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Case report

Recurrent pulmonary cryptococcosis in a patient with idiopathic CD4 lymphocytopenia

ZHU YUANJIE*, GU JULIN*, CHE FUBING & CHEN JIANGHAN
Cryptococcus Lab, Department of Dermatology, Changzheng Hospital, Shanghai, China

A case of recurrent cryptococcosis with idiopathic CD4 lymphocytopenia is reported in this article. After an initial cryptococcal infection in the lung, the patient experienced one episode of cryptococcal meningitis and two more episodes of cryptococcal pneumonia within a period of 12 years. Genetic studies revealed that all isolated microbes were identical, indicating that all subsequent episodes were recurrence instead of re-infection.

Keywords *Cryptococcus neoformans*, cryptococcosis, idiopathic CD4 lymphocytopenia

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Introduction

Cryptococcosis combined with idiopathic CD4 lymphocytopenia (ICL) is an uncommon infectious disease with rare recurrence. The central nervous system (CNS) is the primary site of infection [1]. To our knowledge, there has been no report of >2 relapses, or cryptococcal pneumonia in the relapse.

Here we report a case of cryptococcosis with three relapses within 12 years, among which two episodes were cryptococcal pneumonia. Genetic analysis of isolated pathogens by minisatellite core sequence–PCR (M13-PCR) [2] and IGS1 sequencing [3] demonstrated that all four isolates were identical.

Case report

A 41-year-old female was admitted to the Changzheng hospital after a routine health examination revealed patchy consolidation in the left lung using chest X-ray in 1988. The patient was free of symptoms at the time. The patient received treatment with wide-spectrum antibiotics, but responded poorly. Pulmonary lobectomy of left lower lobe was performed on suspicion of lung cancer. A diagnosis of pulmonary cryptococcosis was established by histopathology and fungal culture. The cerebrospinal fluid (CSF) test was negative. The patient was discharged after one-month treatment with daily intravenous amphotericin B injection. Three months later, she was admitted again on complaint of headache and vomiting. A diagnosis of cryptococcal meningitis was made by mycological microscopy and CSF culture. The sputum test was negative. The patient recovered after 6-week treatment with amphotericin B plus 5-flucytosine. The patient did not receive maintenance therapy, and was free of any sign of cryptococcal infection for 12 years until 2000.

In March 2000, she was hospitalized for fever and cough. A diagnosis of cryptococcal pneumonia was made based on X-ray, computed tomography (CT), fungal microscopy and sputum culture. Magnetic resonance imaging results were normal. Encapsulated budding yeast-like cells were not found in the CSF. The patient was treated with amphotericin B plus 5-flucytosine for 6 weeks. The last three sputum cultures were negative prior to the discharge.

In October 2000 (6 months from the last episode), the patient was admitted again for coughing. Mycological microscopy, sputum culture and endobronchial biopsy were positive for *C. neoformans* (Figs. 1 and 2). X-ray showed patchy consolidation in the right lower

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Correspondence: Chen Jianghan, Cryptococcus Lab, Department of Dermatology, Changzheng Hospital, Shanghai, China. Tel/Fax: +86 21 63610109, ext 73438. E-mail: chenjianghan@126.com
*These 2 authors contributed equally to this work.

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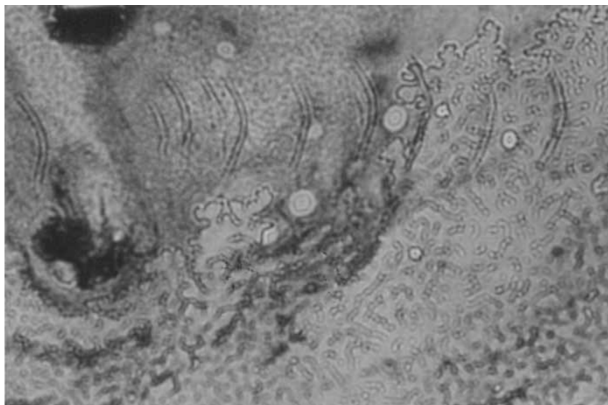

Fig. 1 Encapsulated, yeast-like microbes in sputum under a microscope (×400).

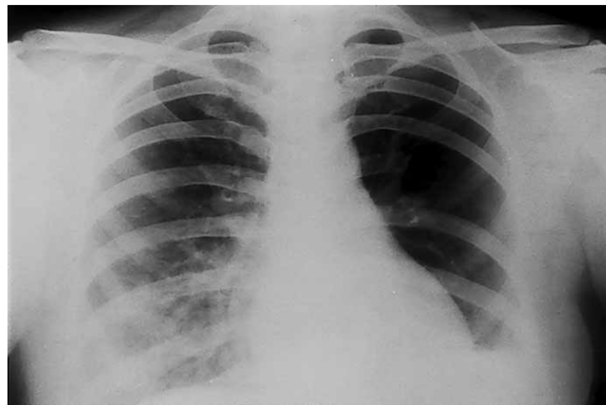

Fig. 3 Chest X ray during the third episode (March 2000), showing patchy consolidation in the right side.

lobe (Fig. 3). Fungal culture and microscopic examination of the CSF and blood were negative. Cryptococcal antigen was positive in serum but negative in the CSF. After 12-week antifungal treatment, symptoms and signs dissipated (Fig. 4). The patient was discharged after a 2-week period during which three consecutive fungal culture and microscopic examination of sputum were negative. The patient was placed on maintenance treatment with oral fluconazole (150 mg per day). Table 1 show the clinical profile, lab results, and treatment of the four episodes.

White blood cell count was abnormally low at a range between 2.61×10^9/l and 2.61×10^9/l since the first episode of infection in 1988. Red blood cell count was also low, within a range between 3.26×10^9/l and 3.26×10^9/l. Platelet count was normal. Since March 2000, the CD4 lymphocyte count in this patient remained constant between 4.5% (81 cells/ml) and 5.1% (94 cells/ml). She was HIV-negative (confirmed repeatedly using ELISA, Western blot and PCR) and never received any immunosuppressive treatment. PPT test for tuberculosis was negative. No evidence of autoimmune disease or malignant tumor was found. Based on these findings, a diagnosis of recurrent pulmonary cryptococcosis with ICL was made.

The follow-up consisted of visits to our clinic every 6 months, routine lab works, and CD4 lymphocyte count. The last visit occurred in December 2007. The

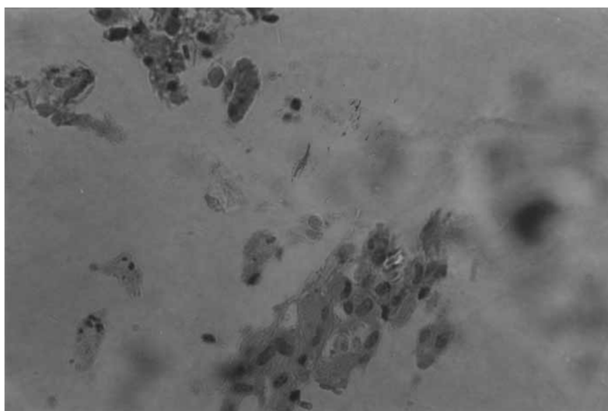

Fig. 2 Encapsulated yeast-like microbes in the endobronchial biopsy sample stained with PAS (×400).

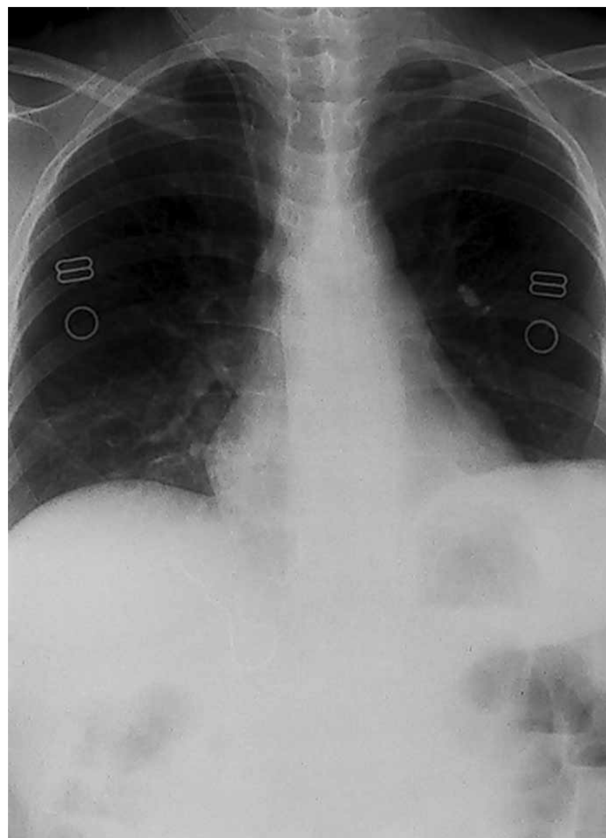

Fig. 4 Chest X ray showed improvement after treatment.

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Table 1 Clinical profile, labs, and treatment of the reported case.

Infection Time	Infection type	CSF findings	Sputum findings	Blood findings	Treatment
Aril,1988	Pneumonia	Culture: Negative LAT: N.A.	Microscopy: N.A. Culture: Positive	CD4 count: N.A. Culture: Negative. LAT: N.A.	Lobectomy; amphotericin B 25 mg /day for 1 month
July,1988	Meningitis	Culture: Positive LAT: N.A.	Microscopy: Negative Culture: Negative	CD4 count: N.A. Culture: Negative. LAT: N.A.	amphotericin B 25 mg/day plus 5-flucytosine 3 g /day for 6 weeks
March, 2000	Pneumonia	Culture: Negative LAT: Negative	Microscopy: Positive Culture: positive	CD4 count: 4.5% Culture: Negative. LAT: 1:1280	amphotericin B 25 mg/day plus 5-flucytosine 3 g/day for 6 weeks
October 2000	Pneumonia	Culture: Negative LAT: Negative	Microscopy: Positive Culture: positive	CD4 count:5.1% Culture: Negative. LAT: 1:1280	amphotericin B 25 mg/day plus 5-flucytosine 3 g/day for 12 weeks and floconazole 150 mg/day as maintenance

CD4 lymphocyte count remained at a steady low level of 4–5% (72–96 cells/ml) throughout the follow-up. Additional HIV tests were all negative. No infection with uncommon microbes, such as herpes zoster virus and pneumocystis, was noted.

Isolate analysis

Phenotypic identification

Analysis of the pathogen phenotype included colony morphology in bird seed agar, urease activity, and ability of the fungi to grow in 37°C. Canavanine-glycine-bromothymol (CGB) blue agar was used to differentiate *C. neoformans* from *C. gattii*. The mating type was determined using a PCR method. Mating type **a** was amplified using an allele-specific primer of the STE12 gene [4]. Serotype (A and D) and mating type (a and α) were amplified with specific primers of the STE20 gene [5]. In a V-8 juice agar, each strain was crossed with B-3501A (α) and JEC20 (a). The serotype was determined by Crypto Check Kit (Iatron Laboratories Inc.; Tokyo, Japan). All four strains were *C. neoformans var. neoformans*, serotype A and mating-type α.

Genetic analysis

The reference strains for PCR fingerprinting were provided by Dr K. J. Kwon-Chung at the National Institutes of Health of the US, and included: WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM 629 (serotype D, VNIV), H99. High-molecular-weight DNA was isolated as described previously [5]. The minisatellite-specific core sequence of the wild-type phage M13 (5′GAGGGTGGCGGTTCT 3′) [2] was used as single primer in the PCR. Amplification was performed in a volume of 50 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH; Mannheim, Mannheim, Germany), 3 mM magnesium acetate, 30 ng primer, 2.5 U Amplitaq DNA polymerase (Applied Biosystems; Foster City, CA), and 25 ng high-molecular-weight genomic DNA. PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler with 20 s of denaturation at 94°C, 1 min annealing at 50°C, and 20 s extension at 72°C, followed by a final extension cycle of 6 min at 72°C. Amplification products were concentrated to approximately 20 µl and separated using 1.4% agarose gel in 1X Tris-borate-EDTA (TBE) buffer at 60 V, and visualized under UV light. The four isolates displayed identical pattern (Fig. 5).

Multilocus sequence typing (MLST)

Multilocus sequence typing was performed using methods previously described for TEF1, SOD1, CAP10, PLB1 and IGS [3]. The PCR primers and amplification conditions are shown in Table 2. PCR products were purified using a QIAquick PCR purification kit (QIAGEN; Valencia, CA). Sequences were generated from DNA strands and manually edited. MLST analysis results indicated that all 4 isolates were identical.

Discussion

In 1992, ICL was defined by the US Centers for Disease Control and Prevention (CDC) as a condition with depressed number of circulating CD4 lymphocytes (<300 cells/ml or <20% of total T cells) on at least two occasions separated by >6 weeks, with no

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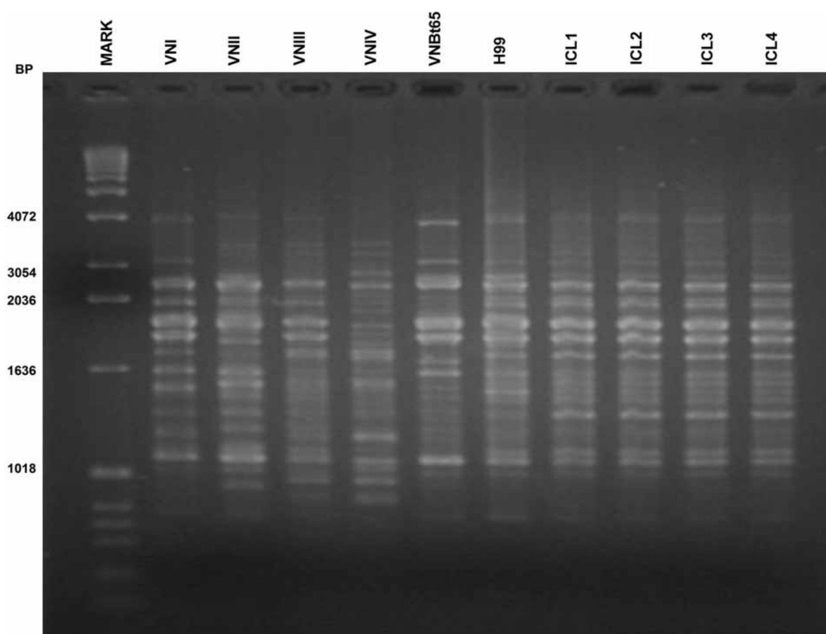

Fig. 5 PCR fingerprints of the four isolates.

identifiable causes such as HIV infection or immunosuppressive therapy [6]. This case clearly met all diagnostic criteria for ICL [7], i.e., low CD4 lymphocyte over a period of close to 20 years, no HIV infection, and no history of immunosuppressive therapy.

Methods commonly used to identify *C. neoformans* strain include electrophoretic karyotyping [10,11], PCR fingerprinting [12], random amplified polymorphic DNA (RAPD) analysis [13,14], multilocus enzyme typing [13], allelic variation of the URA5 locus [15,16], and DNA fingerprinting [17–21]. The methods used in the reported case (minisatellite core sequence PCR and MLST analysis, etc.) are generally more sensitive. In particular, IGS gene is the most rapidly evolving region of rDNA families [22], and thus provided a reliable marker for identification. Results from these experiments demonstrated that the later episodes of infection were relapse in nature and not new infection. This finding is consistent with previous notion that cryptococcosis relapse is mostly due to persistence of the original infecting strain [19,23].

The CNS is the most common site for cryptococcal infection in ICL patients. In a comprehensive review, Zonios *et al.* [1] reported that 30 out 42 cases of cryptococcosis in ICL patients were CNS infection. Among the 42 cases, 5 (12%) had relapse. In all five cases with relapse, the initial episode were CNS

Table 2 MLST primers.

Locus	Primer sequence	Melting temperature (°C)	Product size (bp)	PCR conditions
CAP10-f	5′-CCG GAA CTG ACC ACT TCA TC	56.3	668	12 cycles 62–56°C stepdown 2°C every 2 cycles followed by 20 cycles at 56°C
CAP10-r	5′-GCC CAC TCA AGA CAC AAC CT	58.5		
PLB1-f	5′-CTT CAG GCG GAG AGA GGT TT	57.7	674	
PLB1-r	5′-GAT TTG GCG TTG GTT TCA GT	55.2		
SOD1-f	5′-TCT AAT CGA AAT GGT CAA GG	50.7	680	
SOD1-r	5′-CGC AGC TGT TCG TCT GGA TA	58.1		
TEF1-f	5′-AAT CGT CAA GGA GAC CAA CG	55.9	844	
TEF1-r	5′-CGT CAC CAG ACT TGA CGA AC	56.5		
IGS1-f	5′-ATC CTT TGC AGA CGA CTT GA	55.3	790	30 cycles 56°C
IGS1-r	5′-GTG ATC AGT GCA TTG CAT GA	54.7		

PLB1, Phospholipase; *TEF1*, Translation elongation factor 1α; IGS1, Ribosomal RNA intergenic spacer; *SOD1*, Cu, Zn superoxide dismutase; *CAP10*, Capsular associated protein.

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infection. Also, more than two episodes of relapse have never been documented in any single case [1,8,9]. In our case, relapse occurred three times within a period of 12 years. Another interesting feature of this case is the fact that two out three relapses were cryptococcal pneumonia. A much longer follow-up (20 years vs. an average of 32 months in other reported cases) may have accounted for the unusual number of relapses in our case.

CD4 lymphocyte remained fairly constant at a low level of 4.5 ~ 5.1%, even during the period between the episodes. This is different from the partial recovery of CD4 lymphocyte when infection is under control in previous reports [1]. Since there was no medical history that suggested other risk factors for cryptococcosis in this case (such as cryptococcosis endemic), we believe decreased CD4 lymphocyte is the predisposing factor in this patient as it is for AIDS patients.

It is estimated that 3/4 patients with a defined diagnosis of cryptococcal infection receive long-term antifungal maintenance treatment. The relatively low rate of relapse after the initial episode in ICL patients argues against routine maintenance treatment [1]. Our case had three relapses during a period of 12 years without maintenance treatment. During a 7-year period with floconazole maintenance treatment after 2000, the patient did not have a single episode of infection. Based on this observation, we believe that the best practice is to use antifungal maintenance therapy only after a relapse, and not after the initial episode of infection. For patients not receiving maintenance therapy, the CD4 lymphocyte count should be monitored regularly.

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