Diagnostic potential of (1→3)-ß-D-glucan and anti-*Candida albicans* germ tube antibodies for the diagnosis and therapeutic monitoring of invasive candidiasis in neutropenic adult patients

Carmen Pazos[1], María-Dolores Moragues[2], Guillermo Quindós[3], José Pontón[3] and Amalia del Palacio[1]

[1]Unidad de Micología, Departamento de Microbiología, Hospital Universitario 12 de Octubre, Madrid; Departamentos de [2]Enfermería I and [3]Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Bilbao, Vizcaya, Spain

Summary

The usefulness to diagnose and monitor invasive candidiasis (IC) using ß-glucan (BG) and antibodies against *Candida albicans* germ tubes (CAGT) was evaluated in a twice-weekly screening of 35 episodes in neutropenic adults at high risk. Three proven IC and three probable IC were assessed. Diagnostic levels of both markers were detected in 100% of proven IC and in 66% of probable IC. Sensitivity, specificity, positive and negative predictive values of BG and anti-CAGT antibodies detection were 83.3%, 89.6%, 62.5% and 96.3%, and 83.3%, 86.2%, 55.5%, 96.1%, respectively. False positive reactions occurred at a rate of 10.3% and 13.8% for the detection of BG and anti-CAGT antibodies, respectively. However, the patients with false positive results were different by each test. Both tests anticipated the clinical and radiological diagnosis, and the initiation of antifungal therapy in most patients. Combination of both tests improved specificity and positive predictive value to 100%.

Key words

Candida albicans, (1→3)-ß-D-glucan, Fungitell, Invasive candidiasis, CAGT antibodies, Germ tubes, Diagnosis

Utilidad de la detección de (1→3)-ß-D-glucano y anticuerpos anti-micelio de *Candida albicans* para el diagnóstico y seguimiento terapéutico de la candidiasis invasora en pacientes neutropénicos adultos

Resumen

Se ha evaluado la utilidad de la detección dos veces por semana de ß-glucano (BG) y de anticuerpos anti-micelio de *Candida albicans* (CAGT) para el diagnóstico y el seguimiento de la candidiasis invasora (CI) en 35 episodios de pacientes neutropénicos de alto riesgo. Se diagnosticaron tres casos de CI probada y tres probables. Se alcanzaron resultados positivos para ambos marcadores en el 100% de las CI probadas y en el 66% de las CI probables. La sensibilidad, especificidad, valores predictivos positivo y negativo para la detección de BG y anticuerpos contra CAGT fueron 83,3%, 89,6%, 62,5% y 96,3%, y 83,3%, 86,2%, 55,5% y 96,1%, respectivamente. El porcentaje de falsos positivos para BG y anticuerpos contra CAGT fue del 10,3% y 13,8% para la detección de BG y anticuerpos anti-CAGT, respectivamente. Sin embargo, los pacientes con resultados falsos positivos fueron diferentes para cada prueba. Ambas pruebas se anticiparon al diagnóstico clínico y radiológico, así como al inicio de la terapia antifúngica en la mayoría de los pacientes. La combinación de ambas pruebas mejoró la especificidad y el valor predictivo positivo hasta el 100%.

Palabras clave

Candida albicans, (1→3)-ß-D-glucano, Fungitell, Candidiasis invasora, Anticuerpos anti-micelio, Tubos germinales, Diagnóstico

Dirección para correspondencia:
Dra. Carmen Pazos
Unidad de Micología
Departamento de Microbiología
Hospital Universitario 12 de Octubre
Avenida de Córdoba s/n
28041 Madrid, Spain
Tel.: +34 91 390 8239
Fax: +34 91 565 2765
E-mail: pazos.c@terra.es

The diagnosis of invasive candidiasis (IC) is complex since colonization is difficult to distinguish from invasive disease. In most cases, signs and symptoms are non-specific and patients are unable to undergo invasive diagnostic procedures [36]. Although considerable progress in the detection of candidemia by blood culture has been made in the last decades, Kami et al. [12] have reported that fluconazole prophylaxis is reducing the sensitivity of blood culture in the diagnosis of IC in hematological patients, since only 20 out of 94 patients with IC demonstrated at autopsy, had positive blood cultures.

The numerous problems encountered during the clinical and microbiological diagnosis of IC have prompted the development of non-culture based laboratory methods [40]. These methods rely on the detection of antibodies against a variety of *Candida* antigens, detection of a number of *Candida* antigens and detection of non-antigenic *Candida* components, such as the metabolites D-arabinitol and mannose, the cell wall component (1→3)-ß-D-glucan (BG) or *Candida* nucleic acids [30].

BG is a polysaccharide of the cell wall of fungi except for the Mucorales and *Cryptococcus*. Prokaryotes, viruses, as well as the mammalian cells also lack BG. Thus, its presence in blood and normally sterile body fluids using this panfungal marker could be ideal for detection of invasive fungal infections (IFI), including candidiasis [15,19,20,23]. Fungitell is a new assay for BG detection (Associates of Cape Cod, Inc., USA) that is based on amebocyte enzymes from *Limulus polyphemus* [24]. Assays for BG are based on the activation of the horseshoe crab coagulation cascade [28]. Liposaccharide and BG initiate the coagulation cascade by activating different serine protease zymogens, factors C and G. Liposaccharide specifically activates factor C, while BG activates factor G. *L. polyphemus* amebocyte lysates are made specific for BG by removal of factor C. In most published reports, the diagnostic potential of BG for the diagnosis of IC has been used retrospectively or with a single sample per patient as a tool for screening IFI with high sensitivity and specificity [14,19,23,25,28].

The detection of anti-*Candida albicans* germ tube (CAGT) antibodies takes advantage of the different antigenic composition of blastoconidia and germ tubes of *Candida albicans* to differentiate *Candida* colonization from invasive candidiasis [6,29]. This test has shown a sensitivity of 77-89% and a specificity of 91-100% for the diagnosis of IC in both immunocompetent and immunocompromised patients [7,9,18,21,31,34,38,39]. In addition to its diagnostic usefulness, the detection of anti-

CAGT antibodies may be useful in monitoring the response of the patient to antifungal therapy for IC, since decreasing titers of antibodies to CAGT have been reported in patients responding to antifungal therapy [33]. Although the test detects antibodies against antigens expressed by *C. albicans* germ tube surface, patients with infections by non-*C. albicans Candida* species such as *Candida tropicalis, Candida parapsilosis, Candida krusei, Candida glabrata, Candida guilliermondii* and *Candida dubliniensis* may also have anti-CAGT antibodies due to cross-reactivity with homologous cell wall antigens [2,7,33,35].

In this study we have evaluated and compared the usefulness of two different markers of *Candida* infection, BG and the presence of anti-CAGT antibodies for the diagnosis and therapeutic monitoring of IC in neutropenic adult patients at increased risk for IC.

Patients and methods

Patients selection. During fifteen months, all adult hematological cancer patients (n=154) treated at the "Hospital 12 de Octubre" Madrid, Spain, and stratified as high risk individuals as defined by Prentice et al. [32], were prospectively analyzed twice weekly for the quantitative determination of galactomannan (GM) with the commercially available sandwich ELISA test (Platelia *Aspergillus*; Bio-Rad Laboratories, France) until the high risk condition for developing IFI had subsided. The availability of serial serum samples and the patient's complete clinical records were retrospectively used to assess and compare the BG and anti-CAGT antibodies detectetion value for the diagnosis of IC in 35 selected patients; including three proven IC, three probable IC, and 29 patients with no IC. All assays were performed in a blind fashion. The patient characteristics and sample distribution are summarized in Tables 1 and 2. The study was approved by the Institutional Review Board of the "Hospital 12 de Octubre" and all the subjects in this study agreed to participate.

Definition of invasive candidiasis. IC episodes were classified according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC-IFICG and NIAID-MSG) case definitions [1]. Proven IC was assessed by blood cultures that yielded *Candida* species in patients with temporally related clini-

Table 1. Characteristics of patients with proven and probable invasive candidiasis.

Patient No.	Sex[a] / age (years)	Under-lying disease[b]	Type of IC	Treatment with steroids	Neutro-penia (days)	No. of BG positive samples / No. samples	Highest BG levels (pg/ml)	No. of samples positive for antibodies to CAGT/ No. samples	Highest titer of antibodies to CAGT	Organ involvement of IC	Site/s of isolation / *Candida* species	Fungal superficial colonization	Death in relation with IC
1	M / 70	MM	Proven	NO	12	4/4	>523	4/4	1/160	Blood	Blood / *C. albicans*	NO	YES
2	M / 54	ALL	Probable	YES	29	5/6	238	4/6	1/640	Liver	NO	YES	YES
3	M / 60	AML	Proven	NO	38	5/9	250	3/9	1/160	Blood	Blood / *C. glabrata*	YES	NO
4	F / 53	NHL	Proven	NO	15	5/6	380	3/6	1/320	Blood	Blood / *C. albicans*	NO	NO
5	M / 45	CLL	Probable	NO	32	7/8	>523	6/8	1/640	Liver	NO	NO	YES
6	M / 51	HD	Probable	NO	16	0/4	0	0/4	0	Liver, spleen	NO	YES	YES

[a]M (Male)/ F (Female)
[b]Underlying disease: MM, Multiple myeloma; ALL, Acute lymphocytic leukaemia; AML, Acute myelogenous leukemia; NHL, Non-Hodgkin´s lymphoma; CLL, Chronic lymphocytic leukaemia; HD, Hodgkin disease

ß-glucan and antibodies to CAGT in candidiasis
Pazos C, et al.
211

Table 2. Evaluation of serum in patients at risk for invasive candidiasis (IC).

	Proven IC	Probable IC	No IC	Total
No. of patients	3	3	29	35
Mean age (years) / range	61 (53-70)	50 (45-54)	45 (18-70)	47 (18-70)
Sex (M/F)[a]	2/1	0 / 3	19 / 10	21 / 14
Underlying disease[b]				
ALL		1 (33.3%)		1 (2.9%)
AML	1 (33.3%)		4 (13.8%)	5 (14.3%)
CLL		1 (33.3%)	7 (24.1%)	8 (22.9%)
MM	1(33.3%)		5 (17.2%)	6 (17.1%)
NHL	1 (33.3%)		8 (27.6%)	9 (25.7%)
HD		1 (33.3%)	4 (13.8%)	5 (14.3%)
sAA			1 (3.4%)	1 (2.9%)
No. of patients (%) receiving steroids	0	1 (33.33%)	3 (10.3%)	4 (11.4%)
Median duration of neutropenia (days)	15	29	23	23
Range of duration of neutropenia (days)	12-38	16-32	6-70	6-70
No. of episodes of antifungal therapy	3 (100%)	3 (100%)	6 (20.7%)	12 (34.3%)
No. of samples (total)	19	18	144	181
No. samples/episode	6.3	6	5	5.2
No. (%) of positive BG episodes	3 (100%)	2 (66.6%)	3 (10.3%)	8 (22.9%)
No. (%) of positive antibodies to CAGT episodes	3 (100%)	2 (66.6%)	4 (13.8%)	9 (25.7%)
Positive BG samples (%)	14 (73.7%)	12 (66.6%)	6(4.2%)	32 (17.7%)
Positive antibodies to CAGT samples (%)	10 (52.6%)	10 (55.6%)	7 (4.86%)	27 (14.9%)

[a]M/F, male/female.
[b]Underlying disease: ALL, Acute lymphocytic leukaemia; AML, Acute myelogenous leukemia; CLL, Chronic lymphocytic leukaemia; MM, Multiple myeloma; MDS, Myelodisplastic syndrome; NHL, Non-Hodgkin´s lymphoma; HD, Hodgkin disease; sAA, Severe aplastic anemia.

cal signs and symptoms compatible with IC. Probable IC were assessed by high-resolution CT scan/ultrasound focal lesions (liver, spleen or kidney), or elevated serum levels of alkaline phosphatase, hepatomegaly or splenomegaly, abdominal pain, candiduria, hypotension, embolic skin lesions or septic shock.

Diagnostic work-up of IFI. In cases of clinical suspicion of IFI, or when GM assay was positive, a diagnostic work-up was started including a pulmonary and abdominal HRCT scan, MRI or abdominal ultrasound, followed, when possible, by bronchoalveolar lavage and/or biopsy for bacterial, mycobacterial, fungal and viral cultures. Direct examination for bacteria and fungi (including *Pneumocystis jiroveci*) were performed in all patients. In addition, *Legionella* antigen was tested in urine.

Management of patients. All patients were nursed in rooms with HEPA filtration. Antifungal prophylaxis with fluconazole (200 mg o.d) was given when necessary. Febrile neutropenia was initially treated with a beta-lactam and aminoglycoside; vancomycin was added 48 h later if fever persisted. Antimicrobial therapy was modified according to microbiological findings. Criteria for initiating antifungal therapy with liposomal amphotericin B included: 1) persistent fever after five days of intravenous antibiotic treatment, 2) development of pulmonary infiltrates while receiving antibacterial therapy, 3) isolation of filamentous fungi from respiratory tract and 4) fever relapsing after an afebrile interval of at least 48 h in neutropenic patients still receiving broad spectrum antibiotics.

Collection and storage of sera samples. Blood samples (5 ml) were collected by venipuncture twice weekly until cessation of risk for IFI. Sera were separated and were stored at –70 °C until tested for detection of BG and anti-CAGT antibodies.

BG detection. BG was detected retrospectively with the Fungitell test kit, essentially as recommended by the manufacturer. Briefly, serum samples (5 μl) were pre-trea-

ted for 10 min at 37 °C with 20 μl of a solution containing 0.6 M KCl and 0.125 M KOH and assayed with the Fungitell chromogenic reagent in a kinetic study for 40 min at 37 °C. Optical densities were read at 405 nm. The concentration of BG in each sample was calculated by using a calibration curve with standard solutions ranging from 6.25 to 100 pg/ml. Patients were judged positive when levels of BG in serum were equal or superior to 120 pg/ml in at least one serum sample.

Detection of anti-CAGT antibodies. Antibodies against CAGT were detected by an indirect immunofluorescence antibody assay (Candida IFA IgG, Laboratorios Vircell S.A., Spain), essentially as described by García-Ruiz et al. [7]. Patients were considered positive when reverse titers of anti- CAGT antibodies were ≥160 in at least one serum sample.

Surveillance cultures. Semiquantitative surveillance cultures for yeasts were performed weekly. Oropharyngeal, nasal, perineal skin, vulvovaginal or balanoprepucial, rectum and pericatheter skin specimens were plated onto CHROMagar *Candida* medium and Sabouraud with chloramphenicol (0.4 g/l) agar plates that were incubated at 37 ºC for two weeks. Cultures were evaluated using the following score: negative (0 colonies), light (<10 colonies), moderate (11-20 colonies) and heavy (>20 colonies). The yeast isolates were identified by the API 32 system (Bio-Mérieux, Marcy L'Etoile, France).

Mycological studies. Specimens from clinically infected foci were collected and processed according to the guidelines of Denning et al. [3]. Blood samples for culture were inoculated in a BACTEC Plus aerobic/F bottle and incubated for up to 15 days with the BACTEC 9240 blood culture system (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis. Sensitivity, specificity, and positive and negative predictive values were calculated as described by Kozinn et al. [17]. Calculations of the 95% confidence intervals (CI95) were also included.

Results

BG detection. Eight out of 35 patients (22.9%) showed levels of BG ≥120 pg/ml in at least one serum sample. In most patients, positive BG was detected in two or more samples (median, 4.5 positive serum samples per patient). All three (100%) patients with proven IC and two out of three (66.6%) patients with probable IC tested positive for BG (Tables 1 and 2). In patients with proven IC, BG levels showed a constant rise before any clinical or microbiological confirmation of IC. Those levels later declined and eventually became negative if the patient responded to antifungal therapy (Figure 1a). However, patients that did not respond to antifungal treatment, did not show also a decrease on BG levels (Figure 1b). There was one false negative BG result in a patient classified as a probable IC. The patient had Hodgkin's disease and hepatosplenomegaly with elevated serum values of alkaline phosphatase and multiple abscesses in liver and spleen (Patient 6, table 1) and had not received fluconazole prophylaxis. In this patient surveillance cultures showed high counts for *C. albicans* in three different sites. The patient later died, and permission for a necropsy was denied.

Three out of 29 patients (10.3%) with no IC were positive for BG detection. In these three patients, IFI was excluded after the careful assessment of clinical, microbiological and radiological records of the patients, as well as the surveillance semiquantitative yeast cultures. The first patient (non Hodgkin's lymphoma) was colonized in four different sites (high counts) with both *C. glabrata* and

C. albicans. This patient tested negative for GM in a twice weekly prospective screening for the diagnosis of invasive aspergillosis (IA). Due to the intense colonization, therapy with oral fluconazole was started (100 mg/b.i.d) on day 4 and amphotericin B was started on day 9 because elevated serum levels of alkaline phosphatase and liver nodules on ultrasound examination were detected. Despite maintained treatment with amphotericin B, the patient had a fatal outcome and permission for a necropsy was denied. The second patient (multiple myeloma) had negative surveillance cultures and negative GM and had an *Escherichia coli* bacteremia (Figure 1c). The third patient had acute myeloid leukemia and had one single site colonized with *C. albicans* (low count). The patient had negative GM levels. None of three false positive patients had mucositis neither had received fluconazole prophylaxis. Analysis of the kinetics of BG levels helped in the identification of false positive results since in these patients BG levels showed abrupt rises and falls. An example of this type of kinetics is shown in figure 1c, where high levels of BG were detected in a patient with multiple myeloma at the time *E. coli* was isolated in a blood culture. BG levels became negative during the following days in absence of any antifungal treatment. Most patients with no IC showed very low levels of BG during the period studied (data not shown).

BG positive results preceded (n=4) the development of fever by seven, seven, six and two days, respectively. Positive BG tests preceded (n=3) the development of clinical signs by 14, six and two, days, respectively. A positive BG result preceded the demonstration of abnor-
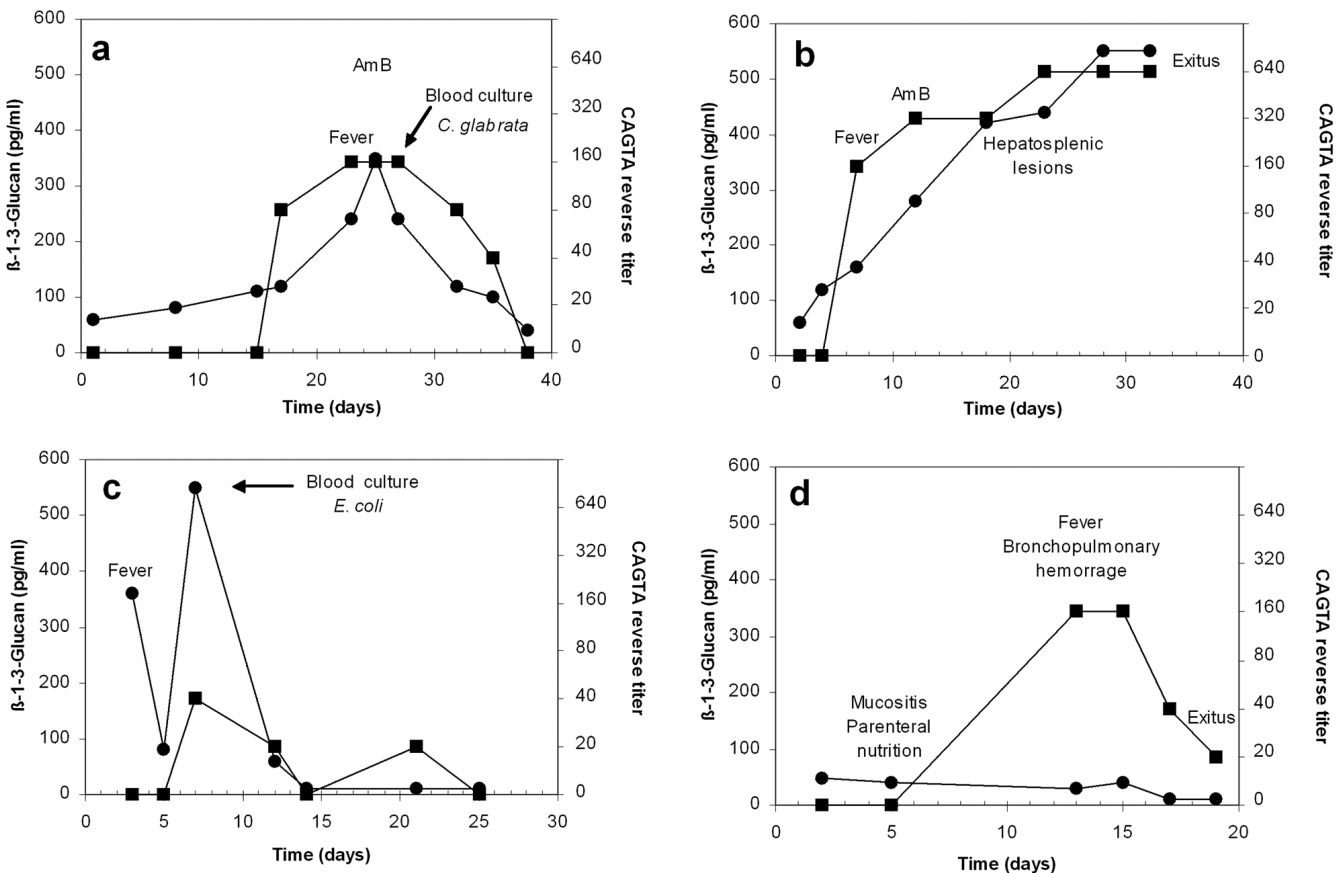

Figure 1. Representative kinetics of BG (●) and antibodies against CAGT (■) in different patients. (a) Proven IC in a patient with acute myeloid leukemia who responded to treatment with AmB. (b) Probable IC in a patient with chronic lymphocytic leukemia who did not respond to treatment with AmB. (c) False positive BG results in a patient with multiple myeloma and no IC. (d) False positive titers of antibodies against CAGT in a patient with non-Hodgkin's lymphoma and no IC.
Abbreviations: AmB, Amphotericin B.

malities on HRCT scan or ultrasound in all five patients (100%) by nine, 14, 10, 4 and 8 days, respectively. Positive BG results preceded the initiation of antifungal therapy in all five patients (100%) by nine, 14, six, four and eight days, respectively.

Considering as true positives only the patients with proven and probable IC, and true negative the group of patients without IC, the sensitivity, specificity and positive and negative predictive values of BG monitoring at a cutoff of 120 pg/ml were 83.3% (CI95: 36.5-99.1%), 89.6% (71.5-97.3%), 62.5% (25.9-89.8%) and 96.3% (79.1-99.8%), respectively. Lowering the cutoff to 80 or 60 pg/ml decreased the specificity and positive and negative predictive values of the test. The sensitivity, specificity, positive predictive value and negative predictive value of BG monitoring at a cutoff of 80 and 60 pg/ml were 83.3% (CI95: 36.5-99.1%), 75.8% (56.1-89.0%), 41.6% (16.5-71.4%) and 95.6% (76.0-99.8%), and 83.3% (CI95: 36.5-99.1%), 65.5% (45.7-81.4%), 33.4% (13.0-61.3%) and 95.0% (73.1-99.7%), respectively.

Detection of anti-CAGT antibodies. Nine of 35 patients (25.7%) tested positive for antibodies against CAGT (≥1/160). In most patients, antibodies to CAGT were detected in two or more samples (median, three serum samples per patient). All 3 (100%) patients with proven IC and two out three (66.7%) of patients with probable IC showed positive levels of antibodies against CAGT (Tables 1 and 2). In patients with proven IC, titers of antibodies against CAGT levels showed a constant rise before clinical and microbiological evidence of IC existed, to decline and eventually become negative if the patient responded to antifungal therapy (Figure 1a). However, maintenance or increase of titers of antibodies against CAGT was associated with a fatal outcome (Figure 1b). Antibodies against CAGT could not be detected on a patient with probable IC (Patient 6, table 1). This patient was also negative for BG and GM detection and was analyzed in the previous section. Four out of 29 patients (13.8%) with no IC had positive titers of antibodies against CAGT. One of these false positive patients had acute myelogenous leukemia with negative surveillance cultures and negative BG and GM detection. This patient had a *Streptococcus pneumoniae* bacteremia with fever, dyspnea and pulmonary condensation in lower right lobe that responded to antibiotics. The patient did not have mucositis neither had received fluconazole prophylaxis. The second patient had Hodgkin's disease, two sites colonized (high counts) with *C. albicans* and had negative results for BG and GM detection. The patient was treated with wide spectrum antibiotics but had not received fluconazole prophylaxis. The third patient had chronic lymphocytic leukemia and high counts of *C. albicans* in two different sites (oropharynx and rectum). The patient had intense mucositis at the time of presentation of a positive titer of antibodies against CAGT but did not receive fluconazole prophylaxis. Detection of both BG and GM were negative. The fourth patient had a non-Hodgkin's lymphoma and was colonized by *C. albicans* (high count) in two different sites (oropharynx and rectum) but had not received fluconazole prophylaxis. Both BG and GM values were negative (Figure 1d). The patient also had intense mucositis and parenteral nutrition at the time of presentation of a positive titer of antibodies against CAGT, with fever and an X-ray image compatible with bronchopulmonary hemorrhage that was treated with high doses of steroids. The patient later died and a necropsy consent was denied.

Considering as true positives only the patients with proven and probable IC, and true negative the group of patients without IC, the diagnostic validity of detection of antibodies against CAGT expressed as sensitivity, specificity and positive and negative predictive value, and their respective 95% confidence intervals, were 83.3% (CI95: 36.5-99.1%), 86.2% (71.5-97.3%), 55.5% (25.9-89.8%) and 96.1% (79.1-98.8%), respectively.

Detection of antibodies against CAGT preceded in one patient (20%) the development of fever by seven days and the development of clinical signs in another patient (20%) by seven days also. A positive titer of antibodies against CAGT preceded the demonstration of HRCT scan or ultrasound abnormalities, or positive blood culture or elevated serum levels of alkaline phosphatase in five patients by a median of five days (nine, seven, four, two and five days, respectively). Detection of antibodies aginst CAGT preceded the initiation of antifungal therapy in five patients (100%) by a median of five days (seven, seven, two, two and five days, respectively).

Combined analysis of both markers. Results obtained with the detection of BG and antibodies against CAGT in each patient, were combined to assess whether the combination of both markers resulted in the early and specific diagnosis of IC. Interestingly, both tests were found positive in the same patients with IC and the kinetics of both markers was very similar in most patients. BG tended to become positive earlier than the antibodies against CAGT. Discrepancies were observed in the false positive patients, since patients without IC but having positive titers of antibodies against CAGT were negative by BG detection and patients with false positive results for BG detection did not have antibodies against CAGT. Interestingly, these discrepancies were of importance in the identification of false positive patients, since only when both markers were positive the patient had IC.

The combination of results of both markers resulted in an improvement of the diagnostic efficacy of the tests to predict IC. The diagnostic validity (sensitivity, specificity and positive and negative predictive values) was 83.3%, 100%, 100% and 96.7%, respectively.

Discussion

Despite the large number of laboratory tests developed for the diagnosis of IC, none of them have been found widespread clinical use, mainly for the difficulties in obtaining consistently reliable results. In recent years, a number of circulating surrogate markers have emerged improving the diagnosis of IC [10]. One of such markers is BG, which due to its presence in the cell wall of most fungi, has been used as a panfungal marker for screening IFI [14,23]. Although fungi differ in their BG content in the cell wall, *Candida* species usually release large amounts of BG that can be detected in the sera of patients with IC [20]. In fact, BG detection seems to be a very sensitive means of detecting candidemia since all the patients studied by Miyazaki et al. [20], Kondori et al. [16] and Odabasi et al. [24] presented BG levels above the cutoff value. Moreover, Pickering et al. [28] found that 13 of 15 patients (86.7%) with candidemia were BG positive (≥80 pg/ml). Mitsutake et al. [19] reported a sensitivity of 84.4% in 39 patients with candidemia. Recently, Ostrosky-Zeichner et al. [25] have reported that 87 out of 107 patients with proven candidiasis had serum BG concentrations ≥60 pg/ml (81.3% sensitivity) and 83 patients ≥80 pg/ml (77.6% sensitivity). The results presented in this paper confirm the high sensitivity of BG detection for the diagnosis of IC, since all three patients with proven IC and two out of three patients with probable IC tested posi-

tive for BG. The reason for the BG false negative result could be related to the formation of immunecomplexes due to the presence of antibodies directed against BG that may be induced in patients with systemic candidiasis [16]. Although the possibility of IC can not be totally excluded since the autopsy was not performed, the negative result may also reflect the limitations of the EORTC-IFICG/ NIAID-MSG case definitions [37]. Other authors [25] have reported a lower sensitivity of BG detection in invasive infections by *C. parapsilosis*, as sera from five out of 18 patients with candidemia by this species had less than 60 pg/ml of BG (and seven patients less than 80 pg/ml). This fungal species could have a lower concentration of BG in its cell wall and this can be the explanation of its lower in vitro susceptibility to antifungal agents, like the echinocandins (caspofungin, anidulafungin, and micafungin), which act by blocking BG synthesis [5,26].

The specificity of BG detection for diagnosing candidemia has been reported to be 87.5-100% using patients with superficial candidiasis and healthy volunteers as controls [16,19-20]. When bacteremic patients were included in the study by Pickering et al. [28], the specificity decreased to 77.2%. These authors reported that 10 of 15 patients (66.7%) with bacteremia by Gram-positive cocci (five with *Staphylococcus aureus*, three with coagulase negative staphylococci, one with *Streptococcus mitis*, and one with *Enterococcus faecium*) had at least one specimen positive for BG. Moreover, of the 10 patients whose blood cultures grew Gram-negative bacilli, three (two with *Escherichia coli*, one with *Salmonella* species) had at least one specimen positive for BG. In the present study, one out of three patients with positive BG without invasive candidiasis suffered from a bacteremia by *E. coli*.

The existence of false positives has been reported in patients undergoing hemodialysis with cellulose membranes [13], patients treated with intravenous human immunoglobulins [8] or antitumoral polysaccharides [11], surgery patients exposed to glucan-containing gauze [22], and the inhalation of fungal glucan from the indoor environment [4]. Albumin, coagulation factors, and plasma protein fraction manufactured by certain vendors for intravenous injection also have been shown to contain high levels of BG [8]. In addition, Pickering et al. [28] showed in a cross-contamination experiment that excess manipulation of a sample can result in its contamination with BG.

In high risk colonized patients, it is particularly difficult to distinguish colonization from invasive disease. However, it is well known that *Candida* spp. colonization is a risk factor for the development of IC in high risk neutropenic patients [32]. Since BG is a panfungal marker it is possible that these patients suffered from a non-*Candida* invasive mycosis. However, they were all negative for both antibodies against CAGT and to GM. In addition, the kinetics of BG in these patients showed abrupt rises and falls in the absence of an antifungal treatment (Figure 1c) as opposed to the more protracted rise in serum BG levels observed in patients with proven IC, a feature that has also been described in patients with invasive aspergillosis [27].

Currently, the kinetics of BG (release, circulation, clearance and metabolic pathways) in humans is poorly understood. However, the data presented in this paper suggest that increasing or persistently high BG levels, in spite of antifungal treatment, could indicate a negative outcome. Conversely, decreasing values of BG were observed in patients showing clinical improvement. This study is in agreement with those observations reported by Obayashi et al. [23] and Odabasi et al. [24]. Thus, these data tend to confirm that BG is a useful marker for monitoring the patient's response to antifungal therapy.

The other surrogate marker studied was the detection of antibodies against CAGT. Results obtained in this study confirm and expand data reported in previous studies [7,9,18,21,29,31,33,34,38,39]. The test allowed the diagnosis of IC with a sensitivity of 83.3% and a specificity of 86.2%. The reason for the four false positive patients who presented antibodies against CAGT is not clear. Although three of the patients were colonized with *C. albicans* in different body sites, *Candida* colonization is unlikely to induce high titers of antibody to CAGT, since the majority of the true negative patients were colonized with *Candida*. Interestingly, all four patients were negative by BG detection. In agreement with previous results [9,31] and data presented in this paper for BG, detection of antibodies against CAGT was useful in predicting the therapeutic outcome of patients with IC. Decreasing titers of antibodies against CAGT were observed in patients who recovered from IC, while patients not responding to antifungal treatment showed a continuous rise in the titer of anti-CAGT antibodies.

Combinations of tests that detect different markers may be useful to overcome the deficiencies of individual tests. Mitsutake et al. [19] demonstrated that combined detection of BG and enolase or BG and mannan increased the accuracy of diagnosis of candidemia. In our study, combination of both markers did not improve the sensitivity obtained with each individual test, but it was very useful in confirming the existence of IC, since both markers were positive in patients with IC and, interestingly, their kinetics were very similar. Given the concordance of both markers in patients with IC, discrepancies in positive readings on each individual test were very useful in identifying false positive results. If the positive concordance on the combined detection of BG and antibodies against CAGT is confirmed in other studies, this approach could be an important tool to improve the diagnosis of IC.

An important feature of the two markers used in this study is that both were usually positive before the diagnosis of IC could be suspected or demonstrated by other means. However, BG had an earlier positive trend than the detection of antibodies against CAGT. This is in agreement with data reported by Odabasi et al. [24] who observed that BG was positive at a median of 10 days before the clinical diagnosis. The precocity of both surrogate markers may be a potential benefit for patients who are screened prospectively, since the diagnostic work-up of IFI could be started earlier and a preemptive therapy could be instituted with a wide-spectrum antifungal.

In conclusion, detection of BG or antibodies against CAGT are very useful as a part of a surveillance strategy for the diagnosis of IC in high risk hematological patients. More importantly, the combination of both tests was essential to identify false positive reactions on each test.

References

1. Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ and the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group of the National Institute of Allergy and Infectious Diseases. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2002; 34: 7–14.

2. Bikandi J, San Millán R, Regúlez P, Moragues MD, Quindós G, Pontón J. Detection of antibodies to *Candida albicans* germ tubes during experimental infections by different *Candida* species. Clin Diagn Lab Immunol 1998; 5: 369-374.

3. Denning DW, Evans EG, Kibbler CC, Richardson MD, Roberts MM, Rogers TR, Warnock DW, Warren RE. Guidelines for the investigation of invasive fungal infections in haematological malignancy and solid organ transplantation. Eur J Clin Microbiol Infect Dis 1997; 16: 424-436.

4. Douwes J. (1→3)-ß-D-glucans and respiratory health: a review of the scientific evidence. Indoor Air 2005; 15: 160-169.

5. Espinel-Ingroff A. In vitro antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature. Rev Iberoam Micol 2003; 20: 121-136.

6. Evans EG, Richardson MD, Odds FC, Holland KT. Relevance of antigenicity of *Candida albicans* growth phases to diagnosis of systemic candidiasis. Br Med J 1973; 4: 86-87.

7. García-Ruíz JC, Arilla MC, Regúlez P, Quindós G, Alvarez A, Pontón J. Detection of antibodies to *Candida albicans* germ tubes for the diagnosis and therapeutic monitoring of invasive candidiasis in patients with hematologic malignancies. J Clin Microbiol 1997; 35: 3284-3287.

8. Ikemura K, Ikegami K, Shimazu T, Yoshioka T, Sugimoto T. False positive result in *Limulus* test caused by *Limulus amebocyte* lysate reactive material in immunoglobulin products. J Clin Microbiol 1989; 27: 1965-1968.

9. Iruretagoyena JR, Regúlez P, Quindós G, Pontón J. Antibodies to *Candida albicans* germ tubes in two intensive care patients with invasive candidiasis. Rev Iberoam Micol 2000; 17: 93-96.

10. Jones BL, Mc Lintok LA. Impact of diagnostic markers on early antifungal therapy. Curr Opin Infect Dis 2003; 16: 521-526.

11. Kakinuma A, Asano T, Torii H, Sugino Y. Gelation of *Limulus* amoebocyte lysate by an antitumor (1→3)-beta-D-glucan. Biochem Biophys Res Commun 1981;101: 434-439.

12. Kami M, Machida U, Okuzumi K, Matsumura T, Mori Si S, Hori A, Kashima T, Kanda Y, Takaue Y, Sakamaki H, Hirai H, Yoneyama A, Mutou Y. Effect of fluconazole prophylaxis on fungal blood cultures: an autopsy-based study involving 720 patients with haematological malignancy. Br J Haematol 2002; 17: 40-46.

13. Kato A, Takita T, Furuhashi M, Takanashi T, Maruyama Y, Hishida A. Elevation of blood (1→3) ß-D glucan concentrations in hemodialysis patients. Nephron 2001; 89: 15-19.

14. Kawazu M, Kanda Y, Nannya Y, Aoki K, Kurokawa M, Chiba S, Motokura T, Hirai H, Ogawa S. Prospective comparison of the diagnostic potential of real-time PCR, double sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1→3) ß-D glucan test in weekly screening for invasive aspergillosis in patients with hematological disorders. J Clin Microbiol 2004; 42: 2733-2741.

15. Kohno S, Mitsutake K, Maesaki S, Yasuoka A, Miyazaki T, Kaku M, Koga H, Hara K. An evaluation of serodiagnostic tests in patients with candidemia: beta-glucan, mannan, *Candida* antigen by Cand-Tec and D-arabinitol. Microbiol Immunol 1993; 37: 207-212.

16. Kondori N, Edebo L, Mattsby-Baltzer I. Circulating ß (1→3) glucan and immunoglobulin G subclass antibodies to *Candida albicans* cell wall antigens in patients with systemic candidiasis. Clin Diagn Lab Immunol 2004; 11: 344-350.

17. Kozinn PJ, Taschdjian CL, Goldberg PK, Protzmann WP, MacKenzie DW, Remington JS, Anderson S, Seelig MS. Efficiency of serologic tests in the diagnosis of systemic candidiasis. Am J Clin Pathol 1978; 70: 893-898.

18. Linares MJ, Javier MR, Villanueva JL, Solis F, Torre-Cisneros J, Rodriguez F, Kindelan JM, Casal M. Detection of antibodies to *Candida albicans* germ tubes in heroin addicts with systemic candidiasis. Clin Microbiol Infect 2001; 7: 218-226.

19. Mitsutake K, Miyazaki T, Tashiro T, Yamamoto Y, Kakeya H, Otsubo T, Kawamura S, Hossain MA, Noda T, Hirakata Y, Kohno S. Enolase antigen, mannan antigen, Cand-Tec antigen and ß-glucan in patients with candidemia. J Clin Microbiol 1996; 34:1918-21.

20. Miyazaki T, Kohno S, Mitsutake K, Maesaki S, Tanaka K, Ishikawa N, Hara K. Plasma (1→3) ß-D glucan and fungal antigenemia in patients with candidemia, aspergillosis and cryptococcosis. J Clin Microbiol 1995; 33: 3115-3118.

21. Moragues MD, Ortiz N, Iruretagoyena JR, García-Ruiz JC, Amutio E, Rojas A, Mendoza J, Quindós G, Pontón J. Evaluación de una nueva técnica comercializada (*Candida albicans* IFA IgG) para el diagnóstico de la candidiasis invasiva. Enferm Infecc Microbiol Clin 2004; 22: 83-88.

22. Nakao A, Yasui M, Kawagoe T, Tamura H, Tamaka S, Tagaki H. False-positive endotoxemia derives from gauze glucan after hepatectomy for hepatocellular carcinoma with cirrhosis. Hepatogastroenterology 1997; 44: 1413-1418.

23. Obayashi T, Yoshida M, Mori T, Goto H, Yasuoka A, Iwasaki H, Teshima H, Kohno S, Horiuchi A, Ito A, Yamaguchi H, Shimada K, Kawai T. Plasma (1→3) ß-D glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 1994; 345: 17-20.

24. Odabasi Z, Mattiuzzi G, Estey E, Kantarjian H, Saeki F, Ridge RJ, Ketchum PA, Finkelman MA, Rex JH, Ostrosky-Zeichner L. ß-D-glucan assay as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clin Infect Dis 2004; 39: 199-205.

25. Ostrosky-Zeichner L, Alexander BD, Kett DH, Vazquez J, Pappas PG, Saeki F, Ketchum PA, Wingard J, Schiff R, Tamura H, Finkelman MA, Rex JH. Multicenter clinical evaluation of the (1-3)-ß-D-glucan assay as an aid to diagnosis of fungal infections in humans. Clin Infect Dis 2005; 41: 654-659.

26. Ostrosky-Zeichner L, Rex JH, Pappas PG, Hamill RJ, Larsen RA, Horowitz HW, Powderly WG, Hyslop N, Kauffman CA, Cleary J, Mangino JE, Lee J. Antifungal susceptibility survey of 2,000 bloodstream *Candida* isolates in the United States. Antimicrob Agents Chemother 2003; 47: 3149-3154.

27. Pazos C, Pontón J, del Palacio A. Contribution of (1→3)-ß-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 2005; 43: 299-305.

28. Pickering JW, Sant HW, Bowles CA, Roberts WL, Woods GL. Evaluation of a (1→3)-ß-D-glucan assay for diagnosis of invasive fungal infections. J Clin Microbiol 2005; 43: 5957-5962.

29. Pontón J, Jones JM. Identification of two germ tube specific cell wall antigens of *Candida albicans*. Infect Immun 1986; 54: 864-868.

30. Pontón J, Moragues MD, Quindós G. Non-culture based diagnostics. En: Calderone R (Ed.) *Candida* and candidiasis. Washington, American Society for Microbiology, 2001: 395-425.

31. Pontón J, Quindós G, Arilla MC, Mackenzie DWR. Simplified adsorption method for detection of antibodies to *Candida albicans* germ tubes. J Clin Microbiol 1994; 32: 217-219.

32. Prentice HG, Kibbler CC, Prentice AG. Towards a targeted, risk-based, antifungal strategy in neutropenic patients. Br J Haematol 2000; 110: 273-284.

33. Quindós G, Moragues MD, Pontón J. Is there a role for antibody testing in the diagnosis of invasive candidiasis? Rev Iberoam Micol 2004; 21: 10-14.

34. Quindós G, Pontón J, Cisterna R. Detection of antibodies to *Candida albicans* germ-tubes in the serodiagnosis of systemic candidosis. Eur J Clin Microbiol 1987; 6: 142-146.

35. Salesa R, Moragues MD, Sota R, Pemán J, Quindós G, Pontón J. Fungemia by *Candida dubliniensis* in a user of intravenous cocaine. Rev Iberoam Micol 2001; 18: 42-44.

36. Stevens DA. Diagnosis of fungal infections: current status. J Antimicrob Chemother 2002; 49 (Suppl 1): 11-19.

37. Subirá M, Martino R, Rovira M, Vázquez L, Serrano D, de la Cámara R. Clinical applicability of the new EORTC/MSG classification for invasive pulmonary aspergillosis in patients with hematological malignancies and autopsy-confirmed invasive aspergillosis. Ann Hematol 2003; 82: 80-82.

38. Torres-Rodriguez J M, Madrenys-Brunet N, Nolla-Salas J, Carceller A, Tur C. Candiduria in non-neutropenic critically-ill surgical patients. Detection of IgA, IgG and IgM antibodies to *Candida albicans* by germ tube immunofluorescence. Mycoses 1997; 40: 439-444.

39. Villalba R, González AI, Linares MJ, Casal M, Torres A. Detection of antibodies to *Candida albicans* germ tubes as a possible aid in diagnosing systemic candidiasis in bone marrow transplant patients. Eur J Clin Microbiol Infect Dis 1993; 12: 347-349.

40. Yeo SF, Wong B. Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev 2002; 15: 465-484.