RESEARCH ARTICLE

Mixed Infections and *In Vivo* Evolution in the Human Fungal Pathogen *Cryptococcus neoformans*

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ABSTRACT **Koch's postulates are criteria establishing a causal relationship between a microbe and a disease that lead to the assumption that diseases are caused by a single strain or its evolved forms. *Cryptococcus neoformans* is a life-threatening human fungal pathogen responsible for an estimated 1 million cases of cryptococcosis/year, predominantly meningoencephalitis. To assess the molecular diversity of clinical isolates and gain knowledge of *C. neoformans* biology in the host, we analyzed clinical cultures collected during the prospective CryptoA/D study. Using molecular analysis of unpurified isolates, we demonstrated that mixed infections in humans are more common than previously thought, occurring in almost 20% of patients diagnosed with cryptococcosis. These mixed infections are composed of different mating types, serotypes, and/or genotypes. We also identified genetically related haploid and diploid strains in the same patients. Experimental infections and quantitative PCR show that these ploidy changes can result from endoreplication (duplication of DNA content) and that shuttling between haploid and diploid states can occur, suggesting *in vivo* evolution. Thus, the concept of one strain/one infection does not hold true for *C. neoformans* and may apply to other environmentally acquired fungal pathogens. Furthermore, the possibility of mixed and/or evolving infections should be taken into account when developing therapeutic strategies against these pathogens.**

IMPORTANCE ***Cryptococcus neoformans* is a life-threatening human fungal pathogen that is present in the environment and is responsible for an estimated 1 million cases of cryptococcosis/year, predominantly meningoencephalitis in HIV-infected patients. To assess the molecular diversity of clinical isolates and gain knowledge of *C. neoformans* biology in the host, we analyzed clinical cultures collected during a prospective study on cryptococcosis. Using molecular analysis of unpurified isolates, we uncovered an unexpectedly high frequency (almost 20%) of mixed infections. We further demonstrated that these mixed infections could result from infestation by multiple strains acquired from the environment. We also made the serendipitous discovery of *in vivo* evolution leading to endoreplication of the yeasts within the host. Thus, the concept of one strain causing one infection does not hold true for *C. neoformans* and potentially for other environmentally acquired fungal pathogens. The possibility of mixed and/or evolving infections should be taken into account when developing therapeutic strategies against these pathogens.**

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Cryptococcus neoformans is a haploid encapsulated yeast responsible for cryptococcosis, an opportunistic infection that occurs in patients with cellular immune defects, especially those with AIDS. Cryptococcosis affects up to 30% of HIV-infected patients in sub-Saharan Africa and Southeast Asia (1). Overall, cryptococcal meningoencephalitis is diagnosed in almost 1 million individuals per year, accounting for more than 600,000 deaths annually (2). Cryptococcosis is still fatal in almost 20% of cases despite adequate treatment, justifying the development of new therapeutic strategies. *C. neoformans* is ubiquitous in the environment, particularly in bird droppings and decaying vegetation. It exists in two varieties, *C. neoformans* var. *grubii* (serotype A), which causes the majority of infections worldwide, and *C. neoformans* var. *neoformans* (serotype D), which is commonly found in Europe.

Cryptococcosis is usually considered to represent the reactivation of a dormant infection (1). A single isolate of *C. neoformans* has been thought to be responsible for the disease. Yet the isolation of strains with different genotypes, serotypes, or mating types at the same geographic site (3, 4) makes infestation by multiple strains likely. Only anecdotal reports of mixed infections have been published to date. In mixed infections involving different species or serotypes of *Cryptococcus*, multiple single-colony isolates were analyzed due to vastly different colony morphologies (5, 6). In other cases, different genotypes of *C. neoformans* var. *grubii* have been identified in temporally separated single-colony isolates during the same episode or during a relapse of infection (7–11). Microevolution during infection has been suggested and shown to occur during long-term storage and repeated passage in the labo-

ratory (12–14). Previous studies have cited the need for large-scale studies to be carried out with unpurified isolates to determine the true incidence of mixed infections (6, 15) because routine clinical practice (storage of a single colony per isolate, one isolate studied per patient, and no routine detection of ploidy or mating type) precludes the discovery of mixed infections.

Identification of sexual cycles in the pathogenic fungi greatly expanded our understanding of their biology (16–18). The sexual cycle of *C. neoformans* involves mating types **a** and α (*MAT***a** and *MAT*α), but sexual reproduction seems limited by apparently rare encounters with sexual partners (17, 19). However, recombination is observed, potentially due to monokaryotic fruiting following same-sex cell fusion or self-diploidization (also called endoreplication) with the production of recombinant haploid or diploid progenies (20–23). AD hybrid strains of *C. neoformans* var. *neoformans* and *grubii* have also been identified. These hybrids can harbor both mating types or result from same-sex mating (24, 25). Hybrids have been observed in nature and in clinical samples and have been generated in the laboratory (26, 27). The ability to undergo mating or endoreplication is assumed to allow response to environmental and host challenges and contributes to genetic diversity (28). To date, experimental infections with laboratory reference strains have not revealed stable ploidy changes (29).

The scarcity of mixed-infection reports could be explained either by their true rarity or by the fact that most clinical isolates are single colony purified prior to molecular analysis. Our objective was to analyze isolates that were not colony purified to determine the frequency of mixed infections and if AD hybrid formation, mating, or monokaryotic fruiting could occur *in vivo* because of the simultaneous presence of multiple strains. For these analyses, we used a collection of clinical isolates that were sent unpurified to the French National Reference Center for Mycoses and Antifungals (NRCMA) during the prospective CryptoA/D study (30). Our analysis represents the first estimate of mixed infections during cryptococcosis. Various combinations of strains with different mating types, serotypes, ploidies, and genotypes were found during the same episode of cryptococcosis, sometimes in the same sample. While mating and hybrid formation could not be demonstrated *in vivo*, diploidization resulting from endoreplication was documented and enhanced the diversity of infecting strains.

RESULTS

Mixed infections are frequent and diverse during cryptococcosis. We examined 100 isolates corresponding to 49 patients for whom *C. neoformans* unpurified original cultures had been stored during the CryptoA/D study (30). Single-colony isolates (4 to 33 single colonies/culture) were screened for all 100 cultures. Original cultures and single-colony isolates were analyzed for ploidy by flow cytometry, and genotypes were determined by amplification of serotype- and mating-type-specific loci by PCR.

Mixed infections were demonstrated for 9/49 patients studied (18.4%). A total of 31 original cultures (15 from cerebrospinal fluid [CSF] samples, 10 from blood cultures, 4 from urine samples, 1 from a skin lesion, and 1 from a bronchoalveolar lavage [BAL] fluid sample) and 286 single-colony isolates from these nine patients were studied. Among the 31 original cultures, 8 were found to be diploid and 23 were haploid (Table 1). Subsequent analysis of single-colony isolates derived from the eight diploid cultures showed that strains with different ploidies, serotypes, or mating types could be found in the same culture for six patients (Table 1). Different strains were also found at different anatomical sites and at different time points during infection (up to 15 days) for seven patients. Using multilocus sequence typing (MLST), infection by two genetically distinct isolates of *C. neoformans* var. *grubii* was found for one patient (no. 188) with differences in 4/8 loci (Table 1). Overall, mixed infections with different serotypes, mating types, ploidies and/or genotypes in various combinations were observed as follows: Aα + Dα (patient no. 188), Aα + D**a** (no. 71), Aα + αAD**a** (no. 10 and 198), Aα + αAAα (no. 161), Aα + αDDα (no. 20 and 23), D**a** + **a**DD**a** (no. 119), and Aα + Dα + αADα + αDDα (no. 177). Patients with mixed infections did not differ significantly from those with single infections in terms of underlying disease and clinical presentation. However, a trend toward more mycological failures after 2 weeks of antifungal treatment but reduced mortality was observed in the mixed-infection group (Table 2).

Multiple infestations are responsible for mixed infections during cryptococcosis. The discovery of haploid and AD hybrid diploid isolates in the same patient suggested that either these ploidy changes occurred in the environment with subsequent inhalation of multiple strains or they occurred within the human host. MLST analysis was used to differentiate between these two possibilities. Three patients (no. 177, 10, and 198) had infections with haploid Aα and diploid αADα or αAD**a** hybrid strains. MLST analysis of these three serotype A polymorphic loci revealed that in all cases the Aα alleles differed between the haploid and the hybrid strains (Table 1). These results ruled out genetic relatedness between the A and AD isolates and showed that the haploid Aα could not have arisen from unstable AD diploids that dropped the D portion of their genome. These results, together with the discovery of serotype A and D strains during the same episode of cryptococcosis in five patients (no. 20, 23, 71, 177, and 188), provided evidence for coinfection with genetically distinct strains acquired from the environment.

Diploidization can occur *in vivo* to generate mixed infections during cryptococcosis. In four patients infected with both haploid and diploid Aα (no. 161), D**a** (no. 71 and 119), or Dα (no. 177) strains, the infections could have resulted from either coinfestation with genetically unrelated or related strains or *in vivo* diploidization through same-sex cell fusion or endoreplication. The Aα haploid and diploid strains had the same MLST profile (Table 1). Haploid and diploid serotype D strains from the same patient shared the same MLST profile, even though each patient was infected with an evolutionarily distinct strain (Table 1). Given the observed genotypic diversity of strains (six genotypes recorded for nine patients infected with serotype A strains and five genotypes for six patients infected with serotype D strains), the most likely explanation for the genetic relatedness between the haploid and diploid isolates is either cell fusion between genetically identical cells or endoreplication within the host or in nature.

To determine whether these ploidy changes could have occurred *in vivo*, we infected mice with the Aα haploid single-colony isolate (AD 7-99, patient no. 161) in two independent experiments. Two diploid Aα strains out of 330 single colonies studied were recovered from two mouse brains at 9 and 15 days postinoculation (Fig. 1a). They were genetically identical to AD 7-99 by MLST analysis. Interestingly, while the patient-derived haploid and diploid single-colony isolates were equally virulent in mice, a

TABLE 1 Molecular characteristics of *C. neoformans* isolates causing mixed infections in nine patients with cryptococcosis

Patient no. and original culture no.	Site of isolation	Days since 1st positive culture	Original culture[a] Ploidy[b]	Original culture[a] Serotype/ mating type	Single colonies[a] No. studied	Single colonies[a] Ploidy[b]	Single colonies[a] Serotype/ mating type	Single colonies[a] MLST profile[c] *CAP59/URA5/GPD1/SOD1/LAC1/IGS1/PLB1/TEF1*
188								
AD 1-26	CSF	0	n	Aα	5	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-35	Urine	0	n	Dα	5	n	Dα	1/1/1/1/1/4/3/2
AD 1-36	Blood	0	n	αADα	24	n	Aα	1/1/1/13/3/21/4/4
AD 1-12	Blood	4	n	Aα	5	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-28	Blood	5	n	Aα	4	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-29	CSF	6	n	Aα	4	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-30	BAL fluid	9	n	Aα	4	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-13	CSF	11	n	Aα	5	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-14	CSF	13	n	Aα	4	n	Aα	7/1/1/13/**9**/19/1/1
AD 1-15	Blood	16	n	Aα	4	n	Aα	7/1/1/13/**9**/19/1/1
119								
AD 1-60	CSF	0	n	D**a**	6	n	D**a**	2/3/1/2/1/4/3/3
					4	2n	**a**DD**a**	2/3/1/2/1/4/3/3
AD 1-61	Blood	1	2n	D**a**	4	2n	**a**DD**a**	2/3/1/2/1/4/3/3
161								
AD 1-77	Blood	0	n	Aα	4	n	Aα	1/1/3/13/5/19/2/5
					1	2n	αAAα	1/1/3/13/5/19/2/5
AD 1-76	CSF	1	n	αADα	26	n	Aα	1/1/3/13/5/19/2/5
AD 1-78	CSF	15	n	Aα	5	n	Aα	1/1/3/13/5/19/2/5
20								
AD 3-28	CSF	0	n	αADα	9	n	Aα	7/1/1/13/**9**/19/1/1
AD 3-29	Urine	0	2n	Dα	5	2n	αDDα	1/4/1/1/2/3/2/44
23								
AD 4-5	CSF	0	n	αADα	17	n	Aα	7/2/1/13/2/19/1/1
					5	2n	αDDα	1/1/1/1/2/4/3/2
AD 4-6	CSF	15	2n	Dα	5	2n	αDDα	1/1/1/1/2/4/3/2
177								
AD 4-20	Skin	0	n	Dα	11	n	Dα	1/4/1/1/2/3/2/4
					2	n	Aα	7/1/1/13/9/19/1/1
AD 4-22	Urine	4	2n	Dα	4	2n	αDDα	1/4/1/1/2/3/2/4
					1	2n	αADα	−/−/−/13/−/−/2/5
AD 4-23	CSF	5	2n	Dα	5	2n	αDDα	1/4/1/1/2/3/2/4
71								
AD 4-24	CSF	0	n	D**a**	10	n	D**a**	3/2/1/1/3/1/1/1
AD 4-25	Urine	0	n	D**a**	5	n	D**a**	3/2/1/1/3/1/1/1
AD 4-26	Blood	0	n	D**a**	4	n	D**a**	3/2/1/1/3/1/1/1
					1	2n	**a**DD**a**	3/2/1/1/3/1/1/1
AD 4-27	CSF	14	n	αAD**a**	2	n	Aα	1/1/1/13/3/21/4/3
					11	n	D**a**	3/2/1/1/3/1/1/1
AD 4-64	CSF	0	n	αAD**a**	10	n	Aα	7/1/1/13/**9**/19/1/1
					1	2n	αAD**a**	−/−/−/13/−/−/3/***20***
198								
AD 6-53	Blood	0	2n	αAD**a**	5	2n	αAD**a**	−/−/−/13/−/−/3/***20***
AD 4-58	CSF	0	2n	αAD**a**	5	2n	αAD**a**	−/−/−/13/−/−/3/***20***
AD 5-13	Blood	7	2n	αAD**a**	5	2n	αAD**a**	−/−/−/13/−/−/3/***20***
AD 5-14	Blood	7	n	Aα	5	n	Aα	1/1/3/13/5/19/2/5

[a] Original culture, unpurified culture from clinical sample; single colony, purified single colony from corresponding clinical culture.
[b] Ploidy was determined by flow cytometry; n, haploid; 2n, diploid.
[c] Genotypes were determined for serotype A and serotype D isolates by using eight loci selected by the MLST method. For AD hybrid isolates, allelic profiles were determined for *CAP10* and *URE1* (27) and for *SOD1*, *PLB1*, and *TEF1* loci with serotype A-specific primers specifically designed for this study. All of the AD hybrid and Aα strains studied had the same nucleotide sequences for the *CAP10* and *URE1* loci. New alleles are in bold italics, serotype D alleles are underlined, and serotype A alleles are without underlining.

mouse-derived diploid strain had attenuated virulence (Fig. 1b, $P < 0.001$).

We then assessed whether diploid strains were stable *in vivo*. Groups of mice were inoculated with the clinical diploid Aα strain

TABLE 2 Characteristics of patients with and without mixed cryptococcal infections

Patient characteristic	Value for patients with:		P value
	Mixed infection (n = 9)	No mixed infection (n = 40)	
No. of males/total[a] (%)	9/9 (100)	34/40 (85)	0.577
Mean age (yr) ± SD	44 ± 12	44 ± 14	0.912
No. HIV infected/total (%)	7/9 (78)	30/40 (75)	1.000
No. HIV positive with antiretroviral therapy /total (%)	2/7 (29)	12/29 (41)	0.681
No. born in Africa/total (%)	1/9 (11)	4/40 (10)	1.000
Presentation at baseline			
No. with abnormal neurology/total (%)	4/9 (44)	15/40 (37.5)	0.720
No. with meningoencephalitis/total (%)	9/9 (100)	29/34 (83)	0.319
No. with fungemia/total (%)	5/9 (56)	17/38 (45)	0.715
No. with dissemination/total (%)	7/9 (78)	24/40 (60)	0.454
No. with high serum antigen titer (≥512)/total (%)	4/9 (44)	13/36 (37)	0.716
No. with abnormal brain imaging/total (%)	2/9 (22)	8/29 (26)	1.000
No. with mycological failure at day 15[b]/total (%)	5/8 (62.5)	10/32 (31.2)	0.126
No. who died within 90 days after diagnosis/total (%)	0/8	9/37 (24)	0.179

[a] Total number of patients evaluated or for whom the information was available.
[b] Persistence of viable cryptococci in cultured samples.

or with one of the mouse-derived diploid Aα strains. Out of the 405 colonies studied from brain and lung homogenates plated at various times after inoculation, one haploid colony was discovered. These data show that both positive and negative shifts in ploidy occur *in vivo*.

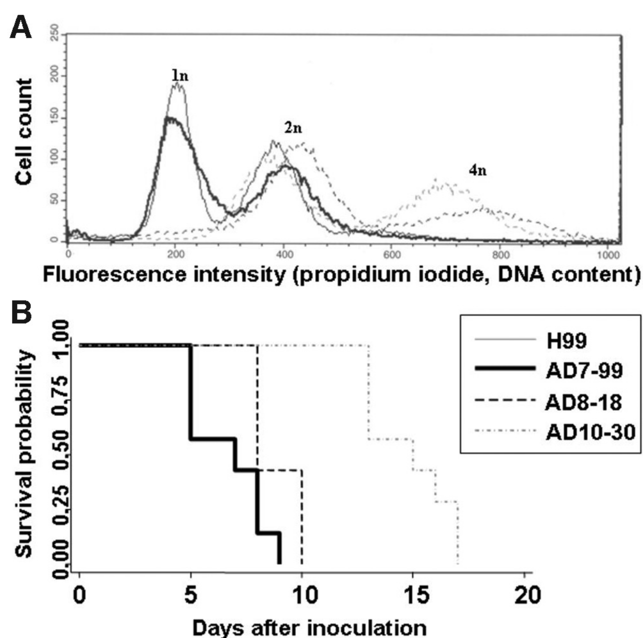

FIG 1 Comparison of DNA contents and virulence of haploid and diploid Aα *C. neoformans* isolates recovered during human or murine infections. (A) Cells were stained with propidium iodide to measure DNA content in comparison with that of reference haploid strain H99. Ploidy was determined for single-colony isolates (AD 7-99 and AD 8-18) purified from clinical sample AD 1-77, as well as a single colony (AD 10-30) recovered from the brain of a mouse inoculated with AD 7-99. (B) Three groups of seven outbred male mice were inoculated intravenously with 10^5 cells of haploid (AD 7-99) or diploid (AD 8-18) strains purified from the clinical sample or the diploid strain (AD 10-30) recovered from the mouse brain. Differences in survival were analyzed using Stata (SE) version 10 (Stata Corporation, College Station, TX). The strains of clinical origin showed no statistically significant differences in virulence (P = 0.056). Mice infected with the diploid strain of murine origin (AD 10-30) survived significantly longer than did mice infected with the strains of clinical origin ($P < 0.001$, log-rank test).

***In vivo* diploidization can result from endoreplication.** For cell fusion to occur *in vivo*, different cell types need to interact. Experimental coinfection with KN99**a** and KN99α inoculated separately revealed that both cell types are visualized in close contact within capillaries (Fig. 2), showing that cell fusion is physically possible. Since ploidy changes could result from cell fusion or endoreplication, a nourseothricin (NAT)-resistant isolate (AD 1-25) was constructed from the wild-type AD 7-99 strain. Analysis of DNA content by flow cytometry following coinfection of six mice with the NAT-resistant and wild-type strains resulted in 11.9% (30/251) diploid NAT-resistant single colonies. These diploid isolates were found in both brains and lungs at days 7 and 15 postinoculation. Quantitative PCR analysis of 30 diploid and six haploid NAT-resistant single-colony isolates was used to determine whether diploid isolates had the same (endoreplication) or half (cell fusion) the amount of *NAT* DNA compared to a control region of the genome present in both cell types (chitin synthase, *CHS1*). Quantitative PCR analysis resulted in equivalent gene copy numbers in both the diploid and haploid isolates (mean crossing point ratio between amplification of the *CHS1* gene and the *NAT* gene = 1.03 ± 0.02 for both haploid and diploid strains [Table 3]), suggesting that the diploid strains resulted from endoreplication and not cell fusion.

DISCUSSION

The concept of one strain/one infection is assumed to be valid for the majority of infectious diseases. Recent studies have used determination of susceptibility to anti-infective drugs, sequence-based approaches, and metagenomic analyses to assess microbial diversity during infection or colonization (31, 32). There is evidence that infections with a number of bacteria, viruses, and parasites may not be clonal. Coinfection with strains of *Plasmodium falciparum* exhibiting different patterns of susceptibility to antimalarial drugs are correlated with treatment failure (33). *Shigella flexneri* strains with multiple plasmids suggest polyclonal infections (34). Sputum samples from patients with active tuberculosis have been shown to contain multiple strains of *Mycobacterium tuberculosis* (35). Different strains of hepatitis C virus or HIV,

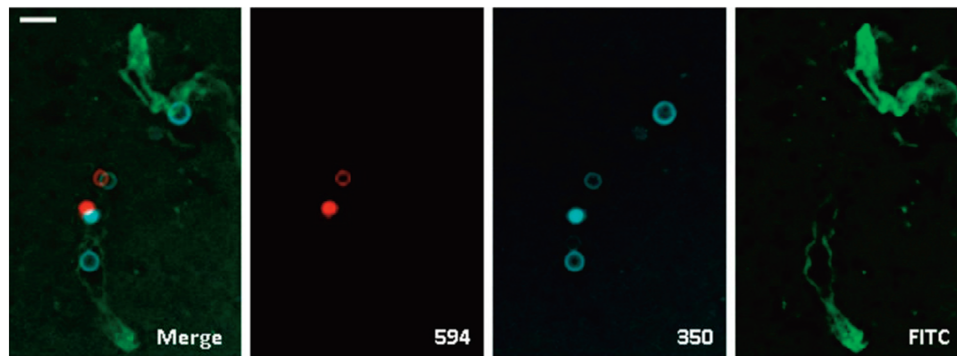

FIG 2 Colocalization of different strains following intravenous inoculation. *C. neoformans* strains KN99**a** and KN99α were stained with Alexa Fluor 594 (red) and 350 (blue), respectively. Mice were inoculated via the tail vein with 5 × 10^6 KN99**a** cells and then 5 × 10^6 KN99α cells. At 24 h postinoculation, the animals were sacrificed and their brains were harvested for immunofluorescence. Frozen sections were immunostained with anti-collagen IV primary antibody with an FITC (green)-labeled secondary antibody to identify capillary endothelial cells. Bar, 20 μm.

possibly representing mixed infections, superinfections, or quasispeciation in the host, have been found in a single patient (36, 37). Here we showed that almost 20% of cryptococcosis cases were associated with multiple strains of the fungal pathogen *C. neoformans*.

Only anecdotal reports of mixed infections have been published previously (6–11). Previous experiments with mice showed that infection with multiple strains of *C. neoformans* inoculated via the intranasal or intravenous route can result in mixed infections in the lungs and/or brain (15, 38, 39). The presence of multiple strains in the environment makes it likely that multiple strains could be acquired by human hosts. Our data showed the presence of multiple varieties of *C. neoformans* (both serotypes A and D), genetically unrelated isolates of the same variety, and AD hybrid strains in the same patient. Because *C. neoformans* can generate latent infections that reactivate upon immunosuppression (1), these different isolates could have been acquired by the patient either simultaneously or over time. Furthermore, less virulent strains (such as serotype D or diploid strains) could be cleared prior to disease manifestation, resulting in the isolation of only the more virulent strains (serotype A *MAT*α) in the majority of patients (6, 20, 27). Furthermore, an experimental model of cryptococcosis has shown that the presence of an established cryptococcal infection does not prevent infection with a second strain, although it can alter the kinetics of the infection (38). The scarcity of mixed-infection reports could also be due to technical bias. Indeed, the routine clinical practice of analyzing single-colony isolates obtained from a single anatomical site would make the discovery of mixed infections unlikely.

In cases where genetically related haploid and diploid strains were found during the course of infection in the same patient, it was important to determine the origin of the diploid strain. These infections could have resulted from either coinfestation with different strains or *in vivo* diploidization. This diploidization could occur through same-sex cell fusion or endoreplication. Coinfection with both haploid and diploid strains could also follow ploidy changes in the environment (21). However, the discovery of stable diploid isolates in murine tissues following inoculation of an Aα haploid isolate demonstrated that changes in ploidy can occur *in vivo*. Previous studies using laboratory-derived isolates had not observed *in vivo* diploidization (29). Our observation of diploidization in clinical isolates is likely due to the overall fitness of the newly isolated clinical strains and their limited subculture in the laboratory. The sources of mixed infections are thus multiple simultaneous or sequential infestations from the environment, as well as ploidy changes during infection progression.

Inoculation of differentially labeled strains and the observation of their proximity within host capillaries show that *in vivo* cell fusion is physically possible. Same-sex fusion has been described *in vitro* for both *C. neoformans* var. *neoformans* (both mating types) and *C. neoformans* var. *grubii* (mating type α) (22, 23). Coinfection with a wild-type Aα haploid isolate and an NAT-

TABLE 3 Evaluation of *CHS1* and *NAT* gene copy numbers for haploid and diploid isolates recovered from infected mouse tissues by quantitative PCR

Strain(s) and DNA amt	*CHS1* Cp[a]	*NAT* Cp	*CHS1*/*NAT* Cp ratio
NAT-resistant haploid AD 1-25			
100 ng	23.99	23.12	1.038
50 ng	25.29	24.99	1.012
20 ng	28.69	27.58	1.040
10 ng	29.70	28.54	1.041
5 ng	30.89	29.88	1.034
2 ng	32.79	31.56	1.039
1 ng	34.78	32.48	1.071
Wild-type haploid AD 7-99	26.27	35	
NAT-resistant haploid isolates from infected tissues ($n = 6$)[b]	27.35 ± 1.48	26.43 ± 1.38	1.03 ± 0.02
NAT-resistant diploid isolates from infected tissues ($n = 30$)[b]	26.43 ± 2.00	25.59 ± 1.78	1.03 ± 0.02

[a] Cp, crossing point.
[b] Values are means ± standard deviations.

resistant construct was used to elucidate whether diploidization *in vivo* resulted from cell fusion and/or endoreplication. Of the 30 diploid NAT-resistant isolates from infected tissue that we analyzed, all had two copies of the *NAT* gene, suggesting that the observed diploidization was due to endoreplication. In a recent report, ploidy changes leading to the production of titan cells with up to eight times the normal DNA content were observed in the lungs of mice (40). It remains unclear whether the diploidization observed in our clinical isolates is related to titan cell formation or occurs via another, unrelated, process.

The demonstration that shuttling between diploid and haploid states occurred *in vivo* reinforced recent findings obtained *in vitro* (21). A mixture of both populations may provide better adaptation to the environment in nature and in the host. Lin and colleagues found that autodiploids represent the most common type of diploid in *C. neoformans* populations in the environment and suggested that their function may be to generate genetic diversity *de novo* during the sexual cycle (21). The ability to produce diploid cells may prove beneficial to cryptococcal cells by allowing rapid evolution of genes (20, 21) or by increasing drug resistance, as shown in *Candida albicans* (41) in response to environmental and host challenges. While we did not detect differences in antifungal susceptibility profiles between our Aα haploid and diploid strains (data not shown), we cannot rule out the possibility that ploidy changes in other strains could explain some treatment failures (Table 2). Interestingly, while drug treatment failure rates may increase in patients with mixed infections, death rates may also decrease, although neither characteristic reached statistical significance. These paradoxical findings could be explained by interactions between coinfecting strains that have been shown to decrease pathogenesis in experimental models (15). However, the relatively small number of patients studied here prevents definitive conclusions about a relationship between a mixed infection and the outcome of the infection. Further studies of humans and in the experimental models are needed to explore this hypothesis.

The discovery that mixed infections are common is a major finding for the management of cryptococcosis and other infections, especially those with environmentally acquired fungi, such as *Histoplasma capsulatum, Blastomyces dermatitidis*, and *Aspergillus* spp. Future drug design—including vaccine—strategies need to take into account the complex nature of cryptococcal infections. The use of unpurified clinical isolates was vital to these experiments. Thus, while Koch's postulates can be used to define that a specific organism is causing a specific class of disease—such as *C. neoformans* causing cryptococcosis—care should be taken in extrapolating these postulates to the clinical setting. Our data suggest, in the context of fungal infections, that clonal infections can cause disease but clinical disease is not always caused by clonal infections. This concept may apply broadly to other microbes and should be considered in the current era when a multiplicity of new tools and approaches can be used to determine the clonality of infections.

MATERIALS AND METHODS

Ethics statement. The CryptoA/D study was approved by the local ethical committee and reported to the French Ministry of Health (registration no. DGS970089). Patients enrolled in the CryptoA/D study gave written informed consent for a systematic workup. Data were analyzed anonymously. All animal experiments were done in accordance with the Institut Pasteur's institutional guidelines (approved protocol CHST no. 03-144).

***C. neoformans* strains.** Clinical cultures were collected during the prospective multicenter CryptoA/D study implemented at the National Reference Center for Mycoses and Antifungals (NRCMA) between 1997 and 2001 (30). The study was approved by the local ethical committee and reported to the French Ministry of Health (registration no. DGS970089). Patients enrolled in the CryptoA/D study gave written informed consent for a systematic workup. This included cultures from CSF, blood, and urine samples, as well as cultures from skin lesions or BAL fluid when appropriate. Original cultures correspond to the cultures obtained in each clinical mycology laboratory after culture of the biological specimens on agar plates or in tubes. These original cultures were directly sent to the NRCMA prior to any purification step or freezing. The original cultures were not purified prior to storage at -80°C and serotyping at the NRCMA. The selection of the 100 original cultures used for the present study was "random" (on a first found/first studied basis) and not based on the patients' characteristics or serotyping results. When available, all of the original cultures from the same patient (recovered from different anatomical sites and at different times during the episode of cryptococcosis) were analyzed. A suspension of approximately 10^3 cells/ml from the original culture was plated on Sabouraud agar plates. Single CFU resulting from the multiplication of a single cell were subcultured for further analysis.

The following *C. neoformans* laboratory reference strains were used for comparison: JEC21 (serotype D *MAT*α [42]), B3502 (serotype D *MAT***a** [43]), H99 (serotype A *MAT*α [44]), KN99**a** (serotype A *MAT***a**), and KN99α (serotype A *MAT*α [19]).

Determination of ploidy using flow cytometry. Cells were prepared for flow cytometry by using the protocol described previously (45). Data were acquired from 30,000 cells using the FL2 channel of a BD FACScan flow cytometer (Becton Dickinson & Company, Franklin Lakes, NJ). The fluorescence-activated cell sorter profile of original cultures or single-colony isolates was compared with that obtained with haploid reference strain H99α to determine ploidy. Analysis was performed using CellQuest software version 3.3 (BD Biosciences, San Jose, CA).

Determination of mating type and serotype using MAT/serotype-specific primers. Original cultures and single-colony isolates were grown for 24 h at 28°C on solid yeast nitrogen base medium. DNA was extracted using the High Pure PCR template preparation kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. PCRs were performed on an iCycler thermocycler (Bio-Rad, Hercules, CA) using primers specific for the serotype A or D SXI1α/SXI2**a** and STE20α/**a** loci to determine the mating type and serotype (25). Results were confirmed using serotype-specific primers for the *PAK1* and *GPA1* genes on all original cultures and selected single-colony isolates (25). Laboratory reference strains were used as positive controls.

MLST. MLST was performed on all Aα isolates from patients with mixed infections. Eight MLST loci (*CAP59, URA5, GPD1, SOD1, LAC1, IGS1, PLB1*, and *TEF1*), including six loci described for *C. neoformans* var. *grubii* by Litvintseva et al. (46) and two chosen by the Isham cryptococcal genotyping working group (47), were examined. Both strands of purified amplified fragments were sequenced at the sequencing facility (PF-8) of the Institut Pasteur on an ABI Prism 3700 DNA analyzer (Applied Biosystems, Courtaboeuf, France), with the same primers that were used in the PCR steps. Sequences edited with Chromas Pro version 1.41 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia) were compared with the allele sequences of the *C. neoformans* var. *grubii* MLST database (http://cneoformans.mlst.net/), and new allelic sequences were submitted.

For serotype D, MLST profiles were determined for the same eight loci. The same primers were used for the PCR amplification and sequencing of seven loci (*CAP59, URA5, GPD1, LAC1, IGS1, PLB1*, and *TEF1*), but for *SOD1*, new primers were designed based on the JEC21 sequences (GenBank accession no. AE017344) (Table 4). For the eight loci, the PCR conditions and the start and end points of the sequences were the same as those for serotype A. The serotype D MLST database is being currently developed at the Imperial College London, and MLST allelic sequences for

TABLE 4 Sequences of primers designed for MLST locus *SOD1* specific for serotype D, for MLST loci *PLB1* and *TEF1* specific for serotype A, for *NAT* insertion, and for quantitative PCR

Locus	Primer	Sequence (5′–3′)	Amplicon size (bp)
SOD1	SOD1Df	TCTAGTCAAAATGGTCAAGG	643
	SOD1Dr	CGCAGCCGTTCGTTTGGACG	
PLB1	PLB1serAf	GCCACGGGTACTTTTGC	821
	PLB1serAr	CGCTCGGGTAAAACATGAG	
TEF1	TEF1f	AATCGTCAAGGAGACCAACG	993
	TEF1serAr	GCTCTTCCCATCGGACTTC	
5′ flanking region for *NAT* insertion	KN0140	TGCTCACATCCTCGCAGCACACCACAGATCCAAAACCC	
	KN0129	GCAACCTTGGTTTGAAGAGC	
3′ flanking region for *NAT* insertion	KN0139	CCGTGTTAATACAGATAAACCCGAAGGGACCAGTCACAAGT	
	KN0122	AACCTGACGGAAGAGCAGAA	
Outside 5′ integration check	KN0177	CGTCATCAAACCTAGCAGCATTCG	
Outside 3′ integration check	KN0176	CTCGAGACCAAGATCAAGATTGCC	
CHS1	CHS1f	GTCCCAGGAGGACTCCTTTC	168
	CHS1r	TGTCGTTCAGGTCGAGTGAG	
NAT	KN0192	GGTGACGCGGAAGACGGTGT	123
	MD001	GTATGGCGGCCGCCACTCT	

serotype D were numbered after discussion with the Isham cryptococcal genotyping working group and will be available online when the serotype D MLST database is established.

For AD hybrid isolates, portions of the *CAP10* and *URE1* loci were amplified according to Lin et al. (27). The MLST locus *SOD1*, amplified with serotype A-specific primers, was also analyzed. In order to improve the typing method for the AD hybrid isolates, new primers specific for serotype A were designed for the *PLB1* and *TEF1* loci (Table 4). For the design of these new primers, nucleotide sequences of serotype D strain B3501 (GenBank accession no. AF238241 for locus *PLB1* and positions 355113 to 355970 of the contig chr13.b3501.040506 from the Stanford database [http://www-sequence.stanford.edu/group/C.neoformans/index.html] for locus *TEF1*) and serotype A strain H99 (GenBank accession no. AF223383 for locus *PLB1*) or M1-106 (GenBank accession no. U81804 for locus *TEF1*) were compared. The PCR conditions were modified as follows: 35 cycles of annealing at 52°C for *PLB1* and 58°C for *TEF1* and then elongation at 72°C for 1 min. For the *SOD1*, *PLB1*, and *TEF1* loci, sequences were also compared with the allele sequences of the MLST database.

NAT-resistant isolate construction. An NAT-resistant isolate of AD 7-99 was generated by gene insertion as previously described (15, 48). The NAT transgene (*NAT*) was inserted into a noncoding region downstream of the *LYS2* gene coding region located on chromosome 5. PCR was used to generate the 5′ (KN0140 and KN0129) and 3′ (KN0139 and KN0122) flanking regions containing linkers to a NAT^r cassette, and overlap PCR was used to generate the full-length *NAT* insertion construct (Table 4). The insertion construct was introduced by biolistic transformation into AD 7-99 to generate NAT-resistant strain AD 1-25. Proper insertion was verified by PCR using primers external to the integration site (KN0177 and KN0176) and Southern hybridization (not shown).

Experimental infections. Yeast suspensions for inoculation were prepared from single-colony isolates after subculture for 18 h in liquid yeast extract-peptone-dextrose (YPD) with or without NAT (100 ng/ml; Werner Bioagents, Germany) and washed three times in phosphate-buffered saline (PBS; Sigma). Six-week-old outbred OF1 male mice (Charles River, L'Arbresle, France) were inoculated intravenously. Mice were euthanized by CO_2 inhalation at various times after inoculation or when unable to reach food because of disease progression. Organs were aseptically removed, homogenized in 1 ml sterile PBS, and plated on Sabouraud agar containing 0.5 mg/ml chloramphenicol and/or on YPD agar containing NAT at 100 ng/ml.

To analyze whether two cells of *C. neoformans* could come into close contact within the host, congenic strains KN99**a** and KN99α (19) were stained as described in reference 40, with Alexa Fluor 594 (red) or 350 (blue), respectively. Mice were inoculated via the tail vein with 5×10^6 KN99**a** (red) cells and then 5×10^6 KN99α (blue) cells. At 24 h postinoculation, animals were sacrificed and perfused with 20 ml PBS and then 20 ml 4% paraformaldehyde (PFA). Brains were harvested, placed in 4% PFA, followed by a 40% (wt/vol) sucrose solution in PBS, frozen in isopentane and liquid nitrogen, stored at −80°C, and cut into 50-μm sections. Slides were washed in PBS for 15 min and then incubated with 100 μl trypsin-EDTA (Invitrogen) at 37°C for 10 min. Slides were then washed in PBS containing 20% fetal calf serum (Invitrogen) for 10 min, blocked with PBS containing 20% fetal calf serum, 0.1% bovine serum albumin (BSA), and 0.1% Triton X-100 (Sigma, St. Louis, MO) for 20 min, and then washed with PBS containing 0.1% Triton X-100. Anti-collagen IV antibody (Chemicon) was diluted 1:50 in PBS with 0.1% BSA and 0.1% Triton X-100. Antibody-treated slides were incubated overnight at 4°C and then washed in PBS. Fluorescein isothiocyanate (FITC; green)-labeled goat anti-rabbit antibody (Invitrogen) was diluted 1:200 and added to the slides. After 3 h of incubation at 37°C, the slides were washed three times in PBS for 15 min and mounted in Vectashield mounting medium. Slides were imaged by fluorescence microscopy, and *z* stack images were captured with an Apotome-equipped Zeiss Axioplan microscope. Extended focus was used to visualize the relationship between cells within the *z* stack using Zeiss AxioVision software.

To study ploidy changes *in vivo*, eight mice were inoculated in two independent experiments with 10^3 cells of AD 7-99, a haploid serotype A *MAT*α single-colony isolate from the original strain AD 1-77 culture (patient no. 161). Mice were sacrificed at 3, 7, 9, and 15 days postinoculation. Spleens, lungs, and brains were plated on Sabouraud agar. The number of CFU was determined, and single-colony isolates (10 to 12 for spleens or lungs, 24 to 56 for brains) were analyzed for DNA content.

For survival studies, mice (seven/group) were inoculated with 10^5 cells of strain AD 7-99 (haploid), a diploid single-colony isolate from patient no. 161 (AD 8-18), or a diploid single-colony isolate from a mouse brain (AD 10-30). Mice were monitored twice daily, and those in pain or sick were sacrificed by CO_2 inhalation.

Coinfection with AD 1-25 and AD 7-99 was used to assess whether diploid isolates resulted from endoreplication or cell fusion. AD 1-25 was compared to AD 7-99 for stability and virulence *in vivo*. CFU counts on Sabouraud agar containing 0.5 mg/ml chloramphenicol and YPD agar containing 100 μg/ml NAT at 7 and 15 days postinoculation were compared and found to be equivalent. Mice ($n = 6$) were then coinoculated with 10^4 cells each of AD 7-99 (wild type) and AD 1-25 (NAT resistant). Mice were sacrificed at 7 and 15 days postinoculation. Lungs and brains were removed, homogenized, and plated on YPD agar containing 100 μg/ml NAT in order to select isolates resistant to NAT. CFU were enumerated, and single-colony isolates (at least 20 per organ) were analyzed as described above for ploidy determination.

To check whether diploid strains were stable *in vivo* or were only a transient stage, two groups of seven mice were inoculated with the diploid strain recovered from the clinical culture (AD 8-18) and from the mouse brain (AD 10-30). Mice were sacrificed at 3, 7, and 15 days postinoculation. Lungs and brains were removed, homogenized, and plated on Sabouraud-chloramphenicol agar. CFU were enumerated, and single-colony isolates (at least 16 per organ) were analyzed as described above for ploidy determination.

Quantitative PCR. To determine if diploid isolates resulted from endoreplication or cell fusion between AD 7-99 (wild-type isolate) and AD 1-25 (NAT resistant), quantitative PCR was performed. DNA was extracted as described above from 30 diploid and 6 haploid NAT-resistant isolates recovered from coinfected mice. Primers were designed to amplify part of the *CHS1* gene, located on chromosome 8, as an internal control (Table 4). Because *CHS1* is present in both the AD 7-99 and AD 1-25 strains, two copies of the gene should be present in the diploid cells, irrespective of whether they were formed by endoreplication or cell fusion. Primers were designed to amplify part of the *NAT* gene (chromosome 5) (Table 4). The *NAT* gene is present only in AD 1-25, so diploid cells generated by endoreplication would have two copies, whereas those generated by cell fusion between AD 7-99 and AD 1-25 would have only a single copy. Quantitative PCR was performed with a LightCycler LC480 (Roche Applied Science, Germany) in a final volume of 20 μl containing 0.75 μl of each primer at 10 μM, 10 μl of Sybr green master mix 2$\times$, 9.25 μl of water, and 2 μl of DNA. The conditions were similar for the amplification of both the *CHS1* and *NAT* insertions: 5 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C, and then 1 min at 95°C and 1 min at 60°C with a melting curve temperature range of 55°C to 95°C. Haploid parental strain AD 1-25 was used for a standard curve using known DNA amounts of 100 ng, 50 ng, 20 ng, 10 ng, 5 ng, 2 ng, and 1 ng determined with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). For each isolate, the ratio of the crossing points obtained for amplification of the *CHS1* gene and the *NAT* gene was calculated to determine if the diploid isolates had the same (endoreplication) or half (cell fusion) the amount of *NAT* DNA compared to *CHS1* DNA.

Statistical analysis. Comparisons between groups were done using chi-square or Fisher's exact tests for categorical variables and Student's *t* test or one-way analysis of variance for continuous variables. Survival curves were compared by the log-rank test. Data were analyzed using Stata Statistical Software (Stata 10.0; Stata Corporation, College Station, TX). *P* values of less than 0.05 were considered significant.

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