Original Article

Atypical *Cryptococcus neoformans* Isolate from an HIV-Infected Patient in Brazil

Jun Uno¹, Reiko Tanaka¹, Maria Luiza Moretti Branchini², Francisco Hideo Aoki²,
Kyoko Yarita¹, Ayako Sano¹, Kazutaka Fukushima¹, Yuzuru Mikami¹,
Kazuko Nishimura¹, Makoto Miyaji¹

¹ Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University,
1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

² Infectious Diseases Division, Faculty of Medical Science, the State University of Campinas,
São Paulo, Brazil

[Received: 9, January 2001, Accepted: 17, March 2001]

Abstract

Cryptococcus neoformans is an important fungal pathogen in immunocompromised hosts. Capsulation, urease and melanin synthesis activity of the fungus are well known virulence factors. Although artificial melanin-deficient mutants of *Cr. neoformans* have been investigated, the clinical mutant is rare. We found a *Cr. neoformans* isolate in the cerebrospinal fluid of an AIDS patient which produced a light tan colony on a caffeic acid cornmeal agar (CACA) plate. The mycological feature of the isolate was as follows; normal capsulation, defective inositol assimilation ability, serotype A; urease-positive; mating type α ; haploid; extremely slow growth in RPMI 1640 medium, Sabouraud dextrose broth, brain heart infusion broth and yeast nitrogen base; lower production of melanin with L-DOPA substrate; and low virulence to ddY mice. We also investigated the partial DNA sequence of *CNLAC1* gene between the 3085th to 3623rd base. There were many substitutions, 3 insertions and 3 deletions in the isolate compared with GenBank accession number L22866. The result indicated some functional disorder in the gene. Although the CACA plate is an excellent selective medium for *Cr. neoformans*, other identification methods should also be used.

Key words: *Cryptococcus neoformans*, HIV, melanin, mutant

Introduction

Cryptococcosis is caused by *Cryptococcus neoformans* and is one of the most important opportunistic fungal infections in immunocompromised hosts¹⁾. In particular, in AIDS patients approximately 6% suffer from cryptococcosis²⁾. As virulence factors of the fungus, capsulation, urease and melanin synthesis activity are well recognized³⁻⁷⁾. Furthermore, there are many reports on artificial melanin-deficient mutants which have lower virulence^{4, 7-9)}. However, there is no study on the relationship between melanin-synthesizing activity and pathogenicity on the clinical melanin-deficient isolate except for a report by Blackstock

*et al.*⁷⁾. This time we succeeded in isolating a poor melanin producing *Cr. neoformans* isolate from an HIV-infected patient. The isolate was a slight tan color on cornmeal agar supplemented with a caffeic acid plate. The present report deals with mycological and physiological characteristics of the isolate and its pathogenicity. In addition, a partial sequence of the laccase gene (*CNLAC1*)¹⁰⁾ which promotes diphenol oxidase of *Cr. neoformans* for melanin production was analyzed.

Materials and methods

Isolate: A *Cr. neoformans* (C14) was isolated from the cerebrospinal fluid of an AIDS patient at the Infectious Diseases Division of the Universidade Estadual de Campinas, São Paulo, Brazil. The patient was a 29-year-old female with AIDS (stage C3), which had been diagnosed in August 1995. She received the anti-retroviral drugs,

Address correspondence to: Jun Uno

Research Center for Pathogenic Fungi and Microbial
Toxicoses, Chiba University.

1-8-1, Inohana, Chiba, Chuo-ku, Chiba 260-8673, Japan.

zidovudine (AZT) and didanosine (DDI). During her follow-up, she suffered oropharyngeal candidiasis and disseminated herpes infection. In June 1996, she complained of symptoms of meningitis with severe headache and underwent a cerebrospinal fluid examination by a lumbar puncture. Then the encapsulated fungal cells were directly observed in the fluid under a light microscope with an Indian black ink mount. Some shiny and mucoid fungal colonies grew on Sabouraud dextrose agar plates. Although the patient was treated with the antifungal drugs fluconazole and amphotericin B, she died of cerebrospinal meningitis in October 1996. The isolate showed no specific feature in the randomly amplified polymorphic DNA analysis as previously reported¹¹⁾.

As control isolates, IFM 5844 (=B-3501 (NIH), mating type α) and IFM 5845 (=B-3502 (NIH), mating type α) were used. All isolates were cultured on potato dextrose agar (Difco, Detroit, USA, PDA) slants at 30°C for 48 hours except for the measurement of melanin synthesizing activity.

Color on cornmeal agar supplemented with caffeic acid: The production of melanin on cornmeal agar (Difco) plate supplemented with 0.03% caffeic acid (Sigma-Aldrich Japan, Tokyo, Japan) described by Kaufman and More¹²⁾ (CACA) with 0.5% agar (Difco) added at 30°C for 4 days was observed. The medium has an advantage for morphological observation of yeasts and production of melanin simultaneously. As a negative color control, a *Candida albicans* isolate (7N=IFM 40009=ATCC 48130) was used.

Mycological study: A capsule was observed under a light microscope with Indian black ink mounting. Identification of the isolate was examined with ID32C (bioMérieux, France). The serotype was determined by CRYPTO Check (Iatron, Japan). Urease activity was performed on Christensen's urea agar slants (Eiken, Japan) at 30°C for 4 days, and a mating test was conducted with IFM 5844 and IFM 5845 on a hay infusion agar plate at 25°C for 4 weeks. The ploidy was measured by flow cytometry with fluorescence staining¹³⁾. Growth rate was measured by Bioscreen C (Labosystem, Finland). Briefly, cell suspensions with 10^4 cells/ml were cultured with RPMI 1640 (Life Technologies, NY, USA), Sabouraud dextrose broth (SDB), 2% glucose added brain heart infusion (BHI, Difco) and antibiotic medium 3 (AM3, Difco). Optical density (OD) was measured every 2 hours at 35°C for 7 days.

Phenol-oxidase activity: Phenoloxidase activity was evaluated by the method of Rhodes¹⁴⁾ with a slight modification. Cryptococcal cells were grown in 20 ml of yeast nitrogen base (YNB) broth containing 0.1% glucose for 48 hours at 25°C on a rotary shaker. The cells were centrifuged and washed once with YNB broth without glucose, then resuspended in 20 ml YNB broth and incubated for 5.5 hours on a rotary shaker at 25°C. The cells were centrifuged and gross volume was measured. One ml of 50 mM sodium phosphate buffer (pH 7) was added to each 50 μ l volume of cells and concentration of the cells was adjusted to 2.0×10^8 /ml. One hundred μ l of toluene: ethanol (1:4, vol: vol) was added for each ml of cell suspension and the suspension was vortexed for 90 sec. Three ml of L-DOPA was added to 200 μ l of the above cell suspension at the final concentration of 1 mM. The mixture was cultured at 30°C for 60 min. Then, the cells were removed by centrifugation and the melanin concentration in the supernatant was determined by the optical density with 480 nm in duplicate. As the negative control, a sample consisting of sodium phosphate buffer with L-DOPA was used.

Pathogenicity in ddY mice: One group was composed of 5 male ddY mice 6 weeks old. The mice received 10^6 fungal cells suspended in physiological saline intravenously. The mortality was recorded up to 28 days after inoculation.

Susceptibility to antifungal drugs: Susceptibility to amphotericin B (AMPH), itraconazole (ICZ), fluconazole (FLCZ), ketoconazole (KCZ), miconazole (MCZ) and flucytosine (5FC), were evaluated by NCCLS (National Committee for Clinical Laboratory Standards) method¹⁵⁾. ATCC 90112 and ATCC 90113 were used as controls. Although the NCCLS method urged the use of RPMI 1640 at 35°C for 3 days, the isolate C14 grew so slowly in the medium that the present evaluation was completed after 7 days, in Sabouraud dextrose broth.

Laccase gene (*CNLAC1*) sequence: DNA was extracted with a DNA extraction kit (Gen Toru Kun™ for yeasts and gram positive bacteria (TaKaRa Shuzo Co. Ltd., Ohtsu, Japan)). The partial sequence of *CNLAC1*¹⁰⁾ (GenBank accession number L22866) was compared. The primer set was designed from the complete sequence of *CNLAC1* from the 3044th to 3668th base. The primers were as follows: S1: 3044-3068; 5'-TGC ACA GCT ACT GGT TCC TCG TCA T-3' corresponding to the 3044th to 3068th base and

AS1: 3668-3644; 5'-CAC AAA GGC TCC TCG TTC GCT ATT G-3' corresponding to the complementary sequence from 3644th to 3668th. Amplification was performed by initial heating at 94°C for 1 min, 30 PCR cycles with annealing at 50°C for 1 min, extension at 72°C for 2 min, denaturation at 94°C for 1 min and final extension at 72°C for 10 min. The amplified DNA was purified with SUPRECTM-02 (TaKaRa Shuzo) according to the manufacture's instructions. Amplified DNA was visualized by electrophoresis in 1.5% agarose in 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) and staining with ethidium bromide. The PCR amplified sample was directly sequenced in both strands using a Thermo Sequence II Dye terminator cycle sequencing kit (Amersham Pharmacia, Piscataway, NJ, USA) and an ABI PRISM™ 377 sequencer. The nucleotide sequence data were analyzed with genetic information processing software, GENETYX-MAC Ver. 10.1.0r3 (Software Development, Tokyo, Japan). We determined that the nucleotide sequence corresponded to from the 3085th to 3623rd base of *CNLAC1*.

Results

The isolate C14 showed a light tan colony on the CACA plate. The control *C. albicans* isolate showed a white color colony and those of *Cr. neoformans* isolates were dark brown on the plate (Fig. 1). The isolate C14 had the lowest phenoloxidase activity with L-DOPA substrate, and had a capsule visible under light microscopy with Indian black ink mount. Thickness of the capsule did no difference from control isolates; its ID32C profile corresponded to *Cr. neoformans*. But, in particular, isolate C14 lacked assimilation ability for inositol. Other characteristics of the C14 isolate were as follows: Serotype was A, urease activity was positive, mating type was α ,

and the ploidy was haploid. The mortality of ddY mice of isolates IFM 5844, IFM 5845 and C14 was 100%, 40% and 0% at 28 days after inoculation, respectively (Table 1).

The isolate C14 grew extremely slowly in RPMI 1640, SDB, BHI and YNB. Figure 2 indicates the growth of *Cr. neoformans* isolates in RPMI 1640 medium. The growth in SDA was the same as in RPMI 1640. The starting time for increasing of OD in RPMI 1640 on isolates IFM 5844, IFM 5845 and C14 was 8, 10 and 58 hours after incubation, respectively. That in SDB was 15, 16 and 26 hours, respectively. The time for double density of OD in RPMI 1640 on isolates IFM 5844, IFM 5845 and C14 was 36, 30 and 157 hours after incubation, while that in SDB was 35, 47 and 120 hours, respectively. Optical densities in BHI and YNB in contrast, showed a slight increase at the ending point (data not shown).

Fig. 1. The production of melanin on cornmeal agar plate supplemented with 0.03% caffeic acid (CACA) cultured at 30°C for 4 days.

Table 1. Characteristics of the isolate

Isolate	IFM 5844	IFM 5845	C14
Color on CACA plate	Dark brown	Dark brown	Light tan
Phenoloxidase activity assay (Mean optical density at 480 nm)	0.038	0.027	0.008
Capsulation	+	+	+
ID32C profile	5576-7653-37	5777-7651-37	5566-7643-11
Serotype	D	D	A
Urease activity	+	+	+
Mating type	α	a	α
Ploidy	Haploid	Haploid	Haploid
Virulence in ddY mice 28 days after inoculation	100%	40%	0%

Fig. 2. Growth rate measured by Bioscreen C. Cell suspensions with 10^4 cells/ml were cultured with RPMI 1640. Optical density (OD) was measured every 2 hours at 35°C for 7 days.

Table 2. Susceptibility for antifungal drugs in Sabouraud dextrose broth

Strain	MIC ($\mu\text{g/ml}$)					
	AMPH	ICZ	FLCZ	MCZ	KCZ	5FC*
C14**	0.25	0.008	1	0.06	0.06	NG***
ATCC 90112	1	0.25	16	0.13	1	0.13
ATCC 90113	1	0.06	4	0.06	0.13	>64

* Evaluated in yeast nitrogen base with 0.5% glucose.

** Isolate C14 was determined at day 7.

*** No growth.

Regarding susceptibility to antifungal drugs, isolate C14 had no resistance against any drug (Table 2).

The partial DNA sequence of isolate IFM 5844 was equivalent to *CNLACI* between the 3085th and 3623rd base. There were 7 substitutions in isolate IFM 5845, while isolate C14 had many substitutions, 3 insertions and 3 deletions (Fig. 3). The homology between IFM 5844 and IFM 5845 was 98.7%, between IFM 5844 and C14 was 87.5%, and between IFM 5845 and C14 was 87.6%.

Discussion

Fortunately, in the present case the fungal cells were observed directly under a light microscope and could be isolated. If isolation were only performed on a CACA plate which is recommended for a selective medium for *Cr. neoformans*¹²⁾, it would be impossible to isolate C14. Furthermore, extremely slow growth also made it hard to

isolate *Cr. neoformans* from the samples of immunocompromised hosts. To date, *Cr. neoformans* mutants with pseudophyphae¹⁶⁾ and with negative urease activity¹⁷⁾ have been reported from AIDS patients, therefore we should watch for various mutations of *Cr. neoformans* isolates from immunocompromised hosts.

Melanin synthesizing and urease activities are known to be important factors for the virulence of *Cr. neoformans*³⁻⁷⁾. Furthermore, there are many reports on the correlation between melanin synthesizing activity and virulence of this pathogen^{2,4,6,7)}. The isolate C14 also had a low virulence in mice and the lowest melanin synthesizing activity. Rhodes *et al.* reported that the growth rate and melanin synthesizing activity were not relevant¹⁸⁾, but our results indicated a correlation between these two factors.

In addition to melanin synthesis, mannitol and inositol assimilation abilities are also important virulence factors in *Cr. neoformans*¹⁹⁾. The action

IFM 5844	1	TCCGGAGATCCAAGTGCCCGTCAACTGCAGGGTTCGTCTGCGCTTTATCAGCGCGACCGC	60
IFM 5845	1		60
C14	1	...C...T..C...A...T.....T.....	60
		.**.*.*****.*****.*****.*****.*****.*****.*****	
IFM 5844	61	CCATCCCATGTACCGCATATCTATCGACAACCACCCTATGGAAGTTGTGGAGGCCGATGG	120
IFM 5845	61		120
C14	61	A.....T.....AA...C..	120
		*****.*****.*****.*****.*****.*****.*****.*****	
IFM 5844	121	TACAGCCGTCTATGGGCCGACAGT-CCATGAAATCTCTGTCGCACCTGGGGAACGGTACT	179
IFM 5845	121	...T.....-.....CA.....C.....	179
C14	121	C...C.....CA.T.....	180
		.**.*****.*****.*****.*****.*****.*****	
IFM 5844	180	CTGCAATTATCAACACCAATGAAGGGAAGAAGGTGATGCGTTTTGGCTGAGGACTAGCG	239
IFM 5845	180		239
C14	181	TCA.....G.....C.....A..T.	240
		*****.*****.*****.*****.*****.*****.*****.*****	
IFM 5844	240	TTGCTCTAAGCTGTATGTTTGGTGCAGTAAGTCAGGAGGGATTGGCGGTGGTGAAGTATA	299
IFM 5845	240		299
C14	241	GG.....G.A..GA...T.....T.....	300
		*****.*****.*****.*****.*****.*****.*****.*****	
IFM 5844	300	CGGGTAATGGAATGGTTAGTACTGAGGAGCCTCAGACTTCTGCTTGGTGAGTTACCGAAA	359
IFM 5845	300	C.....	359
C14	301	A.....A...A.....T.GG.	360
		*****.*****.*****.*****.*****.*****.*****.*****	
IFM 5844	360	AATCTC-AGGTTGAACTGGCACTGACAATATATTCAGGAGTGATCTAGCGGGAGTTACCG	418
IFM 5845	360	-.....G.....	418
C14	361	..C..GT.....C.G.....C...AA	420
		..*****.*.*****.*****.*****.*****.*****	
IFM 5844	419	TTCCATGTACTGGACTGGACCAACATATACTCTTCGTGAGCATTGGTTGATTTCTCTT	478
IFM 5845	419		478
C14	421	C...T...G.....C...C.....T..	480
		..*****.*****.*****.*****.*****.*****	
IFM 5844	479	GTTTGAATTTTTTACTAATAACTACGTAGACCACGAGACAGTCTTAGTGCACC-TCGGG	537
IFM 5845	479	C.....-.....	537
C14	481	T.C..AC..A...ACCACT.--.....G.--.....T...T.	537
		*****.*.**..**.*.*****.*****.*****.*****.*****	
IFM 5844	538	AA	539
IFM 5845	538	..	539
C14	538	..	539
		**	

Fig. 3. The comparison of partial DNA sequence of *CNLAC1* from the 3085th to 3623rd base.

of mannitol has been hypothesized as being either an osmoticum or an antioxidant to promote cryptococcal infection¹⁹⁾, while inositol is a key cellular metabolite that participates in the formation of membrane lipids and the signal transduction mechanism¹⁹⁾. Namely, it promotes the growth of *Cr. neoformans*. Since isolate C14 lacked inositol assimilation ability as determined by the ID32C test, the defect might affect the slow growth and lower virulence. Furthermore, it is reported that melanin exists in the inner cell

wall of *Cr. neoformans*²⁰⁾. There might be some correlation between poor melanin synthesis and lack of inositol assimilation ability. In any event, the biochemistry of melanin and the assembly of melanin on the cell wall remain poorly understood²⁰⁾.

We investigated a partial sequence of *CNLACI* gene of the isolate C14 and there were many substitutions, 3 insertions and 3 deletions. The results indicate that the isolate C14 might have some functional disorder in the laccase gene.

Furthermore, although anti-retroviral drugs have been reported as no influence on fungi²¹⁾, isolate C14 might receive some mutagenic effect on the gene from such drugs. The effects of anti-retroviral drugs of various *Cr. neoformans* isolates are under investigation.

In conclusion, we should recognize that *Cr. neoformans* isolates from AIDS patients might produce mutants.

Acknowledgements

We acknowledge the support of the Japan International Cooperative Agency (JICA) for the Clinical Research Project in the State University of Campinas (UNICAMP) in Brazil, and the coordination of Drs. Kei-ichi Yamamoto, Ademar Yamanaka and Mr. Tatsuo Watanabe. This work was also supported by the Japanese Ministry of Education, Science, Sports and Culture through grant No. 10307006.

References

- 1) Mitchell TG, Perfect JR: Cryptococcosis in the era of AIDS-100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 8: 515-548, 1995.
- 2) Perfect JR, Wong B, Chang YC, Kown-Chung KJ, Williamson PR: *Cryptococcus neoformans*: virulence and host defences. Med Mycol 36: Supplement 1, 79-86, 1998.
- 3) Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect J: Urease as a virulence factor in experimental cryptococcosis. Infect Immun 68: 443-448, 2000.
- 4) Kown-Chung KJ, Rhodes JC: Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect Immun 51: 218-223, 1986.
- 5) Torres-Guererro H, Edman JC: Melanin-deficient mutant of *Cryptococcus neoformans*. J Med Vet Mycol 32: 303-313, 1994.
- 6) Kozel TR: Virulence factors of *Cryptococcus neoformans*: Trends in Microbiol 3: 295-299, 1995.
- 7) Blackstock R, Buchanan KL, Cherniak R, Mitchell TG, Wong B, Bartiss A, Jackson L, Murphy JW: Pathogenesis of *Cryptococcus neoformans* is associated with quantitative differences in multiple virulence factors. Mycopathol 147: 1-11, 1999.
- 8) Kown-Chung K, Polacheck I, Popkin TJ: Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J Bacteriol 150: 1414-1421, 1982.
- 9) Salas SD, Bennett JE, Kown-Chung KJ, Perfect JR: Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans*. J Exp Med 184: 377-386, 1996.
- 10) Williamson PR: Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: Identification as laccase. J Bacteriol 176: 656-664, 1994.
- 11) Aoki FH, Imai T, Tanaka R, Mikami Y, Taguchi H, Nishimura NF, Nishimura K, Miyaji M, Schreiber AZ, Branchini MLM: New PCR primer pairs specific for *Cryptococcus neoformans* serotype A or B prepared on the basis of random amplified polymorphic DNA fingerprint pattern analysis. J Clin Microbiol 37: 315-320, 1999.
- 12) Kaufmann CS, Merz WG: Two rapid pigmentation tests for identification of *Cryptococcus neoformans*. J Clin Microbiol 15: 339-341, 1982.
- 13) Tanaka R, Taguchi H, Takeo K, Miyaji M, Nishimura K: Determination of ploidy in *Cryptococcus neoformans* by flow cytometry. J Med Vet Mycol 34: 299-301, 1996.
- 14) Rhodes JC: A simplified assay for cryptococcal phenoloxidase. Mycologia 78: 867-868, 1986.
- 15) National Committee for Clinical Laboratory Standards (1997) Reference method for broth dilution antifungal susceptibility testing of yeast, Approved Standard. NCCLS Document M27-A Villanova, Pennsylvania, USA.
- 16) Williamson JD, Silverman JF, Mallak LC, Christie JD: Atypical cytomorphic appearance of *Cryptococcus neoformans*. Acta Cytologia 40: 363-370, 1996.
- 17) Ruane PJ, Walker LJ, George WL: Disseminated infection caused by urease-negative *Cryptococcus neoformans*. J Clin Microbiol 6: 2224-2225, 1988.
- 18) Rhodes JC, Polacheck I, Kown-Chung KJ: Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. Infect Immun 36: 1175-1184, 1982.
- 19) Casadevall A, Perfect JR: 6. Virulence factors. In *Cryptococcus neoformans*. (Casadevall A, Perfect JR, ed.) p.71-114, ASM PRESS, Washington, D.C., 1998.
- 20) Casadevall A, Perfect JR: 4. Biochemistry. In *Cryptococcus neoformans*. (Casadevall A, Perfect JR, ed.) p.145-176, ASM PRESS, Washington, D.C., 1998.
- 21) Diz P, Ocampo A: Lack of ritonavir antifungal effect *in vitro*. Antimicrob Agents Chemother 43: 997, 1999.