Case Report

Microscopic, cultural and molecular evidence of disseminated invasive aspergillosis involving the lungs and the gastrointestinal tract

R. S. Jayshree,¹ M. Shafiulla,¹ J. George,² J. K. David,³ P. P. Bapsy² and A. Chakrabarti³

Correspondence

R. S. Jayshree

jayshre@bgl.vsnl.net.in

^{1,2}Departments of Microbiology¹ and Medical Oncology², Kidwai Memorial Institute of Oncology, Bangalore-560 029, India

³Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh-160 012, India

Received 2 November 2005

Accepted 24 March 2006

A patient with acute promyelocytic leukaemia developed invasive aspergillosis post chemotherapy during a pancytopenic episode, clinically involving the lungs and the gastrointestinal tract. Dichotomously branched septate fungal hyphae were demonstrated microscopically in stools and sputa. Cultures of the samples yielded *Aspergillus flavus*, which were identical by RFLP and random amplification of polymorphic DNA analyses and antifungal MICs, proving disseminated disease. To the best of the author's knowledge, this is the first time that boluses of fungal hyphae have been demonstrated microscopically in the stools of a patient with gastrointestinal aspergillosis.

Introduction

Invasive fungal infections (IFIs) are an important cause of morbidity and mortality in patients with haematological malignancies. Invasive aspergillosis (IA) is particularly common during neutropenic episodes following anticancer chemotherapy (Wright *et al.*, 2003). The most common site of involvement is the respiratory tract. However, autopsy studies conducted in immunocompromised patients with disseminated IA have shown the gastrointestinal tract (GIT) to be a frequent site of sub-clinical involvement (Hori *et al.*, 2002). We describe a patient with acute promyelocytic leukaemia (APML) who developed IA post chemotherapy during a pancytopenic episode. This was diagnosed as disseminated IA by conventional methods of both microscopy and culture. Dissemination was proven in addition by DNA fingerprinting analysis of the *Aspergillus flavus* isolates from both sputum and stool. The highlight of this case report is the finding of fungal hyphae in the direct microscopic examination of stools, which although unusual, may be a significant finding in an immunocompromised patient. Microbiologists, mycologists and pathologists involved in patient care may thus be able to offer improved support to clinicians by becoming proficient in recognizing and interpreting such forms in clinical samples.

Case report

An 18-year-old male who received consolidation chemotherapy consisting of daunomycin and cytosine arabinoside for acute promyelocytic leukaemia developed febrile neutropenia on the 11th day. Other associated symptoms were throat pain, cough and nasal discharge. Physical examination, however, was normal. His total leukocyte count (TLC) and absolute neutrophil count (ANC) were $300 \mu\text{l}^{-1}$ and zero, respectively. Chest X-ray was normal. Sputum culture grew *Klebsiella pneumoniae* sensitive to aminoglycosides and third-generation cephalosporins. The patient had no indwelling catheter. Despite receiving intravenous ceftazidime, amikacin and fluconazole, along with other supportive measures, fever persisted and his condition worsened with the development of haemoptysis and epistaxis. Hence an aggressive IFI was suspected, for which fluconazole was substituted with amphotericin B at a dose of about 1 mg kg^{-1} per day (50 mg per day). Two days after a transient improvement in symptoms, the patient's condition deteriorated. He developed diarrhoea and respiratory distress. Physical examination revealed dyspnoea, tachycardia and scattered crackles over the chest. The TLC and ANC continued to reach nadir, and repeat chest X-ray showed two non-homogeneous opacities in the left mid-zone. Direct microscopy of a 10% potassium hydroxide mount of repeated samples of sputa revealed dichotomously branched septate hyphae. In addition, direct mount examination of the stools (Fig. 1) also revealed boluses of similar fungal elements. Cultures of these samples yielded *A. flavus*. With this clinical picture, we strongly suspected an aggressive

Abbreviations: GIA, gastrointestinal aspergillosis; GIT, gastrointestinal tract; IA, invasive aspergillosis; IFI, invasive fungal infection; RAPD, random amplification of polymorphic DNA.

Fig. 1. Direct wet mount of the stool showing a bolus of vegetative dichotomously branched septate hyphae. Bar, 25 μ m.

disseminated fungal infection. In view of the clinical deterioration and the non-availability of voriconazole at the time, the dose of amphotericin B was increased to 2 mg kg⁻¹ per day (100 mg per day). Subsequently, fever subsided and the patient's condition improved. This was followed by a rise in the TLC and ANC. Biopsies of the affected organs were not attempted due to thrombocytopenia, but clinical, radiological and microbiological data (direct microscopy and culture) were correlated to make a diagnosis of probable disseminated IA. Three repeat samples of stools and sputa collected on three different occasions over the next 3 days also grew *A. flavus* in culture. The anti-fungal susceptibility test of the isolates was carried out as per the guidelines of the National Committee for Clinical Laboratory Standards (2002). The MICs of amphotericin B and itraconazole for the isolates from both the sites were 2.0 and 0.125 μ g ml⁻¹, respectively. Amphotericin B was continued at 2 mg kg⁻¹ per day for another 8 days. Fever subsided, and the patient improved after dose escalation. Later, the dose was reduced to 1 mg kg⁻¹ per day and given for a total of 14 days. Subsequently, due to the rising serum creatinine and normalization of TLC and ANC, amphotericin B was stopped. On the basis of the MIC results, the patient was instead started on oral itraconazole at a dose of 4 mg kg⁻¹ per day (200 mg per day) and discharged. The patient underwent one more consolidation chemotherapy with itraconazole prophylaxis, during which he developed febrile neutropenia that responded to anti-bacterial agents. The patient presently has no evidence of leukaemia or fungal infection.

The isolates of *A. flavus* from both sputa and stools were subjected to molecular analysis in order to investigate whether they were genetically identical. The following strains were used for analysis: isolate from sputum, MCCL (Mycology Culture Collection Laboratory, Chandigarh) 760423; isolate from stool, MCCL 760424; standard strain, *A. flavus* ATCC 16883. Whole-cell DNA from the mycelial form of each isolate was extracted following a slightly

modified protocol of a small-scale fungal DNA extraction method (Lee & Taylor, 1990). For evaluating the genetic relatedness of these strains, the genomic DNA was at first analysed by a RFLP assay using restriction enzymes *Eco*RI and *Pst*I. Separate reactions were set up for each enzyme according to its properties. Approximately 5 μ g genomic DNA was subjected to digestion by 10 U of each enzyme in a 30 μ l reaction volume. The incubation time and other reaction conditions were standardized for individual enzymes.

The oligonucleotide primers R-108 5'-GTATTGCCCT-3' (Anderson *et al.*, 1996) and R151 5'-GCTGTAGTGT-3' (Lin *et al.*, 1995) were used to perform the random amplification of polymorphic DNA (RAPD) assay. Fifty-microlitre reactions were set up with 100 ng genomic DNA, 3 U *Taq* DNA polymerase, 200 μ M dNTP mix, 2.5 mM MgCl₂ and 50 pmol primer R-108/R151. The reaction mixture was subjected to an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min. A final extension step of 72 °C for 10 min was also included. The amplicons were resolved by electrophoresis through a 1% (w/v) agarose gel. Isolates were considered similar when the pattern obtained was the same. The isolates from both the sites were genetically identical as determined by RFLP (data not shown) and RAPD analyses (Fig. 2).

Discussion

IA is an opportunistic infection, particularly common in patients who have received chemotherapy for haematological malignancies, and carries a high degree of morbidity and mortality (Denning & Stevens, 1990; Hori *et al.*, 2002; Prescott *et al.*, 1992; Srivastava *et al.*, 1996; Wright *et al.*, 2003). The respiratory tract is the most common site of involvement, and when the GIT is involved, it is most often

Fig. 2. RAPD analysis of *A. flavus* strains isolated from: sputum, MCCL 760423 (lanes 1 and 4); stool, MCCL 760424 (lanes 2 and 5); and standard strain, *A. flavus* ATCC 16883 (lanes 3 and 6). Primers R-108 (lanes 1–3) and R-151 (lanes 4–6) were used. MW, molecular mass marker 100 bp ladder.

in the form of a secondary dissemination. A large autopsy series, involving 1043 patients with haematological malignancies, reported IA in 10.3% (107/1043). Disseminated disease appeared to be common in these patients with IA (55/107), nearly half of whom had histopathological evidence of GIT involvement (25/55) (Hori *et al.*, 2002). However, approximately 50% of these patients with gastrointestinal aspergillosis (GIA) were asymptomatic, and correct ante mortem diagnosis of GIA was not established for any of these patients (Hori *et al.*, 2002). Another autopsy-based study in patients with malignancies found deep mycotic infections affecting the lower intestinal tract in 1.6% (14/890). However, in none of the cases was faecal culture positive ante mortem; two of the three faecal samples collected at necropsy yielded fungi, one of which was *Aspergillus*, which corresponded with the histological diagnosis (Prescott *et al.*, 1992). In contrast, *A. flavus* and other filamentous fungi, such as *Acremonium strictum* (Schell & Perfect, 1996) and *Trichoderma longibrachiatum* (Richter *et al.*, 1999), have been isolated from faeces in the context of disseminated disease. The patient under discussion developed chemotherapy-induced neutropenia and subsequently acquired disseminated IA with symptoms of cough and diarrhoea. The symptoms correlated with the laboratory findings (microscopic demonstration of fungal hyphae in sputa and stools and isolation in culture from both the samples). Hence our present report reiterates the importance of considering GIA as a differential diagnosis in suspected cases and, accordingly, of submitting clinical specimens for microscopy and fungal culture. This case is peculiar in that fungal hyphae were demonstrated microscopically in the stools. The demonstration of the fungal hyphae in sputum is quite common. However, to the best of our knowledge, there are no reports of the microscopic demonstration of fungal hyphae in the stools. *Aspergillus* infection of the GIT most likely caused this phenomenon, and histopathological examination of tissue biopsies from the affected organ would have confirmed this. The international consensus on opportunistic IFI in immunocompromised patients requires histopathological proof for a definitive diagnosis of IFI (Ascioglu *et al.*, 2002). However, in the present case, endoscopic biopsy was avoided due to the poor general condition of the patient and underlying thrombocytopenia. Therefore, as per the guidelines, the patient under discussion is a case of probable IA with the following strong evidence suggesting the involvement of the GIT in addition to the lung. (a) *A. flavus* strains that were isolated from both stools and sputa were genetically identical and also had the same MICs to amphotericin B and itraconazole. (b) The vegetative forms of the fungi demonstrated in the stools were less likely to be commensal, since *Aspergillus* rarely colonizes the GIT. (c) The vegetative hyphae seen in the stools were unlikely to be food contaminants, since even if spores were swallowed in the food, they often pass into the faeces unchanged (Austwick, 1965). (d) DNA fingerprinting techniques provide us with tools for tracking strains and identifying sources of particular infections, and also for assessing genetic relatedness within

the same species (Soll, 2000). RFLP (data not shown) and RAPD (Fig. 2) are the two methods which we have employed here to show that the isolates of *A. flavus* from both the clinical sites were genetically identical.

IA is a life-threatening infection for which early and aggressive multidisciplinary treatment is crucial (Denning *et al.*, 2003). Optimal therapy involves antifungal agents and surgery, along with measures to improve the immune status of the host. Antifungal agents that are currently used to treat aspergillosis are amphotericin B, voriconazole, itraconazole and caspofungin (Denning *et al.*, 2003; Stevens *et al.*, 2000). The clinical response to an antifungal agent may not be adequate, either because the MIC of the agent to the isolate may be high (Lass-Flörl *et al.*, 1997) or because the agent may not attain adequate tissue levels (Paterson *et al.*, 2003). In this case, as the MIC of amphotericin B to *A. flavus* was $2.0 \mu\text{g ml}^{-1}$, we feel that the organism was less susceptible and therefore responded only to a higher dose [$2 \text{ mg (kg body weight)}^{-1}$] of the drug. Patients with haematological malignancies are at highest risk of developing a life-threatening relapse of IA during a subsequent course of cytotoxic chemotherapy (Buchheidt *et al.*, 2000; Working Party of British Society for Antimicrobial Chemotherapy, 1993). IA did not recur during the second course of chemotherapy, probably because chemoprophylaxis with itraconazole was effective. On follow up nearly 3 years after the episode, the patient still lives without evidence of leukaemia or IFI.

Standards of high-quality care for a multidisciplinary approach to diagnosis and management of IFI have been very rightly proposed in a review (Denning *et al.*, 2003). Our present case stresses one point of the review that microscopic examination of the clinical material is very useful, especially in diagnosing IFI, and can at times be life saving. The presence of pseudohyphal forms in the microscopic examination of stools is being taken as indirect evidence of invasive candidiasis in cancer patients at our centre. Surveillance fungal cultures of stools in high-risk patients are routinely taken at some centres. Although the use of faecal cultures cannot be advocated to diagnose hyalohyphomycosis, a mould isolated from the faeces of an immunocompromised patient may be a significant finding and should not be automatically dismissed as a contaminant (Schell & Perfect, 1996). The demonstration of fungal hyphae in sputa and stools microscopically, their speciation by culture, their molecular characterization, their *in vitro* MIC results and the clinical response to antifungal therapy provided strong evidence for a case of disseminated IA involving the GIT and the lungs, even in the absence of histopathological proof. *Aspergillus* infection should be considered in the differential diagnosis of gastrointestinal complications in patients with haematological malignancy. A high index of suspicion, prompt diagnosis and early institution of aggressive antifungal therapy can cure immunocompromised patients with IA without the need for surgery, provided there is resolution of neutropenia.

Acknowledgements

We would like to express our sincere thanks to Dr S. K. Shankar, Professor, Department of Neuropathology, National Institute of Mental Health and Neurosciences, Bangalore, India, for microphotography.

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