

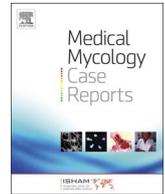
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Microbiological and immunological characteristics of a lethal pulmonary *Aspergillus niger* infection in a non-neutropenic patient



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ABSTRACT

Invasive pulmonary aspergillosis is increasingly described in non-neutropenic patients, such as patients with COPD receiving corticosteroids and the critically ill. Here, we present a case of a lethal pulmonary *Aspergillus niger* infection in a COPD patient. Immunological tests showed an impaired innate and adaptive immune response to *Aspergillus*. A history of COPD, unresponsiveness to antibiotics and especially a suggestive CT-scan should trigger the clinician to consider diseases caused by *Aspergillus*.

1. Introduction

Invasive pulmonary aspergillosis (IPA), defined as lung parenchyma invasion and necrosis due to *Aspergillus*, is known to be a life-threatening infection in severely immunocompromised patients with significant mortality of 25–50% [1,2]. IPA has been increasingly reported in non-neutropenic patients, such as patients with COPD receiving corticosteroids, decompensated liver cirrhosis, and patients in the intensive care unit (ICU) [3–5]. The diagnosis of IPA in non-neutropenic patients is more challenging because signs and symptoms are often non-specific, risking delayed diagnosis. Here, we describe a case of lethal pulmonary aspergillosis due to *Aspergillus niger* complex in a COPD patient and studied the innate and adaptive immune response. Furthermore, we reviewed the microbiological characteristics of invasive pulmonary *A. niger* complex infections.

2. Case

A 55-year-old woman was admitted to a district hospital (day 0) with progressive cough and hemoptysis for four days. She had no relevant prior medical history other than COPD Gold II, recently diagnosed and yet untreated, and she was a heavy smoker. A chest X-ray performed on day 0 showed a cavitary lesion in the apex of the left lung, suggestive for tuberculosis. A broncho-alveolar lavage (BAL) was

performed on day + 1. Ziehl-Neelsen staining for *Mycobacteria* was negative. Pending sputum and BAL cultures, the patient received amoxicillin/clavulanic acid and was discharged from the hospital. On day + 5, she presented to the emergency department of the same district hospital with fever, thoracic pain and severe dyspnea. Blood panel showed leukocytosis ($26.5 \cdot 10^9/L$ ($4.0\text{--}10.0 \cdot 10^9/L$)) with 89% polymorphonuclear cells (PMN), C-reactive protein (CRP) of 436 mg/L (< 10 mg/L), a creatinin level of $282 \mu\text{mol/L}$ ($45\text{--}80 \mu\text{mol/L}$) and lactate of 2.7 mmol/L ($0.5\text{--}1.6 \text{ mmol/L}$). The sputum and BAL cultures taken on day + 1 grew solely *A. niger*, which was considered contamination. The patient was admitted and treated with intravenous broad-spectrum antibiotics (flucloxacillin 1000 mg q8h and ciprofloxacin 400 mg q12h) as well as prednisone 50 mg q24h for exacerbation of COPD. On the same day, she was transferred to the ICU due to progressive respiratory failure and hemodynamic instability, requiring mechanical ventilation and hemodialysis. A thoracic computed tomography (CT) showed severe consolidations with cavities in the apex of the left lung and apical bullae in the right lung, parenchymal damage and pleural thickening (Fig. 1A). A CT-guided aspiration of one of the cavities revealed hyphae, suggestive for *Aspergillus*. The extensive cavitation, pleural thickening and parenchymal damage on the CT scan suggested either a previously undiagnosed chronic cavitary pulmonary aspergillosis (CCPA), presenting with an acute exacerbation, or a sub-acute invasive aspergillosis (SAIA).

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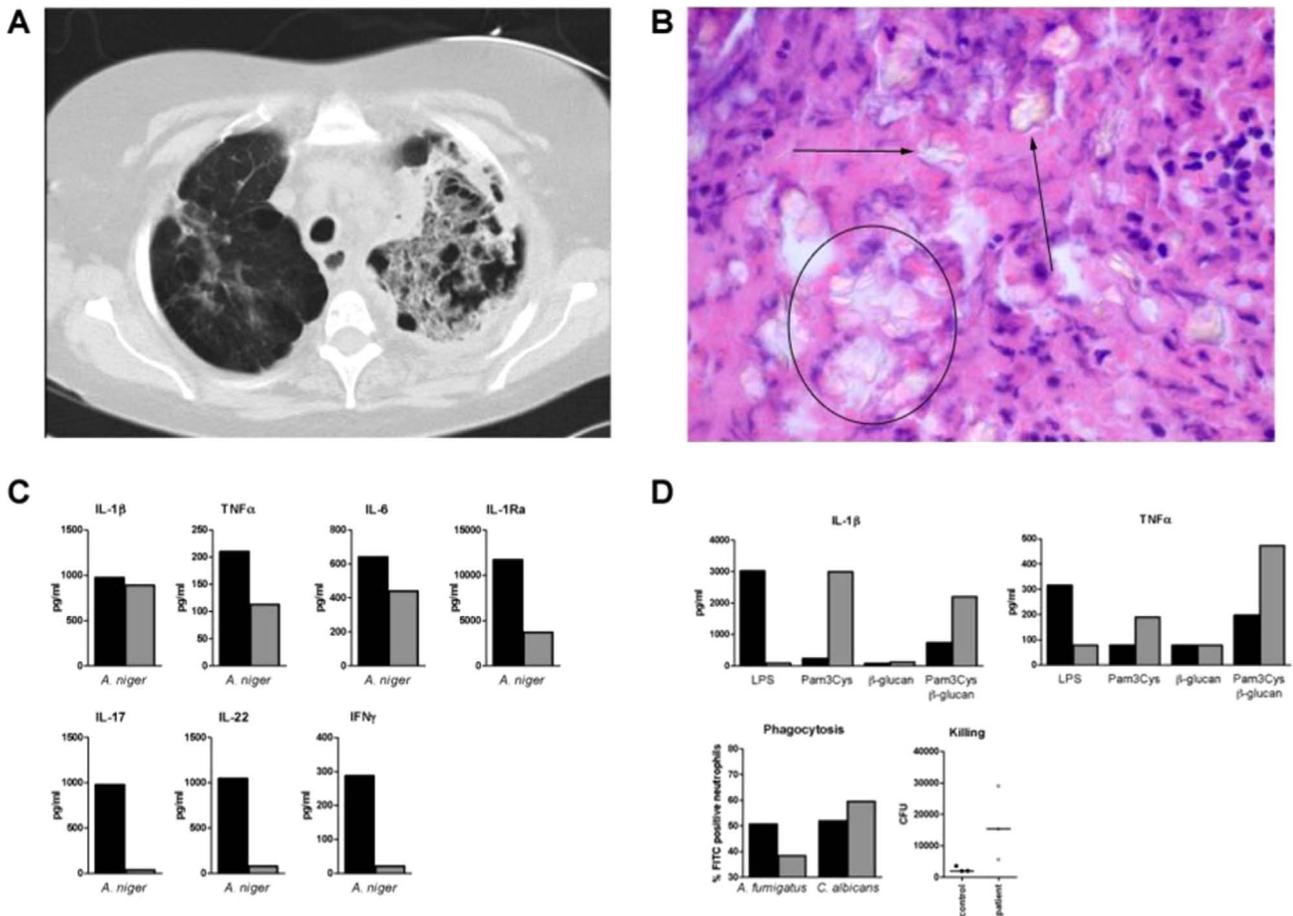


Fig. 1. A. Pulmonary CT-scan on day + 5 of admission showing dense alveolar consolidations in the apex of the left lung with multiple central and subpleural fluid filled cavities and apical bullae in the right lung. B. Calcium oxalate crystal deposition in bronchial debris obtained during broncho-suction. Magnification 400 × ; H&E stain. C. Innate and adaptive cytokine response to *A. niger*. IL-1β, TNFα, IL-6, IL-1Ra (top), IL-17, IL-22 and IFNγ (bottom) levels measured by ELISA in culture supernatants of PBMCs isolated from a healthy volunteer (black bars) and patient (grey bars) stimulated with live (top) or heat-inactivated (bottom) *A. niger* conidia for 24 h (top) or 7 days (bottom). D. Functional assessment of the antifungal host response. Top: IL-1β and TNFα measured by ELISA in culture supernatants of PBMCs isolated from a healthy volunteer (black bars) and patient (grey bars) stimulated for 24 h with LPS, Pam3Cys, β-glucan and β-glucan/Pam3Cys combination. Bottom left: Percentage of FITC-positive neutrophils measured by flow cytometry after 20 min exposure to FITC-labeled *A. fumigatus* conidia or *C. albicans* blastoconidia. Bottom right: Fungal killing capacity, measured by counted CFUs on sabouraud agar plated after exposure of live *A. niger* (1×10^5 conidia) to neutrophils (1×10^6 conidia) from a healthy volunteer (black dots) and patient (grey dots) for 24 h.

That same day (day + 5), intravenous voriconazole 200 mg q12h was started. Blood cultures remained negative. On day + 7 she was transferred to our university medical center ICU due to progressive renal failure for which she needed continuous veno-venous hemofiltration (CVVH). At arrival, she required volume controlled mechanical ventilation, with FiO₂ of 90% for 95% oxygen saturation, and had a respiratory acidosis with severe hypercapnia (pCO₂ 10.3 kPa (4.7–6.4kPa)). Since it was not possible to decrease FiO₂, the patient was ventilated in prone position, initially with good response. A follow-up CT-scan on day + 9 showed increased destruction of the left lung. The antibiotic regimen was switched to piperacillin/tazobactam 4000/500 mg q12h intravenously and the corticosteroids were tapered down. During admission at our ICU, the patient produced excessive amounts of brown sputum, over 500 mL/day. Consecutive sputum cultures taken at our hospital all grew *A. niger*. The *A. niger* isolate cultured from the BAL on day + 1 and all following isolates tested resistant to voriconazole using epidemiological cut-off values of 2 mg/L according to EUCAST (*A. niger* minimum inhibitory concentration (MIC) 4 mg/L). On day + 10, based on the susceptibility testing results, antifungal treatment was switched to intravenous liposomal amphotericin B 3 mg/kg q24h (MIC 0.5 mg/L). As respiratory failure progressed, intravenous micafungin 100 mg q24h was added on day + 12.

Standard immunological tests were performed on day + 8 to determine possible underlying immune deficiencies. Auto-antibody tests,

HIV serology, white blood cell count (differential values and CD4 +), complement system markers (CH50 and AP50), and immunoglobulins (IgA, IgM and IgG including subclasses) showed no abnormalities.

On day + 25, the patient became progressively hemodynamically unstable. Due to lack of respiratory improvement and poor prognosis, it was decided in accordance with the family to cease treatment. The patient was extubated and died soon thereafter. Autopsy was not allowed.

3. Discussion

3.1. Microbiological characteristics of *Aspergillus niger*

Aspergillus spores are amongst the dominant fungal components found during air sampling. The primary route of human infection is via inhalation of airborne conidia, which easily reach the pulmonary alveoli, due to their small size of 2–3 μm [6]. *Aspergillus fumigatus* is the most common species recovered from cases of invasive aspergillosis. It has more favorable characteristics (smaller size of conidia, more thermotolerant) than *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus terreus*, that are responsible for 8–9% of cases [7,8]. Taxonomy of *A. niger* complex is challenging as black-spored aspergilla are difficult to distinguish morphologically. Using sequencing techniques, isolates can be further divided into 19 different taxa, grouped into 5 main clades of

which *A. awamori*, *A. tubingensis* and *A. niger* are most often isolated from clinical samples [9]. Historically, *A. niger* complex has been primarily associated with otomycosis and cutaneous infections. However, an increasing incidence of invasive pulmonary infections with *A. niger* was reported recently [8].

The virulence of *Aspergillus* is multifactorial and depends on both the immune status of the patient and the biological characteristics of the fungus. *A. niger* produces oxalic acid (oxalate) as part of its fermentation process; a feature only rarely described in other *Aspergillus* species [10]. Pyruvate, the end product of glycolysis, is first transformed to oxaloacetate. Then, oxaloacetate acetylhydrolase, located in the cytoplasm of *A. niger*, catalyzes the hydrolysis of oxaloacetate to oxalate and acetate. In the human body, oxalate precipitates with calcium, forming calcium oxalate crystals. These depositions cause tissue damage and necrosis due to obstruction in blood vessels and direct cytotoxicity through oxidative stress [10]. Rapidly developing pulmonary necrosis due to the destructive nature of calcium oxalate produced by *A. niger* complex [11] as well as acute renal failure due to calcium oxalate crystal deposition [12] have been reported.

In our patient, tissue obtained during bronchoscopy was described as necrotic with excessive crystal-like structures (Fig. 1B). In addition, our patient produced excessive amounts of brown sputum, which has been reported in *A. niger* complex infections [11]. Our clinical chemistry department confirmed large amounts of calcium oxalate crystals present in the sputum, which unfortunately was not quantified. As calcium oxalate crystals are hypertonic, this provides a possible explanation for the excessive sputum production. The appearance of black or dark brown sputum with the presence of calcium oxalate crystals on pathological examination may thus be a key feature in the diagnosis of *A. niger* infection. As our patient was anuric, it was not possible to examine a urine sample for the presence of calcium oxalate crystals.

3.2. Immunological defense mechanisms against *Aspergillus niger* invasion

The first defense against inhaled *Aspergillus* conidia begins with their removal by the ciliary action of the mucosal respiratory epithelium. Damage to pulmonary epithelial cells due to inflammation, chronic lung diseases, drugs, chemotherapy, or graft-versus-host disease result in a compromised physical barrier against inhaled fungi. It is now recognized that ICU patients are also prone to IPA. Apart from a critical illness-induced suppressed immunity, mechanical ventilation reduces first line defense of airway clearance and might allow free passage of fungi into the lower segments of the lung [4]. In COPD, impaired ciliary function and chronic inflammation reduce the capacity to clear fungal spores from the lungs and thus provide a natural environment that has a predilection for *Aspergillus* colonization and biofilm formation [13]. Still, it remains unclear why some COPD patients are benignly colonized with *Aspergillus*, whereas other patients develop subacute invasive aspergillosis (SAIA).

To investigate whether the severity of the infection in our patient could be attributed to an immune defect, we tested her *A. niger* innate and adaptive immune responses. First, peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with *A. niger* CBS 101,706 live conidia and heat killed 1×10^7 conidia/mL. A slightly lower, but comparable innate cytokine response of our patient to live *A. niger* conidia was observed when compared to a healthy volunteer. (Fig. 1C).

The function of several pattern recognition receptors, known to be involved in the antifungal host defense to *A. fumigatus* [14], was investigated by stimulating PBMCs with specific ligands, including (lipopolysaccharide) LPS (TLR4), lipopeptide Pam3Cys (TLR2), β -glucan (dectin-1) and a combination of Pam3Cys/ β -glucan. PBMCs of the patient demonstrated an adequate response to stimulation of TLR2 (Fig. 1D). It is known that TLR2 synergizes with dectin-1 [15] that can be observed by stimulating PBMCs with the combination of Pam3Cys and β -glucan (Fig. 1C). Although this synergistic effect was not observed in IL-1 β production, it could be observed for TNF α . In contrast,

stimulation with the TLR4 agonist LPS revealed much less cytokine induction indicating a possible impaired TLR4 function (Fig. 1D). In addition, we observed completely impaired T-helper cytokine production in response to *A. niger*, whereas the control produced significant concentration of T helper cytokines (Fig. 1C).

Impaired neutrophil phagocytosis and killing, as seen in chronic granulomatous disease, might also play a role in the severity of the disease in our patient. PMNs were isolated from whole blood by hypotonic lysis. PMNs of the patient and a healthy volunteer were incubated with fluorescein isothiocyanate (FITC)-labeled *A. fumigatus* V05–27 (heat killed) and FITC-labeled heat-killed *Candida albicans* ATCC MYA-3573 (UC 820) at a final concentration of 2.5×10^7 /mL in a volume of 200 μ L for 30 min at 37 °C and 5% CO₂. The percentage of neutrophils that phagocytosed the fungi was quantified by a FC 500 flowcytometer. Fig. 1D shows 12% less FITC-positivity of the patient neutrophils compared to the healthy control when exposed to *A. fumigatus*, suggesting a slightly lower capacity to phagocytose *A. fumigatus*. Additionally, PMNs were co-cultured with live *A. niger* conidia (1×10^6) to investigate the killing capacity of the patient's neutrophils. After 24 h incubation at 37 °C and 5% CO₂ higher numbers of colony forming units (CFUs) were counted in the patient's cells, indicating a decreased killing ability compared to healthy control. As corticosteroids are known to impair (monocyte) phagocytosis of *Aspergillus* spp. [16], it can therefore not be determined with certainty that our patient had a previously undiagnosed immune deficiency.

4. Conclusion

Invasive pulmonary aspergillosis is increasingly observed in non-neutropenic patients who appear only moderately immunocompromised. We presented a case of pulmonary aspergillosis, caused by *Aspergillus niger* complex, most likely either an acute exacerbation of CCPA or a SAIA, which could not be distinguished as our patient had no prior medical history apart from the very recent diagnosis of COPD. Immunological function tests showed impairment in both innate and adaptive immune system in our patient, which could either be an indication of a previously undiagnosed immune deficiency, or could be attributed to high dose of corticosteroid use. In view of the high mortality, a history of COPD, unresponsiveness to antibiotics and especially a suggestive CT-scan should trigger the clinician to consider diseases caused by opportunistic pathogens, such as *Aspergillus* species, to prevent treatment delays.

Conflict of interest

There are none.

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