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ORIGINAL ARTICLE

Invasive *Aspergillus* infections in allo-SCT recipients: environmental sampling, nasal and oral colonization and galactomannan testing

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A study was performed to investigate the air quality of a haematopoietic SCT ward, colonization of the upper airways with *Aspergillus* spp. and the role of galactomannan (GM) ELISA testing in serum in the diagnosis of invasive aspergillosis (IA). In 102 allo-SCT recipients, two cases of IA (one proven and one probable) were seen. Of 2071 serum samples, 12 were positive, two in a patient with proven IA and 10 in patients without IA. Of the 2059 negative samples, 22 were taken from the patient with probable IA. Of the 245 environmental samples, 20 (8.2%) were positive for filamentous fungi. *Aspergillus fumigatus* was seen in 14 samples. A total of 657 oral and nasal swabs were taken. Seven nasal samples and one oral sample were positive for *Aspergillus* species (*A. fumigatus* 4, *A. niger* 4) in four patients, one of whom had probable IA. In summary, most environmental samples were negative, colonization of the oral and nasal cavities was rare and IA was diagnosed in only 2% of patients. The GM ELISA test remained negative in one of two patients with IA and does not seem useful in a population of patients with a low incidence of IA.
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Introduction

Invasive aspergillosis (IA) is a potentially life-threatening complication in allo-SCT recipients. The incidence of IA in these patients is 2.9–15.1% and the attributable morbidity and mortality is often over 50%.[1–5] IA is diagnosed at a median of 83–131 days after transplantation.[3,6] Most infections are seen in the lungs and inhalation of *Aspergillus* spores is thought to be the usual route of infection. Whether colonization of the upper airways, that is, the oral and nasal cavities with *Aspergillus* precedes or can be used to predict IA, is controversial.[7–9] Placing patients in HEPA-filtered rooms clearly protects patients, as air filtration has been shown to decrease the number of fungal spores in the air and the incidence of IA.[10–12] In HEPA-filtrated rooms, however, low levels of spores sometimes remain.[11] Also, only about 30% of IA infections are nowadays seen in the first month after the transplantation.[4,5] In most cases, IA is diagnosed later when the patients are no longer hospitalized in a protective environment and colonization of the airways cannot be avoided.

Diagnosing IA is challenging because of the difficulty in obtaining appropriate samples as this requires invasive techniques and blood cultures seldom detect *Aspergillus* species. Different serological tests have been developed over the years to support the diagnostics of IA. Several studies have shown the utility of the Platelia ELISA for *Aspergillus* galactomannan (GM) in patients with chemotherapy-induced neutropenia and in allo-SCT recipients.[6,13–15] A single positive result in the GM ELISA test is listed in the revised EORTC/MSG definitions of IA.[16] The specificity of this test ranges from 79 to 100%.[1,6] The sensitivity level varies from 29.4 to 96.5%, depending on the definition of a positive result (the cutoff level and whether one or two consecutive positive results are used in the analysis).[17,18] The use of bacterial antibiotics, such as piperacillin tazobactam or amoxicillin-clavulanate, and the use of certain i.v. fluids and presence of chronic GVHD have been shown to cause false-positive results in this test, whereas the use of antifungal agents has been shown to cause false-negative results.[1,19–24]

The objectives of this study were to determine whether cases of IA can be detected by GM screening in allo-SCT recipients, whether colonization of the upper airways with *Aspergillus* spp predicts IA and whether the air quality of the SCT ward correlates with the risk of IA.

Materials and methods

Patients

All adult allo-SCT recipients transplanted between January 2001 and December 2002 in Helsinki University Central Hospital were eligible for this study. Patients receiving

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reduced-intensity conditioning that does not lead to severe neutropenia were excluded.

Conditioning regimens
The most commonly used conditioning was CYTBI (CY 60 mg/kg once daily i.v. on days 1–2 and TBI of 12 Gy in six fractions on days 3–7). For some patients, i.v. BU 3.2 mg/kg daily in divided doses for 4 days was combined with CY. The third type of conditioning was a combination of treosulphan 10 g/m^2 daily on days 1–3 and i.v. fludarabine 30 mg/m^2 daily on days 1–5.[25] Patients with aplastic anaemia were treated with CY 50 mg/kg on 4 consecutive days. Antithymocyte globulin (Thymoglobulin, SangStat, Fremont, CA, USA) with a dose of 2 mg/kg on 3 consecutive days was added to the CYTBI, BUCY and CY conditioning regimens of patients receiving their graft from an unrelated donor.

GVHD prophylaxis and treatment
CsA and a short course of MTX were used as GVHD prophylaxis. In patients receiving the graft from a sibling donor, methylprednisolone (MP) was used from day +8 to day +110 with a maximum dose of 1 mg/kg.[26] In case of acute GVHD, MP was given as first-line therapy with a minimum starting dose of 2 mg/kg and a maximum dose of 10 mg/kg daily.

Infection prophylaxis and treatment
All patients were placed in HEPA-filtered private rooms from the beginning of the conditioning. As infection prophylaxis, cotrimoxazole or ciprofloxacin was used until engraftment. Acyclovir was given as antiviral prophylaxis from day −4 until day +35. Systemic antifungal prophylaxis was not used routinely. For patients with acute GVHD treated with high-dose MP (10 mg/kg), amphotericin B (AmB) deoxycholate inhalations 25 mg/day were given for 2–3 months after the beginning of high-dose MP.

To control neutropenic fever, ceftriaxone and tobramycin were started. Blood cultures and chest X-rays were taken at the start of neutropenic fever and thereafter according to the clinician's decision. High-resolution computed tomography (HRCT) scanning and bronchoalveolar lavage (BAL) were performed on patients with radiological signs of lung infection. In cases of 5 days of fever unresponsive to broad-spectrum antibiotics, i.v. AmB was started as empirical antifungal therapy.

Study samples
Environmental surveillance samples were taken from five locations inside the HSCT ward at 1–3-week intervals. Environmental sampling was started 6 months before the beginning of patient sampling and continued over the whole study period. The locations were drug dispensary (location 1), bathroom of one of the patient rooms (location 2), vestibule between the double doors of one patient room entry (location 4) and two patient rooms (location 3 and 5). Settled dust analyses were performed using plastic cups that were left at the locations. Fungal cultures were performed on settled dust collected in the plastic containers using the standard techniques for the isolation and speciation of fungi.[27]

The degree of upper airway colonization was studied by taking swab samples for fungal cultures from both nostrils and the dorsum of the tongue of the patients once a week whenever hospitalized.

A total of 1–2 ml of blood was taken for the serum analysis in a prospective way once a week until 12 weeks after transplantation and thereafter 1–2 times a month. The samples were stored at –20 °C. Analyses were performed using the GM ELISA test (Platelia *Aspergillus*, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. An optic density index of $\geqslant 0.5$ in was used as the criterion of test positivity.

The first patient samples were taken before the start of the conditioning regimen and the sampling and follow-up of clinical data were continued until 1 year after the transplantation, death or leukaemic relapse. Informed consent was obtained from all patients. This study was accepted by the Helsinki University Central Hospital Ethics Committee.

Definitions of IA
The revised EORTC/MSG criteria were used to define the cases.[16] Proven, probable and possible cases were included. GM ELISA results were excluded from the criteria for IA. In addition to IA, all other types of invasive fungal diseases were evaluated.

Statistical analyses
Comparison of categorical variables was made by using Fisher's exact test or chi-square test. The difference in numbers of colony-forming units (CFUs) in different locations of environmental sampling was assessed by Kruskal–Wallis test. The sensitivity, specificity and predictive values of the GM ELISA test were calculated from 2×2 tables. P-values <0.05 were considered statistically significant. The statistical analyses were performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

During the study period, 138 patients were transplanted. A total of 36 patients were excluded because of reduced-intensity conditioning or patient refusal and 102 patients were therefore left for the final analysis. The median age of the patients was 44 years (range 18–60). Three patients were retransplanted because of rejection. At 1 year after transplantation, 75 patients (73.5%) were alive. Relapse was the cause of death in 12 patients, and 15 deaths were transplant related. Autopsy was performed on seven patients who died of transplantation complications. The causes of death were GVHD in two patients, post transplant lymphoproliferative disease in one, septicaemia and multiorgan failure in one, disseminated varicella in one, invasive candidiasis (IC) in one and IA in one patient. No new cases of IA were found by autopsy. In the remaining eight patients who died of transplant-related reasons, the probable causes of death were GVHD in four patients, RSV infection in one, IA in one, post transplant lymphoproliferative disease in one and bronchiolitis

Table 1 The main characteristics of the patients

Characteristics	*Total patients* n = *102*
Age, median (range)	(44 (18–60))
Sex (female/male)	45/57
Underlying disease	
AML	30
CML	25
ALL	20
MDS	11
CLL	5
MF	4
MM	2
NHL	2
SAA	2
Diamond–Blackfan anaemia	1
Disease status	
AML/ALL/CML CR1/CP1	52
AML/ALL/CML beyond CR1/CP1	23
Other diagnoses	27
IFD before transplantation	
Probable IC	5
Probable IA	1
Conditioning 1	
CYTBI	93
BUCY	4
Treo—Flu	4
CY	2
Flu–ATG	1
ATG	1
Donor type	
HLA-identical sibling	60
Syngenic	1
Matched unrelated	39
Mismatched unrelated	2
Graft source[a]	
BM	52
PBSC	47
Both	3
Duration of neutropenia, days, median (range)[b]	16 (0–137)
Acute GVHD	
Group I–IV	36
Group II–IV	15
Chronic GVHD	
Limited	21
Extensive	11
OS (1 year)	75 (74.3%)

Abbreviations: ATG = antithymocyte globulin; Flu = fludarabine; IFD = invasive fungal disease; IC = invasive candidiasis; IA = invasive aspergillosis; MDS = myelodysplastic syndrome; MF = myelofibrosis; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; SAA = severe aplastic anaemia; Treo = treosulphan.
[a]Three patients received a second transplantation because of rejection.
[b]ANC $\leqslant 0.5 \times 10^9$/l.

obliterans with organizing pneumonia in one patient. The patient characteristics are shown in Table 1.

Invasive fungal diseases

Three patients were diagnosed with invasive fungal disease. One patient had both a proven IC and *Fusarium* infection and two patients had IA.

One patient with AML had acute GVHD on day +23 after SCT from an HLA-identical sibling and was diagnosed with proven pulmonary IA 109 days after transplantation. The diagnosis was confirmed by a fine needle biopsy of lung tissue. The histological sample was positive for *Aspergillus fumigatus*. All swab samples taken from this patient were negative for *Aspergillus*. A total of 17 serum samples taken before the diagnosis of IA were all negative. The last two samples taken from this patient were positive with optical density indices of 4.5 and 3.2. These samples were taken 3 days before and 4 days after the needle biopsy. Chest X-ray abnormalities, however, were seen 6 days before the first positive serum sample was taken. By the time of IA diagnosis, the patient was receiving prophylactic AmB inhalations that were replaced by i.v. liposomal AmB. The patient died of IA 23 days after the diagnosis.

Another patient had probable IA. This patient had CML in the second chronic phase and received a graft from an HLA-A Ag mismatch unrelated donor, but the graft was rejected. She was retransplanted 97 days later but this graft was rejected as well. The duration of neutropenia was 137 days in this patient. HRCT scan revealed changes indicative of IA and *Aspergillus niger* was cultured from BAL fluid on day +154 after the first transplantation. Nasal swabs taken 2 days before the HRCT and BAL were positive for *A. niger*. Until then, the swab samples had been negative for *Aspergillus*. All 22 serum samples were negative, including the last sample that was taken when signs of IA were otherwise visible. The patient succumbed on day +162 after the first transplantation despite therapy with i.v. liposomal AmB. Postmortem was not allowed.

HRCT abnormalities and BAL

In addition to the two patients with IA, HRCT abnormalities were seen in 16 patients. None of these 16 HRCT findings filled the criteria of possible IA. BAL samples were obtained from 13 patients. Direct microscopy for fungi and fungal cultures were performed each time but remained negative. In the remaining three patients with HRCT abnormalities but no BAL, the clinical diagnoses were clear (two with parainfluenza 3 infections and one with bacterial pneumonia) and the HRCTs became normal without antifungal therapy.

Use of antifungal and antibacterial drugs

With regard to antifungals with anti-*Aspergillus* activity, i.v. AmB was given as the conventional or liposomal form or both to 28 patients during the first year after transplantation with a median duration of therapy of 5 days. Two of these patients received the drug as secondary prophylaxis because of a probable invasive fungal disease (one IA and one IC) before transplantation and in 23 patients the therapy was empirical. The remaining three patients were those with proven or probable invasive fungal disease after transplantation. AmB inhalations were given to 39 patients for a median of 65 days as prophylaxis after acute GVHD. One of these patients developed proven IA while on prophylaxis.

Table 2 Results of environmental surveillance

	No. of positive samples	*Mean no. of CFUs*	*Species*
Location 1	6/49	2	*A. fumigatus* 5 Unidentified *Aspergillus* sp 1
Location 2	5/49	2.4	*A. fumigatus* 4 *C. albicans* 1
Location 3	1/49	1	*Rhodotorula* 1
Location 4	5/49	1	*A. fumigatus* 3 *Rhodotorula* 1 *Penicillium* 1
Location 5	3/49	1.3	*A. fumigatus* 2 *Mucor* 1
All	20/245 (8.2%)	Median 1 (range 1–7)	

Abbreviations: CFU = colony forming unit.
Location 1: drug dispensary.
Location 2: patient bathroom.
Location 3: patient room.
Location 4: entry to a patient room.
Location 5: patient room.

Amoxicillin or amoxicillin–clavulanate was given to 13 patients (12.7%) during the time of follow-up for a median of 7.5 days (range 1–22 days). Piperacillin–tazobactam was given to only one patient for 7 days. Any type of β-lactam antibiotic was given to 85 patients (83.3%).

Environmental sampling

During the 2.5-year period, environmental samples were taken from the five locations 49 times. Of these 245 samples, 20 (8.2%) were positive. A single pathogen was seen in all positive samples. *A. fumigatus* was the most common pathogen, seen in 14 samples. The median number of CFU of positive samples was 1 CFU/m^3 (range 1–7). During a 5-week period 3 months from the beginning of sampling, 2–3 consecutive samples (nine samples altogether) were positive for *A. fumigatus* in four locations. The remaining 11 positive surveillance samples were seen at random instances throughout the surveillance period. During the 2.5-year period, there was no construction activity in the immediate vicinity of the HSCT ward. The differences in the amount of the positive samples or the median numbers of CFUs in the five locations were not statistically significant. The results of the surveillance samples are shown in Table 2.

Colonization of nasal and oral cavities

Swab samples from the nasal and oral cavities were taken 657 times, with a median number of six samples per patient (range 1–23). *Aspergillus* species were seen in seven samples. One patient had *A. fumigatus* in both nostrils in the first samples. Another patient had *A. fumigatus* in one nostril once during the follow-up period. In both patients, all the remaining samples were negative. Neither of these patients was diagnosed with IA. The remaining four samples positive with *A. niger* were taken during the last 2 weeks of life from the patient with probable IA after transplantation. In addition to these aspergillus-positive samples, five other patients had nasal colonization with *C. albicans, C. glabrata* or *Penicillium* but never in more than one sample.

Of the 657 mouth swabs, 92 (13.8 %) samples were positive in 39 (38.2%) patients. *A. fumigatus* was seen in one sample (1.1% of positive samples). This was the first sample taken from a patient who was already receiving i.v. liposomal AmB as secondary prophylaxis because of a probable *Candida* infection before transplantation. Two samples were negative thereafter and the patient was not diagnosed with IA. In the remaining 91 positive oral samples, various species of *Candida* were seen.

GM ELISA results

A total of 2071 blood samples were taken with a median of 22 samples per patient (range 4–38). Of these, 12 samples (0.6%) taken from nine patients were positive in the GM ELISA test. In one patient, two consecutive samples were positive. This patient had proven IA. The remaining 10 positive samples were taken from eight patients in whom IA was not diagnosed. In two patients, the result of the blood test was positive twice but not in consecutive samples. In one patient with a clinical picture of disseminated varicella infection, HRCT abnormalities were seen at the time of false-positive GM ELISA result. BAL samples were negative for fungi. Postmortem confirmed the diagnosis of disseminated varicella. Of the 2059 negative GM ELISA samples, 22 were taken from a patient with a probable IA, the last one when clinical signs of IA were present.

In the per-patient analysis with only two cases of IA, the sensitivity, specificity, positive predictive value and negative predictive value of the GM ELISA test were 50, 92, 11 and 99%, respectively.

Discussion

In the environmental samples, there were no elevated levels of *Aspergillus* spores or seasonal variation at any time point during the follow-up. In the positive samples, the concentration of fungi was low, with a median of 1 CFU/m^3. This finding corroborates a French study where the median

number of CFU/m^3 was 1.4 in a BMT ward and one-third of the positive samples grew *Aspergillus* species with a mean of 1 CFU/m^3.[11] It is somewhat controversial whether the amount of fungal spores in the air correlates with the risk of IA or not, but in some studies this has been the case.[10,28] The HSCT ward air quality issues only relate to early IA infections, diagnosed within the first month after transplantation when patients are hospitalized. The results of air quality monitoring in this study should be interpreted with some reservation as only settled dust analysis was used. To get a more precise picture of the air quality, other methods such as air sampling or particle measurements or both should be used.[29]

Colonization of the oral or nasal cavities of the patients by *Aspergillus* spp. was rare. A similar finding has been reported by others.[30] Three patients were temporarily colonized with *A. fumigatus* but they did not develop IA. The fourth patient, who had *A. niger* in her nasal cavity, developed signs of a probable IA 2 days later. In a study by Aisner *et al*.,[7] nasal colonization by *A. flavus* was a clear risk factor for IA and colonization by *A. fumigatus* a possible risk factor. In that study, 8.8% of the patients were colonized with *Aspergillus* spp. compared with 3.9% in this study. Colonization was more common in a study by Richardson *et al*.,[9] where *A. fumigatus* was seen in 24% of patients. That study, however, included various types of haematological patients, all of whom were not treated in HEPA-filtered rooms. A correlation between colonization and the risk of IA was not seen.

The GM ELISA test was positive in one of two patients with IA, and the sensitivity of the test was thus 50%. This agrees with another study with 121 patients that also used optic density index $\geqslant 0.5$ as the cutoff value.[31] At best, however, the sensitivity has been 81–96.5% and the GM ELISA test has become positive on several days, even weeks before any other signs of IA are visible.[18,32,33] In the only patient with IA and positive GM ELISA test results in this study, the positive result did not precede other clinical signs of infection.

False-positive GM ELISA test results were seen in eight patients but never in two consecutive samples. Overall, only 0.5% of the samples gave false-positive results. Piperacillin–tazobactam, amoxicillin or amoxicillin–clavulanate, which are the best known causes for false-positive results, were given to 13.7% of the patients. None of the patients with false-positive results were receiving these antibiotics when the samples that gave false results were taken.

A few additional points should be made about the design and results of this study. First, the incidence of IA in this study was much lower than in an earlier study from our centre, which included patients transplanted from 1989 to 1995.[3] In that study, 11% of the patients were diagnosed with IA. The performance status of GM ELISA was undoubtedly affected by the low incidence of IA.

Second, 39 patients received AmB inhalations as prophylaxis against IA after therapy with high-dose MP for acute GVHD. This might affect both the degree of nasal or oral colonization and the incidence of IA. To our knowledge, no studies have been published about this type of long-term IA prophylaxis after GVHD, which has been used in our centre since the beginning of 2001.

Third, it is recommended that the GM ELISA test should be performed at least twice a week. In this study, the test was performed once a week for the first 12 weeks and thereafter 1–2 times a month until 1 year. Our objective was to target the Ag testing more towards the most typical time point of IA, which is 3–4 months after the transplantation. In a recent Japanese study, the median time of IA was even later, 204 days after transplantation.[1] This shift in the timing of IA creates more challenges for the use of serological methods.

In conclusion, in this study, the air of the HSCT ward did not contain substantial concentrations of *Aspergillus* conidia, colonization of the nasal or oral cavities of the patients with *Aspergillus* species was rare and IA was seen in 2% of the patients. The GM ELISA test was not helpful for the earlier diagnosis of IA. Overall, the routine use of GM ELISA testing as performed in this study does not seem useful in a population of patients with such a low incidence of IA.

Conflict of interest

M Richardson is the founder and a shareholder of MoBiAir Diagnostics Ltd.

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