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Cryptococcus neoformans α Strains Preferentially Disseminate to the Central Nervous System during Coinfection

Kirsten Nielsen,[1,6] Gary M. Cox,[1,3] Anastasia P. Litvintseva,[1] Eleftherios Mylonakis,[9] Stephanie D. Malliaris,[7,8] Daniel K. Benjamin, Jr.,[4] Steven S. Giles,[2,3] Thomas G. Mitchell,[1] Arturo Casadevall,[7,8] John R. Perfect,[3] and Joseph Heitman[1,3,5,6]*

Departments of Molecular Genetics and Microbiology,[1] Cell Biology,[2] Medicine,[3] Pediatrics,[4] and Pharmacology and Cancer Biology,[5] Howard Hughes Medical Institute,[6] Duke University Medical Center, Durham, North Carolina 27710; Divisions of Infectious Diseases[7] and Microbiology and Immunology,[8] Albert Einstein College of Medicine, Bronx, New York 10461; and Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts 02114[9]

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***Cryptococcus neoformans* is a fungal pathogen that has evolved over the past 40 million years into three distinct varieties or sibling species (*gattii*, *grubii*, and *neoformans*). Each variety manifests differences in epidemiology and disease, and var. *grubii* strains are responsible for the vast majority of human disease. In previous studies, α strains were more virulent than congenic a strains in var. *neoformans*, whereas var. *grubii* congenic a and α strains exhibited equivalent levels of virulence. Here the role of mating type in the virulence of var. *grubii* was further characterized in a panel of model systems. Congenic var. *grubii* a and α strains had equivalent survival rates when cultured with amoebae, nematodes, and macrophages. No difference in virulence was observed between a and α congenic strains in multiple inbred-mouse genetic backgrounds, and there was no difference in accumulations in the central nervous system (CNS) late in infection. In contrast, during coinfections, a and α strains are equivalent in peripheral tissues but α cells have an enhanced predilection to penetrate the CNS. These studies reveal the first virulence difference between congenic a and α strains in the most common pathogenic variety and suggest an explanation for the prevalence of α strains in clinical isolates.**

Over the past decade fungal diseases have increased due to an expanding population of immunocompromised individuals. Rates of human immunodeficiency virus (HIV) infections are rising in many areas of the world, and the regions with the highest AIDS burden often have the least medical resources, resulting in large immunocompromised populations. At the end of 2002, it was estimated that 29 million people in Africa were living with HIV and that 3 million people died of AIDS-related illnesses during 2002 (76). This phenomenon, combined with the development of aggressive surgeries, organ transplantation, and immunosuppressive therapies, has led to an increase in the prevalence of invasive fungal diseases. Fungal diseases, such as oropharyngeal candidiasis and cryptococcal meningitis, are often AIDS-defining illnesses; aspergillosis has emerged as an important cause of mortality following bone marrow transplants; and *Candida* species are now a leading cause of bloodstream infections in hospitalized patients (24). As fungal diseases become more prevalent, understanding the microbial characteristics responsible for virulence becomes increasingly important.

The basidiomycete *Cryptococcus neoformans* is both an important pathogen and an excellent model system for fungal virulence (37). *C. neoformans* infects the central nervous system (CNS) to cause meningoencephalitis that is uniformly fatal if untreated (1). Cryptococcal meningitis occurs in 6 to 10% of AIDS patients in the United States and is the first AIDS-defining illness in 40% of these patients (19, 31, 52, 62). In sub-Saharan Africa, where highly active antiretroviral therapy is less common, *C. neoformans* accounts for 45% of all cases of meningitis in adults; many individuals present with cryptococcosis as their first AIDS-defining illness, and the mean survival rate after diagnosis can be as short as 4 days (29, 32, 35, 50). *Cryptococcus* occurs in three varieties or species that can be distinguished based on genetic and phenotypic differences. While these varieties are pathogenic in humans, they have different disease epidemiologies. *C. neoformans* var. *grubii* (serotype A) strains cause 95% of all *C. neoformans* infections and >99% of infections in AIDS patients in the United States (10). However, *C. neoformans* var. *neoformans* (serotype D) strains can account for up to 20% of clinical isolates in Europe (69). Unlike the first two varieties of *C. neoformans*, which cause disease predominantly in individuals with impaired immunity, strains of the sibling species—*C. gattii* (serotypes B and C)—can infect immunocompetent individuals (63).

C. neoformans is an important human pathogen and has also proven to be an outstanding model system for studying the virulence of fungi. *Cryptococcus* is predominantly haploid, which facilitates genetic analyses, and has a defined sexual cycle that enables classical genetic studies. Genes can readily be disrupted by biolistic transformation and homologous recombination, and essential genes have been examined using stable diploid strains, conditional alleles, and RNA interference (20, 37, 47, 49, 72). *C. neoformans* is a facultative intra-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, 322 CARL Building, Research Drive, Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-2824. Fax: (919) 684-5458. E-mail: heitm001@ duke.edu.

cellular pathogen that can also infect the amoeba *Acanthamoeba castellanii*, the nematode *Caenorhabditis elegans*, the slime mold *Dictyostelium discoideum*, and the insect *Drosophila melanogaster*, allowing identification of genes involved in virulence in heterologous hosts (2, 53, 64–66). Finally, robust animal model systems that enable detailed studies of virulence characteristics, such as the polysaccharide capsule and the antioxidant pigment melanin, have been developed.

Mating type has been proposed to be a virulence factor in *C. neoformans* based on disease epidemiology and animal studies. The majority of clinical isolates are of the α mating type (10, 41). Early experimental work on the role of mating type in virulence was conducted with serotype D var. *neoformans* because of the availability of congenic strains (42; reviewed in reference 34). In serotype D, a comparison of the levels of virulence of nonisogenic, unrelated **a** and α strains revealed that α strains are more virulent at lower inoculum levels than **a** strains (40, 78) and that the α strain JEC21 was more virulent than the congenic **a** strain JEC20 in a murine tail vein infection model of cryptococcosis as well as in the *C. elegans* system (42, 54). In serotype A var. *grubii*, mating type **a** strains are exceptionally rare and have only recently been identified (39, 44, 46, 55, 73, 74). As with serotype D, when the virulence of nonisogenic **a** and α strains was compared, the **a** strains were less virulent than the α strains (39, 44, 55). However, when congenic serotype A strains in the highly virulent H99 background were compared in standard mouse inhalation and rabbit meningitis models of cryptococcosis, no difference in virulence was measured (55).

Here we characterize in detail the role of mating type in pathogenicity by examining the virulence of serotype A var. *grubii* **a** and α congenic strains at various stages in the infective cycle—from their survival in potential environmental predators to their colonization of various organs in animal models—and found no difference between **a** and α strains. Infection by multiple strains has been reported, and studies examining sequential isolates have shown that different strains can be isolated from the same patient (8, 9, 33, 38, 58, 68). These findings suggest that coinfection with strains of opposite mating types may occur. We therefore analyzed the virulence of the congenic serotype A var. *grubii* strains during coinfection with both mating types and discovered that **a** and α congenic strains reached equivalent levels in the lungs and spleen but that a significantly higher proportion of α cells infected the brain. Intracerebral coinfections revealed that the strains have equivalent levels of persistence in the CNS. Thus, the α strain out-competes the **a** strain in entry into the CNS as a consequence of either fungal-fungal or fungal-host interactions.

MATERIALS AND METHODS

Strains and media. *C. neoformans* var. *grubii* strains H99 (70), KN99**a** (55), KN99α (55), KN99**a**NAT, and KN99αNAT were maintained at −80°C. Cultures were initiated by inoculating yeast extract-peptone-dextrose (YPD) solid medium and incubating the plates for 2 days at 30°C, followed by growth in YPD liquid medium at 30°C overnight. Cells were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended at the appropriate concentration in PBS. *Acanthamoeba castellanii* strain 30324 (ATCC) was maintained as previously described (51). *Caenorhabditis elegans* strain N2 Bristol was maintained at 15°C and propagated on *Escherichia coli* strain OP50 (53). The MH-S murine alveolar macrophage cell line (ATCC) was maintained in Dulbecco modified Eagle complete medium at 37°C with 5% CO_2 and transferred to fresh medium every 3 to 5 days.

AFLP. Amplified fragment length polymorphisms (AFLPs) were generated and analyzed as previously described (46). Two different EcoRI primer combinations were used for the selective PCR. Both primer sequences were labeled at their 5′ ends with 6-carboxyfluorescein (6FAM) but differed in the final two bases at the 3′ ends of the primers. The primer sequences were as follows: primer 1, 5′-6FAM-GACTGCGTACCAATTCAC-3′, and primer 2, 5′-6FAM-GACTGC GTACCAATTCTG-3′. Either primer 1 or primer 2 was combined with the MseI primer (5′-GATGAGTCCTGAGTAAG-3′) in the selective PCR. The AFLP reaction and analysis were performed on at least two separate occasions for each strain. Only intense and reproducible bands were scored to identify differences between strains.

Phenotype microarrays. Phenotype microarrays were done as described previously (80) and according to instructions from the manufacturer (Biolog, Hayward, CA) with the following exceptions. Strain cultures were grown overnight in YPD liquid medium; the cells were then collected by centrifugation, washed once with sterile distilled H_2O, resuspended in 12× yeast nitrogen base liquid medium supplemented with histidine, leucine, and uracil, and adjusted to 18% transmittance (T). IFY-0 was the inoculating fluid for all 20 96-well plate phenotype microarrays (PM1 to PM10 and PM21 to PM30). IFY-0 was supplemented with 5 mg/ml ammonium sulfate, 0.85 mg/ml potassium phosphate monobasic, 0.15 mg/ml potassium phosphate dibasic, and 0.5 mg/ml magnesium sulfate for PM1 and PM2; 20 mg/ml D-glucose, 0.85 mg/ml potassium phosphate monobasic, 0.15 mg/ml potassium phosphate dibasic, and 0.5 mg/ml magnesium sulfate for PM3 and PM6 to PM8; 20 mg/ml D-glucose and 1 mg/ml L-glutamate for PM4; or 20 mg/ml D-glucose, 5 mg/ml ammonium sulfate, 0.85 mg/ml potassium phosphate monobasic, 0.15 mg/ml potassium phosphate dibasic, and 0.5 mg/ml magnesium sulfate for PM9, PM10, and PM21 to PM30. Cells were combined with appropriately supplemented IFY-0 at a ratio of 1:11, and 100 μl of this mixture was added to each well. All phenotype microarrays were incubated at 30°C for 36 h in an OmniLog and monitored for color change in the wells every 15 min. Kinetic data were analyzed with OmniLog software using pairwise combinations.

Generation of KN99aNAT and KN99αNAT strains. Strains KN99**a**NAT and KN99αNAT were generated using overlap PCR by following the methods described in reference 27. The nourseothricin transgene (NAT) was inserted into the largest intergenic region in the corresponding MAT locus, where it would be anticipated to have the least effect on surrounding genes. For the serotype A MAT**a** locus (43), the NAT transgene was inserted in a 12-kb region between the *RPL39* and *PRT1* genes. For the serotype A MATα locus, the transgene was inserted in a 4-kb region between the *BSP1* and *RPL39* genes. PCR was used to generate the flanking 5′ (with KN99**a**, JOHE12926 and JOHE12927; with KN99α, JOHE12921 and JOHE12922) and 3′ (with KN99**a**, JOHE12928 and JOHE12929; with KN99α, JOHE12923 and JOHE12924) regions containing linkers to a NAT^r cassette. The NAT^r cassette containing the nourseothricin transgene was amplified using oligonucleotides JOHE8733 and JOHE8738. The products were combined in a PCR overlap reaction (with KN99**a**, JOHE12926 and JOHE12929; with KN99α, JOHE12921 and JOHE12924) to yield the NAT insertion allele. KN99**a** or KN99α was then transformed with the appropriate NAT insertion allele by biolistic transformation and selection for colonies resistant to nourseothricin (100 μg/ml). Transformants were identified by Southern blotting of KpnI-digested DNA using the 5′ fragment as a probe. Primer sequences were as follows: for JOHE8733, GCTGCGAGGATGTGAGCTGGA; for JOHE8738, GGTTTATCTGTATTAACACGG; for JOHE12921, GGTGA GTTGAGTGCGGCTATT; for JOHE12922, **AGCTCACATCCTCGCAGC**GCA TTTCAATTCTGTTAT (NAT^r linker is in bold); for JOHE12923, **CCGTGTT AATACAGATAAACC**CACATTGACTACTTATGACCA; for JOHE12924, GC TATAAACATTCCCGTCGTG; for JOHE12926, GACTCTGCCATCGTCCA AGAA; for JOHE12927, **AGCTCACATCCTCGCAGC**CAGTTTACCTGGATT TCGAGC; for JOHE12928, **CCGTGTTAATACAGATAACC**CACTTCTGTTAT GCGAGATGC; and for JOHE12929, CCACCGCTCTTCCAGATGAAA.

Nematode killing assay. *C. elegans* killing assays were done as described previously (53, 54) with minor modifications. The *C. neoformans* strains were inoculated into 2 ml YPD and grown at 30°C for 24 h, 10 μl of the culture was spread on brain heart infusion agar (Difco), and the plates were incubated at 30°C for 48 h. *C. elegans* animals were transferred to the plates (divided across three plates of each strain) and incubated at 25°C, and then 120 to 150 animals were examined for viability at 24-h intervals. Replicate experiments showed similar results.

Amoeba assay. The survival of *C. neoformans* var. *grubii* in amoebae was as described previously (51). Briefly, *A. castellanii* organisms were collected, washed twice with PBS, suspended in PBS, counted with a hemocytometer, and diluted in PBS to the appropriate density. *A. castellanii* cells were added to a 96-well

tissue culture plate at 1×10^4 cells per well and allowed to adhere for 1 h at 28°C. *C. neoformans* cells (1×10^4) were added to wells containing amoebae or control wells containing PBS alone, and the plates were incubated at 28°C. At 0, 24, and 48 h, the amoebae were lysed and the well contents were serially diluted and plated onto Sabouraud (SAB) dextrose medium. Numbers of CFU were determined after incubation overnight at 30°C. Replicate experiments showed similar results.

Macrophage assay. Macrophages were harvested by removal from monolayers, and cell viability was determined by trypan blue exclusion. Cell number was determined using a hemocytometer, and the macrophage density was adjusted to 5×10^5 cells/ml of culture medium. Macrophages were placed in 96-well plates and stimulated overnight with 50 units/ml gamma interferon (murine) and 0.3 mg/ml lipopolysaccharide (LPS). *C. neoformans* cells were added to the macrophage suspension at a density of 5×10^5 cells/ml of culture medium with 10 μg/ml of monoclonal antibody 1887 as an opsonin. Macrophage-yeast mixtures were incubated for 1 h and washed three times with culture medium. The macrophages were then either lysed with 0.05% sodium dodecyl sulfate (SDS) immediately to determine the phagocytic index or incubated with stimulation for 24 h at 37°C with 5% CO_2. After incubation, the culture medium was aspirated from each well and the macrophages were lysed with two exchanges of 0.05% SDS. The aspirated medium and SDS solutions were combined, serially diluted, and plated onto YPD plates. Numbers of CFU were determined after incubation overnight at 30°C. Replicate experiments showed similar results.

Mouse virulence studies. Groups of 4- to 8-week-old female A/JCr, BALB/c, or C57BL/6 mice (five mice per strain) were anesthetized by intraperitoneal phenobarbital injection. Animals were infected intranasally with 5×10^4 fungal cells in 50 μl of normal saline pipetted slowly into the nares. Animals were infected intracerebrally with 1.5×10^3 fungal cells in 20 μl of normal saline injected slowly into the top of the cerebrum. In further experiments, animals were directly injected in their lateral tail veins with 2.5×10^4 cells. The concentration of yeast cells in the inoculum was confirmed by plating serial dilutions and enumerating CFU. Mice were monitored twice daily, and those that showed signs of severe morbidity (weight loss, extension of the cerebral portion of the cranium, abnormal gait, paralysis, seizures, convulsions, or coma) were sacrificed by CO_2 inhalation.

Quantitative analysis of tissue fungal burden. Groups of 4- to 6-week-old female A/JCr mice were infected either intranasally or intracerebrally. For intranasal infections, animals (five mice per strain per time interval) were sacrificed by CO_2 inhalation at 7, 14, and 21 days postinfection, and lung, spleen, and brain were removed, weighed, and homogenized in 2 ml sterile PBS. Animals were sacrificed by CO_2 inhalation at 4 days postinfection for intracerebral infections (10 mice per strain), and brain and lung were removed, weighed, and homogenized in 2 ml sterile PBS. Serial dilutions of the organ samples were plated on SAB agar plates containing 100 μg/ml chloramphenicol and incubated at 30°C overnight. Colony counts were performed and adjusted to reflect the total number of CFU/g tissue.

Coinfection experiments. Groups of 4- to 6-week-old female A/JCr mice were coinfected with a 1:1 ratio of **a** and α cells either intranasally or intracerebrally. The inoculum was prepared immediately before animals were infected and then plated on SAB medium. Colonies were isolated and analyzed to determine the proportion of **a** cells present in the inoculum. Mice were sacrificed by CO_2 inhalation; the lung, spleen, and brain were harvested and homogenized; and CFU were enumerated as in the previous assay. For the first coinfection experiments (five mice per time interval), animals were coinfected with KN99**a** and H99. Mice were sacrificed at 7, 14, and 21 days postinfection, and single colonies (64 from inoculum, 32 from lungs and spleen for each animal, and 64 from the brain of each animal) were randomly selected from SAB plates and analyzed for mating type based on mating with reference **a** and α strains on V8 medium (55). Coinfections with KN99**a**/KN99αNAT, KN99**a**NAT/KN99α, KN99**a**/KN99**a**NAT, and KN99α/KN99αNAT used 10, 10, 5, and 5 mice, respectively, which were sacrificed at 21 days postinfection. More than 500 colonies from the lung, spleen, and brain from each animal were isolated on SAB plates and screened for NAT resistance on yeast peptone-dextrose plates containing 100 μg/ml nourseothricin to determine mating type. In control experiments, NAT resistance was found to be 100% stable during vegetative culture of KN99**a**NAT and KN99αNAT. A subset of the colonies from coinfections was also analyzed for mating type based on mating with reference **a** and α strains, and nourseothricin resistance cosegregated 100% with the appropriate **a** or α allele of *MAT*. No instances of NAT loss or inactivation during infection were observed. The measured numbers of **a** and α cells were compared to the expected numbers, assuming that both strains remained at the initial infection proportions (based on analysis of the initial inoculum). These raw data were then converted to percentages for presentation in Fig. 5 and 6.

Statistics. *P* values presented are two tailed. *P* values for the nematode and mouse survival data were derived from log rank tests. Wilcoxon rank sum analysis was used to determine significance for the amoeba, macrophage, and organ CFU data. For the coinfection experiments, the measured number of **a** and α cells was compared to the expected number, assuming that both strains remained at the initial infection proportions (based on analysis of the initial inoculum), and Wilcoxon rank sum analysis of the raw data was used to determine *P* values. Thus, for the coinfections, the test of significance compared the ratios of the observed strains to the initial inoculum.

RESULTS

Further evidence that strains KN99a and KN99α are congenic with H99. We recently characterized mating in *C. neoformans* var. *grubii* using the serotype A mating type **a** strain 125.91 (55). A backcrossing scheme was designed using the mating characteristics of strain 125.91 to develop serotype A congenic strains in the H99 genetic background (Fig. 1A) (55). The resulting congenic strains KN99**a** and KN99α were compared to H99. The karyotypes of the KN99**a** and KN99α strains were examined and found to be identical to each other and to H99, indicating that the strains are congenic at the chromosome level (55). Restriction fragment length polymorphisms at two genetic loci were identified in the parental strains and the congenic strains and were found to have the same gene allele at both loci (55).

To further characterize the congenic strains at a molecular level, AFLP analysis with two independent primer pairs was used to create AFLP genotypes for each strain (Fig. 1B). Comparison of the AFLP genotypes of the parental strains 125.91, 8-1, KNA14, and H99 revealed polymorphic amplified DNA fragments between the strains. The congenic strains KN99**a** and KN99α had identical AFLP genotypes and were identical with H99 with both primer pairs tested, indicating a congenic relationship at a higher-resolution genomic level.

KN99**a**, KN99α, and H99 showed no differences in several traits involved in virulence, such as the presence of melanin or capsule, prototrophy, or growth at 37°C (55). To examine the strains in more detail, we performed phenotype microarrays (4, 80; reviewed in reference 3). This analysis allows strain phenotypes to be quantitatively measured over time based on the physiological state of the cell. Cryptococcal cells are combined with a tetrazolium dye that absorbs electrons produced by yeast cell respiration. Reduction of the tetrazolium dye results in a color change that does not require growth of the cell. In the PM assay, >1,000 distinct culture conditions were selected to approximate a comprehensive scan of known cellular pathways. The effects of various carbon, nitrogen, phosphorus, and sulfur sources on the physiological state of the cell were examined. Cell respiration was also monitored in response to various osmotic conditions and in response to differing pHs. Finally, sensitivity to a wide range of chemicals, including antibiotics, respiratory inhibitors, and toxic metals, was examined. We have been able to observe distinct differences between mutant and wild-type *C. neoformans* strains using this method (X. Lin and J. Heitman, unpublished results). However, no consistent differences were observed with the serotype A var. *grubii* congenic strains, suggesting that KN99**a**, KN99α, and H99 are also congenic at the phenotypic level.

Rates of survival of serotype A congenic strains are equivalent in nematodes and amoebae. The serotype A congenic

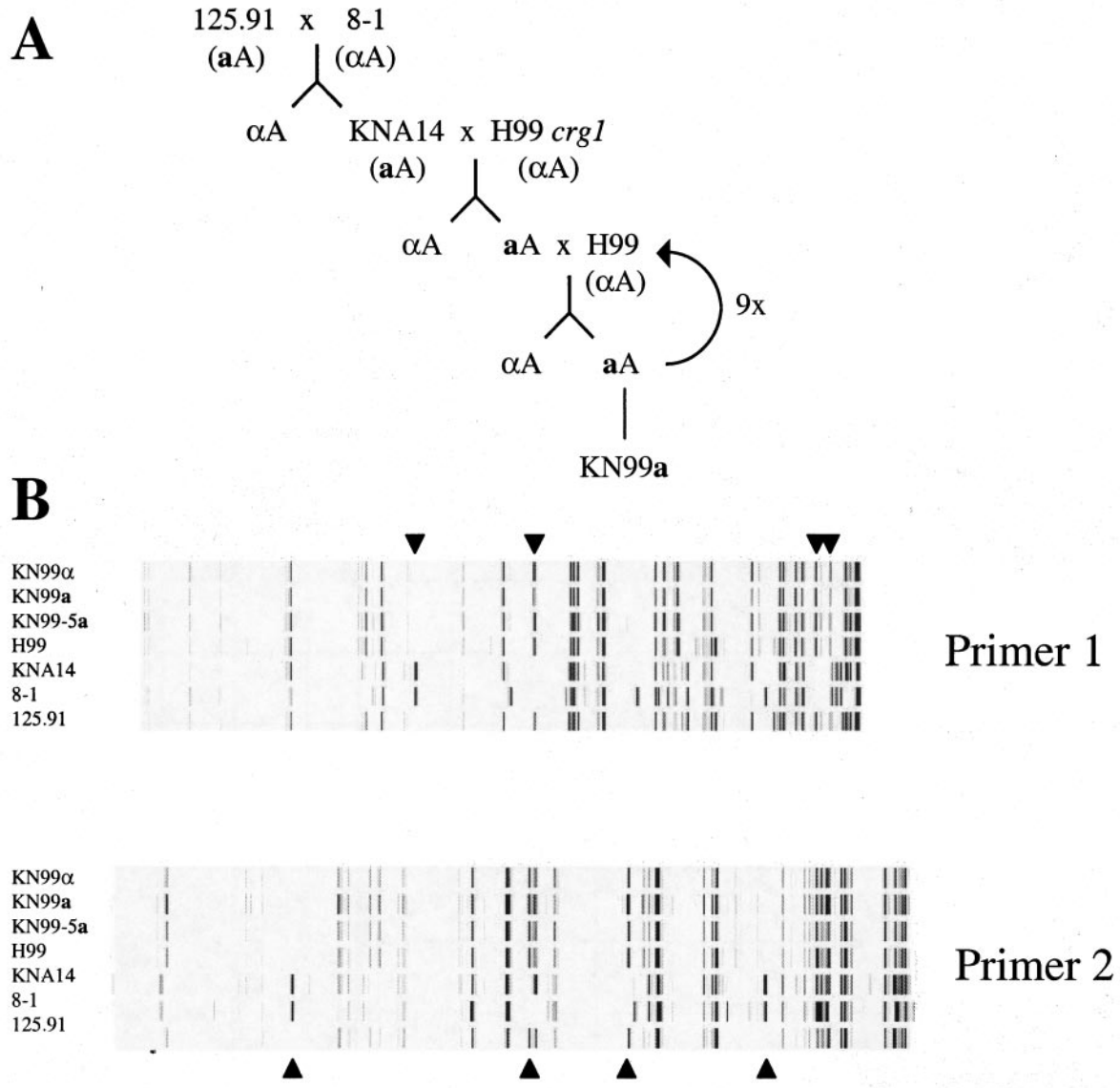

FIG. 1. Characterization of the *C. neoformans* var. *grubii* serotype A congenic strains H99, KN99α, and KN99a. (A) Schematic diagram of the mating scheme used to produce the serotype A congenic strains. (B) Computer-generated AFLPs from the parental α strains H99 and 8–1, the parental **a** strains 125.91 and KNA14, the congenic-progeny strains KN99**a** and KN99α, and the fifth-backcross strain KN99-5**a**. Polymorphic products are indicated by arrows.

strains KN99**a** and KN99α were previously shown to have virulence equivalent to that of strain H99 in two standard animal models of cryptococcosis (55). Because the vast majority of cases of cryptococcosis in humans are associated with mating type α strains, we further characterized the potential role of mating type in the infectious process. *C. neoformans* is a soil-dwelling saprophyte that is not dependent on mammalian infection for reproduction or viability, yet many environmental isolates have the capacity to infect and cause disease in humans and experimental animals. Virulence is considered a complex phenotype that may be maintained as a result of selection. It has been hypothesized that factors that enable infection of mammals may have evolved to allow *C. neoformans* to survive predation by natural environmental predators, such as amoebae, nematodes, and insects, and the human virulence factors capsule and melanin have been shown to be important for the survival of *C. neoformans* in these organisms (2, 11, 53, 64, 66).

To determine whether mating type plays a role in the virulence of *C. neoformans* var. *grubii* strains in nematodes, we examined the survival of *C. elegans* exposed to KN99**a**, KN99α, or H99. Previous studies with less-pathogenic serotype D var. *neoformans* congenic strains in *C. elegans* showed that the α strain was more virulent than the congenic **a** strain (53). However, studies with the var. *grubii* congenic strains showed no significant difference in rates of survival of the nematodes (Fig. 2A) (P values were ≥0.20 for all comparisons). The var. *grubii* congenic strains were also examined for survival in amoebae.

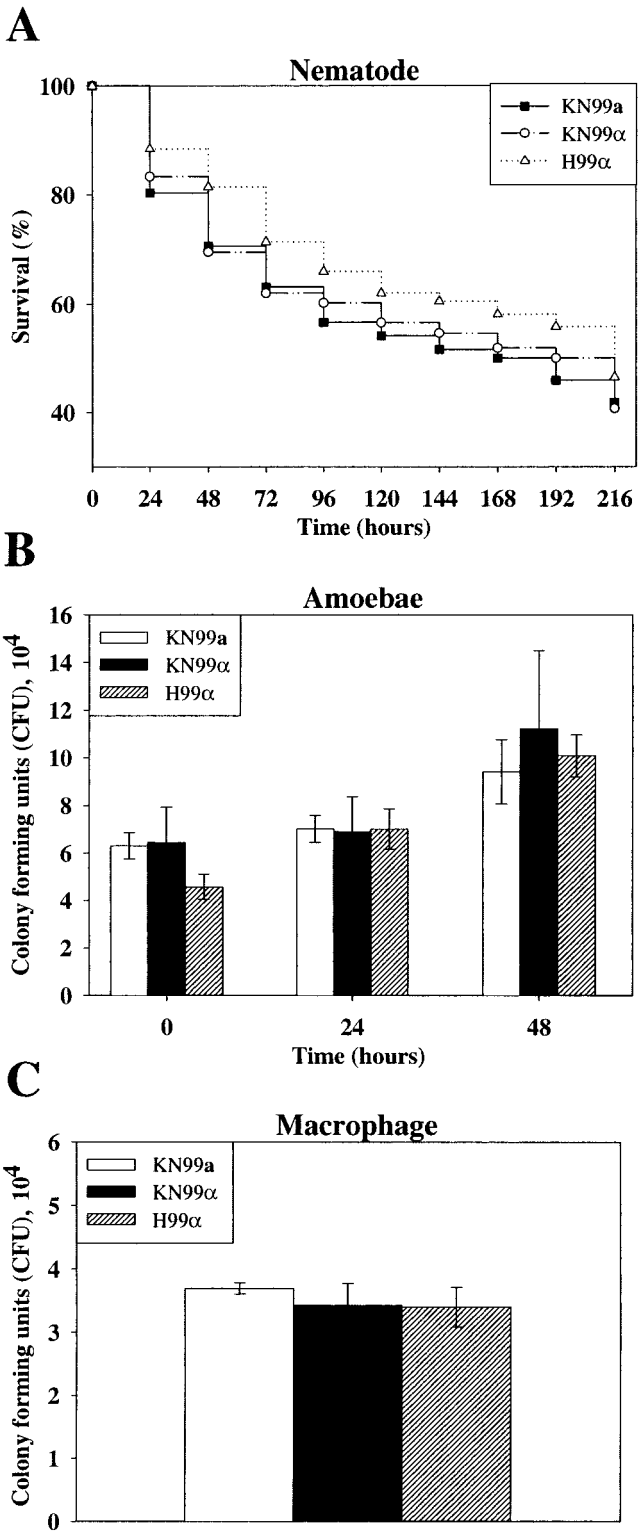

FIG. 2. Survival of *C. neoformans* var. *grubii* serotype A congenic strains in heterologous hosts and macrophages. (A) Virulence of *C. neoformans* in *Caenorhabditis elegans*. *C. elegans* animals were allowed to feed on lawns of the **a** strain KN99**a** or the α strains KN99α and H99. The animals were examined for survival at 24-h intervals, and log rank tests showed *P* values of 0.2, 0.2, and 0.97 for H99/KN99**a**, H99/KN99α, and KN99**a**/KN99α, respectively. (B) Survival in the presence of *Acanthamoeba castellanii*. The **a** strain KN99**a** and α strains KN99α and H99 were incubated with *A. castellanii* or in PBS alone at 28°C. At 24 and 48 h, the amoebae were lysed, diluted, and plated. Numbers of CFU were determined after incubation overnight at 30°C. *P* values were 0.89 for all comparisons at 24 h and 0.49, 0.89, and 0.49 at 48 h for H99/KN99**a**, H99/KN99α, and KN99**a**/KN99α, respectively. (C) Fungal survival in the presence of stimulated murine macrophages. MH-S murine alveolar macrophages were activated overnight with 50 units/ml gamma interferon and 0.3 mg/ml LPS. The activated macrophages were incubated at 37°C in 5% CO_2 with 10 μg/ml monoclonal antibody 1887 and *C. neoformans* strain KN99**a**, KN99α, or H99. After 1 h, the macrophages were washed to remove unengulfed cryptococcal cells. Numbers of CFU were determined. *P* values were 0.70, 1.0, and 0.40 for H99/KN99**a**, H99/KN99α, and KN99**a**/KN99α, respectively.

The congenic strains H99, KN99**a**, and KN99α were incubated with *A. castellanii* cells or a PBS control for 24 and 48 h. As shown in Fig. 2B, no significant difference in survival of the mating type α strains H99 and KN99α was observed from that of the congenic mating type **a** strain KN99**a** in amoebae (*P* values were ≥0.49 for all comparisons).

Survival of serotype A congenic strains in macrophages. Humans are exposed to *C. neoformans* via inhalation of desiccated yeast cells or spores (67). Macrophages play a critical role in the early initial pulmonary immune response to cryptococcal infection and may also play a role in harboring the organism during latent infection as a facultative intracellular pathogen (5–7, 30, 45). Furthermore, the survival of *Cryptococcus* in macrophages has been linked to virulence (18, 22, 25, 26, 71). To study the role of mating type in infection and survival in macrophages, we examined the survival of the serotype A congenic strains KN99**a** and KN99α compared to that of H99 in the murine alveolar macrophage cell line MH-S and culture medium alone (Fig. 2C). Macrophages were stimulated with LPS and gamma interferon for 12 h and cocultured with the appropriate *Cryptococcus* strain for 1 hour, and afterwards, the macrophages were washed with culture medium to remove extracellular cryptococcal cells. After incubation for 24 h, the number of surviving cryptococcal cells, as measured by the number of CFU recovered, was examined for each strain. The serotype A congenic strains exhibited no difference in uptake by macrophages (data not shown) or survival in macrophages in vitro (*P* values were ≥0.4 for all comparisons).

Virulence of serotype A congenic strains in multiple murine models of cryptococcosis. To study the role of mating type in the virulence of the serotype A congenic strains, we compared the levels of virulence of the α (H99) and **a** (KN99**a**) mating type strains in murine inbred lines, which can be divided into two groups: sensitive lines that are deficient in complement C5 secretion and resistant lines that have normal levels of complement C5 (59). Figure 3 shows three inbred lines commonly used for serotype A cryptococcal infections: A/JCr (sensitive), BALB/c (resistant), and C57BL/6 (resistant). A/JCr animals were most susceptible to intranasal infection, BALB/c animals exhibited intermediate susceptibility, and C57BL/6 animals were most resistant to infection by the serotype A congenic strains (*P* values were ≤ 0.009 for all comparisons). While there were significant differences in the susceptibilities of the strains to infection, all three inbred backgrounds showed no difference in levels of virulence for the mating type **a** and α congenic strains (*P* values were ≥0.35 for all comparisons).

There are two predominant murine models of cryptococco-

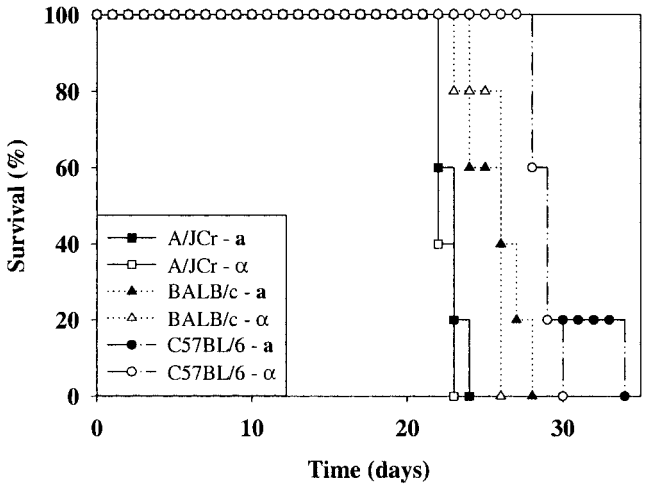

FIG. 3. Virulence of *C. neoformans* var. *grubii* serotype A congenic strains H99 and KN99**a** in A/JCr, BALB/c, and C57BL/6 inbred-mouse lines. Animals were infected intranasally with 5×10^4 cells, and progression to severe morbidity was monitored. *P* values were 0.35, 0.47, and 0.66 for H99AJ/KN99**a**AJ, H99BALBc/KN99**a**BALBc, and H99C57BL6/KN99**a**C57BL6, respectively. *P* values were 0.008, 0.002, 0.004, 0.009, 0.002, and 0.008 for H99AJ/H99BALBc, H99AJ/H99C57BL6, H99BALBc/H99C57BL6, KN99**a**AJ/KN99**a**BALBc, KN99**a**AJ/KN99**a**C57BL6, and KN99**a**BALBc/KN99**a**C57BL6, respectively.

sis based on inoculation via either inhalation or tail vein injection. Our previous studies showing that the serotype A congenic strains have equivalent levels of virulence utilized an inhalational model in which an initial pulmonary infection was followed by extrapulmonary dissemination, resulting in lethal CNS infection. Here we extended this comparison of the serotype A congenic strains H99 and KN99**a** using a mouse tail vein infection model. All animals succumbed to the infection in less than 10 days, and no significant difference in survival was observed between animals infected with the mating type α strain H99 and those infected with the mating type **a** strain KN99**a** (data not shown).

Progression of *C. neoformans* var. *grubii* infection in a murine model. The previous animal models used survival (progression to severe morbidity) as the basis for the virulence of the serotype A congenic strains. While we found no difference in survival when mice were exposed to the serotype A congenic strains, we considered the possibility that the strains might cause disease in different ways. For example, one strain may result in a lethal pulmonary infection, while another strain may spread more quickly to the central nervous system to cause meningoencephalitis. Therefore, we examined the growth and dissemination of the strains in mice early in the infection (7 days), at an intermediate time point (14 days) and late in the infection (21 days). The number of CFU present in the lungs over time was used as a measure of pulmonary infection, while the number of CFU present in the spleen was used as a measure of the ability of the strain to spread hematogenously to other organs. Finally, numbers of CFU present in the brain were used to determine CNS infection (Fig. 4).

When the serotype A mating type α strain H99 was compared to the congenic mating type **a** strain KN99**a**, H99 had slightly higher levels in the lung at the first time interval but no difference was observed in lung fungal burdens at the two later time intervals ($P = 0.02$ at 7 days, $P = 0.15$ at 14 days, $P = 0.41$ at 21 days). Large variability was observed between spleen fungal burdens over time, with KN99**a** having slightly higher levels at early and late time intervals ($P = 0.01$ at 7 days, $P = 0.31$ at 14 days, $P = 0.02$ at 21 days). Interestingly, higher levels of KN99**a** were present in the brain at the earliest time studied (7 days), but fungal burden in the brain increased at a higher rate, such that both strains had comparable numbers of brain CFU at the later times ($P = 0.03$ at 7 days, $P = 0.31$ at 14 days, $P = 0.73$ at 21 days). These data imply that **a** cells may initially disseminate to the brain faster than α cells but that both cell types eventually produce equivalent levels of CNS infection. Based on symptoms, the animals appeared to have cryptococcal meningitis at the late time point and were without signs of a debilitating pulmonary infection. Because these observations correlate with equivalent CNS fungal burdens in the congenic **a** and α strains, the animals appear to succumb to meningitis rather than pulmonary infection.

Coinfections with a and α mating types. The serotype A congenic **a** and α mating type strains have equivalent levels of virulence and similar modes of infection and disease progression when pure cultures are used as the infecting inoculum, yet α strains cause the majority of cryptococcosis in humans. Interestingly, infection by multiple strains has been observed in humans (38, 58). The apparent paradox between animal studies and disease epidemiology could be explained if α strains out-compete or inhibit **a** strains during simultaneous coinfection with both strains.

To examine this hypothesis, animals were infected with a mixture of the serotype A congenic **a** and α strains and the proportions of each strain present in the lungs, spleen, and brain were monitored and compared to the proportions of each strain in the inoculum (Fig. 5 and 6). Infections with unmarked strains showed no difference in the proportions of the mating type **a** strain KN99**a** in the inoculum from the proportion of KN99**a** strains in the lungs and spleen in two independent coinfections at 7, 14, and 21 days postinfection (data not shown). Enumeration of total CFU from the lung and spleen were consistent with those observed in the individual infections (Fig. 4) at all time points. Interestingly, brain infection appeared to be initially delayed during coinfection with low or undetectable numbers of CFU at 7 days postinfection, but by days 14 and 21 postinfection, numbers of CFU increased and were consistent with those observed during pure culture infections (data not shown). At both 14 and 21 days postinfection, the proportions of KN99**a** strains in the brain were low in both assays. In 6 of the 10 mice examined, less than 5% of the strains analyzed were KN99**a** and >95% were H99, indicating a difference in CNS penetration or survival between the two mating types.

The strains analyzed in these coinfections had to be identified based on mating assay or PCR, which is laborious and limited the number of animals and strains analyzed. To extend these findings, large-scale analysis of coinfections was undertaken using marked strains and selective plating. A NAT resistance transgene was introduced into a noncoding region of the mating type locus of strains KN99**a** and KN99α to generate strains KN99**a**NAT and KN99αNAT, respectively.

To verify that the transgenes were phenotypically neutral

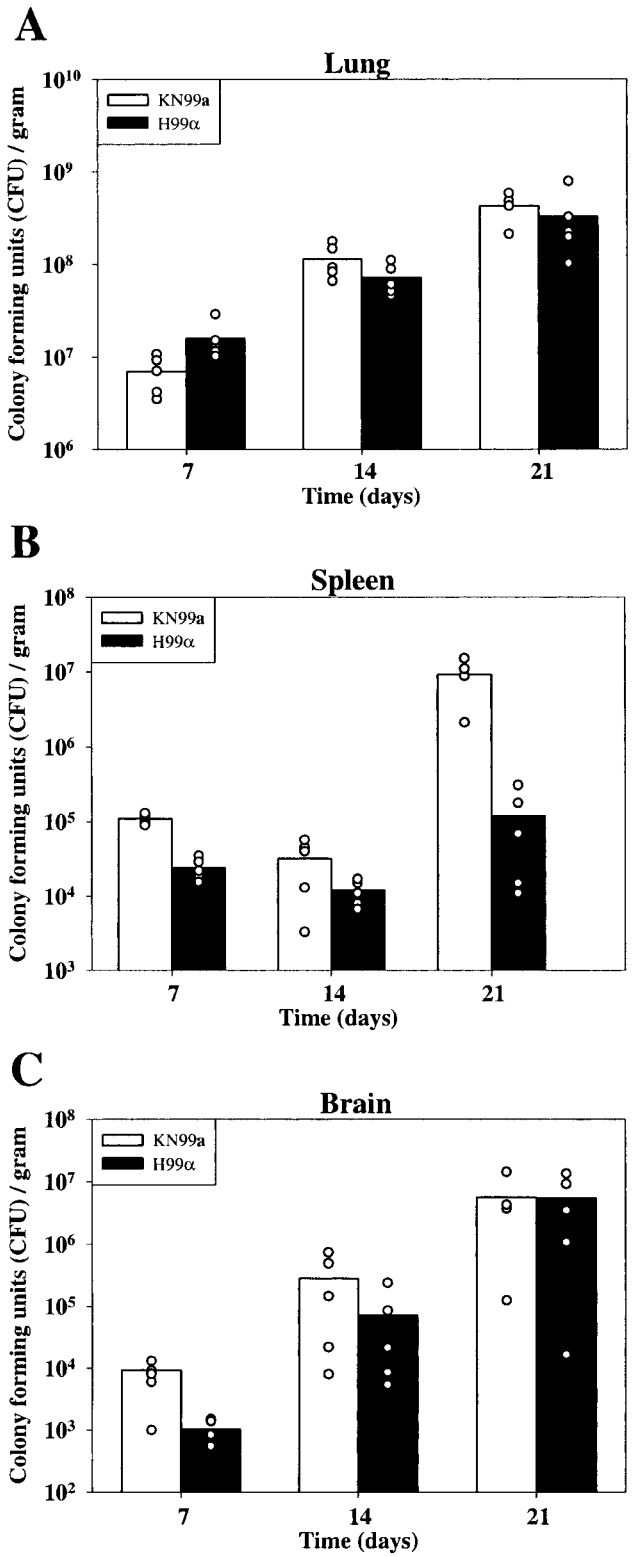

FIG. 4. Tissue burden cultures of *C. neoformans* var. *grubii* serotype A congenic strains H99 and KN99**a**. Mice (A/JCr) were infected with 5×10^4 cells intranasally. Organs were extracted and homogenized, and serial dilutions were plated for tissue burden cultures at 7, 14, and 21 days postinfection. The number of yeast cells present is expressed as the geometric mean of the number of CFU per gram of lungs, spleen, or brain. Open circles denote values from individual animals, and the height of each bar represents the geometric mean.

and did not alter the virulence of the NAT-marked strains, the NAT-marked strains were coinfected with their parent strain and the proportions of NAT-marked strains in the lung, spleen, and brain were analyzed at 21 days postinfection (Fig. 5B). The proportion of KN99**a**NAT cells present in the lungs, spleen, and brain in the **a**NAT/**a** mixture was equivalent to the starting inoculum (represented by the dotted line), demonstrating that KN99**a**NAT survives in vivo at levels equivalent to KN99**a** (lung, $P = 0.75$; spleen, $P = 0.86$; brain, $P = 0.25$). However, most animals had one strain type that predominated in the brain, with roughly half of the animals having a vast majority of marked **a**NAT cells and the other half having unmarked **a** cells in the CNS. This observation is consistent with a single cell or a small group of founder cells producing infection of the brain. Similar results were observed with αNAT/α coinfections (data not shown). Thus, these data demonstrate that the dominant marker transgenes are phenotypically neutral in vivo.

We used the marked strains to compare differences in disease progression between **a** and α using larger numbers of animals and CFU. The KN99**a**NAT strain was coinfected with KN99α, and the proportions of NAT-marked strains in the lung, spleen, and brain were analyzed at 21 days postinfection. More than 500 colonies from each organ were isolated and then individually screened for NAT resistance by selective plating. Figure 5A shows that the proportions of KN99**a**NAT cells present in the lungs and spleen were equivalent to the initial inoculum (represented by the dotted line) but that the level of KN99**a**NAT cells present in the brain was significantly lower than the inoculum level in the **a**NAT/α coinfection (lung, $P = 0.55$; spleen, $P = 0.21$; brain, $P < 0.001$). Similar results were observed with the αNAT/**a** coinfection (data not shown). Thus, this larger data set corroborates the pattern observed in coinfection studies with unmarked **a** and α strains. We conclude that there is a significant difference between congenic **a** and α cells with respect to their ability to establish CNS infection during coinfection of mice.

The difference between **a** and α cell accumulation in the CNS during coinfection may be due to either dissemination to the CNS or growth within the CNS. To distinguish between these two possibilities, animals were infected intracerebrally with either KN99**a** or KN99α or coinfected with KN99**a**NAT/KN99α. Intracerebral infections showed no difference in rates of survival of animals (data not shown) or in brain fungal burdens between **a** and α pure cultures or with **a**NAT/α coinfections of the CNS (Fig. 6A) (P values were 0.81, 0.24, and 0.20 for KN99**a**/KN99α, KN99**a**/KN99**a** coinfection, and KN99α/KN99α coinfection, respectively) and are consistent with the intranasal infections. In contrast to what occurs with intranasal coinfections where α cells predominate in the CNS, Fig. 6B shows that when **a**NAT/α cells were coinfected intracerebrally, no difference in the levels of accumulation of the strains was observed ($P = 0.99$). Analogous results were observed with a rabbit meningitis coinfection model where the strains were inoculated directly into the cerebrospinal fluid (data not shown). These data show that **a** and α cells grow equally well in the CNS and imply that α cells predominate in the CNS during coinfection due to a difference in dissemination to the CNS.

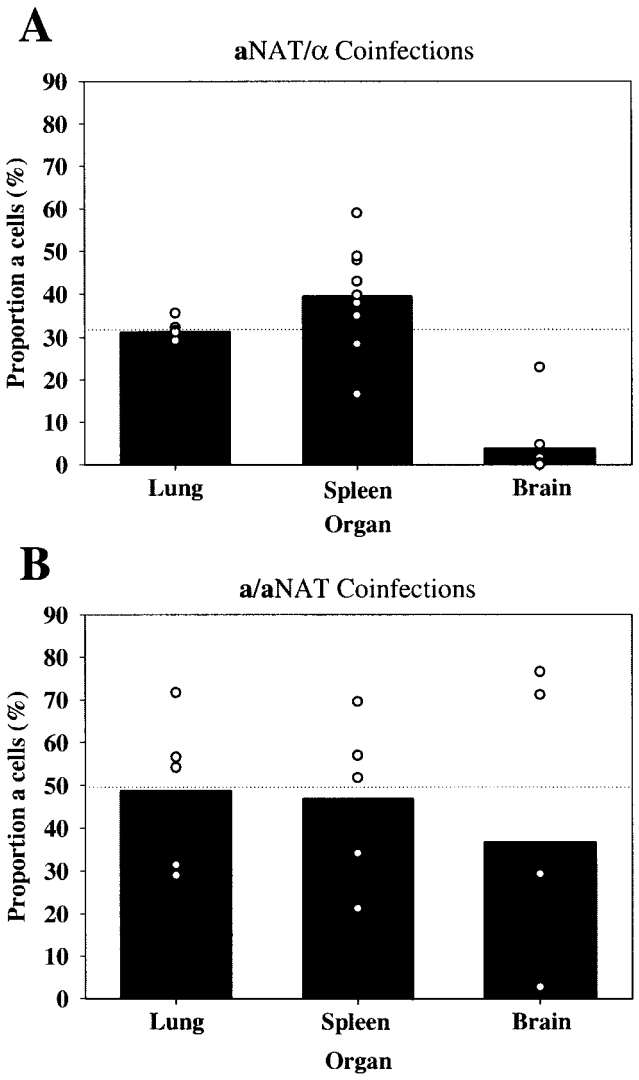

FIG. 5. *C. neoformans* var. *grubii* α strains predominate in the brain during intranasal coinfection with congenic strains. Mice (A/JCr) were inoculated with an approximately 1:1 ratio of (A) KN99**a**NAT and KN99α or (B) KN99**a**NAT and KN99**a** intranasally at a final concentration of 5×10^4 cells. The actual proportion of **a** cells in the infecting inoculum was determined by growth on selective medium and is plotted as a horizontal dotted line. At 21 days postinoculation, animals were sacrificed. The lungs, brain, and spleen from each animal were homogenized, and serial dilutions were plated. More than 500 colonies per organ were isolated from each animal and assayed for NAT resistance to determine mating type. The proportion of **a** cells present in each organ is plotted, with open circles denoting values from individual animals and the height of each bar representing the geometric mean.

DISCUSSION

Rates of AIDS infections are still on the rise in many areas of the world, including Africa and Asia (76, 77). This pandemic has led to an increase in the abundance and severity of infections by opportunistic fungal pathogens. Many virulence factors of one such pathogen, *C. neoformans*, have been identified. Some factors, such as the polysaccharide capsule and melanin, are clearly directly linked to the pathogenicity of *C. neoformans* because cells lacking these factors exhibit a significant decrease in virulence (48, 56, 79). Mating type has been proposed to be a virulence factor in *C. neoformans*. Epidemiological studies have shown that clinical isolates are severely biased towards the α mating type (41) and that some genes

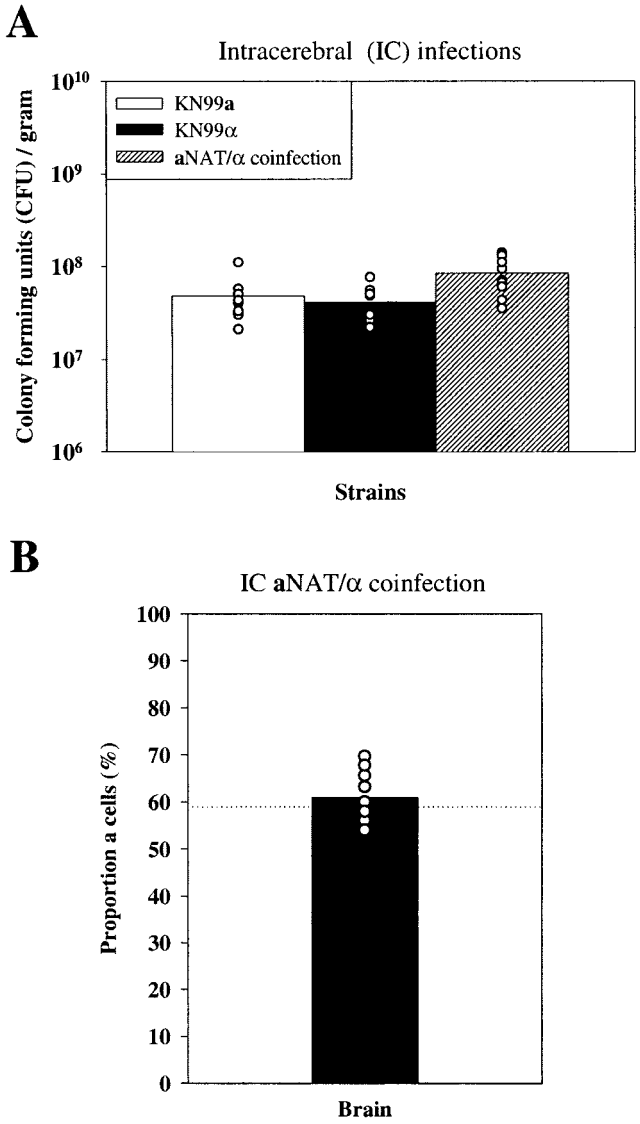

FIG. 6. *C. neoformans* var. *grubii* intracerebral infections show equivalent levels of growth of congenic strains. Mice (A/JCr) were inoculated intracerebrally with either KN99**a** or KN99α or coinfected with an approximately 1:1 ratio of KN99**a**NAT and KN99α at a final concentration of 1.5×10^3 cells. The actual proportion of **a** cells in the coinfecting inoculum was determined by growth on selective medium and is plotted as a horizontal dotted line in panel B. At 4 days postinoculation, animals were sacrificed. The brain from each animal was extracted and homogenized. Serial dilutions were plated for tissue burden cultures (A). The number of yeast cells present is expressed as the geometric mean of numbers of CFU per gram of tissue. Open circles denote values from individual animals, and the height of each bar represents the geometric mean. (B) For coinfection experiments, >500 colonies per organ were isolated from each animal and assayed for NAT resistance to determine mating type. The proportion of **a** cells present in each organ is plotted, with open circles denoting values from individual animals and the height of each bar representing the geometric mean.

associated with mating type, such as *STE12***a** and *STE12*α, have a role in the virulence of the serotype D var. *neoformans* lineage, whereas other genes, such as *SXI1*α, *STE20*α, and *STE11*α, do not (12, 14, 17, 21, 36, 75). In heterogeneous populations of serotype D, no clear distinction between the levels of virulence of **a** and α strains could be determined, although α strains killed more mice at lower inoculums (40). To examine the role of mating type in virulence, congenic strains were generated by repeated backcrossing. Analysis of var. *neoformans* serotype D strains revealed that the α congenic strain was more virulent than the **a** strain (42). However, clinical infections with var. *neoformans* strains account for a limited number of cases compared to cases caused by var. *grubii* serotype A strains. In contrast to serotype D, congenic **a** and α var. *grubii* strains have equivalent levels of virulence in cellular (macrophage), heterologous (nematodes, amoebae), and mammalian (both mouse and rabbit) models of cryptococcosis (reference 55 and this study).

Examination of the virulence of var. *grubii* serotype A congenic strains in multiple inbred-mouse lines showed virulence consistent with the var. *neoformans* serotype D results of Rhodes et al. (59). A/JCr mice were more susceptible to the serotype A congenic strains than the relatively resistant BALB/c and C57BL/6 lines. The serotype A congenic strains were also more virulent in the BALB/c inbred-mouse line than in C57BL/6 mice. While we observed a difference in the overall rates of survival between the inbred-mouse lines, the congenic strains had equivalent levels of virulence in all of the inbred lines tested, showing that the similarity in the virulence potentials of the var. *grubii* congenic strains is not specific to the inbred-animal background.

While cryptococcal meningoencephalitis is the most common life-threatening manifestation of cryptococcosis, the lungs are the second-most-relevant site of cryptococcal infection (10). In animal models of cryptococcal meningitis, neurological symptoms, such as extension of the cerebral portion of the cranium, abnormal gait, paralysis, and lethargy, are observed, whereas animals with pulmonary cryptococcosis exhibit respiratory distress. However, in animal models based on lethal infection, the difference between cryptococcal meningitis and pulmonary cryptococcosis could be overlooked. To differentiate between these two forms of cryptococcosis, numbers of CFU from lung, spleen, and brain tissue (to represent pulmonary infection, hematogenous spread to other organs, and CNS infection, respectively) were compared from animals inoculated with the serotype A congenic **a** and α mating type strains KN99**a** and H99, respectively. Mice infected with KN99**a** had higher numbers of CFU at initial time intervals in the brain, suggesting either a higher rate of dissemination from the initial pulmonary infection to the CNS or reduced clearance in the CNS. However, both KN99**a** and H99 had equivalent numbers of CFU in the brain by the intermediate and final time intervals studied, and all animals exhibited symptoms of CNS infection, indicating that the basis of mortality is the same in both cases.

The most notable virulence difference between the serotype A congenic **a** and α strains was observed during coinfection. In these experiments the ability of one strain to out-compete or inhibit the growth of the other strain would manifest itself as an increase in the proportion of the dominant strain. During intranasal coinfections, the α strain predominated in the brain

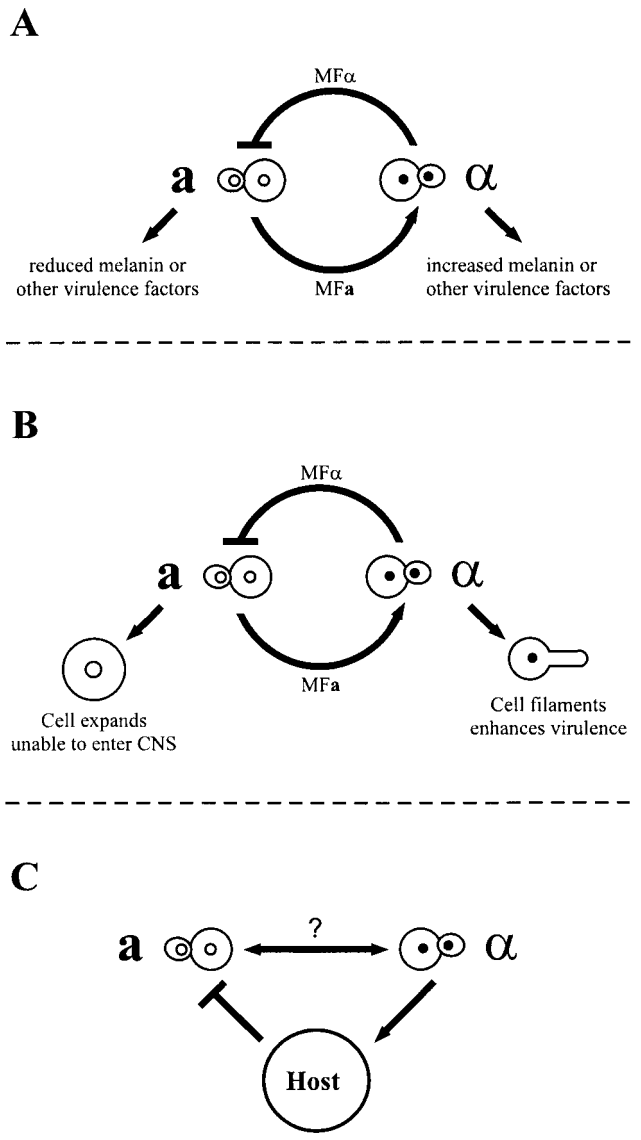

FIG. 7. Proposed models for the role of mating type in the enhanced virulence of α cells in the CNS. (A) Fungal-fungal interactions result in changes in virulence factors; (B) fungal-fungal interactions result in morphological changes which affect virulence; (C) host-fungal interactions result in changes in the virulence potential of the fungus. MF, mating factor/pheromone.

even though the levels of **a** and α strains were equivalent in the lung and spleen. Because intranasal coinfections with the unmarked strains and larger-scale coinfections with marked strains showed that **a** and α strains disseminated equally well from the lung to the spleen, we conclude that the difference observed in the CNS is not simply due to an inability of **a** cells to disseminate from the lung but is specific to the CNS. Intracerebral infections showed that both strains had similar levels of persistence in the brain during coinfection. Thus, α cell predominance in the CNS appears to be due to entry into and not persistence in the brain.

A number of hypotheses could explain the observation that α cells had higher levels of entry into the CNS during coinfec-

tion. First, an interaction between α and **a** cells could enhance the ability of the α cells to invade the brain (Fig. 7A). For example, laccase is critical for the survival of *Cryptococcus* in the brain, and urease has been shown to enhance invasion of the central nervous system by *Cryptococcus* (57, 60). Increased production of these enzymes in the α strain or their decreased production in the **a** strain may potentially result in a higher proportion of α strains in the CNS.

A second hypothesis is that interactions between α and **a** cells results in a physical alteration of the **a** cells that inhibits their ability to enter the CNS (Fig. 7B). In humans, *C. neoformans* replicates in the lung and then spreads via the bloodstream or lymphatic system to other organs as free cells or internalized in mononuclear cells, such as macrophages, that travel to microcapillary beds in the brain (16, 28, 57, 61). At the blood-brain barrier, *C. neoformans* can transverse microvascular endothelial cells either directly or via a Trojan horse mechanism within mononuclear phagocytes (13, 15, 61). Mating type **a** strains are known to generate large, bulbous cells in response to α pheromone in serotype D var. *neoformans*, and the α pheromone *MFα1* gene has been shown to be expressed in the brain during serotype A var. *grubii* infection as well as inside *C. elegans* (23, 53). While enlargement of **a** cells has not been observed in the laboratory for var. *grubii* serotype A, it is possible that **a** cells respond to α pheromone produced by coinfection with α cells in vivo. Pheromone-induced cellular changes in **a** cells may inhibit their entry into microcapillaries in the brain or their subsequent transversal of endothelial cells but not affect their ability to spread to other organs, such as the spleen.

Finally, as a third hypothesis, we cannot rule out the possibility that **a** and α cells do not communicate with each other but interact differently with the host. But as the difference in virulence occurs only during coinfection, the data suggest a three-way dialog between the host and both **a** and α cells of the fungal pathogen (Fig. 7C). For example, one strain could elicit an immune response from the host that affects the other strain disproportionately, which manifests itself as a virulence difference in the brain.

We have detected no evidence of mating or **a**-α cell fusion in vivo to produce **a**/α diploid cells during coinfection (this work and Y.-S. Bahn and J. Heitman, unpublished results). Thus, these conditions may preclude mating or counterselect against **a**/α diploid or dikaryotic products of mating. This said, early events in **a**-α cell signaling that transpire in vitro may occur in vivo, as several conditions conducive to mating (darkness, nutrient limitation, and pheromone expression) are present in vivo and could result in local **a**-α cell signaling that differentially impacts fungal cell interactions with the host.

It is possible that α strains are the predominant clinical isolates because they are more prevalent in the environment. If this is the case, then areas with high levels of environmental **a** strains would be expected to have roughly equal levels of **a** strains as clinical isolates. Examination of serotype A clinical strain populations from sub-Saharan countries show that up to 10% of the clinical isolates can be the **a** mating type, and a population of clinical isolates from Botswana showed evidence of sexual recombination (44, 46). It will be of interest to establish what proportion of environmental strains in sub-Saharan Africa is represented by **a** strains.

The predominance of α cells in the CNS during mixed infections could also contribute to the predominance of α strains in clinical isolates, which are derived predominantly from the cerebrospinal fluid. It is possible that α strains appear to be predominant because coinfections from mixed **a** and α spores, produced as a result of mating, result in CNS infections with a higher proportion of α strains. Furthermore, most clinical isolates are single-colony isolates. Thus, if the cerebrospinal fluid has a higher proportion of α cells, there is also a higher probability that the resulting single-colony isolate will be an α strain. In the few cases where serial isolates have been collected from the same patient, a second strain has been isolated as often as 29% of the time (58). These patients could have been reinfected with a different strain or coinfected with multiple strains. Examination of multiple isolates from the same patient, preferably from lung or sputum cultures from patients in whom multiple strains might be expected to be present in roughly equal proportions, will provide more-accurate data on levels of coinfection and the genetic nature of the coinfecting strains with respect to the mating type locus and other genes linked to the virulence potential of *Cryptococcus*.

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