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Utility of Galactomannan Enzyme Immunoassay and (1,3) β-D-Glucan in Diagnosis of Invasive Fungal Infections: Low Sensitivity for *Aspergillus fumigatus* Infection in Hematologic Malignancy Patients▽

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Previous studies have reported that galactomannan (GM) enzyme immunoassay and 1,3 beta-glucan (BG) assay may be useful diagnostic tools, but their sensitivities are variable. We compared the performances of both tests. Between October 2002 and May 2005, 82 patients were prospectively monitored for 12 weeks. A total of 414 samples were tested by GM assay and 409 samples were tested by BG assay for the following four groups of patients: those with invasive aspergillosis (IA), those with other mold infections (*Fusarium*, scedosporium, zygomycosis, etc.), those with candidemia, and control patients. Blood samples were obtained twice on week 1 and once every other week for a total of 12 weeks. Patients in the invasive fungal infection groups had comparable risk factors. The sensitivity of the GM test was significantly higher for patients with IA due to non-*fumigatus Aspergillus* species than for patients with IA due to *Aspergillus fumigatus* (49% versus 13%; $P < 0.0001$) or with other mold infections (49% versus 6%; $P < 0.0001$). However, the sensitivity range (47% to 64%) and specificity (88%) of the BG assay were comparable among all patients tested, regardless of the infecting pathogen. The performance of GM-based diagnosis appears to be better for detecting non-*fumigatus Aspergillus* species. The diagnostic marker BG was shown to have a higher sensitivity than that of GM in detecting IA and other mold infections in hematologic malignancy patients.

The incidence of invasive fungal infection (IFI) has increased dramatically during the last decade. These infections are associated with high morbidity and mortality, ranging from 60% to 90%, especially in hematologic malignancy patients in the setting of neutropenia and hematopoietic stem cell transplantation (1, 5, 9, 13, 24, 30). The critical problem is the difficulty in making the diagnosis. Unfortunately, delayed diagnosis and therapy for invasive aspergillosis (IA) are associated with poor outcomes and high mortality regardless of the therapeutic modalities used (11). Hence, there has been an increased search for better noninvasive diagnostic methods for IA. Galactomannan (GM) seems to be the most studied diagnostic marker, followed by 1,3 β-D-glucan (BG) (4, 10, 12, 16, 18, 21, 23, 25, 31, 32, 33). Moreover, until now, only a few prospective comparative studies of GM and BG have been performed (14, 25, 29). With regard to GM assay, the test has been commercially available in Europe since the mid-1990s and recently received FDA approval in the United States. However, the reported sensitivity rate has been variable, with a range from 30% to 100%, and the specificity ranges from 38% to 98% (28). This wide range of results may be due to several factors, including various numbers of serum samples collected from patients at different institutions, the severity of infections, and the impact of prior antifungal therapy on the levels of circulating fungal components in the serum (19, 20). Many studies were retrospective in nature and had a limited number of proven fungal infections. Also, the heterogeneity of the study populations is understudied. Similarly, variable results have been reported for BG assay, with a slightly higher sensitivity and specificity, ranging from 70% to 90% (14, 23). We therefore conducted this study to determine the usefulness of GM and BG assays for diagnosis of hematologic malignancy patients.

MATERIALS AND METHODS

Study population. Between October 2002 and March 2005, 82 patients were prospectively monitored for 12 weeks and divided into the following groups: 22 patients with IA (proven or probable), chosen according to the criteria developed by the consensus of the European Organization of the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) (2); 17 patients with other mold infections, such as *Fusarium* infection, zygomycosis, and scedosporium; and 23 patients with candidemia. In addition, we selected 20 control nonneutropenic patients with solid tumors and without any radiological or clinical evidence of IA or risk factors for IFI. Blood was obtained twice on week 1 and once every other week for a total follow-up of 12 weeks. Patient demographics and clinical characteristics were collected, including age, underlying disease, type of transplantation, steroid use, neutropenia, and antifungal therapy used during the study period. This study was approved by the M. D. Anderson Cancer Center Institutional Review Board.

BG analysis of serum. Blood samples were collected in sterile, BG-free clotting tubes. Serum was separated by centrifugation and stored at −80°C until testing. BG levels in the serum were assayed using a Fungitell kit (Associates of Cape Cod, East Falmouth, MA) according to the manufacturer's specifications. BG levels were quantitated against a purified Pachyman standard, which includes a five-point twofold curve ranging from 100 pg/ml to 6.25 pg/ml. The cutoff was 80 pg/ml. In brief, 5 μl of serum per well was dispensed in triplicate and pretreated by addition of 20 ml of 0.125 M KOH–0.6 M KCl and incubation for 10 min at 37°C. This step inactivated protease and other inhibitors present in

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TABLE 1. Demographic characteristics of case patients and negative control patients by infection type

Characteristic	No. (%) of patients[a]		
	Aspergillosis (n = 22)	*Candida* infection (n = 23)	Other mold infection (n = 17)
Gender			
No. of males	16 (73)	12 (52)	9 (53)
No. of females	6 (27)	11 (48)	8 (47)
Age (yr) (median [range])	60 (10–78)	55 (23–81)	51 (22–73)
Underlying disease			
Hematological	22 (100)	11 (48)	17 (100)
Solid tumor	0 (0)	12 (52)	0 (0)
Bone marrow transplantation	12 (55)	2 (9)	9 (53)
Allogeneic hematopoietic stem cell transplant donor type	12 (55)	2 (9)	7 (41)
Site of IFI			
Lung	21 (96)		4 (23)
Sinus	1 (5)		5 (29)
Blood		23 (100)	2 (12)
Skin			6 (35)
Antifungal therapy	22 (100)	21 (91)	17 (100)
Graft-versus-host disease	8 (36)	1 (4)	5 (29)
Neutropenia during prior mo	12 (55)	9 (39)	9 (53)
Neutropenia at infection onset	5 (23)	6 (26)	6 (35)
Use of steroids before infection	15 (68)	9 (39)	11 (65)
Use of steroids during infection	17 (77)	9 (39)	11 (65)
Immunotherapy	18 (82)	10 (44)	13 (77)
White blood cell count	2 (9)	2 (9)	4 (24)
Granulocyte-macrophage colony-stimulating factor	4 (18)	2 (9)	4 (24)
Granulocyte colony-stimulating factor	16 (73)	9 (39)	12 (71)
Gamma interferon	1 (5)	1 (4)	6 (35)
Breakthrough	20 (91)	11 (48)	13 (77)

[a] Data represent numbers (percentages) of patients except where otherwise indicated.

TABLE 2. Performances of diagnostic tests for patients with IFI (per sample and per patient)

Type of comparison and IFI	Assay	Sensitivity (%)	Specificity (%)	PPV (%)[a]	NPV (%)[a]
Per sample					
Aspergillosis (n = 116)	GM	24	99	97	57
	BG	58	88	83	67
Candidemia (n = 102)	GM	2	99	67	54
	BG	61	88	82	72
Other mold infection (n = 77)	GM	6	99	83	62
	BG	47	88	72	72
Per patient					
Aspergillosis (n = 22)	GM	38	100	100	61
	BG	67	90	88	72
Candidemia (n = 23)	GM	0	100	NA	49
	BG	62	90	87	69
Other mold infection (n = 17)	GM	6	100	100	57
	BG	63	90	83	75

[a] PPV, positive predictive value; NPV, negative predictive value.

human serum. The Fungitell BG reagent was then reconstituted and dispensed per manufacturer specifications. A ThermoMax plate reader (Molecular Devices, Sunnyvale, CA) with SofMax Pro software onboard (Molecular Devices, Sunnyvale, CA) was used to accomplish kinetic analysis of the microtiter plate.

GM assay. GM in each sample was measured by using a Platelia *Aspergillus* enzyme immunoassay test kit (Bio-Rad, Hercules, CA). Coded serum samples were thawed and analyzed in batches, as directed by the manufacturer. Optical densities were read at 450 nm to 620 nm. Results were recorded as indexes relative to the mean optical density of the threshold controls. Samples that had an optical density index of >0.5 were considered positive and underwent repeated testing to ensure positive results. Evaluated patients had to have two serum samples available for analysis.

Definitions. Neutropenia was defined as an absolute neutrophil count of <500 cells/ml. Sensitivity, specificity, and predictive values were calculated on both per-patient and per-sample outcome bases. Furthermore, each patient was classified as positive or negative depending on whether the patient had at least two positive test results. The cutoff index value for GM was 0.5, and the cutoff for BG was 780 mg.

Proven or probable IFI was determined on the basis of EORTC/MSG criteria (2). Patients with proven IA had histopathological evidence of tissue invasion by filamentous fungi, the isolation of *Aspergillus* species from a normally sterile but clinically infected body site, and the presence of host factors. A positive test per patient for GM or BG was based on having two consecutive positive tests. The host factors were as follows: (i) recent history of neutropenia of <500 neutrophils/mm^3 for >10 days, if temporally related to the onset of fungal disease; (ii) receipt of an allogeneic stem cell transplant; (iii) prolonged use of corticosteroids at a mean minimum dose of 0.3 mg/kg of body weight/day of prednisone equivalent for >3 weeks; and (iv) treatment with other recognized T-cell immunosuppressants, such as cyclosporine, tumor necrosis factor alpha blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues, during the past 90 days. However, the positive culture for aspergillosis was considered proven if the culture was collected by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine.

Probable invasive pulmonary aspergillosis was considered as the presence of a host factor and a positive culture or cytology for aspergillosis in addition to one major radiological criterion (halo sign, air crescent sign, or cavitary nodules) shown on computed tomography imaging or two of three minor clinical criteria, including symptoms of lower respiratory tract infection (cough, chest pain, hemoptysis, and dyspnea), physical finding of pleural rub, and any new infiltrate not fulfilling major criteria and pleural effusion. Similarly, the identification of a non-*Aspergillus* mold was made according to the above criteria.

Candidemia was defined as the isolation of a *Candida* species from at least one blood culture in the presence of signs and symptoms of systemic infection.

Statistical methods. The sensitivity, specificity, and predictive values of diagnostic tests were calculated according to their definitions, and sensitivity comparisons were performed by chi-square test. The statistical significance was set at P values of ≤0.05 based on two-sided tests. All statistical analyses were performed using SAS, version 9.1 (SAS Institute, Cary, NC).

RESULTS

A total of 414 serum samples were tested with the GM assay, and 409 serum samples were tested with the BG assay. The group of patients with IA or other mold infections was receiving mold-active antifungal therapy (including polyenes, azoles, and echinocandins) before serum sample collection.

All patients in the aspergillosis group and the other mold infection groups had hematological malignancy as their underlying disease (100%), with approximately 50% having undergone bone marrow transplantation. Forty-eight percent of the patients in the candidemia group had hematologic malignancy, and the remaining 52% had solid tumors as their underlying disease (Table 1).

The performances of the GM and BG assays for different groups of IFI patients (per sample and per patient) are shown in Table 2. The BG assay demonstrated a higher sensitivity for aspergillosis, for either per-sample or per-patient calculation, than that of the GM assay. Similarly, with respect to the other mold infections, the BG assay had a higher sensitivity than that of the GM assay.

As expected, GM assay had a very low sensitivity for *Candida* compared to that of BG assay (2% versus 61% per sample and 0% versus 62% per patient). GM assay demonstrated a high specificity (99% per sample and 100% per patient) for IFI. Among the 22 patients with *Aspergillus* infection, 12 infections were due to *Aspergillus fumigatus* and nine infections were due to non-*fumigatus Aspergillus* species. One patient was

TABLE 3. Performances of GM enzyme immunoassay and BG test for patients infected with different organisms (per sample)

Test and organism	Sensitivity (%)	Specificity (%)	PPV (%)[a]	NPV (%)[a]
GM enzyme immunoassay				
A. fumigatus ($n = 69$)	13	99	90	66
Non-*fumigatus Aspergillus* species ($n = 39$)	49	99	95	86
Other mold ($n = 77$)	6	99	83	62
BG test				
A. fumigatus ($n = 69$)	61	88	75	79
Non-*fumigatus Aspergillus* species ($n = 39$)	64	88	64	88
Other mold ($n = 76$)	47	88	72	72

[a] PPV, positive predictive value; NPV, negative predictive value.

excluded from the analysis because his *Aspergillus* species information was unknown. The chi-square test indicated that the sensitivity of the GM test was significantly higher for patients with non-*fumigatus Aspergillus* species infection than for patients with *A. fumigatus* infection (49% versus 13%; $P < 0.0001$) and patients with other mold infection (49% versus 6%; $P < 0.0001$) (Table 3). However, the sensitivities of the BG test were comparable among these three groups of patients, regardless of the *Aspergillus* species or other molds as the source of the infection ($P = 0.14$) (Table 3). We tested 409 samples collected from patients with IA, other mold infections, and candidemia. The overall sensitivities of the BG test were 58%, 61%, and 47%, respectively, with a cutoff of 80 pg/ml.

In this study, all patients with IFI received antifungal therapy, except for two patients with candidemia. Antifungal agents included amphotericin B (Ambisome), caspofungin, fluconazole, voriconazole, itraconazole, and posaconazole. For patients with IA, the impact of different antifungal drugs on diagnostic performance was evaluated. For patients with other mold infections, we found that the sensitivity of the BG test was 69% higher for patients receiving piperacillin-tazobactam ($P = 0.09$). This may be due to false-positive testing in the presence of the drug. For patients with aspergillosis, the impact of polyenes or azoles as well as caspofungin on the diagnostic performance was also evaluated (data not shown). Although no significant difference in the impact on test performance between these agents was found, it was noted that 39% of the caspofungin group samples tested positive based on the GM test, compared to 21% of samples testing positive when patients were not receiving this drug ($P = 0.14$).

Of the patients infected by other molds, 16 were included for evaluating the sensitivity per patient, including 8 patients with disseminated fungal infections and 8 patients without disseminated fungal infections. Based on the BG tests, four patients (50%) with disseminated infections and six patients (75%) with nondisseminated fungal infections tested positive. On the other hand, only one patient with another mold infection tested positive by GM assay.

When we combined these two assays for diagnosis, the sensitivity did not increase much, as most patients (90%) who tested positive by the GM test were also positive by the BG test. For aspergillosis patients, the sensitivity increased to 71%, compared to 67% for the BG test alone. For candidemia patients, since the sensitivity based on the GM test was zero, the sensitivity based on the combination of tests was the same as that of the BG test (62%). Similarly, due to low sensitivity based on the GM test, the sensitivity of the combination of tests for the patients with other mold infections was also the same as that of the BG test alone (63%). The specificity was the same as that based on the BG test alone (90%).

DISCUSSION

Making a definite diagnosis of IFI remains a challenge. Culture of nonsterile fluid, such as respiratory specimens, lacks sensitivity in the setting of invasive mold infection and specificity in the setting of invasive candidiasis (1, 33). Invasive procedures relying on tissue biopsy or histopathological specimens are still considered the gold standard for establishing the diagnosis. However, these procedures are rarely performed, especially in the setting of immunosuppression or patients with thrombocytopenia, where such invasive procedures can be life-threatening.

In our study, we demonstrated that for a population of high-risk hematologic malignancy patients already receiving antifungal therapy, GM assay was significantly better at diagnosing IFI due to non-*fumigatus Aspergillus* species than that due to *A. fumigatus*, whereas BG antigen detection was similar for most fungi.

This is the first clinical study of patients with hematologic malignancy showing that GM assay detection is more frequent for IA due to non-*fumigatus Aspergillus* species than for that due to *A. fumigatus*. This observation is supported by the in vitro study by Mennink-Kersten et al. (22) whereby they demonstrated that the quantity of GM released can vary according to *Aspergillus* species. Higher GM concentrations were seen with *A. terreus*, *A. niger*, and *A. nidulans* than with *A. fumigatus*.

GM assay sensitivity for IA has varied markedly among studies, from as low as 30% to as high as 100% (16, 17, 21, 26, 28). This variability in the assay may be related in part to the hosts and their exposure to antifungal agents. All patients in our study were on antifungal agents, which may explain the lower sensitivity of the GM assay (49%). Several studies reported the impact of antifungal agents lowering the antigen level by decreasing the fungal load (19, 20, 28), making the test less useful for patients receiving mold-active antifungal agents. Marr et al. showed that the sensitivity of GM was reduced from 80% for a nonexposed group to 20% for a group exposed to mold-active antifungal agents (19, 20). In our study, we further investigated the impact of polyenes and azoles, as well as caspofungin, on the diagnostic performance of the GM assay. Although no significant differences in the impact on test performance between these agents were found, it was noticed that testing of samples from patients receiving caspofungin had a slightly higher sensitivity (39% of samples were positive) than did testing of samples collected from patients not receiving this drug (21% of samples were positive) ($P = 0.14$). Similarly, Klont et al. reported a paradoxical increase in circulating GM after caspofungin treatment for proven IA (15).

Furthermore, we evaluated the correlation between the kinetics of serum GM and the clinical outcome of IFI in our study. The GM test was positive 56% of the time among the patients who failed antifungal therapy, compared to 25% for

patients who responded to treatment ($P = 0.20$). This may be helpful in monitoring patient outcomes, as reported by Boutboul et al. (3). In patients with IA, the serum GM values increased for the treatment failure group compared with those for the group of responders ($P = 0.002$), and a GM index increase of >1.0 was predictive of treatment failure. Similarly, Foy et al. (8) reported that a significantly higher response rate was observed among patients whose GM levels normalized posttherapy. In addition, Penack et al. showed that GM testing has a high diagnostic accuracy and especially has implications when test results for optimization of antifungal therapy are correlated with clinical data (26).

In addition to the confounding variables associated with the choice of antifungal agents and their impact on the GM test, our study demonstrated that the type of *Aspergillus* species (*Aspergillus fumigatus* or non-*fumigatus Aspergillus* species) played a major role in the quantity of antigen released by different organisms. The overall sensitivity of the GM test in our study was 49% for patients infected with non-*fumigatus Aspergillus* species, which is similar to the results reported by Marr et al. (20), while the sensitivity of the GM test was only 13% for patients infected with *A. fumigatus*. This significant difference might have been attributed to several confounding factors, including the site of infection, fungal localization, or angioinvasiveness. However, these factors were evenly distributed among our study population, particularly among those patients in the *A. fumigatus* and non-*fumigatus Aspergillus* species groups. Several studies reported that the sensitivity of GM detection depends on the site of infection. Furthermore, the sensitivity of detection for the circulating antigen is significantly higher for patients with disseminated aspergillosis than for those with pulmonary aspergillosis (14). However, in our study, there were no differences among the patients with IA, irrespective of the species, regarding disseminated or local infections.

Another possibility that contributed to the low sensitivity was the low frequency of sampling. Although twice-weekly sampling has been recommended for patients at risk for IA, some authors propose daily monitoring owing to the intermittent presence of GM antigen in the bloodstream (18, 33). However, for patients undergoing treatment for aspergillosis, our study, as well as that of Sulahian et al. (29), recommends monitoring antigenemia at 2-week intervals, without having an impact on the test performance. Hence, the optimal sampling strategy remains unknown. A meta-analysis conducted by Christopher et al. showed that GM testing has moderate diagnostic accuracy for IA in immunocompromised patients (28).

The other noninvasive diagnostic method we used in our study was the BG assay (Fungitell; Associate of Cape Cod), which is widely used in the diagnosis of systemic mycosis. For BG assay, we did not observe any variability in sensitivity with respect to the fungal species like that seen with the GM assay. Moreover, the specificity of the test was above 90% for all tested fungi, regardless of the species, thus making BG a good marker for detecting most fungi.

Ostrosky-Zeichner and colleagues conducted a multicenter trial on a group of 163 patients with proven or probable IFI and on 170 healthy control subjects. The sensitivity of the test was 70%, and the specificity was 87% (23). Pazos et al. (25) also reported results similar to ours. For patients with IA, the sensitivity and specificity of BG testing were 87.5% and 89.6%, respectively. Similarly, Persat et al. (27) reported that for overall IFI, the BG assay had 77.8% sensitivity and 92.5% specificity. In contrast to our results and those reported in the above studies (6, 23, 25, 27), there was a low specificity of BG testing observed for fungal infections in a study involving 46 patients in intensive care units. This may have been due to the low BG cutoff level of 20 pg/ml used in that study. Overall, the BG assay had a high sensitivity, high specificity, and high positive predictive value. Hence, this test appears to be useful for measuring serum BG in clinical specimens from patients hospitalized with suspected fungal infections or as part of a surveillance strategy for hematologic malignancy patients at high risk for developing fungal infections. Furthermore, Ellis et al. reported the clinical usefulness of the BG assay for hematologic malignancy patients with neutropenia and fever who were not responding to antibiotics if the result was interpreted in the context of a thorough clinical, microbiological, and radiological assessment (7).

Our study had several limitations. First, we did not test the performance of the GM or BG test on samples of bronchoalveolar lavage fluid, knowing that the majority of the patients had invasive pulmonary aspergillosis. It is possible that these specimen tests may improve the sensitivities of the diagnostic assays. Second, samples were collected less frequently, which may have contributed to the lower detection level of the antigen. Third, there was a trend toward lower index values for the GM assay among patients who responded to therapy than those among patients who failed therapy. However, this difference did not reach statistical significance and may have been due to the small sample size.

The performance of GM-based diagnosis appears to be better for detecting non-*fumigatus Aspergillus* species than for detecting *A. fumigatus*. Although host-specific differences might exist, these data suggest differences in GM production between *Aspergillus* species. There was no significant difference in test sensitivity between the patients with *A. fumigatus* infection and those with other mold infections. However, the other diagnostic marker (BG assay) was shown to have a better sensitivity than that of GM testing in detecting IA, candidemia, and other mold infections in hematologic malignancy patients on antifungal therapy. Hence, BG testing might be beneficial as part of a surveillance strategy for high-risk patients as a fungal detection method for IFI. Therefore, for high-risk hematologic malignancy patients on antifungal therapy, BG assay is the more useful test. However, the combination of both tests may be the best approach. BG testing is associated with a high sensitivity, while GM testing is associated with a high specificity, particularly for infections caused by non-*fumigatus Aspergillus* species.

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