Case Report

Disseminated cryptococcosis in an AIDS patient caused by a canavanine-resistant strain of *Cryptococcus neoformans* var. *grubii*

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A case of disseminated cryptococcosis caused by *Cryptococcus neoformans* var. *grubii* is presented in a male diabetic who had AIDS. The diagnosis was based upon the isolation and identification of the aetiological agent from a lymph-node biopsy, cerebrospinal fluid and sputum. The isolate formed spherical, encapsulated yeast cells, produced cherry-brown colonies on niger-seed agar, grew on canavanine-glycine-bromothymol blue (CGB) medium, changing its colour from greenish yellow to blue, and hydrolysed urea weakly in the presence of 100 µM EDTA. The strain was unable to assimilate D-proline and, serologically, it was untypable. The identity of the isolate as *C. neoformans* var. *grubii*, serotype A, possessing a mating-type allele Aα, was confirmed by crossing with standard laboratory test strains and by performing PCR with the mating-type α allele-specific primer of the *STE12* gene and with serotype (A and D)- and mating type (a and α)-specific primers of the *STE20* gene. To the best of our knowledge, this is the first report of disseminated cryptococcosis in an AIDS patient caused by a canavanine-resistant strain of *C. neoformans* var. *grubii*, serotype A, possessing mating type allele Aα; the strain is probably a hybrid. The report suggests that, in the absence of a clear-cut serotyping result, a positive CGB reaction alone is not sufficient for intervarietal discrimination and additional confirmatory evidence is required.

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Introduction

Cryptococcosis is a leading mycological cause of morbidity and mortality among patients with AIDS (Powderly, 1993). About 5–10% of AIDS patients develop cryptococcosis in the United States, Europe and Australia, whereas its incidence is much higher in countries of sub-Saharan Africa (15–30%) (Mitchell & Perfect, 1995). It is estimated that 10–25% of AIDS patients with cryptococcosis die in spite of antifungal therapy, and 30–60% succumb within the first year of onset. Based on phenotypic and genotypic characteristics, the aetiological agent *Cryptococcus neoformans* represents three varieties, *grubii* (serotype A), *gattii* (serotypes B and C) and *neoformans* (serotype D) (Casadevall & Perfect, 1998; Franzot *et al.*, 1999; Xu *et al.*, 2000). Being a heterothallic organism, the mating system in *C. neoformans* is controlled by one locus with two alternative functional mating-type alleles, *MATa* and *MATα*. Determination of mating types by crossing with standard

test strains and performing PCR with mating type allele-specific primers is being used as the most reliable tool for characterization of *C. neoformans* strains (Casadevall & Perfect, 1998; Halliday *et al.*, 1999; Yan *et al.*, 2002). In this communication, we report a case of disseminated cryptococcosis in an AIDS patient caused by a canavanine-resistant strain of *C. neoformans* var. *grubii*, serotype A, with the *MATα* allele.

Case report

A 56-year-old diabetic Kuwaiti male reported at Al-Amiri Hospital, Kuwait, in the first week of May, 1996, with complaints of progressive loss of weight and appetite, fever and dysphagia of 2 months duration. Physical examination revealed swelling on the right side of the neck involving cervical lymph nodes and sliding axial hiatal hernia. Barium swallow and endoscopic examinations of the oesophagus revealed narrowing of the passage and presence of patchy, whitish plaques throughout its length. Biopsies taken from the oesophageal mucosa for direct microscopic examination and culture established the diagnosis of *Candida* oesophagitis due to *Candida albicans*. Examination of a fine-needle

Abbreviations: CGB, canavanine-glycine-bromothymol blue; CT, computerized tomography; HIV, human immunodeficiency virus; SDA, Sabouraud's dextrose agar.

aspirate from the right upper cervical lymph node showed granulomatous changes, and a diagnosis of tuberculosis was suspected.

On readmission 7 weeks later, an excision biopsy taken from the same site revealed many encapsulated yeast cells suggestive of *C. neoformans* (Fig. 1). Culture of the biopsy material on Sabouraud's dextrose agar (SDA) yielded pure growth of *C. neoformans*, confirming the diagnosis of cryptococcosis. At this point, he was tested for human immunodeficiency virus (HIV) infection and was found to be positive. His CD4⁺ lymphocyte count was very low (50 µl⁻¹) and the CD4⁺/CD8⁺ ratio was severely reversed (1:62). In the meantime, he had developed productive cough and neurological symptoms that included headache, dizziness and occasional syncopic attacks. Cerebrospinal fluid examination showed many encapsulated yeast cells in an India ink preparation and *C. neoformans* was isolated in culture on SDA. Computerized tomography (CT) of the head revealed an ill-defined hypoechoic lesion at the right dorsoparietal area and cerebral atrophy. Fundi could not be examined due to early onset of cataract. X-rays and a CT scan of the chest revealed bilateral pulmonary infiltrates at the mid and lower zones and a thick-walled cavity (about 10 cm in diameter) in the left mid and lower zones (Fig. 2a–c). Direct microscopic examination of the sputum in 20% KOH showed many yeast cells with a well-developed capsule and culture yielded growth of *C. neoformans*, along with many colonies of *Staphylococcus aureus*. Culture for acid-fast bacilli was negative and *Pneumocystis carinii* was not seen in Gomori methenamine silver-stained smears. The patient was moved to the Infectious Diseases Hospital in mid-July. On admission, he was conscious, oriented and febrile (38 °C). His pulse was 96 min⁻¹, respiration 24 min⁻¹ and blood pressure 140/90 mmHg. He had a haemoglobin of 12.6 g dl⁻¹, a white blood cell count of 6000 mm⁻³ (neutrophils 80%, lymphocytes 20%) and an erythrocyte sedimentation rate (ESR) of 38 mm h⁻¹. His blood sugar was 11.6 mmol l⁻¹ and serum albumin 32.7 g l⁻¹. He was started on intravenous amphotericin B (40 mg day⁻¹) and fluconazole (400 mg day⁻¹) for the first 3 days and 200 mg day⁻¹ subsequently. Despite treatment for 2 weeks, his condition deteriorated rapidly and he succumbed to infection due to acute cardio-respiratory failure.

Fig. 1. Lymph-node biopsy showing many large, encapsulated yeast cells of *C. neoformans*. Haematoxylin and eosin stain; original magnification ×400.

tericin B (40 mg day⁻¹) and fluconazole (400 mg day⁻¹) for the first 3 days and 200 mg day⁻¹ subsequently. Despite treatment for 2 weeks, his condition deteriorated rapidly and he succumbed to infection due to acute cardio-respiratory failure.

Description of the isolate

The isolate formed mucoid, creamy colonies on SDA and, on microscopic examination in India ink, showed encapsulated yeast cells. The isolate was initially identified as *C. neoformans* var. *gattii*, since it changed the colour of the medium from greenish-yellow to blue when grown on canavanine-glycine-bromothymol blue (CGB) medium (Min & Kwon-Chung, 1986). However, on serotyping by the Crypto Check kit (Iatron Laboratories Inc.), the isolate reacted with factors 1, 5 and 7, and it was untypable in our laboratory as well as in the laboratory of Dr K. J. Kwon-Chung (Molecular Microbiology Section, NIH, USA). This warranted further tests for D-proline assimilation (Nishikawa *et al.*, 1996) and inhibition of urease activity by 100 µM EDTA (Kwon-Chung *et al.*, 1987). While the isolate showed weak urease activity in the presence of EDTA (changing the colour of the medium to pink), it failed to assimilate D-proline. Upon crossing with standard laboratory test strains JEC20 and JEC21 on V8 agar medium, it produced hyphae with JEC20 (*MATa*) and thus scored as a *MATa* strain (Yan *et al.*, 2002). Identification of the mating-type allele of the isolate as *Aa* was determined by performing PCR with the mating-type α allele-specific primer of the *STE12* gene and with serotype (A and D)- and mating type (a and α)-specific primers of the *STE20* gene (Chaturvedi *et al.*, 2000; Yan *et al.*, 2002). The characteristics of the isolate are listed in Table 1.

Discussion

Although our patient had persistent dysphagia due to *Candida* oesophagitis, the diagnosis of HIV infection or cryptococcosis was missed due to the low index of suspicion and the absence of neurological symptoms. In fact, his cervical lymphadenopathy was considered to be of tuberculous origin until *C. neoformans* was observed in the biopsy material, which subsequently led to the diagnosis of HIV infection. In many patients, cryptococcosis has been reported to be the first indication of AIDS, and it is regarded as one of the AIDS-defining opportunistic infections (Powderly, 1993). Although there is no direct evidence, cryptococcosis in AIDS patients is presumed to be the outcome of newly acquired infection rather than the reactivation of a pre-existing disease. Once the yeast cells or basidiospores (Ellis & Pfeiffer, 1992) are inhaled, they reach the alveolar spaces, where the intact cell-mediated immune system of an immunocompetent host controls the infection (Mitchell & Perfect, 1995). However, in an AIDS patient with impaired cellular immunity, the organisms may infect the lung to a variable extent and may disseminate haematogenously to other organs, with a predilection for the central nervous system, as seen in the present case. About 6–10% of patients with AIDS are known to develop cryptococcal meningitis

Fig. 2. (a) X-ray of the chest, day 1, showing a thin-walled cavity in the left mid and lower zones. (b) CT scan of the chest, day 4, showing a thick-walled cavity with irregular margins. (c) X-ray of the chest, day 9. The right lung shows evidence of widespread patchy areas of consolidation, in addition to the cavity in the left lung. Note the significant increase in the size and wall thickness of the cavity in the short span of 9 days.

(Powderly, 1993), and it is usually associated with profound immunodeficiency, with $CD4^+$ counts mostly < 100 cells mm^{-3} (Crowe *et al.*, 1991). Chest radiographs typically show bilateral diffuse alveolar or interstitial pneumonitis, although focal or nodular patterns, cavitary lesions and lymphadenopathy have also been described (Gallant & Ko, 1996). The presence of a rapidly enlarging cavitary lesion, not commonly seen in AIDS patients, was a noteworthy observation in our patient (Fig. 2a–c).

The bulk of current evidence appears to favour the use of amphotericin B at a relatively high dose (0.7 mg kg^{-1} for more than 2 weeks) with flucytosine (100 mg kg^{-1} , given orally in four divided doses) as an initial treatment of choice in AIDS patients with meningitis (van der Horst *et al.*, 1997; Saag *et al.*, 2000). It needs to be followed by fluconazole (400 mg day^{-1}) for a minimum of 10 weeks, which may then be reduced to 200 mg day^{-1} , depending on the patient's clinical status (Saag *et al.*, 2000). However, there is no consensus as to when anticytotoxic suppressive therapy should be stopped. Perhaps a sustained rise in $CD4^+$ counts and undetectable HIV RNA levels could be helpful in such a decision. In our patient, amphotericin B was administered at

a dose of 0.8 mg kg^{-1} day^{-1} and the treatment was combined with fluconazole instead of 5-flucytosine, which was not available locally. Incidentally, the isolate was resistant to 5-flucytosine by Etest performed on buffered RPMI medium, but demonstrated intermediate susceptibility (8 μg ml^{-1}) in a broth microdilution test read at 72 h (NCCLS, 1997). Whether fluconazole can be combined with amphotericin B to achieve synergy is not clear (Sugar, 1995). Experimental studies in animal models of mycoses using amphotericin B in combination with fluconazole/itraconazole have mostly reported synergistic/additive effects (Sugar *et al.*, 1995) with a few exceptions (Schaffner & Bohler, 1993).

In Kuwait, cases of cryptococcosis have been reported in renal transplant recipients (Nampooray *et al.*, 1996; Khan & Chugh *et al.*, 2000). Environmental sources for this pathogen have not been investigated, although pigeons as well as eucalyptus trees are present in large numbers in the urban areas of Kuwait. Whether our patient acquired cryptococcosis from a local source or elsewhere is not clear. He was a frequent visitor to South-East Asian countries, where cryptococcosis due to *C. neoformans* serotype A is predominant among AIDS patients (Sukroongreung *et al.*, 1996).

Table 1. Characteristics of the canavanine-resistant isolate of *C. neoformans* var. *grubii*

Test	Observations
Morphology	Encapsulated, spherical yeast cells
Phenol oxidase activity	Intense brown colonies on niger-seed agar
CGB reaction	Turned medium blue within 3 days
D-Proline assimilation	Negative (no growth)
Inhibition of urease activity by 100 µM EDTA	Turned the medium pink; weak reaction
Serotyping with Crypto Check kit	Reacted with factors 1, 5 and 7; untypable
Mating type experiments with tester strain JEC20 (<i>MATa</i>)	Produced hyphae and scored as <i>MATa</i>
Mating type as determined by PCR	<i>Aα</i>

Since our isolate initially grew on CGB medium, we misidentified it as *C. neoformans* var. *gattii*. Although D-proline assimilation was negative, results of serotyping and inhibition of urease activity by 100 µM EDTA were inconclusive, necessitating further confirmatory tests, such as crossing of the isolate with standard test strains JEC20 and JEC21 on V8 agar medium and/or performing PCR with mating type allele-specific primers (Yan *et al.*, 2002). Both of the latter tests established the identity of the isolate as serotype A with mating type allele *Aα*. Here, it may be mentioned that *MATa* strains have shown greater virulence than congenic *MATa* strains in a mouse model of systemic cryptococcosis (Kwon-Chung *et al.*, 1992). The isolation of a *MATa* strain from a fatal case of disseminated cryptococcosis in this report supports this observation. Concerning the origin of our strain, it may be reasonable to speculate that it is a hybrid. This inference is derived from the results of a recent study, where fragments of four genes of each of 34 strains originating from different locations around the world were sequenced and significant incongruences were observed among the gene genealogies (Xu *et al.*, 2000). As many as five (14.7 %) of the strains in this study were inconsistently placed in the four genealogies, and each of these strains was grouped by different genes with at least one other serotype or variety. Interestingly, one of these strains, CN 111.97, reacted with antiserum factors 1, 5 and 7 and was found to contain gene sequences characteristic of different serotype groups. In contrast, although our strain reacted with antiserum factors 1, 5 and 7, none of the four sequences obtained belonged to the serotype A group. It has been suggested that the existence of multiple gene genealogies among the varieties and serotypes of *C. neoformans* reveals recent dispersion and hybridization, a phenomenon that may be more marked among virulent strains because of increased global migration of susceptible hosts (Xu *et al.*, 2000). In this context, the recent observation by Chaturvedi *et al.* (2002) that *MATa* strains from any of the three *C. neoformans* varieties can mate and hybridize in nature with a *MATa* strain of *C. neoformans* var. *neoformans* reinforces this view.

Although *C. neoformans* var. *grubii*, serotype A, is the major causative agent of cryptococcosis in AIDS patients, even in geographical areas where var. *gattii* is predominant (Mitchell & Perfect, 1995), our isolate is noteworthy in that it showed

resistance to canavanine. Nakamura *et al.* (1998) described two isolates of *C. neoformans* serotype A from pigeon droppings that grew on CGB medium with the ability to resist a high concentration of canavanine (3.6 mM) besides assimilating a high concentration of glycine (133 mM). In an earlier study, Min & Kwon-Chung (1986) reported that about 15 % of serotype A isolates assimilated glycine and also showed resistance to canavanine, but failed to grow on CGB medium. Discrepancies in the intervarietal differentiation of *C. neoformans* strains by colorimetric agar tests have also been reported by other investigators (Shadomy *et al.*, 1987). However, consistent with the observation of Nakamura *et al.* (1998), our isolate showed good growth on the medium with a positive CGB reaction. This report suggests that, in the absence of a clear-cut serotyping result, a positive CGB reaction alone is not sufficient for intervarietal discrimination, and additional confirmatory evidence is required.

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