CLINICAL ARTICLES

Stage-Specific Manifestation of Mold Infections in Bone Marrow Transplant Recipients: Risk Factors and Clinical Significance of Positive Concentrated Smears

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Potassium hydroxide–concentrated smears, prepared from sedimented remains of clinical specimens, were used to distinguish between mold infection and exogenous contamination in fungal culture–positive specimens. This method was applied in the study of 3,857 clinical specimens from 230 bone marrow transplant recipients who were followed up prospectively for infectious complications. Concentrated smears of only 86 (from 21 infected patients) of 149 fungal culture–positive specimens were positive for hyphae; 82 of the strains were *Aspergillus* species. Concentrated smears of the remaining 63 fungal culture–positive specimens were negative; the strains identified by culture were considered as exogenous contaminants (87% of which were *Penicillium* species). A stage-specific manifestation of mold infection was observed: 67% of mold infections occurred during acute graft-vs.-host disease (GVHD) a median of 47 days after transplantation, whereas 9% of mold infections occurred as rapidly fatal invasive disease before engraftment. Overall, of the 21 patients with mold infection, 17 (81%) had invasive mold disease, and four (19%) had mold colonization of airways secondary to chronic GVHD after day 100. The significant risk factors for mold infection were total-body irradiation and grade 2–4 acute GVHD. Because of our high mortality rate (82%), the consideration of antimold prophylaxis for such patients may be warranted.

Since the last decade, molds are gaining prominence as opportunistic pathogens. In immunocompetent hosts, molds rarely cause serious illnesses, except for indwelling device–associated infections, posttraumatic mycetoma, aspergilloma in patients with preexisting chronic lung diseases, and self-limiting diffuse pneumonitis after massive inhalation of spores. However, molds cause life-threatening diseases in immunosuppressed hosts, especially patients with AIDS, neutropenic patients, and solid organ and bone marrow transplant (BMT) recipients [1, 2]. Most invasive diseases due to molds are caused by *Aspergillus* species. In one study [3], infections with this mold occurred in 8.7% of allogeneic BMT patients. In another study [4], the mortality rate associated with aspergillus infection in patients with pulmonary involvement and persistent neutropenia was 95%.

The successful management of mold infections is hampered by difficulties in establishing diagnoses. Molds are ubiquitous in the environment and are often disregarded in cultures of nonsterile specimens such as sputum, urine, stool, and superficial swabs. In the absence of clinical evidence, the presence of molds in these specimens usually indicates contamination by fungal spores during collection and processing or colonization of fungal spores on damaged airways, mucosal lesions, and superficial skin [5].

Several laboratory criteria (e.g., the presence of characteristic fungal elements in smears and multiple cultures of the same species) have been proposed to exclude exogenous contamination. However, the sensitivity of detection by smear examination is low, and consecutive specimens for culture are usually not available. The sensitivity or specificity of alternative diagnostic approaches, such as the detection of fungal antigens or DNA amplification, has also been found to be low [6].

The definitive diagnosis of invasive disease requires the isolation of mold in culture and histological demonstration of its presence [7]. Yet, in one study [8], the predictive value of positive cultures of respiratory tract specimens from neutropenic patients with invasive pulmonary aspergillosis varied from 40% to 100%, and tissue biopsy is invasive and requires necessary expertise to minimize the associated morbidity in immunosuppressed patients.

Hence, the clinical significance of a single positive culture with a negative direct smear is hard to determine. Since early and appropriate therapy is crucial, we used the concentrated

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smear as a rapid and sensitive diagnostic tool to differentiate genuine mold infection from contamination. A prospective study of 3,857 clinical specimens from 230 BMT patients was conducted; direct and concentrated smears of fungal culture–positive specimens were prepared and examined. The clinical correlation of positive concentrated smears, the temporal sequence of the onset of infection, and the risk factors for mold infections were analyzed. The results of this study are presented herein.

Patients and Methods

Patients and antimicrobial prophylaxis. Two hundred thirty patients who underwent bone marrow transplantation at the Queen Mary Hospital in Hong Kong during a 5-year period (1991–1995) were included in this study. Before transplantation, all patients were admitted to protective isolation rooms with high efficiency particulate air filtration [9]. Patients who were receiving BMTs from mismatched siblings or matched unrelated donors (MUDs) were admitted to rooms that were also equipped with laminar airflow [10]; these patients underwent total-gut decontamination with tobramycin (80 mg q.i.d.), vancomycin (500 mg q.i.d.), and nystatin (10 mL q.i.d.).

From day 0 until stable engraftment, all patients were treated with chlorhexidine mouthwash, amphotericin B lozenges, ciprofloxacin (500 mg b.i.d.), and acyclovir (200 mg t.i.d.) [11, 12]. When patients developed neutropenia and fever, they were treated empirically with iv imipenem/cilastin (10 mg/kg q6h). If fever persisted for 48 hours and the workup for sepsis was negative, iv vancomycin (10 mg/kg q6h) was added to the treatment. On the fifth day of neutropenia and fever, iv amphotericin B therapy (0.5 mg/[kg·d]) was initiated. If mold infection was documented or suspected on clinical and microbiological grounds, the dosage of amphotericin B was increased to 1.5 mg/(kg·d).

All patients undergoing allogeneic or MUD transplantation received the following as prophylaxis for acute graft-vs.-host disease (GVHD): 15 mg of iv methotrexate/m^2 on day 1; 10 mg of iv methotrexate/m^2 on days 3, 6, and 11; and 5 mg of oral cyclosporine/kg b.i.d. from day 1 to day 50 or 1.5 mg of iv cyclosporine/kg q12h from day 1 to day 50 when the patient could not tolerate oral medication.

Microbiological methods. One week before and up to 1.5 years after transplantation or until death, each patient's medical progress was recorded, and clinical specimens from each patient were collected. Routine surveillance cultures of stool, nasal swabs, and throat swabs were performed weekly to detect fungi and multidrug-resistant bacteria. All respiratory secretions, including sputa, endotracheal aspirates, and bronchoalveolar lavage (BAL) fluids, were cultured for bacteria, mycobacteria, *Legionella,* and fungi. Direct smears of only tissue and respiratory specimens undergoing fungal culture were prepared for microscopic examination for mold. Concentrated smears of all fungal culture–positive specimens were retro-

spectively prepared and examined; only concentrated smears of BAL fluid specimens were prepared directly and examined.

Before microscopic examination and culture of the specimens were performed, respiratory secretions were pretreated with Sputasol (Oxoid, Basingstoke Hampshire, United Kingdom), and tissue biopsy specimens were homogenized in microfuge tubes containing PBS. For microscopic examination, tissue biopsy specimens were further digested in 20% (w/v) KOH solution at 37°C for 15 minutes. To prepare a direct KOH smear by wet mounting, 20 μL of the specimen was mixed with an equal volume of 20% KOH solution, placed on a glass slide, and mounted under a coverslip. The slide was screened at low-power magnification (×100) under dark-field illumination; septate hyaline hyphae with uniform diameters (4 μm) and dichotomous branching at 45° were highly suggestive of *Aspergillus.*

Each specimen was inoculated onto two plates with Sabouraud dextrose agar supplemented with chloramphenicol (50 mg/L); the plates were incubated at 25°C and 37°C. The remains of each specimen were kept at 4°C for 2 weeks before disposal. Mold isolates were identified by standard morphological criteria [13]. The day on which positive growth was visually observed and the site and number of cfu on the agar were recorded.

The corresponding refrigerated clinical sample of the fungal culture–positive specimen was retrieved for the preparation of concentrated smears. The maximal amount of each specimen was digested in an equal volume of 20% KOH solution and centrifuged at 5,000 rpm for 10 minutes at room temperature. Swab specimens positive for fungi were vortexed in Eppendorf tubes containing PBS to recover fungal elements and concentrated by centrifugation. All but ~100 μL of the supernatant fluid was decanted; an aliquot of the fluid was mounted on a glass slide and examined as described above.

Definitions. Invasive mold disease was defined either by culture and histological examination of a biopsy or autopsy tissue specimen revealing mold or by culture and examination of a direct smear showing mold, a compatible clinical and radiological picture, and no response to antibacterial therapy. A positive culture without documented evidence of invasive disease could be due to colonization or exogenous contamination. Acute myeloid leukemia and acute lymphoblastic leukemia in the first complete remission, chronic myeloid leukemia in chronic phase, lymphoma in the first complete remission, myeloma in complete remission, and severe aplastic anemia in patients younger than 25 years of age who were not receiving immunosuppressive therapy were considered as early underlying diseases. Any other underlying diseases were considered as late diseases.

Data analyses. A comparison of microbiological characteristics between concentrated smear–positive, fungal culture–positive specimens and concentrated smear–negative, fungal culture–positive specimens was performed by the χ^2 test. Univariate logistic analysis was performed to identify risk factors for infections by molds. On the basis of the results of the

univariate logistic analysis, variables with P values of $<.05$ or those known to be biologically important were included in a multivariate logistic analysis. The backward elimination method was used to select variables. The importance of each factor to the model was assessed by the likelihood ratio; a two-tailed χ^2 test was used to determine this ratio.

Results

Fungal cultures of 149 of 3,857 clinical specimens (of which 1,072 were derived from febrile neutropenic patients) were positive; concentrated smears of 86 of these 149 fungal culture–positive specimens were positive for mold (table 1). Not unexpectedly, there was an absolute correlation between the results of the examinations of concentrated smears and those of the examinations of the direct smears. Several statistically significant differences ($P < .05$) were noted between concentrated smear–negative specimens and concentrated smear–positive specimens. Concentrated smear–positive specimens were taken mainly from sinopulmonary sites or biopsied tissues.

Most isolates (95%) from concentrated smear–positive specimens were *Aspergillus* species, while 87% and 3% of the fungal isolates from concentrated smear–negative specimens were *Penicillium* and *Aspergillus* species, respectively. The

median period from inoculation to detection of visible growth for the concentrated smear–positive specimens was 3 days less than that for the concentrated smear–negative specimens. Multiple colonies were growing on the agar for all concentrated smear–positive specimens; these colonies were all found on the primary streak line. In addition, 12 specimens from patients with invasive diseases who were receiving antifungal therapy were culture-negative but concentrated smear–positive, a finding with implications for therapeutic monitoring (these implications are reviewed under Discussion).

During the study period, 21 (9%) of 230 BMT patients had culture-documented infection by molds (table 2). These infections were diagnosed a median of 67 days after transplantation. Invasive diseases occurred in 17 patients (81%), while four (19%) had colonization. The respiratory tracts of these four patients were colonized with *Aspergillus* species (one with three *Aspergillus* species) after day 100, and all four did not have evidence of mold infection earlier in their clinical courses when they were neutropenic.

Most of the infections were caused by *Aspergillus flavus* (38%), *Aspergillus fumigatus* (19%), and other *Aspergillus* species (28%). The lung and paranasal sinuses (86%) were the predominant sites of infection [14]. Extrasinopulmonary infections involved the brain, skin and soft tissue, gastrointestinal tract, and ear. Most infections occurred after engraftment (me-

Table 1. Comparison of results of examinations of concentrated smears of clinical specimens from BMT recipients that were positive for mold by culture.

Result	Culture-positive specimens		
	Concentrated smear–positive ($n = 86$)	Concentrated smear–negative ($n = 63$)	P value*
No. of patients (no. of specimens)	21 (402)	209 (3,455)	
No. of patients with positive direct KOH smears (no. of specimens)	7 (29)	0 (0)	<.0001
No. of indicated culture-positive specimens			
Respiratory secretion (sputum, ETA, BAL fluid)	49	2	<.0001
Nasal discharge or swab	13	11	NS
Throat swab	0	18	<.0001
Stool or rectal swab	2	20	<.0001
Tissue	10	0	<.05
Others†	12	12	NS
No. of specimens with indicated isolates			
Aspergillus species‡	82	2	<.0001
Fusarium species	2	0	NS
Penicillium species	1	55	<.0001
Others§	1	6	NS
Median duration (d) until visible growth (range)	2 (1–4)	5 (1–12)	
Presence of mold on Sabouraud dextrose agar			
Single colony	11	48	<.0001
Multiple colonies on primary streak line	86	18	<.0001

NOTE.   BAL = bronchoalveolar lavage; BMT = bone marrow transplant; ETA = endotracheal aspirate; NS = not significant.
* Statistical analysis by the χ^2 test.
† Includes antral washout, ear discharge, wound swab, catheter tip, and urine.
‡ Multiple species of *Aspergillus* were isolated from some specimens.
§ Includes *Acremonium* species and members of Mycelia sterilia.

Table 2. Clinical characteristics of 21 BMT recipients with mold infections.

Characteristic	Value*
Median duration (d) until diagnosis (range)	67 (0–634)
Type of mold infection	
Invasive mold disease	17 (81)
Colonization, invasive disease doubtful	4 (19)
Type of mold isolated	
Aspergillus flavus	8 (38)
Aspergillus fumigatus	4 (19)
Other *Aspergillus* species	6 (28)
Fusarium solani	1 (5)
Penicillium funiculosum	1 (5)
Acremonium falciforme	1 (5)
Organ(s) involved[†]	
Lung	13 (62)
Paranasal sinuses	5 (24)
Brain	3 (14)
Skin and soft tissue	2 (10)
Gastrointestinal tract	1 (5)
Ear	1 (5)
Median time (d) of onset of infection (range)	47 (0–634)
Infection before engraftment	2 (9)
Infection after engraftment during the period of acute GVHD	14 (67)
Infection after engraftment during the period of chronic GVHD	5 (24)
Concomitant infection	
Cytomegalovirus	11 (52)
Bacteria[‡]	7 (33)
Concomitant neutropenia (neutrophil count, $<500 \times 10^9$/L)	4 (19)
Death due to invasive mold disease	14/17 (82)

NOTE. BMT = bone marrow transplant; GVHD = graft-vs.-host disease.

* Unless otherwise stated, data are no. (%) of patients.

[†] More than one organ was involved in four patients.

[‡] Includes methicillin-resistant *Staphylococcus aureus,* coagulase-negative staphylococci, *Streptococcus pneumoniae, Pseudomonas aeruginosa, Pseudomonas cepacia, Mycobacterium avium/Mycobacterium intracellulare* complex, and *Mycobacterium chelonae.*

dian time of onset of infection, 47 days); 14 patients (67%) were receiving immunosuppressive treatment for acute GVHD.

Only two patients with severe neutropenia (9%) developed infection before engraftment; both had severe diffuse pulmonary disease with extrapulmonary dissemination. Thirteen patients (62%) developed neutropenia and fever early after transplantation, but none of these patients had any other clinical or radiological evidence of invasive disease. Concomitant cytomegalovirus (CMV) disease occurred in 11 patients; these patients received ganciclovir therapy. Two of these 11 patients developed severe neutropenia secondary to ganciclovir therapy just before the onset of invasive mold disease and required therapeutic support with growth factors; however, none of these patients became more neutropenic after the diagnosis of mold disease.

Of the 17 patients with invasive mold disease, 14 were treated with iv amphotericin B (1–1.5 mg/[kg·d]), and three were treated with iv liposomal amphotericin B (3 mg/[kg·d]).

Three patients received additional treatment with oral itraconazole (600 mg/d for 3 days and then 400 mg/d until death) because of poor response to amphotericin B therapy. Despite treatment of these 17 patients, 14 (82%) died of mold infection and uncontrolled GVHD. The underlying diseases in these patients were in remission. Only three patients infected with *Penicillium funiculosum, Acremonium falciforme* [15], and *Fusarium solani,* respectively, survived. Their neutrophil counts at the onset of infection were $>1.5 \times 10^9$/L, and two received granulocyte macrophage colony-stimulating factor therapy for augmentation of phagocytic function. Of the four patients who were colonized with *Aspergillus,* three received oral itraconazole therapy for 2 weeks. None of these patients had additional evidence of invasive disease, despite continuous isolation of *Aspergillus* from their sputa.

The possible risk factors for infection by molds are presented in table 3. Univariate logistic analysis found that acute lymphoblastic leukemia, MUD, total-body irradiation (TBI), and grade 2–4 GVHD were significant risk factors. However, multivariate logistic analysis revealed that only TBI and grade 2–4 GVHD were significant risk factors.

Discussion

The differentiation of infection from exogenous contamination by mold is often difficult but would be lifesaving in the setting of bone marrow transplantation. We used concentrated smears of fungal culture–positive specimens to distinguish between the two entities and found that the presence of characteristic hyphae was often detected in clinically compatible cases (table 1). This finding was expected since the concentrated smear in effect screened the entire specimen for the presence of the mold, while only a fraction of the specimen was inoculated for culture.

Examination of concentrated smears for hyphae is rapid, and the entire field of the smear can be completely evaluated for these large fungal elements at a magnification of ×100 within 2 minutes. However, it would not be practical and cost-effective to incorporate this method into routine mycology procedures because there would be a tremendous increase in the work load. Therefore, immediate preparation and examination of concentrated smears of specimens from patients suspected of having mold infection or of selected specimens (e.g., BAL fluid) would definitely help in establishing a diagnosis earlier. Furthermore, the concentrated smear may also be useful in monitoring the progress of treatment, since cultures become less sensitive after the initiation of antifungal therapy.

After ascertaining the value of the concentrated smear, we explored the clinical aspects and possible implications for mold infection. A stage-specific pattern of mold infection was found. Most mold infections occurred after engraftment during the period of acute GVHD; only two of the 17 episodes of invasive disease with extrasinopulmonary dissemination occurred in the neutropenic period during the first 30 days, and colonization of airways in four patients with chronic GVHD

Table 3. Possible risk factors for mold infections in BMT recipients.

Risk factor	Infected BMT recipients (n = 21)	Uninfected BMT recipients (n = 209)	P value
Male:female ratio	13:8	120:89	NS
Mean age in y (range)	29 (1–43)	28 (0.25–54)	NS
No. (%) with diagnosis			
Acute myeloid leukemia	8 (38)	59 (28)	NS
Acute lymphoblastic leukemia	8 (38)	21 (10)	<.05*
Chronic myeloid leukemia	3 (14)	62 (30)	NS
Lymphoma	0	26 (12)	NS
Others	2 (10)	41 (20)	NS
No. (%) with stage of underlying disease			
Early	9 (43)	92 (44)	NS
Late	12 (57)	117 (56)	NS
No. (%) with indicated transplant			
Allogeneic	15 (71)	156 (75)	NS
Autologous	0	34 (16)	NS
Match unrelated donor	6 (29)	16 (8)	<.05*
Syngeneic	0	3 (1)	NS
No. (%) with conditioning regimen			
With TBI	19 (90)	108 (52)	<.005*†
Cyclophosphamide	5 (23)	40 (20)	
Busulfan and cyclophosphamide	9 (43)	54 (26)	
Melphalan	1 (5)	3 (1)	
Etoposide and cyclophosphamide	4 (19)	11 (5)	
Without TBI	2 (10)	101 (48)	
Busulfan and cyclophosphamide	1 (5)	55 (26)	
Cyclophosphamide, busulfan, and etoposide	1 (5)	25 (12)	
Others	0 (0)	21 (10)	
Mean duration (d) of neutropenia‡ ± SEM	18.8 ± 1.2	21.4 ± 0.6	NS
No. (%) with indicated acute GVHD			
Grade 0–1	3 (14)	126 (60)	NS
Grade 2–4	18 (86)	83 (40)	<.05*†

NOTE.　BMT = bone marrow transplant; GVHD = graft-vs.-host disease; NS = not significant; TBI = total-body irradiation.

* By univariate logistic analysis.

† By multivariate logistic analysis.

‡ Engraftment failed in two patients in the infected group and in 11 patients in the uninfected group; these patients were not included in the analysis.

manifested as bronchiolitis obliterans after day 100. The median time of the onset of mold infection in our patients was 47 days, a period comparable with that (42 days) reported in a previous study [16].

The emergence of this pattern of mold infection might be associated with the policy for and practice of antifungal therapy in our hospital. Antifungal prophylaxis during the preengraftment period and the early use of amphotericin B as empirical treatment for neutropenia and fever on day 5 might have prevented the onset of overt mold infection in most of our patients during the preengraftment period.

After engraftment, no routine antifungal prophylaxis was given to patients who were receiving pulse steroid and cyclosporine therapy for acute GVHD. The risk of mold infection was increased further by the transfer of these patients from protective isolation rooms to general wards after engraftment and by the mild neutropenia (neutrophil count, >500 to <1,500 × 10^9/L) associated with ganciclovir therapy for CMV disease in some of these pa-

tients [17]. The incidence of mold infection decreased after day 120. The lower risk of invasive disease in patients with chronic GVHD than in those with acute GVHD could be associated with the relatively smaller doses of immunosuppressive therapy as well as with the relatively lower rate of CMV infection (and therefore less need for ganciclovir therapy).

Infection by molds, especially *Aspergillus* species, is an increasing problem in most tertiary care hospitals. Known risk factors (in the order of decreasing importance) are prolonged severe neutropenia, neutrophil and/or macrophage dysfunction, steroid therapy, GVHD or graft rejection, and its associated treatment [18–20]. Like other investigators, we found that grade 2–4 acute GVHD was important [21]. However, more interestingly, TBI was found to be another independent risk factor for mold infections (table 2). To our knowledge, TBI has never been directly recognized as a significant risk factor for mold infection, although it has been cited as a risk factor for increased development of invasive fungal infection in granulocytopenic patients [22].

The use of TBI may reflect the severity of the underlying malignancy, or in effect, TBI may impose additional immunosuppression on patients. Since BMT patients undergoing TBI have a greater risk of developing mold infection, we propose that this group of patients should receive antimold prophylaxis during acute GVHD.

The incidence of culture-documented mold infection in our hospital approached 10%, which is comparable with that (8.7%) reported in a previous study [3]. However, the incidence of mold infection in this study could be underestimated. An aspect not analyzed in this study was the absence of fungal growth in specimens from patients with focal pulmonary lesions. There were six patients who were treated empirically for possible pneumonia due to mold in the absence of a positive culture of BAL fluid. They did not consent to an open lung biopsy because the operative risk was considered unacceptable. This non-compliance for further invasive testing could not be overlooked since most Chinese patients are reluctant to undergo diagnostic operative procedures and postmortem examinations.

In this prospective study of fungal culture–positive specimens from BMT recipients, *Aspergillus* was the most important pathogen. Unlike most previous reports [14, 23], *A. flavus,* instead of *A. fumigatus,* was the dominant isolate. This occurrence might be a regional phenomenon (as reported by other investigators [24]) or might be related to the use of methotrexate prophylaxis for GVHD, since this agent is known to inhibit the germination of spores of *A. fumigatus* but not those of *A. flavus* [25]. The most frequent environmental contaminant was *Penicillium* species. Similar findings were obtained by routine environmental sampling (data not shown). The only *Penicillium* species that was isolated and associated with invasive disease was *P. funiculosum,* a finding consistent with its ability to grow at 37°C and its minute-sized conidia (which are known virulence factors of mold [26]).

In conclusion, concentrated smears are useful for differentiating infection from exogenous contamination by mold. Before empirical antifungal therapy is considered for patients whose specimens are concentrated smear–negative but culture-positive (especially for *Aspergillus*), multiple additional specimens should be retaken and examined. Furthermore, the documentation of an increased risk of mold infection in patients receiving treatment for acute GVHD who do or do not have histories of TBI may be important in targeting those patients who should receive antifungal prophylaxis.

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