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Autopsy-Controlled Prospective Evaluation of Serial Screening for Circulating Galactomannan by a Sandwich Enzyme-Linked Immunosorbent Assay for Hematological Patients at Risk for Invasive Aspergillosis

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Efforts to improve the diagnosis of invasive aspergillosis (IA) have been directed towards the detection of fungal antigens, including galactomannan (GM). However, previous evaluations of GM detection have been hampered by a lack of proven cases of IA and by a nonserial study design. This prospective study assessed the diagnostic value of serial screening for circulating GM by using a recently developed sandwich enzyme-linked immunosorbent assay (ELISA) for prolonged-neutropenic and/or steroid-treated patients with hematological disorders. Serum GM levels were monitored twice weekly for 186 consecutive patients at increased risk for IA. The patients were stratified according to the likelihood of IA (proven, probable, possible, and no evidence of IA) by using stringent criteria. Proven IA was defined by characteristic histopathological findings together with a positive culture for *Aspergillus* species. Autopsy and culture from autopsy specimens was used to verify both positive and negative test results. A total of 2,172 serum samples were tested from 243 episodes (mean, 9 samples/episode). Based on the analysis of 71 patients with confirmed disease status (culture and histology), the sensitivity and specificity of serial GM monitoring were 92.6 and 95.4%, respectively. The positive predictive value was almost 93%, the negative predictive value was 95%, and the efficacy was 94%. False-positive reactions occurred at a rate of nearly 8%, although this figure might have been overestimated. Less than 1% of all tested sera were considered inconclusive. In more than half of the cases, antigenemia was detected before clinical suspicion of IA (median, 6 days before). Serial determination of serum GM by the sandwich ELISA technique is a sensitive tool for the diagnosis of IA in hematological patients at risk. This approach may substantially influence clinical management with regard to preemptive and empirical antifungal therapy.

Invasive aspergillosis (IA) is an increasingly recognized condition in immunocompromised hosts. Patients with prolonged and deep granulocytopenia following chemotherapy for hemato-oncological disorders and steroid-treated allogeneic bone marrow transplant recipients are particularly at risk (3). The crude mortality rate of IA approaches 100% and results at least partly from difficulties in obtaining a reliable diagnosis at an early stage of the disease, often leading to a fatal delay in adequate therapy (1, 4, 5). Definite proof of IA implies the demonstration of hyphal invasion in tissue specimens obtained by invasive procedures together with a positive culture for *Aspergillus* species from the same specimen (25). However, the performance of invasive diagnostic procedures, such as open lung biopsy or stereotactic brain biopsy, is often precluded by profound cytopenia or by the critical condition of the patient. Consequently, in daily clinical practice, physicians combine clinical, radiological, and/or microbiological criteria to define the level of probability of IA. However, these criteria either lack sensitivity or specificity or depend largely on a high fungal burden (26). The detection of circulating fungal antigens has been advocated as a promising indirect diagnostic method to overcome these drawbacks. One such component is galactomannan (GM), a major aspergillar exoantigen released during invasive disease. A number of techniques, such as enzyme immunoassays (14), radioimmunoassays (21), and latex particle agglutination tests (9), have been evaluated for the detection of this cell wall constituent (18). However, their routine use has been hampered by poor sensitivity, resulting in the detection of serum GM only at advanced stages of the disease, when antifungal therapy may have become useless (6).

Recently, Stynen et al. have introduced a sandwich enzyme-linked immunosorbent assay (ELISA) (17). This test employs the rat monoclonal antibody EB-A2, which recognizes the (1→5)-β-D-galactofuranoside side chain of the GM molecule (18). Since each GM molecule harbors several epitopes, the same monoclonal antibody can function as capture and detector antibody. This sandwich technique results in a significantly lower limit of detection of GM of 0.5 to 1.0 ng ml of serum^{-1}, whereas the latex agglutination test has a 15-ng ml^{-1} threshold. Detection of circulating GM at a lower threshold should allow earlier diagnosis of IA, which is of paramount importance in determining outcome. Preliminary studies have documented the superior sensitivity of this ELISA (Platelia Aspergillus; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) compared to that of the latex agglutination test (13, 19, 24). However, large clinical studies establishing the value of this sandwich test in a prospective and serial way are lacking. Furthermore, given the rigid definition of proven IA, stringent criteria should be used in the analysis of sensitivity, specificity, and predictive values; this should preferably include the histopathological control of positive and negative test results. In

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TABLE 1. Prospective evaluation of serum GM in patients at risk for IA

Parameter	Value for patients with:				Total
	Proven IA	Probable IA	Possible IA	No IA	
No. of episodes	27	6	77	133	243
No. of neutropenic episodes	24	6	74	120	224 (92%)
No. of patients	27	6	61	92	186
Underlying disorder (no. of episodes)					
Acute myelogenous leukemia	8	1	35	18	62 (25%)
Acute lymphocytic leukemia	2	1	6	7	16 (6.5%)
Chronic myelogenous leukemia			1	1	2 (0.8%)
Aplastic anemia	1		2	7	10 (4%)
Myelodysplastic syndrome		1	10	17	28 (11.5%)
Multiple myeloma	1	1	5	4	11 (4.5%)
Non-Hodgkin's lymphoma	9		6	3	18 (7.4%)
Autologous transplantation			1	54	55 (22.5%)
Chronic granulomatous disease			1		1 (0.4%)
Allogeneic transplantation (inpatient)	2	2	9	9	40 (16%)
Allogeneic transplantation (outpatient)	4		1	13	
No. of episodes of corticosteroids	15 (55%)	1 (16%)	20 (26%)	20 (15%)	56 (23%)
Neutropenia					
Mean duration (days)	28.8	16.5	21.2	12.4	17
Range (days)	0–180	0–38	0–60	0–75	
No. of episodes of antifungal therapy	22 (81%)	5 (83%)	69 (89%)	6 (4.5%)	102 (42%)
No. of samples (total)	276	66	644	1,186	2,172
Mean no. of samples/episode	10	11	8	9	9
No. of positive samples	168 (61%)	15 (23%)	21 (3.2%)	39 (3.2%)	243 (11%)

this prospective and pathology-verified study, we have assessed the value of twice-weekly screening for circulating GM by the sandwich ELISA technique in a mixed population of hematological patients at increased risk for IA.

MATERIALS AND METHODS

Eligibility and sample collection. In a prospective study performed between January 1997 and March 1998, serum GM levels were measured in 243 consecutive treatment episodes for 186 hematological patients (116 male; median age, 44 years; range, 8 months to 76 years) considered to be at increased risk for developing IA. Eligible patients were undergoing intensive myelosuppressive or immunosuppressive therapy for a number of underlying disorders: acute myelogenous and lymphocytic leukemia (primary, relapsed, or refractory) (n = 78), high-risk myelodysplastic syndrome (n = 28), blast crisis of chronic myelogenous leukemia (n = 2), relapsing non-Hodgkin's lymphoma receiving third-line therapy (n = 18), relapsing myeloma (n = 11), and severe and very severe aplastic anemia (n = 10). Patients undergoing allogeneic (n = 40) or autologous (n = 55) bone marrow and/or peripheral blood progenitor cell transplantation and one patient with chronic granulomatous disease were also included. The mean duration of neutropenia—defined as an absolute neutrophil count (ANC) below 0.5×10^9/liter—was 17 days in 224 episodes (92.2%). In the remaining 19 nonneutropenic episodes, therapy with anti-thymocyte globulin or high-dose corticosteroids was administered. Corticosteroids were given in 23% of all studied episodes (Table 1).

Serum samples were collected at entry and then twice weekly until death or discharge from the hospital. In addition, allogeneic transplant recipients were screened at least once per week as outpatients up to a minimum of 6 months posttransplant. Serum samples were stored at −70°C and analyzed weekly by the same technicians. For ethical reasons, the results of the ELISAs were not kept confidential, but decisions regarding treatment or preemptive therapy were left to the discretion of the attending physician.

Antifungal prophylaxis with itraconazole at 100 to 200 mg twice a day was routinely administered to all patients. Around 75% of the patients were nursed in reverse-isolation rooms with HEPA filtration from the start of therapy until recovery of the ANC to above 0.5×10^9/liter. The remainder were nursed in reverse isolation without HEPA filtration. Anti-infective therapy was instituted according to the criteria of the Immunocompromised Host Society. Amphotericin B was added for all patients with fever refractory to 4 or 5 days of broad-spectrum antibacterial coverage and to those developing recurrent fever while still neutropenic. Surveillance cultures for fungi and gram-negative organisms were performed twice weekly on stool samples and oral washes. In cases of clinical suspicion of invasive fungal infection, a diagnostic work-up was initiated, including a high-resolution pulmonary computed tomography (CT) scan, followed by bronchoalveolar lavage (BAL) and/or biopsy, unless these procedures were considered too great a risk during cytopenia.

Cultures for fungus were performed by plating clinical specimens onto Chromagar or Sabouraud agar and incubating the plates at 37°C for 2 days and at room temperature for another 19 days. *Aspergillus* species were identified by their culture characteristics and morphologies.

Autopsy of patients who died during the study period was mandatory, with the exception of explicit refusal by the patient or the family. Specimens of the lungs and pericardium were sent for culture. All autopsy specimens (including six pulmonary samples) were stained with periodic acid-Schiff or Gomori stain for the detection of hyphal invasion.

A study episode was defined either as a period of hospitalization or, following allogeneic transplantation, as the entire period between engraftment and the end of the sixth month posttransplantation.

Hospital construction and renovation activity took place during the entire study period.

Stratification of episodes and definitions of IA. Episodes were stratified in four groups according to the likelihood of IA. Proven IA (group I) implied (i) the histopathological evidence of tissue invasion by filamentous fungi, disclosing typical septated acutely branching hyaline hyphae, in specimens obtained by biopsy or autopsy, together with a positive culture for *Aspergillus* species from the same site or (ii) a positive culture for *Aspergillus* from an otherwise-sterile body fluid (not including BAL fluid) obtained by a sterile procedure. In cases of pulmonary aspergillosis, at least one BAL fluid or two sputum samples with positive culture for *Aspergillus* species in the absence of other pulmonary pathogens (e.g., cytomegalovirus) and in addition to a positive histopathology was also defined as proven pulmonary aspergillosis. Probable IA (group II) referred to the presence of characteristic clinical signs and symptoms (e.g., pleuritic chest pain) in the presence of pleural wedge-like pulmonary lesions or newly appearing lung infiltrates or with highly suggestive radiological (computerized axial tomography scan) evidence of invasive infection in the sinuses or central nervous system in neutropenic (ANC, $<0.5 \times 10^9$/liter) or steroid-treated patients receiving adequate broad-spectrum therapy and at least one positive culture (two for sputum) or cytology for *Aspergillus*. A second category of probable aspergillosis included the presence of characteristic radiographical lesions (computerized axial tomography scan), such as a halo sign or air crescent sign, in the lungs or pacification of the paranasal sinuses with extension to adjacent structures in the aforementioned patient population with or without positive cytology or culture. Possible IA (group III) was defined as fever not responding to 5 days of adequate broad-spectrum antimicrobials or relapsing after initial defervescence in persistently neutropenic patients or in patients receiving high-dose corticosteroids with negative cultures for bacteria and without evidence of viral disease (with or without pulmonary infiltrates).

Finally, neutropenic or steroid-treated patients without any clinical clue and with no radiological abnormalities and no microbiological isolation of *Aspergillus* species served as an internal control group, not suspected of having IA (group IV). In addition, sera from 50 healthy blood platelet donors (18 to 60 years old) were analyzed once for circulating GM. Patients defined as having probable or

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possible disease could be upgraded retrospectively on the basis of a later surgical procedure or necropsy.

Antigen detection. The ELISA was performed as described previously (19). Briefly, 300 μl of test serum was mixed with 100 μl of 4% EDTA treatment solution and boiled for 3 min. After centrifugation at 10,000 × *g* for 10 min, 50 μl of the supernatant was added to 50 μl of a reaction mixture containing horseradish peroxidase-conjugated anti-GM monoclonal antibody EB-A2. The 100-μl mixture was placed in the wells of a microtitration plate previously coated with the same monoclonal antibody EB-A2 and incubated at 37°C. After 90 min of incubation, the plates were washed extensively before 100 μl of buffer containing orthophenylenediamine dihydrochloride solution was added. Then the plates were incubated for another 30 min in darkness at room temperature, followed by the addition of 100 μl of 1.5 M sulfuric acid to stop the reaction. The optical density (OD) was read at 450 and 620 nm. All reagents were purchased from Sanofi Diagnostics Pasteur. Positive and negative controls were included in each assay. Doubtful or positive samples were retested in parallel with recent samples in the next assay. The OD index for treated samples was calculated by dividing the OD value of each serum sample by the OD of a control serum at 1 ng of GM/ml (threshold positive control). Although not recommended by the manufacturer, an index of 1.0 or more was considered positive while an index of <1.0 was negative. A result was considered true positive when two consecutive samples for that patient tested positive, including the retesting of the first sample.

Analysis. The sensitivity of the test for IA was calculated from the results for group I; the specificity was calculated from the results for a necropsy- and culture-verified negative group. The positive and negative predictive values were estimated from the combination of these two groups. Results that were not biopsy or autopsy verified could not be accurately validated, since the true disease status of the patient was unknown (10).

RESULTS

Entire study group. Fifty isolated serum samples from as many healthy blood platelet donors tested negative for GM. Of 2,172 consecutive patient samples, 243 (11%) tested positive. The distribution of underlying disorders and results for all patient groups are detailed in Table 1. A total of 1,186 samples were obtained from 133 episodes in group IV (mean, 9 serum samples/episode). Of these, sera from 121 episodes (91%) tested consistently negative; in the remaining 12 episodes, 39 of 210 sequential sera tested positive. However, in eight episodes, positivity was limited to a single sample out of 49, 31, 20, 11, 11, 7, 7, and 9 assays, respectively, not exceeding an OD value of 1.7. Multiple consecutive positive results were found in four cases, with 12 positive sera out of 17 assays, 3 of 6, 7 of 30, and 9 of 12, respectively. One of these patients had been treated for probable *Aspergillus* pneumonia several months before. An inverse relation between serum positivity, granulocyte recovery, and increased antifungal therapy was noted in all four cases.

Considering the definition of a true-positive sample (i.e., confirmation with a second test), the positivity rate in this control group equalled 3% (31 sera, or four episodes).

A total of 276 sera were analyzed from 27 episodes in group I (mean, 10 sera/episode); 60% of the serially analyzed samples tested positive in the ELISA (ranging from 1 of 2 to 20 of 20 tested sera). However, two patients remained consistently negative in five and nine assays, respectively. In the remaining 25 cases, sequential sera tested after the first positive result remained consistently positive; transient or alternating positivity was not observed. Antigenemia was positive before clinical suspicion of IA (median, 6 days before) in 18 patients (66%). Details of the patients are given in Table 2.

Group II consisted of only six patients. Twelve cases originally categorized in this group were later upgraded to proven IA. Sixty-six sequential serum samples were analyzed (mean, 11 samples/episode); 15 sera (22.7%) tested positive for two patients. The first patient became negative after 12 of 15 positive samples; however, during a new episode, antigenemia reappeared; the diagnosis was confirmed at autopsy. The other patient with relapsed acute myelogenous leukemia developed a halo sign on CT scan, while *Aspergillus fumigatus* and *Saccharomyces cerevisiae* were isolated from sputum. After three consecutive positive sera, antifungal therapy with amphotericin B was increased to 1.5 mg/kg of body weight, supported by the infusion of primed-donor granulocytes; thereafter, the ELISAs were consistently negative (10 additional samples). GM was not detected in any of the 38 samples from the four remaining episodes of probable IA; however, two patients recovered without antifungal therapy (making the diagnosis unlikely) while biopsy specimens taken from the other two patients (open lung biopsy and sinus biopsy) did not disclose hyphal presence.

A total of 644 serum samples were analyzed during 77 episodes of possible IA in group III (mean duration of neutropenia, 21 days). A total of 21 samples (3.2%) were positive in five distinct episodes. In two cases of multiple positive ELISA results (11 of 13 and 6 of 9 sera), a complete autopsy (including brain examination) could not establish the diagnosis of IA. The remaining three positive episodes included two patients with one positive result out of six and eight sequential assays, respectively (OD values, 1.0 and 1.2) and one episode of two positive results (OD value, 1.4) within seven samples. Excluding the last three results, the rate of unconfirmed positivity in this group equalled 2.6% (17 sera or two episodes). Autopsy and culture were performed on 42 additional patients who were highly suspected of invasive fungal disease (group III patients with unexplained pulmonary infiltrates) but demonstrated consistently negative ELISAs: fungal invasion was identified in six cases, including three cases of culture-proven mucormycosis, one case of disseminated fusariosis, and two cases of *Candida* pneumonia. From another patient, *Penicillium* sp. was recovered from lung tissue; however, since microscopic examination did not demonstrate tissue invasion, the isolate was considered a laboratory contaminant. In the remaining 35 cases, the clinicoradiological picture interpreted as possible fungal disease could be attributed to a variety of nonfungal etiologies, including bacterial pneumonia; tumor involvement; hemorrhage; bronchiolitis obliterans; viral, mycobacterial, or parasitic infections; botriomycosis; or diffuse alveolar damage without specific etiology.

Autopsy-verified cases. Both histology and culture results from tissue specimens obtained by autopsy were available for a group of 71 patients (27 from group I and 44 from group III); this subpopulation was analyzed separately. A total of 619 sera (mean, 8.7 sera/episode) were obtained. Two patients with proven IA, screened with 14 samples, were found to be consistently GM negative and were labelled as false negative. A total of 185 sera tested positive, of which 17 samples were considered false positive for two autopsy- and culture-negative patients. However, for at least one patient, the clinical picture and the gradual increase in GM titer (maximal OD, 6.0), followed by a progressive decline to undetectable values (11 of 13 sera) after neutrophil recovery and antifungal therapy, makes the diagnosis of IA highly suspect. The absence of fungal invasion on autopsy and the normalization of ELISA values 2 weeks prior to autopsy might represent a complete cure of IA. Based on the results for this completely verified subgroup, the sensitivity and specificity of serial GM monitoring were 92.6 and 95.4%, respectively. The positive predictive value of the test was 92.6% (25 of 27), the negative predictive value was 95.4% (42 of 44), and the efficiency was 94% (67 of 71) (Table 3).

For the entire study group, 10 samples (0.46%) from 10 different patients were isolated positives and were not confirmed by the results of previous or subsequent samples. These sera were considered inconclusive. Their OD values never ex-

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TABLE 2. Characteristics of patients with proven invasive aspergillosis[a]

Patient no.	Sex/age (yr)	Neutropenia (days)	Steroids[b]	No. of pos. samples/ total no. of samples	Time between first pos. test and clinical suspicion[c]	Underlying disorder or condition(s)	Site(s) of *Aspergillus* isolation	Fungal infection site at autopsy
1	Fe/42	6	−	9/12	+3	Relapsed ALL	Sputum, BAL, autopsy specimen lung	Right lung, myocardium, thyroid gland
2	Ma/10	18	+	8/12	−7	Relapsed ALL, alloBMT, GVHD	Autopsy specimen lung	Lung bilateral
3	Fe/43	10	+	2/5	−3	Relapsed NHL	Autopsy specimen lung	Lung bilateral, myocardium, liver
4	Ma/61	4	+	1/2	−2	NHL	Autopsy specimen lung	Right lung upper lobe
5	Ma/41	19	+	20/20	−3	MDS, alloBMT, GVHD	Sputum, BAL, autopsy specimen lung	Lung bilateral
6	Ma/54	18	+	5/7	0	Relapsed NHL	Sputum, BAL, autopsy specimen lung	Lung bilateral, myocardium, pericardium, stomach, thyroid gland
7	Fe/70	54	−	2/2	−3	HCL	Sputum	Lung bilateral, liver
8	Ma/38	6	+	5/9	−15	AML, alloBMT, GVHD	Sputum, BAL, autopsy specimen lung	Lung bilateral
9	Fe/16	>82	−	6/10	−12	Relapsed AML	Autopsy specimen lung	Lung bilateral, pericardium
10	Ma/33	3	+	17/18	−22	NHL post-liver Tx	Autopsy specimen Pericardium	Left lung, pleura, myocardium, Pericardium, kidney, thyroid gland
11	Ma/35	0	+	11/25	−13	ALL, alloBMT, GVHD	Sputum, BAL	Cerebral, lung bilateral
12	Ma/2.5	20	−	9/11	+4	AML, alloBMT	Autopsy specimen lung	Lung bilateral
13	Ma/53	24	+	7/12	−23	NHL-CLL	Autopsy specimen lung	Lung bilateral
14	Ma/72	>18	−	5/6	+6	AML	Autopsy specimen lung	Lung bilateral
15	Fe/65	22	−	3/7	+6	AML	BAL	Lung bilateral
16	Ma/71	12	−	2/3	−1	sAA	Autopsy specimen lung	Right lung and pancreas
17	Fe/13	>40	−	3/11	+2	Relapsed AML	Sputum, BAL	Lung bilateral
18	Ma/35	0	+	14/26	−22	AML, alloBMT, GVHD	Sputum, BAL, autopsy specimen lung	Cerebral
19	Ma/52	79	+	2/18	−6	Relapsed ALL	Autopsy specimen lung	Lung bilateral
20	Ma/62	23	+	0/5	NA	NHL-CLL	Sputum, BAL	Bronchus biopsy
21	Ma/69	>180	−	0/9	NA	HCL	Sputum, BAL, autopsy specimen	Lung bilateral, trachea
22	Ma/64	>40	−	5/7	−2	AML	Autopsy specimen	Lung bilateral, trachea
23	Fe/67	>60	+	3/3	−8	AML	Sputum, autopsy specimen	Left lung upper lobe
24	Ma/45	9	+	2/4	−6	Relapsed myeloma	Autopsy specimen lung	Lung bilateral
25	Ma/57	19	−	6/9	+4	Relapsed AML	Autopsy specimen lung	Lung bilateral
26	Ma/69	24	−	8/10	−5	Refractory AML	Autopsy specimen lung	Lung bilateral
27	Ma/53	0	+	13/13	−6	Relapsed NHL	Sputum, BAL, autopsy specimen lung	Lung, pleura, thyroid gland

[a] AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; CML, chronic myeloid leukemia; sAA, severe aplastic anemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; HCL, hairy cell leukemia; AlloBMT, allogeneic bone marrow transplantation; GVHD, graft-versus-host disease; Tx, transplantation; BAL, bronchoalveolar lavage; Ma, male; Fe, female; NA, not applicable; pos, positive.
[b] +, present; −, absent.
[c] Values are numbers of days elapsed between first positive test result and clinical suspicion of IA. Negative numbers indicate the number of days ELISA results were positive before clinical suspicion.

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TABLE 3. ELISA results in 71 pathology-controlled cases

ELISA result	No. of patients		
	Invasive aspergillosis (n = 27)	No invasive aspergillosis (n = 44)	Total
Positive	25	2	27
Negative	2	42	44
Total	27	44	71

ceeded 1.7. The percentages of inconclusive results were 0.3 and 0.6% in groups III and IV, respectively.

No *Aspergillus* species was recovered from surveillance cultures from stool and oral washes. With the exception of a single isolate that was identified as *Aspergillus flavus* (patient no. 24), all *Aspergillus* isolates cultured from proven and probable cases were identified as *A. fumigatus*.

DISCUSSION

Diagnosing invasive aspergillosis in immunocompromised hosts remains problematic. Cultures may require days or weeks to grow, while the histopathological examination of tissue specimens obtained by invasive procedures—still considered the "gold standard"—is often precluded by profound cytopenia. Theoretically, these drawbacks could be overcome by measuring fungal antigens or metabolites as surrogate markers for invasive infection. In this prospective study, we have assessed the value of serial screening for circulating GM in the diagnosis of IA in hematological patients, using a standardized, commercially available sandwich ELISA technique. In contrast to previous surveillance studies that used clinical, radiological, and/or microbiological criteria for disease classification or that relied on a negative control group without histological verification (13, 15, 19, 24), this analysis implemented gold standard criteria for proven IA (defined by histology and culture from the same tissue site). In addition, we verified a series of consistently negative test results in clinically highly suspect patients by autopsy and culture. A subgroup of 71 patients in which *Aspergillus* infections were discriminated from other etiologies was identified for determining major statistical endpoints. By using a cutoff OD value of 1 and after confirming a first positive sample by a second one, this ELISA proved to be a highly sensitive and specific diagnostic tool.

A sensitivity value of 92.6% agrees with earlier results obtained by Stynen et al. and Verweij et al. (90%) (17, 24) and is considerably better than the 76% reported by Sulahian et al. for confirmed aspergillosis and 82.5% when probable and confirmed IA are considered together (19). The higher proportion of false-negative results in the French study compared to our results (4.5%) may be due to the use of more stringent definitions in our study. Although *Aspergillus* sp. was cultured from all of their patients, the absence of histological confirmation does not allow discrimination between invasive disease and colonization. In the latter, GM detection will remain negative (15). By analyzing our six cases of probable IA as (unverified) definite cases of IA, the sensitivity would indeed decrease to 82%. This underlines once more the importance of uniform and widely approved case definitions of IA for the evaluation of new diagnostic and therapeutic tools. However, truly false-negative sera, resulting from limited angioinvasion, high antibody titers, or low-level release of GM by the fungus, have been documented (24), although they did not exceed 5% in our series. Furthermore, as evidenced in animal models, the prophylactic or preemptive use of amphotericin B may suppress the expression of GM (7), a phenomenon that appears to be due to reduced mycelial growth (15). Whether itraconazole prophylaxis might result in a similar effect remains to be examined.

Others have reported that the improved sensitivity of the sandwich ELISA was associated with the occurrence of false-positive results, reducing the specificity to 81 to 82% in two prospective studies (13, 19) and 84% in a retrospective study, at least when a positive result was defined as two consecutive positive sera (24). In our series, we found a false-positive rate of 7.4% and a specificity of 95.4%. The false-positive figure of approximately 8%, also reported by others (17, 24), contrasts with the approximately 3% observed positivity in group III and IV patients. Whether this figure really represents the false-positive fraction is difficult to determine in the absence of histological evidence. However, the obtained value of 7.4% may be overestimated, since it includes 11 positive sera from a patient who demonstrated a protracted rise during prolonged febrile neutropenia, followed by a gradual decline after hematopoietic recovery and antifungal therapy, suggesting an (occult) *Aspergillus* infection (20). Moreover, GM detection became negative several weeks prior to autopsy. Since circulating GM correlates with the extent of tissue burden (17) and seems to correspond to clinical response (22), autopsy may have failed to reveal a recent *Aspergillus* infection.

The nature of these persistent false-positive reactions remains undetermined. Cross-reactivity with transfused blood products or antiglobulin sera has not been observed (19). It remains an open question whether this positivity results from subclinical aspergillosis, intestinal fungal colonization, or cross-reactivity with an unidentified serum compound. Earlier reports have indicated cyclophosphamide as a potential inducer of false-positive reactions (8); however, none of our patients receiving cyclophosphamide tested positive (data not shown). Cross-reactivity was also not observed in cases of proven mucormycosis, disseminated fusariosis, or invasive candidiasis. These data are consistent with recent in vitro findings (20). Although *Penicillium* sp. was cultured from a lung specimen and cross-reactivity has been demonstrated, GM detection was consistently negative; however, in our case the fungus should probably be considered a contaminant given the negative histopathology (11, 18). Since intestinal *Aspergillus* colonization might result in false-positive GM levels, we routinely performed stool surveillance cultures on Chromagar; no fungal pathogens other than *Candida* sp. were identified. In accordance with previous authors, we further confirm that most persistent positive reactions occur within the first month post-transplant or within the first 2 weeks after cytoreductive therapy (22), which happens to be the period of maximal mucosal damage. The passage of dietary GM into the blood circulation via these lesions of the gastrointestinal tract has been hypothesized by some investigators (2, 12), while others thought that cross-reactivity with exoantigens from bacteria or yeast was responsible for the false positivity within this period. Recently, an abnormally high percentage of false-positive reactions (15%) was demonstrated in sera from bacteremic or fungemic patients without evident concurrent aspergillosis (20), although no cross-reactivity with cultured pathogens was observed. Using a similar group of 99 patients with bloodstream infections, we found less than 1% reactivity after exclusion of all patients with proven invasive aspergillosis (data not shown). The large discrepancy between the analyses may result from a major difference in sample size. This results in a specificity of 99.5% in non-*Aspergillus* bloodstream infections, similar to an earlier report of 98.7% (19). As such, this assay offers an

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additional benefit in polymicrobial situations, when a concomitant *Aspergillus* infection can be obscured by bacterial infections. However, the nature of the serum compound(s) that results in false-positive reactions needs to be further clarified. It should be noted that isolated positive samples, composing 0.45% of all tested samples in our series, are inconclusive and may also result from laboratory contamination.

The high positive (92%) and particularly excellent negative (95.4%) predictive values may affect the clinical approach to febrile immunocompromised patients, especially when these tests are substantiated by CT findings (23). On the one hand, confirmed positive test results may stimulate physicians to initiate maximal antifungal coverage, even before the appearance of clinical signs (preemptive) (19, 22) or to switch to new antifungal drugs, new preparations, or adjunctive measures. On the other hand, persistently negative test results should convince clinicians to look for alternative etiologies of neutropenic fever or unexplained lung infiltrates, with the aim of preventing the indiscriminate and liberal use of antifungals. As evidenced by a recent strategy analysis, the likelihood of withholding therapy incorrectly based on GM detection and imaging studies appears to be very low (16). A minor drawback for clinical monitoring, however, remains the species specificity of the assay; non-*Aspergillus* fungal pathogens are not covered by the test.

In conclusion, these prospective data confirm the diagnostic utility of serial GM screening by sandwich ELISA for the diagnosis of IA in patients with underlying hematological disorders. However, a positive assay should always be confirmed to exclude isolated positive results and, whenever possible, should be substantiated by additional radiological and/or microbiological examinations. How antigenemia corresponds in time with clinical and radiological findings and what its value is in therapeutic monitoring remains to be established in prospective trials.

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