Disseminated Infection and Pulmonary Embolization of *Cunninghamella bertholletiae* Complicated with Hemophagocytic Lymphohistiocytosis

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Abstract

A 22-year-old Japanese woman was diagnosed with hemophagocytic lymphohistiocytosis and subsequently was treated with etoposide and cyclophosphamide. On Day 22, multiple nodular lesions appeared in the bilateral lungs. Neither the administered antibiotics nor the antifungal agent were effective, and she died suddenly of respiratory failure on Day 35. An autopsy revealed disseminated zygomycosis and a pulmonary infarction due to the embolization of an angioinvasive fungus, which was later identified as *Cunninghamella bertholletiae* using in situ hybridization of 18S rRNA. *C. bertholletiae* is aggressive as well as resistant to antifungal agents. This rare species should therefore be taken into consideration as a potential causative agent of zygomycosis.

Key words: hemophagocytic lymphohistiocytosis, *Cunninghamella bertholletiae*, Epstein Barr virus, in situ hybridization


Introduction

Zygomycosis is rare, and it is often complicated with diabetes mellitus, HIV infection or hematological malignancies (1). The diagnosis is difficult, and the patients usually follow an unfavorable course. We herein report a case of disseminated zygomycosis complicated with hemophagocytic lymphohistiocytosis (HLH). The patient died suddenly of respiratory failure due to the embolization of a fungal infection. The fungus was identified as *Cunninghamella bertholletiae*, which is highly aggressive among the many species that can cause zygomycosis.

Case Report

Clinical history

A 22-year-old Japanese woman presented with a high fever of 39°C. She was treated with a non-steroidal anti-inflammatory drug, and her symptoms improved. One month later, she presented again with a fever and was subsequently admitted to the hospital. She had no significant medical history. The laboratory data showed pancytopenia (RBC 372×10⁴/μL, WBC 2,300/μL, Plt 11.1×10⁴/μL) and increases in both sIL2R (2,456 U/mL) and ferritin (898 ng/mL). The DNA copy number of the Epstein Barr virus (EBV) was elevated to 5.4×10⁵ copies/mL. All other viral antigens and antibodies results were negative. There were no abnormalities in either the chest X-ray or CT scan (Fig. 1A, B). Her bone marrow was hypoplastic (Fig. 2A), and active hemophagocytosis was observed in the smear specimen (Fig. 2B). Scattered EBV-positive cells were revealed using in situ hybridization of EBV-encoded RNA [EBER (Fig. 2C)].

Under the diagnosis of EBV-associated HLH, she was treated with prednisolone at a dosage of 30 mg/day. An additional steroid pulse therapy of 1 g prednisolone for 3 days was also administered. Her fever persisted, and she was...
Figure 1. Chest X-ray and CT scan. No abnormal shadows were found in the chest X-ray (A) or CT scan (B) on Day 1. Multiple nodular lesions (arrowheads) were noted in the X-ray (C) and CT scan (D) on Day 22.

Figure 2. Histology of the bone marrow. (A) The bone marrow taken on Day 22 was markedly hypoplastic. (B) Numerous macrophages with phagocytosis were observed on the marrow smear. (C) EBER-positive cells were observed in the bone marrow. (D) The bone marrow at autopsy was hypoplastic, and there were numerous macrophages with features of phagocytosis. (Original magnification: A, ×400; B, ×1,000; C, ×400; D, ×400.)
treated with prulifloxacin and fluconazole. The pancytopenia was progressive (RBC 252×10⁴/μL, WBC 1,300/μL, Plt 2.0×10⁴/μL), and her ferritin level increased to 15,704 ng/mL. Etoposide (16 mg/day) and cyclophosphamide (230 mg/day) were therefore administered per the HLH-94 protocol. The antimicrobial treatment was changed to cefepime and fluconazole. On day 22, she began to exhibit dyspnea, and multiple nodular shadows were noted on the chest X-ray and CT scan (Fig. 1C, D). The laboratory results for fungal antigens for *Candida*-mannan and *Aspergillus* were negative, and the level of β-D-glucan was 2.9 pg/mL (normal: <20.0 pg/mL). The bacteriological culture tests of the sputum and blood were negative. Ciprofloxacin was added to the treatment regimen. She subsequently exhibited abdominal pain and gastrointestinal bleeding. A transfusion of RBCs and fresh frozen platelets was performed. On day 35, her oxygen

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**Figure 3.** Autopsy findings. (A) Nodules with hemorrhaging in the lung. (B) Multiple ulcers of the intestine. (C) The loupe image of the lung. The emboli are indicated with arrowheads. (D) The Grocott staining demonstrated the angioinvasive growth of the fungus. (E) The loupe image of the intestine. The fungal emboli are indicated with arrowheads. (F) A high magnification of the Grocott staining demonstrated ribbon-like hyphae of approximately 10 μm in width. The septum was not apparent (arrowhead). (G) Periodic acid-Schiff staining demonstrated only faint staining. (H-J) *In situ* hybridization with 18S rRNA probes: (H) *Cunninghamella bertholletiae*, (I) *Mucor/Rhizomucor/Rhizopus/Saksenaea* spp. and (J) *Absidia* sp. (Original magnification: D, ×200; F-J, ×400).
saturation level decreased due to dyspnea. The laboratory results showed severe pancytopenia (RBC 280x10^12/L, WBC 100x10^9/L, Plt 1.7x10^10/L) and multiple organ failure. Her ferritin level was further elevated to 47,776 ng/mL. She died of respiratory and circulatory failure.

**Autopsy findings**

Multiple nodular lesions with hemorrhaging were found in both lungs (Fig. 3A). Necrotic lesions due to infarction were also noted. There were multiple hemorrhagic ulcers in the stomach, the small intestine and the colon (Fig. 3B). Her adrenal glands were swollen and degenerated.

A histological examination disclosed the formation of emboli that led to infarctions of the lung (Fig. 3C). Grocott staining revealed the widespread growth of fungi throughout the lung and the formation of emboli caused by the angiinvasive growth of the fungi (Fig. 3D). The fungal emboli were present in the vessels that were located in the bottom of the intestinal ulcers (Fig. 3E). Using Grocott staining, the fungi were shown to be filamentous with wide ribbon-like hyphae of approximately 10 μm in width with no apparent septum (Fig. 3F). The fungi showed occasional branching at wide angles. The fungi could only be faintly stained with periodic acid-Schiff (Fig. 3G). The bone marrow at autopsy was hypoplastic, and hemophagocytosis was evident (Fig. 3H). The pathogen was determined to be *C. bertholletiae*.

**In situ hybridization of 18S rRNA of Zygomycetes**

The *in situ* hybridization (ISH) was performed using the method reported by Hayden (2) with the modification of using digoxigenin-labeled synthetic RNA probes for 18S rRNA of *Zygomycetes*. The probes were 5'-UCA AUG AAG ACC AGG CCA C-3' for *Mucor/Rhizomucor/Rhizopus/Saksenaea* spp., 5'-ACC UGA CCA AAG GTC AAG GC-3' for *Absidia* sp., 5'-UGG CUA GAC CGA AAU CUA GAA AC-3' for *Cunninghamella bertholletiae*. The ISH was processed using a Ventana Discovery HX System (Roche Diagnostics K.K., Tokyo, Japan). After deparaffinization, the slides were fixed in RiboPrep at 37°C for 30 minutes. They were sequentially treated with EZ buffer and CC2, and then with Protease 2. After denaturation at 70°C for 10 minutes, the hybridization was done in 200 μL RiboHyb with 15 ng probes at 70°C for 6 hours. The slides were then washed three times with 0.1XSSC at 65°C for 6 minutes and fixed in RiboFix for 10 minutes. They were incubated with alkaline phosphatase-labeled anti-digoxigenin antibody (Sigma-Aldrich Japan, Tokyo, Japan) for 30 minutes. The labeled alkaline phosphatase was visualized using a BlueMap Kit (Roche Diagnostics K.K.).

The fungal hyphae yielded positive signals with the probe for *C. bertholletiae* (Fig. 3H), but were negative for the probes for *Mucor/Rhizomucor/Rhizopus/Saksenaea* spp. (Fig. 3I) and *Absidia* sp. (Fig. 3J).

**Discussion**

The patient was diagnosed with EBV-associated HLH. During chemotherapy, multiple nodular lesions were found in the bilateral lungs. Despite the administration of intensive chemotherapy and multiple antimicrobial treatments, she died suddenly of pulmonary failure. The autopsy revealed a disseminated infection of filamentous fungi, which had caused multiple areas of embolization of the pulmonary vessels. The pathogen was determined to be *Zygomycetes* on the histological sections, and was later identified as *C. bertholletiae* using ISH.

Zygomycosis is an infectious disease caused by filamentous fungi of the *Zygomycetes* order (1). The infection generally occurs in patients who have diabetes, are on hemodialysis or are otherwise immunocompromised. The association of zygomycosis with HLH is very rare, and there have only been 3 such cases reported thus far in the literature (Table) (3-5). Neutropenia is one of the risk factors for the development of zygomycosis (1). The zygomycosis case reported by Inagaki (5) as well as the current case were both associated with severe neutropenia caused by HLH. It has also been reported that two diabetic patients have had HLH-associated zygomycosis (3, 4). Although repeated transfu-
sions and the treatment with iron chelating agents could be risk factors for the development of zygomycosis, the clinical significance of these treatments was not noted in the reported cases of HLH that were complicated with zygomycosis. Despite the administration of intensive chemotherapy and multiple antimicrobial treatments, the clinical courses were aggressive, and all four patients died within five weeks. The causative species was not identified in the previously reported cases, and our case is the first to identify the precise infectious agent.

Cunninghamella sp. is an environmental organism, and it can be isolated from the soil, air and water (1). Most of the reported cases of this fungal infection were in immunocompromised patients (6). The respiratory tract and lungs are the major routes of infection for these cases. Embolization due to the angioinvasive growth is a feature of C. bertholletiae infection, and sudden death caused by the emboli of pulmonary vessels has been reported (7). The mortality of C. bertholletiae infection is greater than the mortality of infections caused by other members of the Zygomycetes order (6). It has also been reported that C. bertholletiae is one of the more common isolates from the clinical samples in Japan, although the number of cases was limited (8). Because the pathogen is so highly virulent, it is vital that the species be identified promptly so that a sensitive antifungal therapy can be administered immediately.

An accurate diagnosis of zygomycosis is often challenging. There is no useful serum marker for the diagnosis. The lung is frequently involved in zygomycosis, but it is difficult to distinguish the condition from invasive pulmonary aspergillosis. It has been reported that multiple nodular shadows and areas of pleural effusion are more frequently associated with pulmonary zygomycosis, as compared to invasive pulmonary aspergillosis (9). In the current case, multiple nodular lesions appeared on the chest X-ray and CT scan during the treatment. These lesions were shown by autopsy to be necrotic and hemorrhagic, and were caused by the angioinvasive growth of the fungi. Although the radiological findings may not be specific, the possibility of zygomycosis should therefore be taken into account in cases presenting with multiple nodular lesions.

Zygomycetes show variable susceptibility to antifungal agents. Currently, amphotericin B (AMPH-B) with lipid formulations and posaconazole are considered to be effective for the treatment of zygomycosis. In vitro susceptibilities of Zygomycetes against antifungal agents showed that most of them are susceptible to AMPH-B and posaconazole (10). C. bertholletiae showed susceptibility to posaconazole, but only limited sensitivity to AMPH-B (10). Therefore, for the appropriate treatment of zygomycosis, the species of Zygomycetes needs to be identified. Polymerase chain reaction analysis is useful for the definite diagnosis of zygomycosis and the identification of the species (11). When histological samples are available, ISH using species-specific oligonucleotide probes is an alternative method for diagnosis (2). An early initiation of treatment with an effective antifungal agent is of great importance. Treatment with AMPH-B or posaconazole should thus be considered even before a diagnosis of zygomycosis can be definitively made.

The authors state that they have no Conflict of Interest (COI).

References