Case Report

Cryptococcus gattii: immunological and microbiological study in a patient with neurocryptococcosis

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Introduction: Cryptococcal meningitis is a disease that remains a significant cause of morbidity and mortality. This report describes the importance of conducting a detailed clinical investigation and the treatment challenges in cases of meningitis caused by *Cryptococcus gattii*. In recent years this species has received considerable interest due to its increased emergence and virulence.

Case presentation: A patient with apparent good health (a fitness practitioner) showed symptoms including intermittent headache that became more intense and frequent when he began experiencing nausea, vomiting, dizziness and temporal headache without nuchal rigidity. The patient had human immunodeficiency virus-negative serology and had no chronic disease. Analysis of cerebrospinal fluid was performed and cryptococcal meningitis was diagnosed. Immunophenotyping by flow cytometry evidenced the presence of an anomalous lymphoid population. RFLP analysis of the *URA5* gene indicated *Cryptococcus gattii* genotype VGII and considerable virulence was observed for the isolated strain.

Conclusion: This case suggests the importance of a detailed investigation in patients who apparently have a competent immune system with meningitis caused by *Cryptococcus* spp., particularly *C. gattii*.

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Introduction

Cryptococcosis is a serious medical problem, particularly amongst immunocompromised patients, and remains the most important cause of fungal meningitis (Li *et al.*, 2012). *Cryptococcus neoformans* is the most common human pathogenic species; however, non-*neoformans* *Cryptococcus* have been reported (Saijo *et al.*, 2014). Another species that has been observed in cases of cryptococcal meningitis is *Cryptococcus gattii*, which can affect immunocompetent and non-immunocompromised hosts (Chen *et al.*, 2014), and the infections may be more difficult to treat (Perfect *et al.*, 2010). In some cases the infection caused by *Cryptococcus gattii* was fatal (Favalessa *et al.*, 2014).

Amongst the risk factors for infection by *C. gattii* we should highlight environmental exposure as an important factor (Barreto de Oliveira *et al.*, 2004; Randhawa *et al.*, 2008; Jesus *et al.*, 2012; Gullo *et al.*, 2013), although human immunodeficiency virus (HIV) infection and altered immune status of the human host may facilitate the infection (Lizarazo *et al.*, 2014). Here, we report the case of a clinical patient with neurocryptococcosis, and describe the immunological features and microbiological characteristics of the strain in question.

Case report

This report concerns the case of a 68-year-old male patient, living in São Paulo, Brazil, who was previously healthy and who habitually jogged in a eucalyptus garden. The first symptoms appeared on 16 July 2010 (day 0) with

Abbreviations: 5-FC, 5-fluorocytosine; HIV, human immunodeficiency virus; NK, natural killer.

intermittent headaches and dizziness. Initially, labyrinthitis was suspected, but the diagnosis was negative. The physical examination did not show signs of meningitis. With the improvement of the symptoms the patient did not seek further health care. However, on 5 February 2011 (day +205), his symptoms became more intense and frequent when he began experiencing vomiting, nausea, dizziness and temporal headache without nuchal rigidity. On 9 February (day +209), the patient was admitted to a hospital, where a lumbar puncture was performed and cryptococcal meningitis was diagnosed in the cerebrospinal fluid. India ink staining revealed the presence of encapsulated yeasts. Concomitantly, on day +209, he underwent an endoscopy and oesophageal candidiasis was diagnosed. The patient had no history of major or chronic disease; he was HIV-negative and used no medications. His father had died of prostate cancer and his mother of breast cancer.

On day +219, the species *Cryptococcus gattii* was identified by morphological and physiological tests (Kurtzman *et al.*, 2011), including positive assimilation for inositol and glucose, and negative for lactose, assimilation of nitrogen sources for peptone and negative for KNO_3 ; no fermentation of carbon sources (glucose); growth in Sabouraud medium at 37 °C; positive for urease; phenol oxidase production on Niger seed agar; and growth in canavanine/glycine/bromothymol blue. A study of virulence factors was positive for phenoloxidase and phospholipase, and strongly positive for proteinase (Price *et al.*, 1982; Rùchel *et al.*, 1982). The molecular type was determined by RFLP analysis of the *URA5* gene (Meyer *et al.*, 2003) and was shown to be genotype VGII (Fig. 1).

Fig. 1. RFLP profiles of the *URA5* gene from *C. neoformans* obtained by double digestion of the gene with *Sau96I* and *HhaI*. Lane 1, patient isolate; lanes 2–9, standard genotypes (VNI serotype A, VNII serotype A, VNIII serotype AD, VNIV serotype D, VGI serotype B, VGII serotype B, VGIII serotype B and VGIV serotype C, respectively); lane M, 100 bp DNA ladder (Thermo Scientific).

On 9 February (day +209), the patient was initially treated with amphotericin B ($1 \text{ mg kg}^{-1} \text{ day}^{-1}$) and 5-fluorocytosine (5-FC; $150 \text{ mg kg}^{-1} \text{ day}^{-1}$) up to 21 February (day +221), when amphotericin B was changed to liposomal amphotericin ($4 \text{ mg kg}^{-1} \text{ day}^{-1}$) due to the lack of improvement. He continued being treated with 5-FC for a further 2 days, completing 14 days of treatment. On 28 February (day +227) it was necessary to change liposomal amphotericin to fluconazole due to nephrotoxicity. He started with intravenous fluconazole 800 mg day^{-1} for 14 days. On day +241, due to an increase of cryptococcal antigen levels in the cerebrospinal fluid (1/1024), the treatment was changed to liposomal amphotericin ($4 \text{ mg kg}^{-1} \text{ day}^{-1}$) plus 5-FC ($150 \text{ mg kg}^{-1} \text{ day}^{-1}$). This change caused a decrease of cryptococcal antigen (1/8) after 2 days (day +243).

On 28 March (day +255), liposomal amphotericin plus 5-FC was changed to fluconazole 800 mg day^{-1} orally, with good evolution and the patient has been clinically well ever since. Following discharge, high-dose fluconazole (800 mg day^{-1} for 10 weeks) was prescribed for consolidation therapy followed by secondary prophylaxis with low-dose fluconazole (200 mg day^{-1}) for 6 months. The patient has shown no sequelae and is doing well after 3 years of follow-up.

On day +274, the patient was referred to the primary immunodeficiency outpatient clinic for investigation and flow cytometry was performed conforming to the H43-A2 guidelines for immunophenotypic analysis (CLSI, 2007; Wood *et al.*, 2007). The results of the flow cytometry showed leukocytes of 8600 mm^{-3} and lymphocytes of 2055 mm^{-3} , whilst the lymphocyte subpopulations were: CD3^+ , 1572 mm^{-3} ; CD4^+ , 769 mm^{-3} ; CD8^+ , 600 mm^{-3} ; CD19^+ , 208 mm^{-3} ; $\text{CD3}^+\text{CD16}^+\text{CD56}^+$, 258 mm^{-3} ; $\text{CD3}^+\text{CD16}^+\text{CD56}^+$, 123 mm^{-3} ; $\text{CD4}^+/\text{CD8}^+$ ratio, 1.28. The evaluation of T-cell subsets showed a population of double-negative T-cells expressing $\gamma\delta$ T-cell receptors, which, following more detailed phenotyping, showed aberrant cells expressing $\text{CD19}^+\text{CD16}^+\text{CD56}^+\text{CD123}^+$ in 6 % of peripheral blood lymphocytes. His exams also demonstrated the presence of IgG λ paraprotein. Subsequently, on day +364, the patient was referred to the haematological unit for more detailed investigations.

The myelogram confirmed the presence of an abnormal clone in the bone marrow; immunophenotyping by flow cytometry evidenced 15 % of lymphocytes expressing the lymphoid B-cell antigen CD19^+ (weak intensity), and high expression of natural killer (NK)-associated antigens CD56^+ and CD57^+ , 9 % of NK cells ($\text{CD3}^-\text{CD19}^-\text{CD56}^+$) and 20 % large granular lymphocytes ($\text{CD3}^+\text{CD56}^+\text{CD57}^+$). Light chain restriction of B-lymphoid populations was not demonstrated in the bone marrow. The results indicated the presence of an anomalous lymphoid population expressing B and NK markers CD56^+ and CD19^+ .

Despite the evidence of these abnormal cells, the patient was clinically well except for the cryptococcal meningitis, and the recommendation of the haematology team was to wait and watch the patient's evolution. The present study was approved by the research ethics committee CAPESQ-USP (no. 0478/2011) and the patient provided written informed consent.

Discussion

Cryptococcal meningitis is a disease that usually occurs in patients presenting some type of abnormality in the immune system (Prado *et al.*, 2009). This disease remains a significant cause of morbidity and mortality (Meya *et al.*, 2015). Numerous studies have reported that the patient's immune status is critical to the clinical course of cryptococcal infection (Gullo *et al.*, 2013). In this case, we identified *C. gattii* genotype VGII, which in recent years has received considerable interest due to its increased emergence and virulence (Bovers *et al.*, 2008; Hagen *et al.*, 2010; Chaturvedi & Chaturvedi 2011; Morales *et al.*, 2015). Throughout the diagnostic process, the studied patient was apparently healthy; however, a more detailed investigation detected a population of aberrant lymphoid cells. These cells could have caused a secondary immunodeficiency that contributed to the patient's meningitis by *C. gattii*.

The yeast in question is the most aggressive form of the genus *Cryptococcus* and suggests that whenever a patient with cryptococcal meningitis is diagnosed, it is important to investigate the underlying primary or secondary immunodeficiency. In this case, the patient presented cryptococcal meningitis and oesophageal candidiasis, suggesting a possible T-cell immunodeficiency. It is also worth noting that whilst evaluating the lymphocyte phenotyping, no decrease in the number of T- or B-lymphocytes or NK-cells was verified, but an expanded double-negative T-cell population that was shown to be aberrant, coexpressing CD19⁺CD16⁺CD56⁺, was evident amongst the double-negative $\gamma\delta$ T-cells.

This fact was corroborated by the result of the bone marrow analysis, which showed the presence of the anomalous lymphoid CD56⁺CD19⁺ population. These cells presented double staining for B-lymphocytes and NK markers. This lineage of lymphoid cells is fundamental in defending against fungal infection, which suggests that the replacement of immunocompetent cells by aberrant neoplastic cells that are functionally inefficient could be the culprit for the patient's immunosuppression. In this case, the patient presented a fungal infection even before he showed any clinical manifestation of the neoplasm of the lymphoid lineage. The phenotype of large granular lymphocytic leukaemia can be divided into two major types: NK-cells (15 %) and T-cells (85 %). The first type comprises typical NK-cells that can be divided into two types, i.e. chronic lymphoproliferative disorder of NK-cells (also known as chronic NK-cell lymphocytosis), generally associated with viral infections, and aggressive

NK-cell leukaemia. This latter disease is highly malignant and the patient needs urgent chemotherapy. However, T-cell large granular lymphocytic leukaemia is derived from antigen-activated effector-memory cytotoxic T-cells. There is an association with autoimmune diseases such as rheumatoid arthritis, Sjögren's syndrome and autoimmune haematologic diseases. This disease is indolent and the therapy is indicated only when the patients are symptomatic (Dhodapkar *et al.*, 1994). Moreover, associations of large granular lymphocytic leukaemia and B-cell dyscrasias are also rather frequent (Howard *et al.*, 2010; Zhang *et al.*, 2010).

This case suggests the importance of a detailed investigation in patients with meningitis caused by *Cryptococcus*, particularly *C. gattii*, in a patient with an apparently competent immune system.

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