains. The virulent control strain, H99, is shown in black. *P* values reflect the probabilities of rejecting the null hypothesis of no difference in survival times among strains. Groups of five mice were challenged intranasally with 5×10^5 CFU. The body weight of each mouse was monitored for 60 days, and mice with a weight loss of $\geq 20\%$ were sacrificed. The experimental strains of each genotype were selected randomly from our large collection of environmental and clinical isolates. (A) Mice were challenged with A1/M1 strains; (B) mice were challenged with A2/M2 strains (no clinical strains with A2/M2 genotype have been found); (C) mice were challenged with A4/M4 strains; (D) mice were challenged with A5/M5 strains; (E) mice were challenged with strains of VNII molecular type; (F) combined survival data for the environmental (red) and clinical (blue) strains with different genotypes.

A1-84-14, caused disease within 60 days postinfection. Conversely, 10 clinical isolates were tested: 3 (C12, C22, and C54) were nonpathogenic through 60 days, and 7 strains were lethal for mice within a median time of 19 to 40 days (Table 1). Our results are consistent with those of Clancy et al. (7), who found a wide range in the murine virulences of 18 isolates of *C. neoformans* from AIDS patients. They also confirm the report of Fromtling et al. (14), who reported that environmental isolates were less virulent than clinical strains in intravenously infected mice.

Environmental and clinical strains with identical AFLP/MLST genotypes differ in murine virulence. We selected seven pairs of environmental and clinical strains (14 strains total) with identical or closely related AFLP/MLST genotypes and representing five diverse phylogenetic clades: A1/M1, A2/M2, A4/M4, A5/M5, and VNII (Fig. 1) (28). For example, environmental strain A1-38-2 has the same genotype as clinical strain C23, and environmental strain A5-35-17 is identical to the clinical strain C8 (Fig. 1). In vitro, all of these strains were capable of growing at 37°C and produced similar, medium-sized capsules and comparable amounts of melanin (data not shown).

The survival curves of BALB/c mice infected with the 14 strains of serotype A are shown in Fig. 2A to D. With the exception of strain A1-84-14, isolated from pigeon droppings in California, mice infected with environmental strains did not develop any symptoms of cryptococcosis and remained healthy for at least 60 days. Conversely, with the exception of strain C12, as well as C22 and C54 (data not shown), mice infected with clinical strains of *C. neoformans* exhibited severe weight

loss consistent with cryptococcosis and were sacrificed. There was considerable variation in the signs and symptoms of mice infected with different strains of *C. neoformans*. The clinical strains (C44, C8, C23, C45, and C11) were more lethal, and the mice died 19 to 30 days after the infection; median times are listed in Table 1. Mice infected with environmental strain A1-84-14 and a clinical strain C27 developed symptoms at a much lower rate with median times to death of 34 and 40 days, respectively (Table 1).

Viable yeast cells of *C. neoformans* were recovered from the lungs and brains of asymptomatic mice infected with the environmental strains, indicating that these mice acquired chronic or latent infections (Fig. 3). Approximately 10^3 to 10^5 CFU were isolated from the lungs, and many fewer viable yeasts were isolated from the brains of asymptomatic animals (<20 viable CFU/brain for most of the strains [Fig. 3B and Table 1]). (It is possible that the few yeasts recovered from brain tissue resulted from contamination with blood or other organs during necropsy; however, each deceased mouse was prewetted with disinfectant to minimize contamination by fur, and sterile instruments were used.) The census of yeasts in the target organs varied considerably with the strain of *C. neoformans*. For example, at day 60, mice infected with strain A2-19-13 only had a minuscule number of viable yeast cells in their lungs (84 CFU/lung [Table 1 and Fig. 3]) and almost no detectable yeasts in their brains (<1 CFU/brain [Table 1 and Fig. 3]), which suggests that they probably cleared the infection. Conversely, three of five mice infected with strain C12 contained $>1,000$ CFU/brain and $>10^5$ CFU/lung (Table 1 and Fig. 3), which indicates that they developed chronic, progressive infections

FIG. 3. Infection load in the lungs (A) and brains (B) of the mice infected with different strains of *C. neoformans*. Surviving mice were sacrificed on day 60 after inoculation; the lungs and brains were harvested, homogenized, serially diluted, and plated on YPD medium. Mice that succumbed before 60 days were sacrificed after a weight loss of $\geq 20\%$, and the lungs and brains were similarly harvested, homogenized, and plated on YPD medium. Total CFU in the brains and lungs were determined.

and would likely have developed more severe cryptococcosis beyond 60 days.

Isolates with identical A1/M1 genotypes differ in murine virulence. To further compare the pathogenicity of *C. neoformans* strains with identical genotypes, we infected groups of mice with 10 clinical and environmental strains that have the same A1/M1 (i.e., AFLP and MLST) profiles (Fig. 1). Five of

these strains were isolated from pigeon excreta (viz., A1-46-3, A1-19-11, A1-35-8, A1-38-2, and A1-84-14), the remaining five strains were isolated from patients at Duke Hospital (C11, C23, C22, C54, and C36), and the H99 strain was included as a positive control (Table 1). Survival curves of BALB/c mice infected with these A1/M1 strains are presented in Fig. 4. All strains segregated into the following two distinct phenotypic groups: (i) the pathogenic group, including three clinical strains (C23, C11, and C36) and an environmental strain from California (A1-84-14), of which the infected mice died within 50 days after infection, and (ii) the nonpathogenic group, consisting of four environmental strains (A1-46-3, A1-19-11, A1-35-8, and A1-38-2) and two clinical strains (C22 and C54), of which most of the infected mice remained healthy at 60 days.

Repeated passaging in mice. It has been suggested that prolonged incubation in the host may enhance the virulence of *C. neoformans* (30). To test this hypothesis, two environmental strains, A1-38-2 and A7-35-23, were recovered from the lungs of asymptomatic mice at 60 days postinfection and used to infect new groups of mice. As shown in Fig. 5, with one exception, all the mice infected with the mouse-passaged yeast cells of strain A1-38-2 or A7-35-23 remained asymptomatic at 60 days. The exception was one mouse infected with A7-35-23 that developed severe weight loss (25%) and was sacrificed at day 48 postinfection. Yeast cells of *C. neoformans* were cultured from the brain of this mouse and used to infect a new group of mice. However, all of the mice infected with this isolate survived for 60 days without developing symptoms of cryptococcosis. These data suggest that propagation in the mammalian host does not enhance the virulence or acute lethality of *C. neoformans*. However, it is possible that 120 days of incubation in mice (two 60-day experiments) is insufficient time to select or acquire a more virulent phenotype. There is clinical evidence that cells of *C. neoformans* remain dormant in the lungs for years prior to the development of active cryptococcal disease (1). This change could be precipitated by an impairment of host defenses, such as infection with HIV, and/or a genetic change in the yeast cells that enhances virulence. More experiments are required to test these hypotheses.

Isolates with identical A1/M1 genotypes can be differentiated by retrotransposon-specific probes. The results presented

FIG. 4. (A) Survival of mice infected with 11 strains of *C. neoformans* with the A1/M1 genotype, including the H99 control. Groups of 10 mice were challenged intranasally with 5×10^5 CFU. The weights of the infected mice were monitored for 60 days, and animals with a weight loss of $\geq 20\%$ were sacrificed. Survival curves for the A1-35-8, A1-84-14, and C23 strains represent independent experiments from those for which the results are shown in Fig. 2. *P* values reflect the probabilities of rejecting the null hypothesis of no difference in survival times among strains. (B) Combined survival data for the environmental (red) and clinical (blue) strains with the A1/M1 genotype.

FIG. 5. Repeated passaging in mice. Two environmental strains, A7-35-23 (A) and A1-38-2 (B), were isolated from the brains of mice that did not succumb to the infection and were reinoculated into naive mice. A1-38-2 and A7-35-23 represent strains obtained from the environment. A1-38-2-i and A7-35-23-i represent single passage; A1-38-2-ii and A7-35-23-ii represent two passages.

above demonstrate that strains with identical A1/M1 genotypes have dramatically different virulence phenotypes. Although both the AFLP and MLST methods of genotyping have high discriminatory power, they are clearly unable to distinguish strains of *C. neoformans* with phenotypic differences in murine pathogenicity. The genome of *C. neoformans* is replete with transposons and retrotransposons (17, 29). Probes specific to the TCN1 retrotransposon have been used to genotype *C. neoformans* and *C. gattii* as well as to differentiate strains of serotypes A and D (24). We used DNA probes developed against the TCN2 and TCN4 retrotransposable elements (17) to discriminate among closely related clinical and environmental strains. Genomic DNA was isolated from the same 10 strains with identical A1/M1 genotypes described above (Fig. 4), digested with restriction endonuclease and hybridized with either a TCN2 or TCN4 probe. As shown in Fig. 6A and B, hybridization with retrotransposon-specific probes differentiates among strains with the same AFLP/MLST genotype; however, there was no apparent correlation between the retrotransposon pattern and virulence (data not shown). In addition, the retrotransposon banding patterns were unstable after prolonged incubation in the laboratory (Fig. 6C). For this experiment, six separate colonies were selected, and all produced identical banding patterns (data not shown).

DISCUSSION

In several reports, the pathogenicities of strains of *C. neoformans* have been compared by observing experimental infections in mice following intravenous or intracerebral challenge (2, 7, 14, 19, 39). However, mammals develop cryptococcosis by inhaling infectious propagules from the environment, and the abilities to survive, proliferate as yeasts, and establish a nidus in the lungs are crucial for the successful development of cryptococcal infection. From the lungs, the yeasts may disseminate to many organs but preferentially to the central nervous system (4, 33). We hypothesized that the replication of this process in experimental hosts can be used to differentiate virulent and avirulent strains. Therefore, we used the intranasal method of establishing experimental cryptococcosis in mice (9) and evaluated the subsequent development of systemic symptoms, as well as infection of the lungs and central nervous system.

There may be several explanations for the relative avirulence of strains isolated from pigeon excreta compared to that of clinical strains. First, it is possible that pigeon excreta do not represent the major source of human isolates. The growth conditions in pigeon guano are dramatically different from the environment in the mammalian host. For example, pigeon excreta are rich in glucose and nitrogen (34), whereas the mammalian host provides limited nutrients (8, 13). Perhaps, humans acquire *C. neoformans* from a different ecological niche. For example, there is now abundant evidence that both varieties of *C. neoformans*, as well as *C. gattii*, are associated with decayed wood (18, 20, 36), which has a much lower concentration of nutrients than avian excreta. Consequently, arboreal isolates may be better adapted to survival under conditions of suboptimal nutrition and may represent a more plausible source of human infection. Alternatively, perhaps neither wood nor avian habitats, both of which are sites of enrichment for *C. neoformans*, represent its true environmental reservoir.

Second, it is possible that not all strains from pigeon excreta are equally capable of causing infection and that routine genotyping methods are not sensitive enough to differentiate among virulent and avirulent strains. Our data using TCN2/TCN4 hybridization patterns for genotyping (Fig. 6) demonstrate that this method distinguishes among individual strains with identical AFLP/MLST genotypes. Furthermore, every strain tested had a unique retrotransposon hybridization profile. However, the TCN2/TCN4 retrotransposon patterns were not stable over time, which diminishes their value as tools for molecular identification. Other probes or molecular methods, ranging from microsatellites to whole-genome approaches, may be necessary to identify virulent and avirulent strains (32).

Third, numerous studies have confirmed that mice are ideal hosts for acute experimental cryptococcosis. Mice are less expensive than larger laboratory mammals, and their genetics, specific immunological responses, and innate immunity are well defined. However, inbred strains of mice vary in their susceptibility (or resistance) to *C. neoformans* (4, 22, 33, 37, 41). Experimental cryptococcosis in rats is more subacute and reminiscent of human disease (16). Although our intranasal model of experimental murine cryptococcosis has proven to detect strain differences in virulence (9), it is possible that the

FIG. 6. Southern hybridization with probes specific to TCN2 (A) and TCN4 (B) retrotransposons. Polymorphic bands are indicated with arrows. Genomic DNA was isolated from 10 strains with identical A1/M1 genotypes, digested with restriction endonucleases, electrophoresed on agarose, transferred to a nylon membrane, and hybridized with the probes. (C) Changes in TCN4 retrotransposon binding pattern after prolonged incubation in culture. Strain A1-38-2 was subcultured in YPD broth for 40 consecutive days at 37°C, individual colonies were obtained, genomic DNA was isolated, and Southern blot hybridization with TCN4 probe was performed and compared with the TCN4 banding pattern from the original isolate stored at -80°C . The arrow indicates a change in the hybridization pattern that occurred after the propagation.

use of another animal strain or species, or immunocompromised mice, might have evinced a correlation between genotype and lethality.

Fourth, it is also possible that the strains deemed “non-pathogenic” because they failed to produce symptoms in mice within 60 days actually establish latent infections that could become life-threatening after a period of months or years. Although we have not observed any increase of virulence in strains passaged in murine lungs for 120 days, a longer incubation time may be necessary. Regardless, the recovery of

viable yeasts from the brains of healthy mice may portend a significant state of cerebral latency in the pathogenesis of cryptococcosis. Our protocol for the passing experiments involved the selection and purification of single colonies from primary streak plates of the brain tissue of surviving, infected mice for the subsequent reinfection of naive mice. We assumed that using individual cerebral isolates for passing experiments ensured that we tested cells that disseminated from the lungs, were adapted to in vivo cerebral growth conditions, and were genetically identical. Between isolation and reinfection, the passaged strains were subcultured and stored for a short period. It is possible that these in vitro manipulations resulted in a loss of putatively enhanced virulence.

Fifth, perhaps the observed differences in virulence between clinical and environmental strains are attributable to prior growth and acclimation of the clinical strains in mammalian hosts because the clinical strains were performed “passaged” through humans. There is serological and pathological evidence that healthy humans may carry latent asymptomatic infections (1, 15, 38). As our experiments showed that yeast cells were cultured from the lungs and brains of apparently asymptomatic mice (Table 1), the clinical strains may have propagated in human hosts for several years prior to their isolation, and this exposure enhanced their pathogenicity. If this hypothesis were proven to be the common natural history of infection with *C. neoformans*, then the anomaly may be that any environmental isolate is lethal on “first passage” in mice.

To our knowledge, these results reflect the first investigation of the virulence of molecularly typed natural isolates of *C. neoformans* using the natural route of infection. The data confirm observations by others that isolates of *C. neoformans* vary in their ability to cause disease in mice, and even genetically similar isolates demonstrate a wide range of pathobiological phenotypes. Overall, the data suggest that clinical isolates are more virulent than environmental strains and question the commonly accepted notion that “fully virulent strains are readily isolated from the environment” (21).

This investigation raises questions about the source of human cryptococcosis. The results also challenge the validity of experimental murine cryptococcosis, which is a common phenotype used to compare the pathogenicity of laboratory strains of *C. neoformans*. A third telling observation is that molecular genotypes, even those generated by robust 12-locus MLST markers, are not sufficiently discriminatory to differentiate strains with diametrical lethality for mice.

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