Preparation of Combined Extract of Cell Wall and Cytosol Antigens of *Candida albicans* for Immunoblot Analysis

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Immunoblot analysis is not in wide use for diagnosis of invasive candidiasis, mostly because the procedure is not standardized and hence not reliable. This work describes a standardized method for *C. albicans* antigen extract preparation and immunochemical detection. The major improvement of the method is the preparation of combined antigen extract—consisting of the cell wall and cytosol antigens. The fungal cells and lysis buffer were mixed at a 1:3 ratio and disintegrated by ultrasound for six cycles of one minute each. After centrifugation, cytosol antigens were

obtained in the supernatant and cell wall antigens were in the precipitate. Precipitate was dissolved in lysis buffer with 2% sodium dodecyl sulfate (SDS) and boiled at 100°C for 2 min. After centrifugation, the supernatant was combined with the previous one, so the extract of the combined antigens was obtained in the mixture. With those combined antigen extracts and with sera of three different groups of patients, immunoblot analysis showed sensitivity of 90.2%, specificity of 84.4%, and accuracy of 88.0%. J. Clin. Lab. Anal. 21:406–412, 2007.

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INTRODUCTION

Invasive fungal disease is one of the most common infective complications among immunocompromised patients (1), and is associated with high morbidity and mortality (2,3). In North America, *Candida* is the fourth most common bloodstream pathogen (4), and in Europe *Candida* spp. rank eighth (5,6).

Early diagnosis of invasive candidiasis is quite difficult because the blood cultures often lack sensitivity (30–50%); they also require a long incubation time (7,8). Needless to say, quick diagnosis is of great importance for adequate treatment. The most frequently used methods for detection of the yeast cells in the blood are the commercial tests based on the isolation and germination of *C. albicans* (9), an enzyme-linked immunosorbent assay (ELISA) (10), and the test of indirect immunofluorescence (TIIF) (11). Many attempts have been made to detect the *C. albicans* cytosol antigens using molecular methods, such as polymerase chain reaction (PCR), nucleic acid probes, and restriction enzymes analysis; however, the results have varied in the detection limit, with poor sensitivities and specificities (2).

Although immunoblot analysis is the standard diagnostic method for many infective diseases such as acquired immunodeficiency syndrome (AIDS) and borreliose or coxsackie infection, it is not in wide use for the diagnosis of invasive candidiasis. This is mainly because a standard methodology for preparation of the *C. albicans* antigens extract does not exist, so the results obtained often vary in sensitivities and specificities (2). The immunoblot technique and sodium dodecyl sulfate (SDS) polyacrylamide gel (PAG) electrophoresis (SDS-PAGE) in the analysis of *C. albicans* are mainly focused on the analysis of the cell wall structure (12–14) and on the examination of antigen determinants (15,16). In the recent few years attempts have been made to detect the specific immunodominant antigens, rather than

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undefined antigenic preparations, so that the detection of antibodies of such antigens may prove suitable for the diagnosis of invasive candidiasis (17).

As we have seen in our previous work (18), patients with invasive candidiasis do not have antibodies against one or more specific antigens, they have antibodies against both types of antigens (cytosol and cell wall), so the immune response is rather individual. Therefore, the immunoblot test based on the combined extract of as much cell wall and cytosol antigens of *C. albicans* as possible might be a good starting point in developing a standardized immunoblot detection procedure.

The aim of this work was to standardize the antigen extract preparation of *C. albicans* for immunoblot diagnosis of invasive candidiasis, which should be a mixture of as much *C. albicans* antigens as possible, and derived from a wild-type *C. albicans* specific for the region in which it occurs. Such an extract could be a reliable tool for early diagnosis of invasive candidiasis.

MATERIALS AND METHODS

Materials and Reagents

All reagents and solvents used were of the highest available purity, mostly from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO). Alkaline-phosphatase-conjugated goat anti-human immunoglobulin G (IgG) was from Sigma-Aldrich. The *C. albicans* wild-type strains were isolated from the sera, sputum, pharyngeal swab, and stool of the patients with invasive candidiasis.

Cultivation of the *C. albicans* Strains

To examine various morphological phases of *C. albicans* (blastoconidia and the germling cells) the ATCC 24433 strain was cultivated at various temperatures (25, 27, 32, 37, and 42°C) in Parker liquid medium (Torlak, Serbia) using the gyratory shaker for 1, 2, and 3 hr. The cells were collected from the culture medium by centrifugation at 5,000 g for 10 min and washed twice using sterile distilled water. Morphological phases were examined by light microscopy.

Preparation of the *C. albicans* Extracts

The *C. albicans* strains cultivated at 37°C for 3 hr were mixed in lysis buffer (0.0625 M Tris HCl, pH 6, 10% glycerol, 5% 2-mercaptoethanol) in two ratios; 1:3 and 1:20 wt/vol. All ultrasound destruction was done by disintegrator (22 Hz, three to six 1-min cycles, with a 30-sec cycle pause) while cooling on the ice-water bath. The lysate was centrifuged at 5,000 g for 15 min. Cytosol antigens of *C. albicans* were located in the supernatant and the cell wall antigens remained in the precipitate.

The precipitate, separated from the supernatant, was mixed with lysis buffer with 2% SDS 1:1 wt/vol, boiled at 100°C for 2 min, and centrifuged at 5,000 g for 10 min. After the centrifugation, the supernatant obtained (the cell wall antigens) was combined with the previous supernatant (the cytosol antigens) obtained after the ultrasound disintegration.

SDS-PAGE Electrophoresis

The combined extract was separated in 10% SDS-PAGE according to Laemmli (19). The samples were subjected to the electrophoresis at the constant current of 15 mA until the tracking dye reached near the end of the gel.

Immunodetection

After SDS-PAGE, proteins were transferred from the gel to the nitrocellulose (NC) membrane (20). The membranes were blocked with 3% bovine serum albumin (BSA) for 2 hr. The sera and secondary antibodies were diluted with 3% BSA. The sera of the patients with *C. albicans* invasive candidiasis (14 samples), the patients colonized by *C. albicans* (30 samples), and the blood donors (27 samples) were included in this study. The blotted NC strips were incubated for 90 min in the patient's sera at the different dilutions. The sera dilutions were standardized using several samples from the patients with *C. albicans* candidiasis and several from the gastrointestinally colonized persons. The antigens of *C. albicans* were analyzed with the 1:10, 1:100, 1:500, 1:1,250, and 1:2,500 sera dilutions. After washing, the strips were incubated with the alkaline-phosphatase-conjugated goat anti-human IgG in a dilution of 1:1,000 for 1 hr. The antibody-antigen complex was detected after the incubation of the strips with the 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium. The reaction was stopped by the addition of 20 mM ethylene diamine tetraacetic acid (EDTA). The washed strips were dried in a dark chamber.

Statistical Analysis

The sensitivity of the assay was defined as TP/(TP+FN), the specificity as TN/(FP+TN), positive predictive value as TP/(TP+FP), and negative predictive value as TN/(TN+FN). Accuracy was defined as (TP+TN)/(P+N), where TP = true-positive results, FP = false-positive results, TN = true-negative results, FN = false-negative results, P = total positive results, and N = total negative results (21).

RESULTS

Most of the *C. albicans* cells demonstrated germ tubes; i.e., an invasive form, at 37°C after 3 hr of cultivation

(results not shown), which is why they were chosen as a source of the antigens for the immunoblot.

To obtain the antigen profiles consisting of both cell wall and cytosol proteins, 2-mercaptoethanol combined with the ultrasound disintegrator for cell lysis was used. Fig. 1A represents cycles 3 and 6 (1 min each) of the disintegration. The protein profiles obtained were the same, but the electrophoretic profile after six cycles had more proteins with higher molecular weight (Mw) in comparison to three cycles. To perform the standardization of the extract preparation, it was necessary to obtain not only the antigen quality, but also the optimal dilution of antigens that were subjected to the electrophoresis. Ratios 1:3 and 1:20 of the cells and the lysis buffer for the extraction were used. The sample prepared from the 1:3 ratio was diluted and compared with the sample obtained from the 1:20 ratio. The resulting protein profiles were different in quantity and quality (Fig. 1B). The proteins of very high molecular masses were visible in the mixture of the *C. albicans* cells and the sample buffer at a ratio of 1:3.

The antigen content of the wild types of *C. albicans* strains were compared with the antigens from the commercial ATCC 24433 strain—a standard diagnostic issue. The 45 wild types (Wt) were tested; the immunoblot of the four of them and of the ATCC strain are shown on Fig. 2. These four different types are shown as just a model—all other 41 Wt antigen extracts are the same as

one of those four representative types. The sera for mentioned immunodetection was taken from the patient with invasive candidiasis confirmed by the TIF (antibody titer 1:1,280). It is obvious that there is difference in the quantity and quality of major antigens (>100, 85, 52, 47, 35, 30 kDa) and in many other antigens between the ATCC and Wt strains. The Wt strains also differ from each other. The antigens extracted from the ATCC cells lack the ~14 kDa antigen. To obtain an antigen extract that contains as much antigens as possible, one unique antigen extract was prepared combining all four of the antigen extracts from the Wt strains.

For the antigen extract preparation described in this work, three different groups of patients were examined: patients with invasive candidiasis (14 patients), colonized patients (30 patients), and blood donors (27 patients). The immunoblot test was performed as described in Materials and Methods, Cultivation of the *C. albicans* Strains. The optimal sera dilution for all of the three groups of patients was found to be 1:500. Major (defined) *C. albicans* antigens were analyzed in all of the three groups of patients. Results of the immunoblot for 14 patients with invasive candidiasis, 26 colonized patients, and five blood donors are shown in Table 1. The calculated sensitivity was 90.2%, specificity was 84.4%, positive predictive value (PV+) was 90.2%, negative predictive value (PV-) was 84.4%, and accuracy was 88.0%.

Fig. 1. SDS-PAGE of the proteins from the *C. albicans* extract. **A:** Obtained by the ultrasound disintegrator: 1 = 3-min cycle; 2 = 6-min cycle. **B:** Prepared at different ratios of the cells in lysis buffer: 1 = Ratio of the *C. albicans* cells in the lysis buffer 1:20; 2 = Ratio of the *C. albicans* cells in the lysis buffer 1:3; Mw = molecular markers.

Fig. 2. Immunoblot of the antigens derived from the different *C. albicans* strains: 1 = Extract from the ATCC strain of *C. albicans*; 2 = Extract from the wild-type strain 1 of *C. albicans* isolated from the patient's blood; 3 = Extract from the wild-type strain 2 of *C. albicans* isolated from the patient's blood; 4 = Extract from the wild-type strain 3 of *C. albicans* isolated from the patient's blood; 5 = Extract from the wild-type strain 4 of *C. albicans* isolated from the patient's blood.

TABLE 1. Comparison of the three different groups of patients using the combined antigen extract in immunoblot analysis

Major antigens ^a	>100	100–80	75	60–47	58	47	35	20–10	Sum of major visible bands
No. of ICP sera									
1					+		+	+	3
2		+	+				++ ^c	+	4
3			+			+		+	3
4	+	+		+	+	+			5
5	+					+			2
6				+		+	+	++	4
7	+				+				2
8	+	+		+					3
9	++	+							2
10	++					+	+		3
11	++					+	+		3
12	+						+		2
13	+	+				+	+		4
14	+			+			++	++	4
Abundance of major antigens	60%	33%	13%	27%	20%	47%	53%	33%	
No. of PBD sera									
1	+								1
2	+								1
3	+								1
4									0
5									0
No. of PCP sera									
1			+						1
2								+	1
3									0
4									0
5									0
6								+	1
7		+					++	+	3
8							+		1
9								+	1
10									0
11							+	+	2
12				+					1
13							+		1
14									0
15									0
16									0
17									0
18									0
19								+	1
20									0
21									0
22									0
23						+		+++	2
25								+	1
28							+		1
30							+		1
Abundance of major antigens	0%	3%	3%	3%	0%	3%	20%	27%	

^aAntigens that are found in the immunoblot in more abundance than the others that are signed as weak bands; weak bands are found in all sera in the whole Mw range.

^b10 times greater intensity than the weak bands.

^c100 times greater activity than the weak bands.

ICP, invasive candidiasis patients; PBD, positive blood donors; PCP, positive colonized patients.

DISCUSSION

Many different mechanical, physical, and chemical methods including an enzyme treatment have been used for the preparation of *C. albicans* antigens (12,16,22). These methods are based on extracting two distinct types of antigens, from the cell wall (mannoproteins) and from the cytosol. Major cell wall *C. albicans* antigens are glyceraldehyde 3-phosphate dehydrogenase (23), the proteins of Mw from 110 to 170 kDa (24), and the mannoproteins of Mw 260 kDa (25) and 58 kDa (26). Major cytosol *C. albicans* antigens are enolase Mw 47 kDa (27) and the proteins of Mw 52 kDa, from 64 to 67 kDa and 70 kDa (15,28).

The described *C. albicans* cell wall antigens were obtained using either chemical methods (14), an enzyme treatment (13), or by the combination of these two methods (12,25). The mechanical (22) and physical methods (16) were used for the extraction of the antigens from the cytosol. There were also some attempts to get both antigens in one extract (29). However, these procedures gave less and more diffuse antigen content compared to our results.

For the reproductive immunoblot detection (due to high molecular masses and hence poor blot transfer) the cell wall antigens need to be very abundant. The previously described methods for *C. albicans* homogenization are not convenient for the extraction of the mannoproteins. That is why we have been focused on obtaining combined extract with a sufficient quantity of cell wall proteins; because of that we have chosen six cycles of 1 min each as a method for the cell disintegration, and a 1:3 ratio of the sample dilution, as shown in Fig. 1.

In recent years attempts have been made to detect specific immunodominant antigens to detect antibodies that may serve diagnostic purposes for invasive candidiasis (17). The main reason for that is the existence of an individual human immune response, which could be overcome by the findings of marker antigens that are found in every person suffering from invasive candidiasis. Recently (30), about 85 immunoreactive protein species were detected with the systemic candidiasis patients' serum specimens using immunoproteomics. Those species were identified through a combination of matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), electrospray-ion trap/mass spectrometry (ESI-IT/MS), and genomic database searches. These findings suggest that the production of antibodies to *C. albicans* phosphoglycerate kinase and alcohol dehydrogenase during systemic candidiasis could be associated with a differentiation of the human immune response. Until these results become reality in routine diagnostic practice, we believe that the combined antigen extract of *C. albicans* could serve as an additional tool for

the diagnosis of invasive candidiasis. By the term combined antigen extract, we presume the extract consist of as much cell wall and cytosol antigens as possible clearly visible as defined bands in the SDS-PAGE or immunoblot, gained by the procedure described in Materials and Methods, under Cultivation of the *C. albicans* Strains. This term also means that the antigen combination contains all of four antigen extract types from the Wt strains (Fig. 2).

For effective distinguishing of forms of *C. albicans* (colonization and the invasion form) optimal dilution of serum for the immunoblot (31) is very important. Sensitive techniques, such as the immunoblot, could give false-positive results in cases of invasive candidiasis in patients who are only colonized by *C. albicans* if the dilution of the patient's sera is inappropriate. The most commonly used dilutions of the sera for performing the immunoblot for the diagnosis of invasive candidiasis are 1:10 (15) and 1:100 (16). The optimal dilution for each serum was determined as the dilution that resulted in the appearance of the maximal number of clear bands in the immunoblot. Optimal serum dilution in our investigation was 1:500. The dilution of 1:10 was rejected because of nonreproductive results, as a consequence of the NC membrane background due to a high content of primary antibody. The number of the detected bands in all positive sera rapidly decreased at a dilution higher than 1:500. Bands with Mw 45–29 kDa were not detected in the dilutions higher than 1:500 with the alkaline phosphatase chromogen kit (BCIP/NBT; Sigma-Aldrich, St. Louis, MO) detection system.

From the results of the immunoblot of the three different groups of patients it can be seen that some important differences can be distinguished between the patients with invasive candidiasis and the positive colonized patients. First of all, comparing the sum of all visible bands in Table 1 between those two groups of patients, we can notice that in the group with invasive candidiasis there are a greater number of bands visible in the immunoblot than in the group of colonized patients. Second, the combined extract that we prepared and used in the immunoblot covers a wide protein Mw range. In the group of patients with invasive candidiasis, antigens of the Mw > 100 kDa are the most abundant antigens, so it is very important that the antigen extract contain proteins of that (high) Mw. As it can be seen from Table 1, the abundance of the low Mw antigens 10–20 kDa is 33%. However, they must not be neglected because there are some patients (1, 3, and 6) that recognized them and have not been able to recognize some major antigens. The advantage of this procedure for antigen preparation is the fact that, unlike the ATCC strain, it does have these low Mw antigens, as shown in Fig. 2. This could be a possible explanation of why some

patients whose clinical manifestations strongly suggests invasive candidiasis showed negative results when tested with the THIF test (32)—because the THIF test is also based on the same ATCC strain that lacks low Mw antigens. In the group of colonized patients, these low Mw antigens are the most abundant and the clinical manifestations of these patients (7, 11, and 23), strongly suggests the beginning of invasive candidiasis. Considering the immunoblot data gained from the group of blood donors, it is clear that using only the antigens with Mw >100 kDa as a marker for invasive candidiasis could not be justified.

It is important here to point out that besides the major antigens (Table 1), all patients showed positive results for a great number of the other antigens (results not shown). These antigens were recognized with less intensity and were signed as weak bands. They were visible over the whole Mw range, with more abundance in the group of patients with invasive candidiasis. This also supports our theory that the antigen extract should contain as many antigens as possible.

The defined methodology for the preparation of the antigens for SDS-PAGE and the immunoblot is a good starting point but a more complex study should be undertaken to define all criteria for the diagnosis of invasive candidiasis. It is clear that the combined antigen extract with a wide Mw range for the antigens used for the immunoblot is necessary because of the high individual immune response that occurs among the patients. We believe that immunochemical detection based on the combined antigen extract could be a helpful tool in the standard methods of diagnosis or as a method of distinguishing invasive candidiasis from the colonization form of the disease.

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