DIAGNOSING AND MONITORING INVASIVE CANDIDIASIS

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DIAGNOSING AND MONITORING INVASIVE CANDIDIASIS

DIAGNOSTIEK VAN INVASIEVE CANDIDIASIS

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Voor Michiel

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Epidemiology of invasive candidiasis

Candidiasis can be divided into superficial and invasive candidiasis. Superficial candidiasis strictly means "on the surface". The respiratory, urinary, and gastrointestinal tract are continuations of the body surface, thus, candidose of these sites should be regarded as superficial candidiasis. *Candida* lesions in all other sites are classified as systemic or invasive infections. Invasive forms of candidiasis may arise in one organ only, or as a truly disseminated candidiasis in which *Candida* is spread by the blood stream to invade multiple organs (1).

Candida albicans is an opportunistic pathogen of the human gastrointestinal system that takes advantage of factors which decrease the normal host resistance to invade across the mucosa of the gastrointestinal tract (1, 2, 3). Haematogenous dissemination of *C. albicans* can lead to infection in virtually every organ or tissue of the human body (1). These infections are among the most important causes of morbidity and mortality among patients with cancer and other severely immunocompromised hosts (4).

C. albicans is the most frequently isolated fungal pathogen (59.7%) followed by other *Candida* species (18.6%) including *C. glabrata, C. tropicalis, C. krusei,* and *C. parapsilosis* (5, 6). This is in correlation to their virulence factors in terms of their capacity to adhere to both epithelial, and prosthetic surfaces (7), their proteolytic potential and the production of phospholipases (6).

The frequency of invasive *Candida* infections has increased by 219 to 487% in U.S. teaching hospitals and by 75 to 370% in non-teaching hospitals during the past two decades (8, 9, 4, 10). *Candida* species are now the fourth most common organism recovered from blood of hospitalized patients (11). Although this increase may be due partly to increased awareness there is little doubt that a true increase in the number of *Candida* infections has occurred. Factors that have altered the epidemiology of candidal infections include prolonged mucosal damage due to chemotherapy or viral infections, increased use of broad-spectrum antibacterial agents, central vein catheterization, and increased periods of prolonged neutropenia consequent to the use of intensive chemotherapeutic regimens (8, 12).

Diagnosing invasive candidiasis.

The problem

Despite recognition of the above mentioned increase in *Candida* infections, diagnosis of invasive candidiasis remains difficult. Unfortunately, the clinical signs and symptoms associated with invasive candidiasis are non-specific and not helpful in distinguishing between bacterial or candidal infections (13).

Other sources of difficulties in diagnosing invasive candidiasis arise from the problem of distinguishing clinically insignificant yeast colonization from invasive candidiasis with regard to the risk of antifungal chemotherapy. The drugs available for treatment of invasive candidiasis carry either high risk of host toxicity (Amphotericin B), a high probability that drug resistance will develop (fluconazole) or uncertain therapeutic efficacy (azole antifungals).

The diagnosis of invasive candidiasis partly depends on culture and/or histological evidence from appropriately collected blood and/or tissue biopsies of normal sterile body cavities (1). On the other hand, non-culture techniques are helpful in confirming and establishing the diagnosis invasive candidiasis which include detection of anti-*Candida* antibodies, antigens and metabolic products of *Candida*, and, recently, the detection of *Candida* species-specific DNA or RNA sequences in blood or other body fluids (13, 14, 15). The non-culture-dependent diagnostic approaches will be discussed separately.

Anti-Candida antibodies

For the detection of specific antibodies several serologic tests have been developed; whole cell agglutination (16), latex agglutination (17), counterimmunoelectrophoresis (CIE) (18), indirect immunofluorescence (19), passive haemagglutination (20), radioimmunoassay (RIA) and enzyme immunoassay (EIA) (21). Several *Candida* antigenic determinants have been used in such tests. They include polysaccharides (cell wall antigens, mannan), proteins (cytoplasmic extracts) and glycoproteins (antigenic components released by *Candida* cells into their growth medium) (21-25).

There are two major problems that undermine the clinical use of anti-Candida antibody assays. Due to the ubiquity of *C. albicans* as a human commensal anti-*Candida* antibodies are commonly present within patients and cause diagnostically "false-positive" results. In contrast the lack of an immuneresponse within immunocompromised patients results in diagnostically "false-negative" reactions (26). Alternatively, antibody production may occur so late in the course of the disease that it lacks diagnostic value, in clinical practice.

Immunodominant antigens have been adapted for use in immunoassays to increase specificity of the antibody detection assays, based on the assumption that the host is exposed to these antigens solely in invasive candidiasis (26). Several immunodominant antigens of *C. albicans* of similar molecular weight, correlated to invasive infection, have been described (27). The two major immunodominant antigens of interest for the development of antibody detection assays, are the 47 kDa breakdown product of a 90 kDa heatshock protein (28) and the 45 kDa enzyme enolase (29, 30).

None of the above mentioned tests have been commercialized or standardized internationally which makes comparison between various studies using these methods difficult.

Structural antigens

Several immunologic and chromatographic principles have been employed to detect cell wall and cytoplasmic antigens of *C. albicans*. At least three assays for detection of different *Candida* antigens have been made available by commercial companies. These assays include a latex agglutination (LA) assay for an undefined heat-labile antigen (CAND-Tec, Ramco, Houston) (31), an enzyme immunoassay for *Candida* enolase (Becton Dickinson, Microbiology Systems, Baltimore) (32) and tests for the detection of cell wall mannan (LA-Candida Antigen Detection System , Immuno-Mycologics, Norman, OK; Pastorex Candida, Diagnostics Pasteur, Marnes-la-Coquette, France; Hybritech EIA, San Diego) (33-35) (Table 1).

Table 1. *Antigen detection tests for the diagnosis of invasive candidiasis.*

antigen/test	patient population	percentage (%)		references
		sensitivity	specificity	
heat-labile[#]/LA	Mixed	21-91	29-98	[36-44]
enolase/EIA	Neutropenic	85	96	[32]
mannan/LA or EIA	Mixed	47-66	89-100	[33, 35, 45-48]

[#] at a cutoff point of ≥ 1:4
LA = latex agglutination; EIA = enzyme immunoassay

The CAND-Tec is currently the most widely available and used assay. The test however, is rather insensitive and antigen tends to be detectable only late in the disease. Also, the wide range of antigen titers detected by the CAND-Tec assay among healthy donors and patients reduces the applicability of the test in a clinical laboratory (Table 1). The Becton Dickinson kit for enolase has already been withdrawn from the market. Detection of mannan is troublesome since mannan is eliminated rapidly from the bloodstream and circulates at very low concentrations as soluble immune complexes which have to be dissociated before detection.

Metabolic antigens

Assays have been developed for the detection of circulating *Candida* sugar metabolites such as mannose and arabinitol (49) which are both markers for disseminated candidiasis (50). Both can be quantified by gas liquid chromatography (15).

Much effort has been given into the detection of arabinitol. The enantio-selective measurement of D-arabinitol in human serum was originally performed by microbiologic-gas chromatographic (GC) (51) or enzymatic-GC techniques (52). Although these techniques are specific for D-arabinitol they are expensive, technically demanding and therefore not useful for routine diagnostic laboratoria. The use of an automated quantitative enzymatic assay for the detection of serum D-arabinitol using the enzyme D-arabinitol dehydrogenase, seems promising (53-55). However, arabinitol is cleared by the kidneys so that the normal range of serum concentration has to be adjusted in patients with renal insufficiency. Subsequently, D-arabinitol/creatinine ratios were used to avoid false positive results due to renal impairment (56, 57).

Recently, Walsh et al. (58) established in an experimental rabbit model of disseminated candidiasis a direct correlation between D-arabinitol/creatinine ratio's and tissue concentrations of *C. albicans*. They also suggested the use of the D-arabinitol/creatinine ratios in monitoring the efficacy of antifungal therapy. The diagnostic and prognostic validity of this assay has still to be determined in patients.

DNA detection

The specific diagnosis of invasive candidiasis depends primarily on the repeated recovery of the organism from blood specimens or normally sterile tissue sites. Unfortunately, it is apparent that *Candida* species are recovered from only 15-50% of patients with invasive candidiasis and cultures become positive only at a late stage of the disease (13). Although the lysis-centrifugation blood culture system increases the detection rate it is still not adequate (59). The detection of fungal species-specific DNA

sequences, as a non-culture technique, has the potential to be sensitive, specific, and rapid. Most work in this area has focused on the epidemiologic typing of *Candida* species (60, 61); however, application of the polymerase chain reaction (PCR) to the detection of *Candida* species in clinical specimens has been recently described (Table 2) (13).

Table 2. *Yeast DNA detection using different genes, specimens and methods.*

detection threshold (CFU/ml)	gene	target	specimens*	ref.
100	cytochrome $P_{450}L_1A_1$	*Candida* spp.	U, S, W, B	[62]
30	EO3 DNA fragment	*C. albicans*	U, B	[63, 64]
100	ribosomal RNA gene	yeast DNA	B	[65]
15	ribosomal RNA gene	yeast DNA	B	[66, 67]
100	*Candida* actin gene	*Candida* spp.	B	[68]
100	ribosomal RNA gene	yeast DNA	PBS	[69]
1000	*C. albicans* HSP 90	*C. albicans*	sw, U, P, pus, B, se	[70]

* U = urine, S = sputum, W = wound fluid, B = blood, sw = swabs, P = peritoneal fluid, se = serum.

PCR may be used to simultaneously detect and identify *Candida* species in clinical specimens which is another advantage over blood culture. Several techniques have been described for the isolation of yeast DNA from blood, but large volumes of blood (5 ml) have never been used. Generally, the techniques described in Table 2 are still too complex to be used in a routine setting. Further simplification is necessary while maintaining or preferably improving the detection limit of about 100 cells/ml blood.

Although several studies have used patient material for evaluation (often culture positive) there is as yet no large clinical study in which PCR is compared with blood culture. The diagnostic or prognostic usefulness has, therefore, still to be established in a well designed patient study.

Aim of the present study.

It is evident that accurate diagnosis of disseminated candidiasis is fraught with difficulties. The aim of this thesis was to investigate whether accurate diagnostic assays could be developed, in combination with their use in monitoring the progression of infection and response to treatment. In the first place we evaluated the use of more specific and sensitive antibody detection systems. As discussed in the diagnostic section, the detection of antibodies directed against *C. albicans* is limited by false positive and false negative results. In order to overcome these problems, we used purified cytoplasmic antigens (Chapter 2) and an immunodominant 45 kDa enolase antigen (Chapter 3), to determine specific antibodies using sensitive determination techniques such as ELISA. The diagnostic contribution made by these assays was investigated in different groups of patients. Initially, to determine the prevalence of these antibodies in a normal population a group of healthy donors was investigated. Simultaneously, a group of patients colonized by *C. albicans* but without signs of an invasive *Candida* infection, and a group of patients with a proven invasive candidiasis was investigated. Separate evaluations were made for immunocompetent and immunodeficient patient groups. Patients from whom consecutive serum samples were available were used to study the prognostic value of these assays in comparison to existing serologic assays (Chapter 4).

The detection of fungal species-specific DNA by PCR was evaluated for its diagnostic usefulness (Chapter 5). We also monitored the results of PCR on blood samples from neutropenic mice with invasive candidiasis treated with various concentration of liposomal Amphotericin B (Chapter 6).

Finally, we evaluated the contribution of antibodies directed against a crude antigen and the immunodominant enolase antigen, in protection against a *Candida* infection in an experimental mouse model (Chapter 7). To gain insight into the protective effect of anti-enolase antibodies, the opsonic capacity with anti-enolase antibody for *C. albicans* was investigated using macrophage monolayers.

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CHAPTER 2

Increased specificity of antibody detection in surgical patients with invasive candidiasis with cytoplasmic antigens depleted of mannan residues.

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ABSTRACT

In several studies it was shown that cytoplasmic antigens of *Candida albicans* were more frequently found in the sera of patients with invasive candidiasis, than in the sera of patients with superficial candidiasis. In this study, therefore, it was examined whether the specificity and the sensitivity, of a counter-immunoelectrophoresis technique (CIE), used in our laboratory, could be improved, by using cytoplasmic antigens depleted of mannan residues, and also by staining the precipitated complexes. Three successive serum samples, if possible, were collected from 44 patients with major surgical problems (but without haematological malignancies) of which 30 had invasive candidiasis and 14 superficial candidiasis. The CIE using the cytoplasmic antigen depleted of mannan was better in discriminating between invasive and superficial candidiasis. The specificity of the CIE increased from 28.6 to 78.6% when cytoplasmic antigens depleted of mannan were used, while the sensitivity slightly decreased from 80 to 70%. Diagnostic accuracy did not improve with addition of an antigen detection assay (Cand-Tec).

INTRODUCTION

The frequency of invasive candidiasis as a major cause of morbidity and mortality among hospitalized patients has increased. An antemortem diagnosis of invasive candidiasis is very difficult because there is no specific clinical picture. Although isolation of *Candida* spp. from clinical specimens and serodiagnostic tests for antigen and antibody detection may help in diagnosis, the following problems are inherent. In 56% of the cases, blood cultures of patients with autopsy-proven disseminated candidiasis, are negative for *Candida albicans* (20). Tests for the detection of circulating *Candida* antigens lack sensitivity and have been controversial (3, 4, 12, 13, 17, 21), while antibody detection systems mainly detect antibodies against *Candida* cell wall antigens which are ubiquitous in sera of patients with systemic candidiasis as well as in the sera of patients with superficial candidiasis (11, 15). To obtain a more specific test, investigators have evaluated the usefulness of measuring antibodies to cytoplasmic antigens (9, 23, 24, 25). The hypothesis behind these studies was that cytoplasmic antigens are only exposed during invasive infection and are therefore predictive and specific for invasive candidiasis (23, 25). For instance, Syverson et al. (24) and Vanmali et al. (26) used a crossed immunoelectrophoresis technique (XIE) with concanavalin A intermediate gel and a double immunodiffussion assay to evaluate the diagnostic usefulness of the antibody response to cytoplasmic antigens. The XIE was superior over the double immunodiffusion assay. Despite its high specificity, the test was not recommended for a routine setting because of the complexity of the technique.

In the present study, we evaluated the antibody response against cytoplasmic antigens from *C. albicans* in the presence and absence of mannan residues in the sera of surgical patients with either invasive or superficial candidiasis. To evaluate whether combinations of antibody detection and antigen detection assays could improve diagnostic accuracy, antigen titers in the same sera were determined by the Cand-Tec test.

MATERIALS AND METHODS.

Patient sera. Sera from patients included in this study were collected between october 1982 and january 1991. The majority of the patients underwent major abdominal surgery. None of the patients in this study had haematological malignancies or had received treatment with corticosteroids, immunosuppressive or cytostatic drugs or total body irradiation. On the basis of clinical findings and culture results these surgical patients were subdivided into two groups according to earlier-defined criteria. Group I consisted of 30 patients with proven invasive candidiasis. The criteria for this diagnosis were based on obtaining positive *Candida albicans* cultures from tissue biopsy, needle aspiration of normally sterile body cavities, or at

autopsy. One or more positive blood cultures was not regarded adequate to classify a patient as having invasive candidiasis. Group II consisted of 14 patients with a superficial *C. albicans* infection. Superficial candidiasis was determined by more than one positive *C. albicans* culture which had been isolated from different parts of the gastrointestinal tract in the absence of visible lesions.

Serological tests were done with fresh or fresh-frozen (-80°C) sera. In this study, 3 serum samples per patient, if available, were included for serological evaluation. The sera were obtained, at weekly intervals. The first sample was defined as the serum obtained at the time of the first microbiological evidence of invasive candidiasis.

Preparation of the cytoplasmic antigen with mannan residues. The cytoplasmic antigen was prepared by growing *C. albicans* (CBS 5982 serotype A, Centraal bureau voor schimmelcultures, Delft, The Netherlands) overnight at 37°C in minimal medium (11.7 g/l Yeast Carbon Base (Difco laboratories, Detroit , Michigan USA), 10 g/l D-Glucose, 20 g/l Bactopeptone (Difco)). Cells were harvested by centrifugation and washed twice in distilled water. The final pellet was dissolved in 42 ml icecold ammonium carbonate (20mM) followed by disruption with glassbeads (2mm) for 15 min in a Braunshaker (Kobold, FRG) under continuous CO_2 cooling. Cell walls were spun down at 5000 x g at 4°C for 45 min (Beckman JA20), and the supernatant was further centrifuged at 40.000 x g at 4°C for 30 min (Beckman JA20). The final supernatant was lyophilized and stored at -20°C (10), and will be referred to as M(+) antigen.

Preparation of the cytoplasmic antigen depleted of mannan. The cytoplasmic antigen depleted of mannan was prepared from the lyophilized M(+) antigen by removing mannan determinants using affinity chromatography on concanavalin A-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) according to the method of Ellsworth et al. (6). The eluent, containing the unbound material was concentrated by ultrafiltration (Amicon PM10 filter, Pharmacia), stored at 4°C, and will be referred to as the M(-) antigen. Before applying the M(-) antigen in the CIE it was examined for remaining mannan, by using a commercial latex kit (Pasteur diagnostica, France) and by using XIE.

Immunoelectrophoresis. Counterimmunoelectrophoresis was performed according to the method of Marier (16). Precipitating antibodies were scored before and after staining with Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, Mo.). Results were designated as CIE and CIE-stained, respectively. Antibody titers of 1:2 and 1:4 or higher, using the M(+) antigen, were regarded as positive in the CIE and CIE-stained, respectively. Antibody titers of 1:1 and 1:2 or higher, using the M(-) antigen were regarded as positive in the CIE and CIE-stained, respectively.

Characterization of the M(+) antigen and the M(-) antigen by XIE. The method applied was originally evaluated by Axelsen (1). Briefly, glassplates (5 x 5 cm.) with 1% agarose (Sigma) in Barbital buffer (pH 8.6, μ=0.02) were used. The M(+) antigen or M(-) antigen was electrophoresed (10 V cm^{-1}) in the first dimension. The run in the second dimension against a hyperimmune rabbit serum (5 $\mu l/cm^2$ DAKO) was performed for 18 h at 2 V cm^{-1}. After electrophoresis gels were washed first in 0.9% NaCl overnight then in distilled water for 1h. Finally the gels were bound to agarose gelbond film (Bio-Rad Laboratories, Richmond, CA) and then stained with Coomassie Brilliant Blue R-250 (Sigma).

For comparison of the CIE using M(-) antigen (M(-)CIE) with the CIE using M(+) antigen (M(+)CIE), antigen concentrations were equalized by titration in such a way that the areas under six of the same precipitins of both antigens were the same when electrophoresed against a rabbit hyperimmune serum (5 $\mu l/cm^2$ DAKO) in the XIE. This titration actually

resulted in a concentration of 100 μg/ml of protein for both the M(+) and M(-) antigen, as used in the particular CIE.

Antigen detection assay. Titers of circulating antigens were determined by the Cand-Tec assay (Ramco laboratories Inc., Houston, Tex.) (2, 5), according to the directions of the manufacturer. A serum dilution of 1:4 or higher was regarded as a positive titer.

Statistical analysis. Correlations between the three serum samples obtained from each patient were assessed using the Intraclass correlation coefficients (r_i). The capacity to discriminate between invasive and superficial candidiasis was evaluated by the Mann Whitney test (MW). Logistic regression was used as multivariable analysis, to evaluate various serological assays simultaneously, with regard to their discriminating capacities.

RESULTS

Characterization of the M(+) and M(-) antigen. Before applying the M(-) antigen in the CIE it was examined for remaining mannan by crossed immunoelectrophoresis (XIE) (1). When M(+) antigen was electrophoresed against a patient's serum in XIE, different precipitins were observed (Fig. 1A). As indicated by the arrow very strong but indistinct precipitins could be observed, but also very sharp but less intens precipitins. By tandem XIE the large precipitin appeared to be identical to a purified immunodominant antigen which in our hands had enolase activity (data not shown). When M(-) antigen was used in the XIE the strong but indistinct precipitins due to mannan residues disappeared (Fig. 1B) (25). Also, with the commercial mannan kit (Pasteur diagnostica, France) the M(-) antigen did not react. However, in order to correlate antibody titers obtained by the CIE using M(-) antigen (M(-)CIE) with the CIE using M(+) antigen (M(+)CIE), concentrations of antigens other than mannan, were equalized by titration in such a way that the areas under six of the same precipitins of both antigens were the same when electrophoresed against a rabbit hyperimmune serum (5 μl/cm^2 DAKO) in the XIE. This titration actually resulted in equal protein concentrations of 100 μg/ml for both the M(+) and M(-) antigen, as used in the particular CIE.

Discriminative capacity of the Cand-Tec, M(+)CIE, and M(+)CIE-stained. The M(+)CIE results showed that for patients with invasive candidiasis, there was an increase in the percentage of positive antibody titers in the three serum samples examined (Table 1). For patients with a superficial *Candida* infection, the titers were either stable (without staining), or decreased (with staining), with time. Antigen titers, measured by Cand-Tec, however, decreased from 50 to 36% in the same sera from patients with invasive candidiasis, or remained stable in the sera from patients with superficial candidiasis.

Despite these increases in CIE titers as determined by M(+)CIE (for patients from whom three serum samples were available, n=13), statistical analysis on the sera from

all patients (n=30) showed no significant differences with time. The titers for serum samples 1, 2, and 3 of each patient appeared strongly correlated without (r_i=0.76), and with (r_i=0.78) staining.

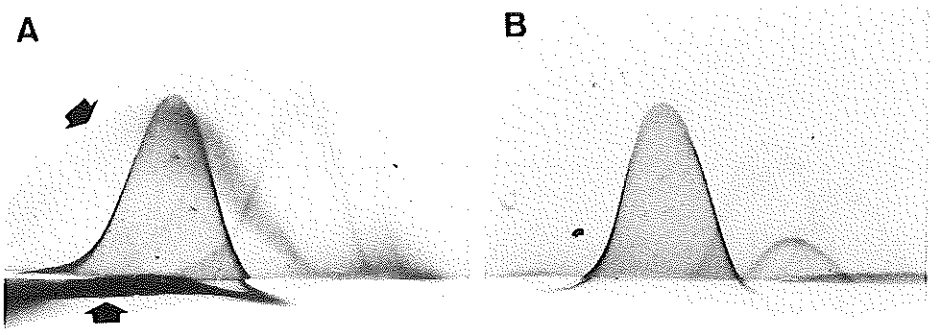

Figure 1. *XIE demonstrating antibodies against the M(+) (A) and M(-) (B) antigens. Antibodies against mannan are indicated by arrows.*

Table 1. *Percentage of positive titers by M(+)CIE for patients with invasive (group I, n=13) or superficial (group II, n=6) candidiasis from whom three serum samples were available*

Serum sample no.	% of positive titers by M(+)CIE[a]			
	Without staining		With staining	
	I	II	I	II
1	77.0	60.0	46.2	80.0
2	92.4	60.0	61.6	60.0
3	92.3	60.0	84.7	20.0

[a] Cutoff values for positive titers were ≥ 2 without staining and ≥ 4 with staining.

Therefore, to evaluate the capacity of the CIE test to discriminate between invasive and superficial candidiasis, the mean titers of serum samples 1, 2, and 3 were used, after logarithmical transformation. Figure 2 shows the distribution of the mean logarithm(titer + 1) obtained for each patient.

On the basis of statistical analysis, it was observed that the M(+)CIE test without staining could not discriminate between groups I and II (MW P = 0.09). However, significant differences were found between groups I and II (P = 0.04) when the gels were stained before scoring. Generally higher values were obtained for group I. At a cutoff value of 1:4 M(+)CIE-stained had a sensitivity of 66.7 and a specificity of 57.2%. The Cand-Tec assay on the other hand did not discriminate between the two patient groups (P = 0.12).

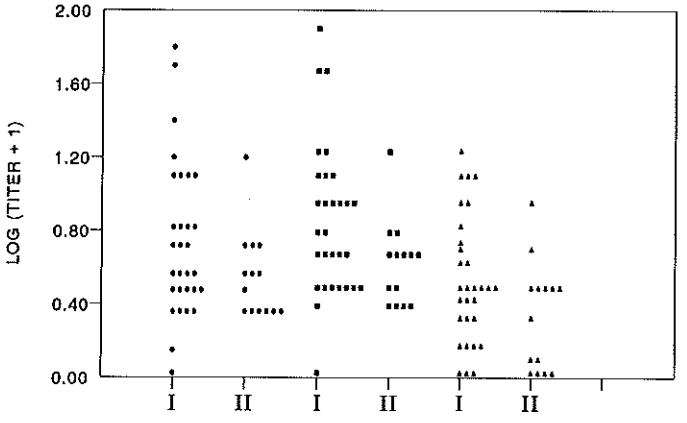

Figure 2. Distribution of the mean logarithm(titer + 1) obtained for each patient with invasive (group I) or superficial (group II) candidiasis. ●, M(+)CIE without staining; ■, M(+)CIE with staining; ▲, Cand-Tec.

Discriminating capacities of the M(-)CIE and the M(-)CIE-stained. By M(-) CIE, titers were lower (or absent) than those by M(+)CIE for all serum samples from patients with either invasive or superficial candidiasis (Table 2).

Table 2. *Percentage of patients with invasive (group I, n=30) or superficial (group II, n=14) candidiasis with the indicated mean titer value for each test.*

Test	% of patients with indicated titer in group:					
	I			II		
	0-1	2	≥4	0-1	2	≥4
M(+)CIE						
Without staining	6.7	30.0	63.3	0.0	50.0	50.0
With staining	3.3	26.6	70.0	0.0	42.9	57.1
M(-)CIE						
Without staining	56.7	20.0	23.3	85.7	14.3	0.0
With staining	30.0	40.0	30.0	35.7	57.1	7.1

Table 3. *Percentage of positive titers by M(-)CIE for patients with invasive (group I, n=12) or superficial (group II, n=6) candidiasis from whom three serum samples were available*

Serum sample no.	% of positive titers by M(-)CIE			
	Without staining		With staining	
	I	II	I	II
1	50.0	20.0	50.0	20.0
2	66.7	20.0	83.4	20.0
3	66.7	20.0	66.6	20.0

For the patients from whom three serum samples were available (n=12), an increase in positive titers between the first and the second serum was observed, by M(-)CIE with and without staining (Table 3). However, the mean values of titers for serum samples 1, 2, and 3 for all patients did not significantly differ. Again, the serum titers were transformed as mentioned before and are depicted in Fig. 3. By statistical analysis, the mean levels of titers obtained by M(-)CIE without staining were significantly higher for invasive candidiasis than for superficial candidiasis, irrespective of a chosen cutoff value (P=0.005). At a cutoff value for a positive titer of 1:1, the test had a sensitivity of 70% and a specificity of 78.6%. M(-)CIE with staining, on the other hand, could not discriminate between groups I and II (P=0.06). From the results given in Table 4, there is an actual increase in specificity of the test, by removing mannan, from 28.6 to 78.6% or from 57.2 to 85.7% by CIE without and with staining.

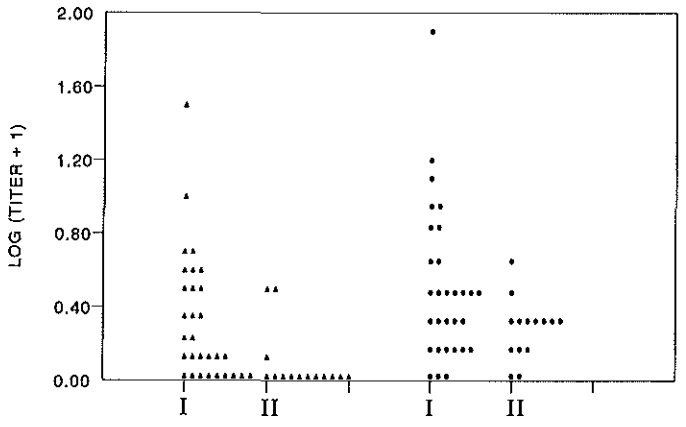

Figure 3. *Distribution of the mean log(titer + 1) obtained for each patient with invasive (group I) or superficial (group II) candidiasis. ▲, M(-)CIE without staining; ●, M(-)CIE with staining.*

Table 4. *Sensitivity and specificity of the different tests determined at a chosen cutoff value by using the mean log(titer + 1) value for groups I and II*

Test	Cutoff value	Sensitivity (%)	Specificity (%)	*P* value[a]
Cand-Tec	≥ 4	26.7	85.7	0.46
M(+)CIE				
Without staining	≥ 2	80.0	28.6	0.43
With staining	≥ 4	66.7	57.2	0.19
M(-)CIE				
Without staining	≥ 1	70.0	78.6	0.0037
With staining	≥ 2	50.0	85.7	0.0013

[a] The *P* value was determined by the Fisher exact test by comparing the percentages of patients with a titer above cutoff levels for groups I and II.

DISCUSSION

It is known, from literature data and from our own investigations that several cytoplasmic antigens, including the 47 kDa antigen of *C. albicans*, are the most frequently found inducers of antibodies in the sera of patients with invasive candidiasis (18, 22, 27). Antibodies directed against the 47 kDa antigen, in addition to other cytoplasmic antigens, were either rare in uninfected controls or, were infrequently found in superficially infected patients. Therefore, these cytoplasmic antigens could serve as the basis of a reliable diagnostic test. The purpose of this study was to develop a serological procedure suitable for a routine microbiological laboratory. In this study, it is found that 30.0% of the patients (n=9), with invasive candidiasis gave a negative response to the M(-)CIE, whereas only one patient did not respond in the M(+)CIE. In contrast, 78.6% of the patients with superficial candidiasis (n=11) responded negatively to the M(-)CIE but all responded to the M(+)CIE. These results confirm the findings of Matthews et al. (18) who found that, a negative response in an antibody test may be due to the small number of antigens applied and therefore, not always due to the inability of some patients to produce antibodies (19). However, in accordance with Jones et al. (11), we found that the use of more antigens including mannan antigens, lead to low specificities in antibody detection assays.

It was further shown that during the first two weeks after the first clinical signs of invasive candidiasis, titers increased, leading to higher sensitivities of the test, confirming the findings of Kostiala et al. (14). Increasing titers over time in our data, however, were not significant because of the large number of patients for whom only one or two successive serum samples were available.

Despite the fact that the M(-)CIE had a diagnostic value, the test has only moderate sensitivity. This is because nine patients (30%), with invasive candidiasis, did not have an antibody response against the M(-) antigen. From six of these nine patients, only one or two serum samples were available, because of early death within 6 to 16 days after the clinical onset of disease. From the 21 patients who did produce antibodies against M(-) antigen, 8 died despite antibody production. However, 13 of the 14 patients who died produced antibodies against the M(+) antigen. Perhaps these patients produced antibodies against mannan initially but were not able or did not have the time, to develop an antibody response to the M(-) antigen including the 47 kDa antigen. Matthews has suggested that antibodies against 47 kDa antigen might be protective (18).

Staining of the precipitated complexes led to a general increase in the sensitivity of the test for both groups I and II. In order to maintain the discriminative capacity of the test, higher cutoff values were needed. Eventually, because of this procedure the sensitivity of the test with staining decreased in comparison with the test without staining. By removing mannan residues from the cytoplasmic antigen applied, the

specificity of the CIE increased from 28.6 to 78.6%. Fisher et al. (7) found a specificity of 90% and a sensitivity of 92% for their EIA with the commercially supplied Hollister-Stier sonicate antigen of *C. albicans*. Probably the differences are due to the fact that they used healthy volunteers or patients without signs of a *Candida* infection as a control group to calculate sensitivites and specificities. In the same study, it was claimed that a combination of both antibody and antigen detection assays considerably improved diagnostic accuracy. Logistic regression analysis of our data did not show any improvement when the antigen and antibody results were combined.

The data obtained with the Cand-Tec test were not as favourable as claimed in other studies (5, 8). Discrepancies again may be due to different selection criteria used for determining invasive candidiasis, since our criteria were more restrictive. Since the presence of antigens in patient sera induce the synthesis of antibodies, a decrease in antigen titers could therefore be explained by the formation of antigen antibody complexes or by renal excretion, suggesting, that antigen titers are probably best determined in the early onset of invasive candidiasis.

In conclusion, we believe that for patients with surgical problems without haematological malignancies, the M(-)CIE can assist the clinician in the diagnosis of invasive candidiasis at an early stage of infection. Further study is obviously needed to assess the use of the M(-)CIE in immunocompromised patients.

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CHAPTER 3

Diagnostic value of anti-*Candida* enolase antibodies.

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ABSTRACT

An immunodominant antigen with enolase enzyme activity was purified and used for the development of an assay to detect antibodies directed against this antigen in sera of patients with either invasive candidiasis or *Candida* colonization. The enzyme-linked immunosorbent assay established with the *Candida* enolase antigen was able to discriminate significantly between invasive candidiasis and colonization in both immunocompetent and immunodeficient groups of patients. The test had a sensitivity of 50% and a specificity of 86% in the immunocompetent patient group. In the immunodeficient patient group a sensitivity of 53% and a specificity of 78% were established. Antibody levels determined by a counterimmunoelectrophoresis assay with the same set of sera resulted in a better sensitivity for sera from the immunocompetent patient group but a lower specificity, i.e. 80% and 29% respectively. The counter-immunoelectrophoresis assay of sera from the immunodeficient patient group was not able to discriminate significantly between invasive candidiasis and colonization. With the use of more serum samples per patient, the sensitivity of the antibody detection assays increased while the specificity was maintained. The increase, however, was statistically not significant. Combining the results of the antibody assays with antigen titers obtained by the Cand-Tec assay did not improve the predictive value with respect to invasive candidiasis, as determined by multivariance regression analysis. Furthermore, it was

demonstrated by performance of Western blots (immunoblots) that sera of patients as well as a rabbit antiserum cross-reacted with the *Candida* enolase and baker's yeast enolase enzyme. However, by tandem crossed immunoelectrophoresis it was demonstrated that the antibodies were directed towards different epitopes of the antigen.

INTRODUCTION

The use of antibacterial, immunosuppressive, and cytotoxic drugs increases the incidence of lethal invasive candidiasis in hospitalized patients (13). Despite many attempts to develop reliable serological tests, the laboratory diagnosis of deep-seated *Candida* infection still remains a problem (3, 11). Until now, antibody detection tests with crude antigens have produced suboptimal sensitivities and/or specificities (11). However, the detection of antibodies directed against specific antigens seems promising. Matthews et al. (17) demonstrated that the presence of antibodies against an immunodominant antigen of *Candida albicans* with a molecular mass of 47 kDa correlated with invasive infection. Since cytoplasmic antigens are released during invasive infection, it is conceivable that cytoplasmic antigens applied in an antibody detection assay could discriminate between invasive candidiasis and colonization. Recently, Zöller et al. (23) reported in a mixed immunocompromised and immunocompetent patient population a sensitivity and a specificity of 81.5 and 96.4%, respectively, for an antibody detection assay that made use of the partially purified 47 kDa antigen.

In the present study, we purified an immunodominant antigen of *C. albicans* and used it to develop an enzyme-linked immunosorbent assay (ELISA) which discriminates positively between invasive candidiasis and colonization. Since several contradictory results have been published concerning the presence or absence of enolase activity in the immunodominant antigen, we also analyzed the purified antigen by Western blot (immunoblot) and tandem crossed immunoelectrophoresis (XIE) and subsequently tested it for enolase activity.

To determine whether the kinetics of the antibody response is more important than the antibody titer itself, three successive serum samples from each patient, when available, were included for serological evaluation. The results obtained by the ELISA were compared to titers of antibodies directed against a cytoplasmic antigen-containing mannan [M(+) antigen] and to antibody titers directed against a cytoplasmic antigen depleted of mannan and glycoproteins [M(-) antigen] as determined by a counterimmunoelectrophoresis (CIE) technique. Besides antibody detection, the same serum samples were analyzed for circulating antigens by the Cand-Tec assay.

MATERIALS AND METHODS

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (19). In brief, proteins were separated electrophoretically in a discontinuous system as described by Laemmli (15). The electrophoresis system consisted of a 10% running gel and a 4% stacking gel, and the proteins were subsequently transferred to a polyvinyldifluoride membrane (Immobulon-P; Millipore) by the method of Towbin et al.(20). For immunoblotting, diluted rabbit antiserum raised against a crude antigen prepared from *C. albicans* CBS 5982 serotype A (Centraal Bureau voor Schimmelcultures, Delft, the Netherlands) and patient sera were used. Blots (bands) were developed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-human IgG, respectively, by using 5-bromo,4 chloro-3-indol phosphate (Sigma Chemical Co., St. Louis, MO.) and nitroblue tetrazolium (Sigma) as substrate.

Purification of the immunodominant *Candida* antigen. For purification procedures *C. albicans* CBS 5982 serotype A was grown for 48 h at 37°C in 2 liters of minimal medium (11.7 g of yeast Carbon base per liter, 10 g of D-Glucose per liter, 20 g of Bacto Pepton [Difco laboratories, Detroit, Mich] per liter). Cells were harvested by centrifugation and were washed twice in distilled water. The final pellet was dissolved in 42 ml ice-cold distilled water and was then disrupted with glassbeads (diameter,2 mm) for 15 min in a Braun shaker (B. Braun, Melsungen, Germany) under continuous CO_2 cooling. The suspension was spun down at 40,000 x g at 4°C for 20 min. Supernatants were stored overnight at -80°C and after being thawed were centrifuged at 40,000 x g for 20 min at 4°C. Supernatants were brought to 50% saturation by adding a saturated solution of $(NH_4)_2SO_4$. Further saturation to 67% was obtained by adding solid $(NH_4)_2SO_4$ to the supernatant. The solution was then centrifuged at 40,000 x g at 20°C for 20 min, and solid $(NH_4)_2SO_4$ was added to the supernatant until 100% saturation was accomplished. After 20 min at room temperature, the solution was further centrifuged at 40,000 x g at 20°C for 20 min. The pellet was resuspended in a Tris buffer (0.067 M; pH 7.5) and desalted by using a Sephadex G25 Column (Pharmacia LKB, Uppsala, Sweden). The protein-containing fraction was further purified by a fast-performance liquid chromatography (FPLC) on a Highload Q-Sepharose high performance column (FPLC HQSHP; Pharmacia) by using a 0 to 10% gradient of 2 M NaCL in a 0.067 M Tris buffer (pH 7.5). Gradients were created using 325 ml (1 to 3%) or 150 ml (3 to 10%) of 2 M NaCl at a flow rate of 4 ml/min. Aliquots of each fraction were analyzed by SDS-PAGE, and the enolase activity in each fraction was determined. Samples containing enolase activity were pooled and exchanged with a 20 mM piperazine buffer (pH 6.0) on a Sephadex G25 column. The pooled fraction was loaded on a FPLC Mono P column (H 5/20; Pharmacia), and proteins were separated by a linear gradient from pH 6 to pH 4.5 in polybuffer.

Fractions containing enolase activity were analyzed by SDS-PAGE, pooled and exchanged with a concanavalin A starting buffer (0.02 M Tris, 0.1 M NaCl, 1 mM $CaCl_2$, 1mM $MnCl_2$ [pH 7.4]) before they were applied to the concanvaline A-Sepharose column (Pharmacia) in order to remove mannan by the method of Ellsworth et al. (5). Removal of mannan was confirmed by a negative result in the mannan kit of Pasteur (Diagnostics Pasteur, Marnes-la-Coquette, France). Finally, the immunodominant antigen containing enolase activity was stored at -80°C in a 0.01 M Tris buffer (pH 7.5).

Enolase activity. During the purification procedure, fractions were analyzed for enolase activity by the method of McAllistor and Holland (18).

ELISA. Optimal concentrations of the antigen, sera, and conjugate were predetermined by checkerboard titrations with known positive and negative sera obtained by Western blotting.

Highly activated polystyrene microtiter plates (Nunc immunoplate Maxisorp F96) were coated with 1.5 μg per well of *Candida* antigen in 50 μl of coating buffer (0.13 M sodium carbonate and 0.03 M hydrogenated sodium carbonate [pH 9.6]) per well and were subsequently incubated overnight at 4°C. The plates were washed twice with 200 μl of TBS-block (0.01 M Tris, 0.15 M NaCl [pH 7.6], 0.1% gelatine) per well and were then incubated with 200 μl of TBS-block per well at 37°C for 2 h. The plates were then drained. Sera were diluted twofold in TBS (0.01 M Tris, 0.15 M NaCl [pH 7.6]) starting at a dilution of 1:200. A total of 50 μl of each serum was added to each well, and the plates were subsequently incubated at 37°C for 60 min. After three cycles of washing with TBSt (TBS with 0.04% [wt/vol] gelatin and 0.05% [vol/vol] Tween 20), 50 μl of an alkaline phosphatase-labelled goat anti-human IgG conjugate (diluted 1:1,000 in TBSt [Sigma]) was added to each well; this was followed by incubation for 1 h at 37°C. A 100 μl-volume of acid enzyme substrate solution (9.7% diethanolamine, 3mM NaN_3, 5 mM $MgCl_2.6H_2O$, 1 mg/ml *p*-nitrophenylphosphate [Sigma]) was added after three cycles of washing with TBSt and the plates were incubated at 37°C in the dark. Color development was stopped after 30 min of incubation by adding 100 μl of 3 M NaOH to each well. The optical densities at 405 nm were determined by using an automated plate reader (Bio-Tek Instruments, Inc., Winooski, Vt.), and it was assumed that the results of the ELISA, expressed as optical densities at 405 nm of the reference sera, might fluctuate at the maximum of 10%. A cutoff point was determined by analysis of 60 serum samples from healthy individuals of different age groups. The cutoff point defined the upper 5% of this population as positive by the test. Patient sera were regarded as positive when the results obtained by the *Candida* enolase ELISA were above this cutoff point at a serum dilution of 1:200. Sera with positive test results were twofold diluted until the test results became negative, a dilution of 1:200 was regarded as undiluted. Titers were defined as the highest serum dilution with a positive test result.

Tandem XIE. Tandem XIE was performed as described by Axelsen et al. (1). Briefly, glassplates (5 by 5 cm) coated with 1% agarose (Sigma) in barbital buffer (pH 8.6; $\mu = 0.02$) were used. The *Candida* enolase antigen (0.1 mg/ml) was subjected to tandem electrophoresis (10 V cm^{-1}) with the purified bakers' yeast enolase antigen (20 mg/ml; Sigma) in the first dimension. The run in the second dimension against a hyperimmune rabbit serum (7,5 μl/cm^2; DAKO Glostrup, Denmark) or patient serum (10 μl/cm^2) was performed for 18 h at 2 V cm^{-1}. After electrophoresis, gels were washed first in 0.9% NaCl overnight and then in distilled water for 1 h. Finally, the gels were bound to agarose gelbond film (Bio-Rad Laboratories, Richmond, Calif.) and stained with Coomassie Brilliant Blue R-250 (Sigma).

Standard serological tests. Antibody responses against cytoplasmic antigens in the presence or absence of mannan, were performed as previously described (21). Briefly, titers of antibodies against M(+) antigen and M(-) antigen were measured by CIE and referred to as M(+)CIE and M(-)CIE respectively. Titers of circulating antigens were determined by the Cand-Tec assay (Ramco laboratories Inc., Houston, Tex) (2, 4) according to the directions of the manufacturer. A serum dilution of 1:4 or higher was regarded as a positive titer.

Patient sera. The patient sera included in the present study were collected between October 1982 and January 1991. Patients were divided into two groups on the basis of clinical findings and culture results. Group I consisted of 76 patients with confirmed invasive candidiasis. The criteria for this diagnosis were based on obtaining positive *Candida albicans* cultures from tissue biopsy specimens or needle aspirates of normally sterile body cavities or at autopsy. One or more positive blood cultures were not adequate to classify a patient as having invasive candidiasis. Group II consisted of 32 *C. albicans*-colonized patients. Colonization was established by the presence of several positive *C. albicans* cultures of samples from different parts of the

gastrointestinal tract in the absence of visible lesions.

On the basis of the immune status of the patients, both groups were subdivided into patients with immunodeficiencies caused by therapy or underlying diseases and patients without immunodeficiencies caused by therapy or underlying diseases. The criteria for immunodeficiency were histologically confirmed neoplasms, kidney function disorders requiring dialysis, treatment with corticosteroids, total-body irradiation, or the use of immunosuppressive or cytotoxic drugs. The majority of the patients without signs of immunodeficiencies underwent major abdominal surgery. None of these patients had haematological malignancies or had received treatment with corticosteroids, immunosuppressive or cytostatic drugs or total-body irradiation. On the basis of these criteria, group I patients were divided into 46 patients with signs of immunodeficiency (group IA) and 30 immunocompetent patients (group IB); the 32 colonized patients were divided into those with signs of immunodeficiency (group IIA; $n=18$) and those who were immunocompetent (group IIB; $n=14$).

Not enough serum was available from some patients to be analyzed by all serologic assays. Therefore, sera from only 41 patients with invasive and 18 colonized patients could be evaluated by the M(+)CIE and the M(-)CIE, and sera from 44 patients with invasive candidiasis and 18 colonized patients could be evaluated by the ELISA. Three serum samples per patient, when available, were included for serological evaluation. The sera were obtained, at weekly intervals. The first serum sample was defined as that obtained at the time of the first microbiological evidence of invasive candidiasis.

Statistical analysis. Correlations between the three serum samples obtained from each patient were assessed using intraclass correlation coefficients (r_i). The capacity to discriminate between invasive candidiasis and colonization was evaluated by the Mann-Whitney test. Percentages of patients in groups I and II with a titer above cutoff levels were compared by Fisher's exact test. The significance level used was $P=0.05$ (two-sided) was used. Logistic regression was used as multivariable analysis, to evaluate simultaneously, the discriminating capacities of the various serological assays.

RESULTS

Isolation and characterization of the immunodominant antigen. Samples obtained from different stages of the purification procedure were examined by SDS-PAGE and western blotting (Fig. 1). The enzyme activities in the pooled fractions obtained during the different purification steps were determined in an enolase assay. After each purification step, the specific activity of the immunodominant protein increased (Table 1). Five milligrams of pure *C. albicans* enolase could be recovered from a 2-liter culture.

Immunologic characterization of the *C. albicans* enolase antigen. By using the techniques SDS-PAGE, Western blotting and tandem XIE, the *C. albicans* enolase was compared with the baker's yeast enolase. The baker's yeast enolase had a molecular mass of approximately 49 kDa, and the *Candida* enolase is about 45 kDa (Fig. 2). These findings were confirmed by the Western blotting, in which the applied polyclonal rabbit anti-*C. albicans* antiserum cross-reacted with the baker's yeast enolase.

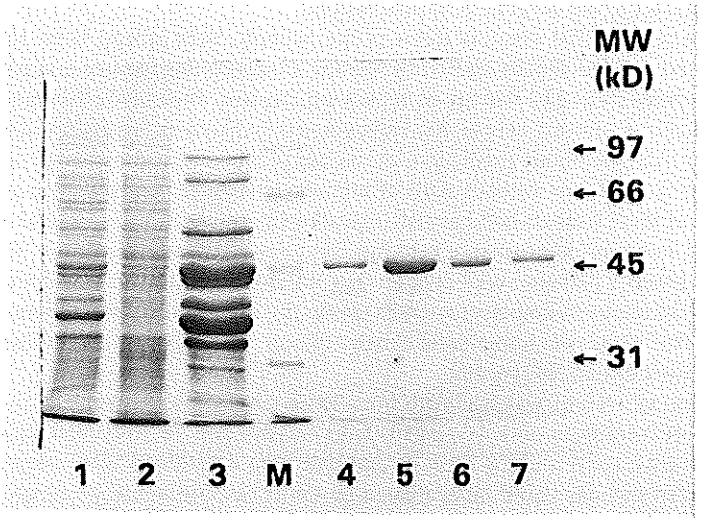

Figure 1. *SDS-PAGE of pooled fractions obtained during the antigen purification procedure. Lanes: 1, supernatant after shaking on a Braun shaker; 2, supernatant after 67% $(NH_4)_2SO_4$ saturation; 3, dissolved pellet after 100% $(NH_4)_2SO_4$ saturation; 4, Candida enolase fraction after FPLC HLQSHP; 5, Candida enolase fraction after FPLC Mono P; 6, pooled fraction after concanavalin A-Sepharose chromatography; and 7, pooled Candida enolase fraction used in the ELISA. M, molecular mass markers are indicated on the right (in kilodaltons).*

Table 1. *Enolase activities of different fractions during the Candida enolase antigen purification procedure.*

Sample no.[a]	Total amt. of protein (mg)	Total acitivity (units)	Sp. act. (units/mg)	Relative purification	Yield (%)
1	900	1,050	1.17	1	100
2	210	347	1.65	1.4	33
3	50	149	2.98	2.6	14.2
4	17	96	5.65	4.8	9.1
5	14	93	6.64	5.7	8.9
6	5	61	12.2	10.4	5.8

[a] Sample numbers: 1, supernatant after shaking on a Braun shaker; 2, dissolved pellet after 100 % $(NH_4)_2SO_4$ saturation; 3, *Candida* enolase fraction after FPLC HLQSHP; 4, *Candida* enolase fraction after FPLC Mono P; 5, pooled fraction after concanavalin A-Sepharose chromatography; and 6, pooled *Candida* enolase fraction used in the ELISA.

The antibodies of some of the patients with deep-seated candidiasis also cross-reacted with the baker's yeast enolase, as determined by Western blotting (Fig. 3). This cross-reactivity was further analyzed by performing tandem XIE with patient sera as well as rabbit serum. The observed cross-reactivities on Western blots must be due to antibodies directed toward different epitopes, because the precipitated line formed by the *Candida* enolase antigen crosses the precipitated line formed by the baker's yeast enolase (Fig. 4).

Immunocompetent patient group. In an earlier study, we scored by Western blotting the quantitative and qualitative aspects of patient sera against the immunodominant antigen (19). To study the diagnostic accuracy of specific antibodies directed towards the immunodominant *Candida* enolase antigen, sera of different patient groups were examined in an ELISA. Only IgG antibody titers were determined.

Antibodies directed towards the *Candida* enolase antigen studied in the immunocompetent patient group showed increased antibody titers over time. However, the increase in antibody titers with time was not significant. Since these titers were strongly correlated (r_i=0.72), the mean titers of serum samples 1, 2, and 3 from each patient was calculated, after logarithmic transformation, and were used to discriminate between invasive candidiasis (group IB) and colonization (group IIB).

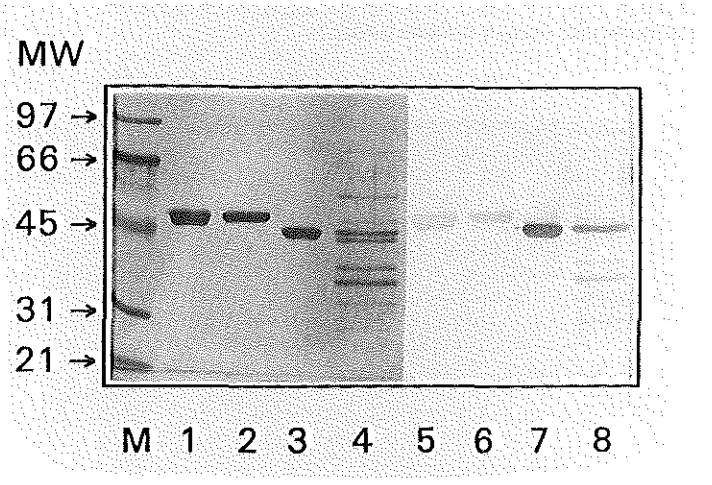

Figure 2. *Comparison of molecular masses obtained by SDS-PAGE and antigenicity by Western blotting of 1 and 5, baker's yeast enolase; 2 and 6, baker's yeast enolase after Mono Q Sepharose column chromatography; 3 and 7, Candida enolase antigen; 4 and 8, partially purified Candida extract. Lanes 1 to 4 were stained with Coomassie brilliant blue, and lanes 5 to 8 were incubated with hyperimmune rabbit serum. M, molecular mass markers are indicated on the left (in kilodaltons)*

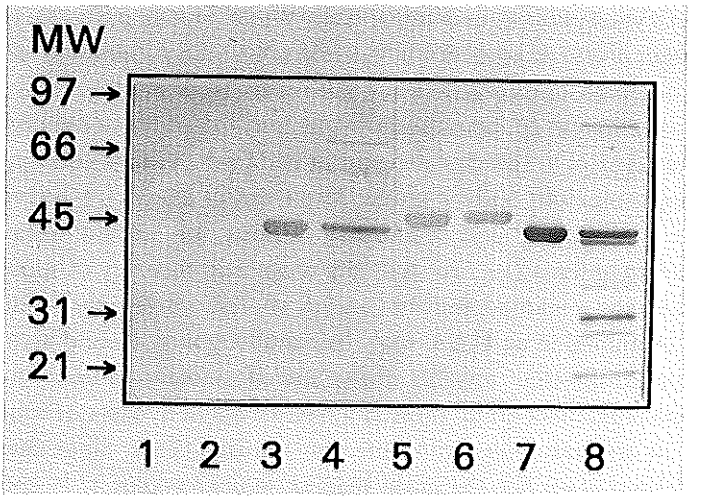

Figure 3. *Western blots of baker's yeast enolase and C. albicans enolase obtained by exposure to sera from two patients with invasive candidiasis. Lanes: 1 and 5, baker's yeast enolase; 2 and 6, baker's yeast enolase after Mono Q Sepharose column chromatography; 3 and 7, Candida enolase antigen; 4 and 8, partially purified Candida extract. Lanes 1 to 4 and 5 to 8 were incubated with sera obtained from two patients with invasive candidiasis, respectively. Numbers on the left are molecular masses (in kilodaltons).*

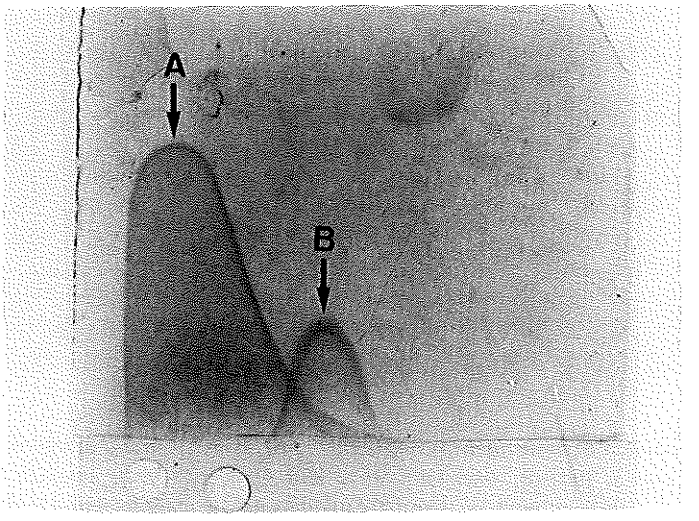

Figure 4. *Tandem XIE demonstrating rabbit antibodies directed toward the Candida enolase (arrow A) and the baker's yeast enolase (arrow B).*

Figure 5 shows the mean logarithm(titer + 1) for each individual patient in groups IB and IIB. A cutoff value of ≥2 for anti-*Candida* enolase antibody levels gave the best sensitivity and specificity, i.e., 50 and 86%, respectively (Table 2). When sera from healthy individuals were used as negative control group, the specificity increased to 100%.

Antibody titers determined with the M(+)CIE, which were conducted on the same sera from each patient from groups IB and IIB, gave a better sensitivity but a lower specificity, while the M(-)CIE also had a better sensitivity but a specificity comparable to that of the enolase ELISA at the indicated cutoff values (Table 2). When the mean log(titer+1) of antigen of the serum samples per patient was calculated, a sensitivity of 27% and a specificity of 86% were established.

Table 2. *Sensitivities and specificities of the different tests determined at a chosen cutoff value by using the mean logarithm(titer+1) values obtained for groups I and II of the immunocompetent patient group.*

Serological assay	Cutoff value	Sensitivity (%)	Specificity (%)	*P* value[a]
M(+)CIE	≥2	80	29	0.4
M(-)CIE	≥1	70	79	0.004
Enolase ELISA	≥2	50	86	0.02
Enolase ELISA	≥2	50[b]	100[b]	3.3×10^{-9}
Cand-Tec	≥4	27	86	0.46

[a] *P* values were determined by the Fisher exact test by comparing percentages of patients in groups IB and IIB whose sera had titers above the cutoff levels.
[b] Sera from healthy individuals were used to calculate the sensitivity, the specificity, and the *P* value.

When calculating the sensitivity and specificity for the first serum sample obtained from each patient group from which three serum samples were available, a sensitivity of 47% and a specificity of 71% were established. However, if the mean value for three serum samples for the same selected group of patients was calculated, the sensitivity decreased to 21% and the specificity was 100%. In both situations, the Cand-Tec did not discriminate between invasive candidiasis and colonization ($P=0.33$ and $P=0.53$, respectively). Three serum samples were available from 13 immunocompetent patients with invasive candidiasis and 6 immunocompetent colonized patients.

Multivariable analysis showed that the antibody assays, i.e. *Candida* enolase ELISA, or the M(+)CIE and the antigen detection assay Cand-Tec did not improve the discriminative capacity relative to that of the M(-)CIE.

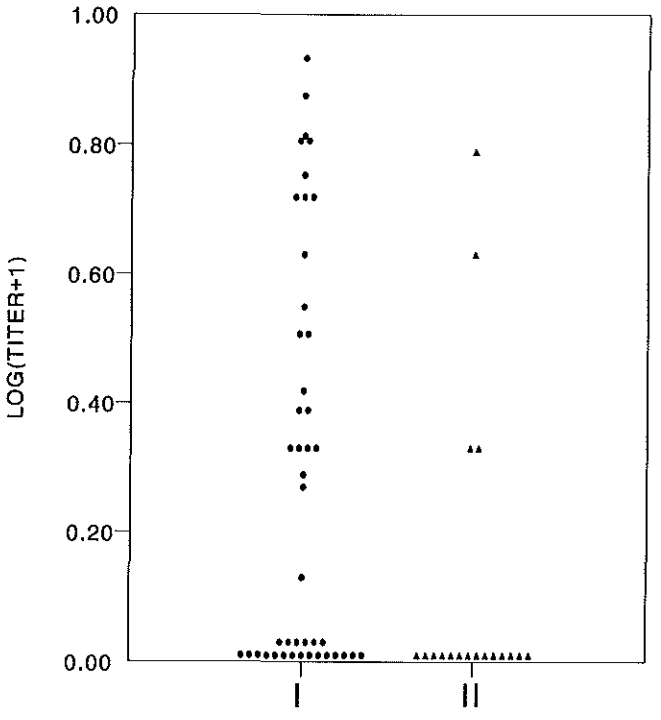

Figure 5. *Distribution of the mean logarithm(titer + 1) of anti-Candida enolase antibodies in the sera of each immunocompetent patient with invasive candidiasis (I) or Candida colonization (II).*

Immunodeficient patient group. In comparison with the titers in the sera from the immunocompetent patient group, anti-*Candida* enolase antibody titers in sera of the immunodeficient patients did not increase significantly over the 2-week interval of observation. Again, the titers in serum were transformed as mentioned above, and the mean logarithm(titer + 1) values are depicted in Fig. 6. Statistical analysis of these data showed that the mean logarithm(titer + 1) levels, obtained by the *Candida* enolase ELISA were significantly higher for patients with invasive candidiasis than patients colonized with *C. albicans* ($P = 0.03$; Mann-Whitney test) without a chosen cutoff value. At a cutoff

value of ≥ 1, the test had a sensitivity of 53% and a specificity of 78% (Table 3). Again the specificity of the assay increased when sera from healthy individuals were used as a negative control group (Table 3). Although, the increase in antibody titers with time was not significant, the sensitivity of the test increased from 42 to 54% for the first serum sample obtained from each patient, while the specificity remained constant (78%).

The results obtained by the M(+)CIE, M(-)CIE, and Cand-Tec antigen detection assays in the immunodeficient patient group are depicted in Table 3. The *Candida* enolase ELISA was the only antibody detection assay that was able to discriminate between invasive candidiasis and colonization. By multivariable analysis, it was found that the antigen detection assay Cand-Tec was somewhat better at discriminating invasive candidiasis and colonization. Combining the results of Cand-Tec with those of *Candida* enolase ELISA did not improve the discriminative capacity relative to that of the Cand-Tec alone.

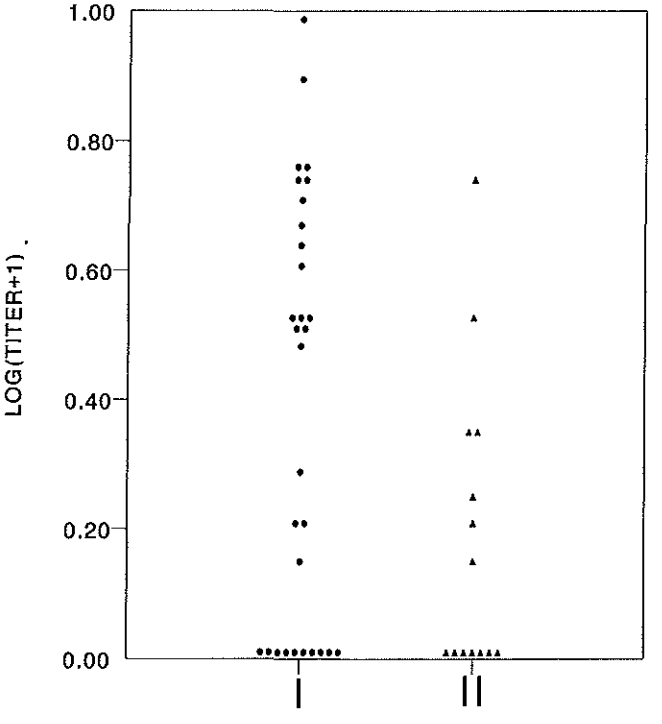

Figure 6. *Distribution of the mean logarithm(titer + 1) of antibody directed toward the Candida enolase in the sera of immunodeficient patients with invasive candidiasis (I) or colonization (II).*

Table 3. Sensitivities and specificities of the different tests determined at a chosen cutoff value by using the mean logarithm(titer+1) values obtained for groups I and II of the immunodeficient patient group.

Serological assay	Cutoff value	Sensitivity (%)	Specificity (%)	*P* value[a]
M(+)CIE	≥2	69	39	0.79
M(-)CIE	≥1	31	83	0.35
Enolase ELISA	≥1	53	78	0.046
Enolase ELISA	≥1	53[b]	95[b]	2.0×10^{-8}
Cand-Tec	≥4	54	78	0.026

[a] *P* values were determined by the Fisher exact test by comparing percentages of patients in groups IB and IIB whose sera had titers above the cutoff levels.
[b] Sera from healthy individuals were used to calculate the sensitivity, the specificity, and the *P* value.

DISCUSSION

It is known, from the literature and from our own investigations that antibody responses against the 47 kDa antigen are frequently found in sera of patients with invasive candidiasis (17, 19). In order to develop a quantitative assay, we purified the immunodominant antigen and coated it onto ELISA plates in an attempt to try and detect specific antibodies directed against this antigen. Furthermore, the purified antigen was characterized by different techniques to solve the enigma as to whether we were dealing with the 47 kDa immunodominant antigen or an enolase antigen or whether both characteristics were gathered into one and the same antigen.

From the results that we observed, we must conclude that the molecular mass of the immunodominant antigen is 45 kDa and that our purified immunodominant antigen also has enolase activity. These findings have also been reported by others (7, 12, 16, 22). In accordance with the results found by Mason et al. (16), we also conclude from the results of the immunoblot analysis that antigenic cross-reactivity exists between our 45 kDa antigen and a commercial baker's yeast enolase. Recently, Kortekangas-Solvainen et al. (14) also demonstrated by Western blotting the cross-reactivity of IgE antibodies directed toward a 48 kDa band of *Saccharomyces cerevisiae*, a 46 kDa band of C. *albicans*, and the purified baker's yeast enolase. However, from our tandem XIE results we concluded that these cross-reacting antibodies are directed against different epitopes,

which was also reported by Franklyn et al. (7).

The *Candida* enolase antibody ELISA was reproducible, as measured by day to day variation tests, and was specific for antibodies directed against *Candida* enolase antigen, as determined by inhibition experiments (data not shown). The *Candida* enolase antibody ELISA had a diagnostic value at a cutoff point of ≥ 2 in the immunocompetent patient group with an acceptable specificity (86%) but a low sensitivity (50%). Multiple serum samples were necessary to maximize detection, as observed by the fact that sensitivity increased slightly, from 47 to 50%, when more than one serum sample was used to determine diagnostic accuracy of the test, whereas the specificity was maintained (86%). In this immunocompetent patient group, an increase in specificity was observed when compared with the data obtained by the M(+)CIE and, to a lesser extent, the M(-)CIE. Despite the use of a more sensitive detection assay, i.e., ELISA as opposed to the CIE, the increase in specificity of the enolase test was counteracted by a loss in sensitivity, i.e., a decrease from 80 to 50%, compared to the M(+)CIE.

For the immunodeficient group, the *Candida* enolase ELISA showed significantly higher titers in the sera of patients with invasive candidiasis than in the sera of patients colonized by *C. albicans*. However, the sensitivity of the assay for sera from the immunodeficient patient group was also low (53%), with a specificity of 78%. In the immunodeficient patient group, patients with leukemia did not respond at all with antibodies directed toward the *Candida* enolase antigen. Consequently, when all leukemic patients in both subgroups IA and IIA were excluded from the immunodeficient patient group for analysis, the assay's sensitivity (64%) as well as specificity (90%) increased ($P = 0.0036$; Fisher exact test).

Greenfield et al. (9) also reported a low sensitivity (25%) and a high specificity of (97.8%) for their assay, which was based on a purified 54 kDa major cytoplasmic antigen. On the other hand, Zöller et al. (23) reported a sensitivity of 81.5% and a specificity of 96.4% for their ELISA with 47-29 kDa antigens of *C. albicans*. The difference between their data and our own is probably due to different criteria used for patients with invasive candidiasis as well as the fact that patients with immunodeficiencies and immunocompetent patients were regarded as a homogeneous group. The use of different cutoff values, which is necessary for the correct evaluation of the titers obtained for immunocompetent patients and immunodeficient patients, also explains the different interpretation of the results obtained for these groups.

The Cand-Tec assay had a good discriminating capacity for sera from the immunodeficient patient group. However, the sensitivity and specificity of the Cand-Tec assay were not as favourable as those determined by other investigators (4, 8); the sensitivity of the test for sera from both patient groups (IA and IB) was low, while the specificity was acceptable. Discrepancies may be due to different selection criteria used for determining invasive candidiasis. The test is probably more useful during the early stage of disease, since additional sera from each patient, which were obtained later in the

course of invasive *Candida* infection, did not improve diagnostic sensitivity of the test. This was also found by Herent et al. (10).

Previously reports of antibody detection assays with crude antigens for the detection of deep-seated candidiasis always showed that the tests suffered from suboptimal sensitivities or specificities (11). The lack of specificity is due to the fact that most healthy individuals already have antibodies directed against cell wall components of *C. albicans* (6). Therefore, it is conceivable that the use of a purified, well-defined antigen rather than a crude extract containing cell wall polysaccharide provides a more specific test for systemic candidiasis. Since it has already been demonstrated that the enolase antigen is released during the progression of infection and that it elicits a specific antibody response (17), it would be conceivable that this particular antigen could be applied in an antibody detection assay. In the present study, we have demonstrated that the detection of anti-enolase antibodies results in a discriminative test with a low sensitivity but an acceptable specificity. Because of its good specificity, results of this particular test, in contrast to previously described assays, supports the clinician in making the decision whether to start with toxic antifungal agents. Whether anti-enolase antibodies can be used in monitoring the response to treatment and in determining the duration of therapy must be investigated.

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CHAPTER 4

Kinetics of anti-mannan antibodies in immunocompromised patients with invasive candidiasis.

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ABSTRACT

In studying the anti-mannan antibodies longitudinally in serial serum samples of three immunocompromised patients, it was observed that anti-mannan antibodies started to increase shortly after the moment that cultures of deep-tissue sites became positive with *C. albicans*.

The mean anti-mannan antibody titers determined in a group of 36 immunocompromised patients with invasive candidiasis increased within two weeks after the probable onset of invasive candidiasis. In contrast, anti-mannan antibody levels in serial serum samples of 14 immunocompromised patients who were only colonized with *C. albicans* remained stable or decreased in time. The HA test measuring the anti-mannan antibodies was 64% sensitive and 89% specific in determining invasive candidiasis. In contrast, antibodies specific for candidal cytoplasmic antigens or enolase alone were of little value in confirming invasive candidiasis in these immunocompromised patients.

INTRODUCTION

The yeast *C. albicans* is a commensal in the human digestive tract and rarely causes invasive disease in the normal individual. However, when the bacterial flora is changed or the host immune system weakened, *C. albicans* can overgrow and cause infection (11). It is currently difficult, if not impossible, to define a point in time at which a mass of yeasts makes the transition from commensalism to a pathogenic state (1). Since biopsies cannot be performed routinely, and the sensitivity of blood cultures is limited (2), it would be helpful to have reliable serological assays that support or refute the clinical diagnosis of invasive candidiasis.

Unfortunately, in immunocompromised patients results of serological tests, especially antibody detection tests, are often difficult to interpret due to the suppression or even complete absence of an immune response (19, 20). Furthermore, serology based on a single serum sample is biased by the time of sampling during a given stage of the *Candida* infection, thus giving rise to false-negative results. This particular shortcoming of serology can be overcome by serial sampling, in which the increase or fall of an antibody titer is a more reliable reflection of the correct diagnosis (8, 10, 13). In this manner we evaluated the levels of several antibodies and antigens in relation to the pathogenesis of candidiasis in immunocompromised patients.

MATERIALS AND METHODS.

Haemagglutination assay. Antibodies directed against cell wall mannan were determined by haemagglutination (HA) (15). The mannan containing antigen was prepared from *C. albicans* CBS 5982 by means of natamycin degradation as described earlier (7), and coupled to sheep erythrocytes with concanavalin A.

Counterimmunoelectrophoresis. Antibodies directed against a mixture of cytoplasmic antigens with and without mannan were measured by counterimmunoelectrophoresis (CIE) as described previously (22). These assays will be referred to as M(+)CIE and M(-)CIE, respectively. Depletion of the cytoplasmic antigens from mannan and glycoproteins was accomplished by use of a concanavalin A-Sepharose column as described by Ellsworth et al. (4).

Antigen detection by Cand-Tec. For antigen detection the Cand-Tec test (Ramco Laboratories Inc., Houston, Tex.) was used as described by the manufacturer.

Patient sera. Patient sera were collected between October 1982 and January 1992. All results, including underlying diseases, therapy as well as of serological data were collected retrospectively. Only patients infected by the species *Candida albicans* were included in the present study. All patients included in this study had evidence of immunodeficiencies caused by therapy or underlying diseases. The criteria for immunodeficiency were histologically confirmed neoplasms, kidney function disorders requiring chronic dialysis, treatment with corticosteroids, total-body irradiation, or the use of immunosuppressive or cytotoxic drugs. Patients were divided into two groups on the basis of clinical findings and culture results. Group I consisted of 36 patients with clinically confirmed invasive candidiasis. The criteria for this diagnosis were based

on obtaining positive *C. albicans* cultures from tissue biopsy specimens or needle aspirates of normally sterile body cavities. Biopsies from such sites taken at autopsy which grew *C. albicans* were likewise taken as evidence for invasive candidiasis. If only one or more positive blood cultures were obtained these were not regarded sufficient to classify a patient as having invasive candidiasis and these patients were not included in this study. Group I included 9 leucopenic patients receiving either chemotherapy, corticosteroids, immunosuppressive drugs or a combination therapy; 4 patients with kidney function disorders requiring dialysis combined with therapy with corticosteroids; 8 patients with histologically confirmed neoplasms; 2 patients with HIV; 2 patients with cancer receiving corticosteroids; 4 patients after organtransplants receiving corticosteroids and immunosuppressive drugs, and 7 patients on corticosteroids or receiving immunosuppressive drugs after surgical interventions.

Group II consisted of 14 patients colonized by *C. albicans*. Colonization was deemed to exist if more than 2 cultures of samples from different parts of the gastrointestinal tract were positive for *C. albicans* in the absence of visible lesions. As invasive candidiasis might have been present subclinically in the group of colonized patients, patients were excluded from this study if they were treated with antifungal agents or if their fever did not react to antibacterial agents within three days. This group included 8 leucopenic patients receiving either chemotherapy, corticosteroids, immunosuppressive drugs or a combination therapy, one patient with kidney function disorders requiring dialysis combined with corticosteroids, two patients with cancer receiving corticosteroids, two patients with histologically confirmed neoplasms, and one patient underwent livertransplantation receiving immunosuppressive agents and corticosteroids.

Serial serum samples taken at weekly intervals from each patient, were included for serological evaluation. The first serum sample was defined as the sample obtained at the time of the first microbiological evidence of invasive candidiasis or *Candida albicans* colonization. The HA, M(+)CIE, and the Cand-Tec assay were performed on freshly obtained serum samples, and serum samples were kept frozen at -70°C thereafter. Since it was not always possible to collect three multiple serial sera from each patient, group I included 23 patients with three sera, and 13 with two sera. Group II included six patients with three sera, and eight with two sera.

To determine sensitivity and specificity of the serological assays the mean of the logarithmically transformed titer values of the sera of each patient were used. Titer values from the sera of each patient were first logarithmically transformed, logarithm(titer + 1), before the mean logarithm titers of the sera was calculated for each patient.

Statistical analysis. Correlation between the three or two serum samples obtained from each patient were assessed by using intraclass correlation coeffients (r_i). The capacity to discriminate between invasive candidiasis and *C. albicans* colonization was evaluated by the Mann-Whitney test. The percentage of patients in groups I and II with titers above the cutoff levels were compared by Fisher's exact test. A significance level of $P=0.05$ (two-sided) was used.

RESULTS

Antibody response and antigen titers in three immunocompromised patients. To study titer kinetics of anti-mannan and anti-cytoplasmic *C. albicans* antibodies and antigen levels three immunocompromised patients with invasive candidiasis were monitored over a period of 11 weeks.

Patient 1. An 18-year-old man was readmitted to the hospital for residual myeloid leukemia. On hospital day two, serology revealed an antibody titer of 1:2 in the M(+)CIE and an anti-mannan antibody titer of 1:64 as determined by HA (Fig. 1). The antigen assay initially gave a titer of 1:8 and a negative result was obtained in the M(-)CIE (data not shown). The patient developed fever on hospital day 33 and blood cultures were taken that grew *C. albicans*. Treatment with amphotericin B and flucytosine was started. Flucytosine treatment was stopped after 10 days because of hepatotoxicity. An X-ray of the thorax taken on day 39 showed infiltrations that were compatible with the diagnosis of pulmonary candidiasis. During the period of treatment anti-mannan antibodies further increased and after three weeks stabilized at a titer of 1:8 as determined by M(+)CIE. Anti-mannan antibody titers detected by HA had increased to 1:4096. However, anti-cytoplasmic antibody, [M(-)CIE], titers remained undetectable, and Cand-Tec antigen titers were low. After a total accumulated dose of 1 gram of amphotericin B the treatment was stopped. On hospital day 70 *C. albicans* was cultured from a broncho alveolar lavage specimen and treatment with itraconazole was started. At day 73 blood cultures again grew *C. albicans* and itraconazole was switched to amphotericin B. On day 78 the patient died but no autopsy was performed.

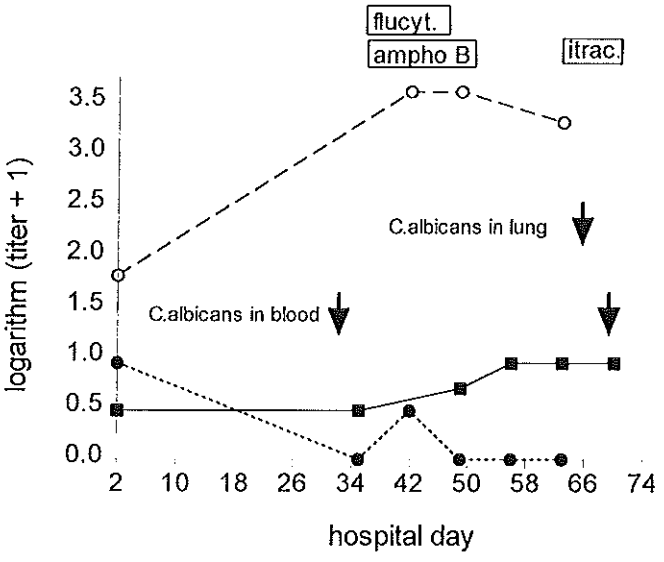

Figure 1. *C. albicans antibody and antigen titers determined by HA, M(+)CIE, and Cand-Tec in sera of immunocompromised patient 1 during his hospital stay, are transformed to logarithm(titer + 1) values. Boxes indicate the time the patient received flucytosine (flucyt.), amphotericin B (ampho B), and itraconazole (itrac.). C. albicans was cultured twice from blood (days 33 and 73) and once from broncho-alveolar lavage (lung) (day 70) as indicated by arrows. (HA = -○-, M(+)CIE = -■-, Cand-Tec = -●-).*

Patient 2. A 36-year-old woman with acute myeloid leukemia was hospitalized for chemotherapy. During chemotherapy she received oral amphotericin B prophylaxis. Despite the prophylaxis cultures from throat, feces, urine, and vagina were positive for *C. albicans*. Antibodies as determined by M(+)CIE, were 1:1 (Fig. 2). On day 72, antibodies directed towards mannan increased to 1:2 which was accompanied by an increase in antigen titers. The mannan-antibody titers as determined by HA were low (1:2) or could not be detected during the rest of the hospitalization period. On day 70 blood cultures became positive with *C. albicans* as well as cultures from feces and urine. Treatment with amphotericin B was started but nine days later patient died. Cultures taken at autopsy revealed *C. albicans* in spleen, liver, and gastrointestinal tissue.

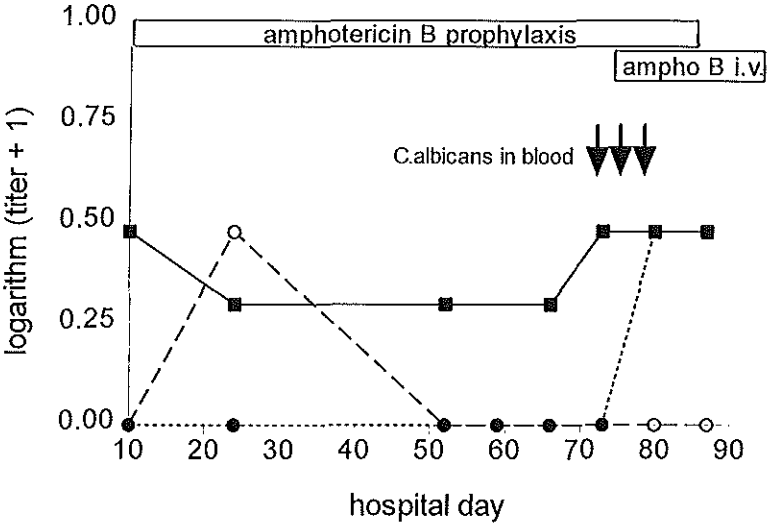

Figure 2. *C. albicans antibody and antigen titers determined by HA, M(+)CIE, and Cand-Tec in sera of immunocompromised patient 2 during her hospital stay, are transformed to logarithm(titer + 1) values. Boxes indicate the time the patient received amphotericin B profylaxis and amphotericin B i.v. treatment. C. albicans was cultured several times from blood (day 70) as indicated by arrows, and from throat, feces, urine, and vagina during the whole hospital stay. (HA = -o-, M(+)CIE = -■-, Cand-Tec = -●-)*

Patient 3. A 24-year-old woman with systemic lupus erythematosus was hospitalized because of fever, nephrotic syndrome and anemia. She did not respond to

the antimicrobial agents administered emperically, and on day four she developed respiratory insufficiency which was due to infection with *Legionella pneumophila*. Erythromycin treatment was started. Initially, the patient seems to improve after erythromycin therapy. However, on day 8 *Candida* oesophagitis was visualized by gastroscopy and treatment with ketoconazole was started. Anti-mannan-antibodies, detected by HA, increased from 1:4 at day 0 to 1:512 at day 11 (Fig. 3). Antibody titers in the M(+)CIE also increased from undetectable to 1:4 in the same period and fluctuated thereafter. The Cand-Tec antigen titers were low, but fluctuated apparently contrary to the M(+)CIE antibody titers. *Candida* was still present in the throat, feces, and vagina on day 70. Eventually, she died on hospital day 77. At autopsy *C. albicans* was cultured from the lungs and the duodenum.

Thus, the kinetics of anti-mannan antibody response as determined by HA and M(+) CIE seemed to be associated with the onset of invasive candidiasis. Therefore we decided to study these responses in a larger population of immunocompromised patients.

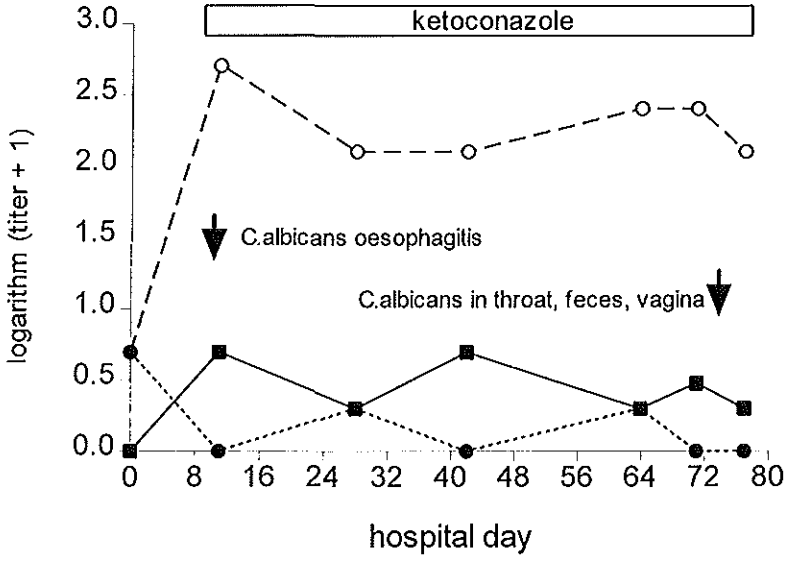

Figure 3. *C. albicans antibody and antigen titers determined by HA, M(+)CIE, and Cand-Tec in sera of immunocompromised patient 3 during her hospital stay, are transformed to logarithm(titer + 1) values. Boxes indicate the time the patient received ketoconazole. C. albicans oesophagitis was established at day 8 indicated by the arrow and high numbers of C. albicans from throat, feces and vagina at day 70. (HA = -o-, M(+)CIE = -■-, Cand-Tec = -●-).*

Kinetics of mannan-antibody response in immunocompromised patients. In the group of patients with invasive candidiasis (I) the mean titer of anti-mannan antibodies, as determined by HA, increased from the moment that cultures became positive (serum 1, mean of 1.04) up to 14 days thereafter (serum 3, mean of 1.48) (Fig. 4). Kinetics of the anti-mannan antibody response expressed as a change from a negative titer to a positive titer or a doubling of a positive titer result, occurred in 14/36 (38.9%) of the paired sera. However, this increase in titers determined by HA was less than twofold and did not reach statistical significance ($r_i = 0.73$). In contrast, the level of mannan-antibody in the sera of colonized patients (group II) was lower and did decrease rather than increase over time (Fig. 4).

The anti-mannan antibody titers of the first serum samples, were already significantly higher from patients with invasive candidiasis than those colonized with *C. albicans* ($P < 0.01$; Mann-Whitney test). The anti-mannan antibody titers of the sera one and two weeks after the probable onset of the invasive infection were also significantly higher in patients with invasive candidiasis ($P < 0.01$; Mann-Whitney test).

Statistical analysis of all patients showed that the mean logarithm(titer + 1) of HA antibody levels are significantly higher in patients with invasive candidiasis than in those colonized with *C. albicans* irrespective of a cutoff value ($P = 0.01$; Mann-Withney test). Using a cutoff value of ≥ 8 the HA test had a sensitivity of 64% and a specificity of 89% ($P = 0.004$; Fisher exact test).

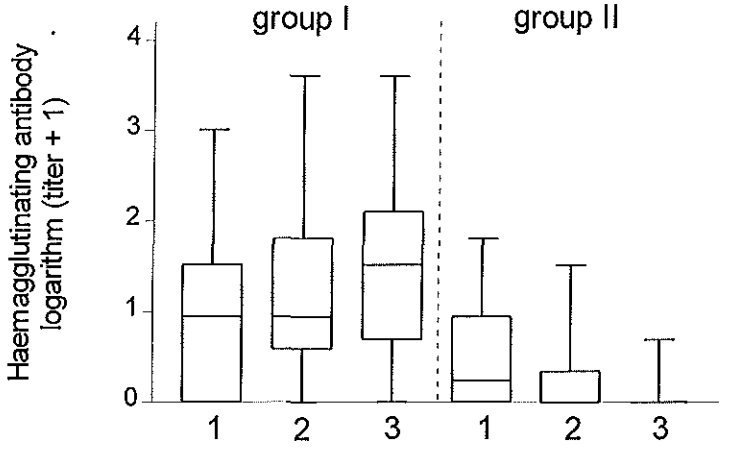

Figure 4. *Box plots of anti-mannan antibody titers in immunocompromised patients with invasive candidiasis (group I, n = 64) and in immunocompromised patients only colonized with C. albicans (group II, n = 18). The boxes represent the 25th percentile to the 75th percentile of anti-mannan antibody titers as determined by HA in serial sera (1, 2, and 3) taken at weekly intervals with a horizontal line at the median (50th percentile). Whiskers indicate the total range of the titer values observed.*

Kinetics of antibody response in immunocompromised patients determined by CIE. The M(+)CIE detects precipitating antibodies directed against a crude mixture of *C. albicans* antigens including mannan. The mean M(+)CIE antibody titer in group I patients tended to increase but this increase was not statistically significant ($r_i = 0.76$) (Fig. 5). Again, in 14 of the 36 paired sera (38.9%), a change from a negative titer to a positive titer or a doubling of a positive titer in the M(+)CIE occurred. Only a few patients in the colonized patient group II had precipitating antibodies and they did not increase in titer over a period of two weeks (Fig. 5).The M(+)CIE antibody levels were significantly higher in the first serum samples from patients with invasive candidiasis than in those colonized with *C. albicans* ($P < 0.01$; Mann-Whitney test). The M(+)CIE antibody titers in serum samples 2 and 3 are significantly higher in patients with invasive candidiasis. Taken the mean logarithm(titer + 1) of the two or three sera per patient and using a cutoff value of ≥ 2 a sensitivity and specificity of 58 and 79% was obtained ($P = 0.03$; Fisher exact test).

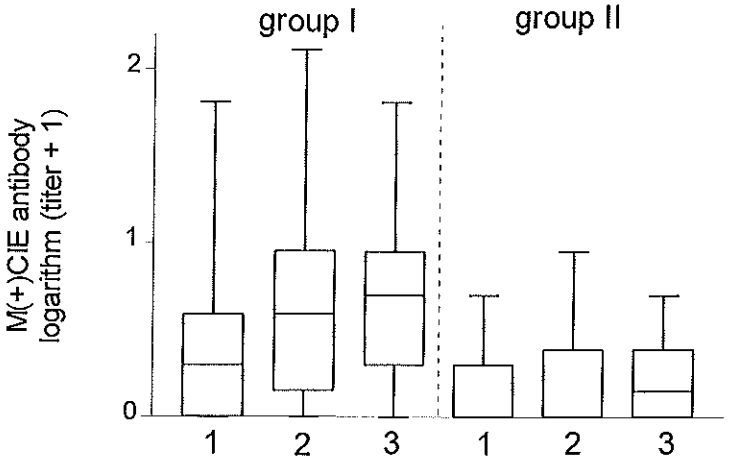

Figure 5. *Box plots of anti-mannan antibody titers in immunocompromised patients with invasive candidiasis (group I, n = 64) and in immunocompromised patients only colonized with C. albicans (group II, n = 18). The boxes represent the 25th percentile to the 75th percentile of anti-mannan antibody titers as determined by M(+)CIE in serial sera (1, 2, and 3) taken at weekly intervals with a horizontal line at the median (50th percentile). Whiskers indicate the total range of the titer values observed.*

To evaluate the contribution of antibodies directed against cytoplasmic antigens as opposed to cell wall mannan the crude *C. albicans* extract was depleted of mannan and glycoproteins. Since antibody detection by M(-)CIE was performed on serum samples that had been kept frozen at -70°C, we also repeated the M(+)CIE on the frozen samples to rule out discrepant results due to degradation of antibodies or antibody-antigen complexes during storage. In general the titers obtained in the M(+)CIE performed on frozen serum samples were somewhat higher than those in the M(+)CIE performed prior to freezing (data not shown). Removal of the mannan moiety from the crude *C. albicans* antigen mixture resulted in low anti-cytoplasmic antibody titers (mean of 1:1) for the patients with invasive candidiasis and less than 1:1 for the colonized patient group (data not shown).

Kinetics of antigen titers in immunocompromised patients determined by Cand-Tec. Our results with the Cand-Tec antigen test showed that antigen titers remained constant during the two week observation period in sera of patients with invasive candidiasis (data not shown). Only in 6 of the 36 paired sera (16.6%), a change from a negative titer to a positive titer or a doubling of a positive titer occurred. For colonized patients the antigen titers were significantly lower and in general decreased with time. The mean (logarithm(titer + 1)) of antigen levels, were significantly higher in patients with invasive candidiasis than in patients colonized with *C. albicans* ($P<0.01$; Mann-Whitney test), and resulted at a cutoff value of ≥ 2 in an assay sensitivity and specificity of 69 and 64%, respectively ($P=0.05$; Fisher exact test).

DISCUSSION

In the present study we gained insight into which assay is most valuable in the confirmation of invasive candidiasis by monitoring the kinetics of the antibody response and of antigen titers.

It is remarkable that in two of the three immunocompromised patients studied longitudinally over 11 weeks, antigen titers were low despite the fact that all three were clearly suffering from invasive candidiasis. These findings indicate that the antigen is not sufficiently detected by the Cand-Tec assay, possibly because free antigen is not released into the circulation; this may be due to insufficient processing of *Candida* cells due to the impaired cellular response of these patients, as suggested by Mohart et al. (16). Therefore, antigen detection systems may not automatically be the serological test of choice in immunocompromised patients as suggested by others (12, 21). In contrast, anti-mannan antibody was detected by M(+)CIE and HA, and started to increase shortly after the moment that cultures of deep-tissue sites became positive with *C. albicans*. Although antibodies directed against mannan are known to be universally present in normal human sera, most likely as a consequence of mucocutaneous colonization (3, 5, 14), changes in

the titers of anti-mannan antibody were positively correlated with the occurrence of invasive candidiasis in immunocompromised patients. Greenfield et al. (6), also demonstrated the utility of mannan-serology for candidiasis in patients treated for acute leukemia. The diagnostic use, however, of anti-*Candida* antibodies in immunocompromised patients remains a controversial issue (9, 18, 19, 24). Immunoblot analysis has been extensively used to identify specific immunogenic components of *C. albicans* that correlated well with invasive candidiasis. Despite the fact that specific immunogenic components provided greater specificity for invasive candidiasis than mannan antigens did, the sensitivity of these assays for invasive disease decreased for patients profoundly immunocompromised (19). On the other hand, titers for antibodies to *C. albicans* germtubes or blastospores, which are anti-mannan antibodies predominantly, were detectable in sera of immunocompromised patients with invasive candidiasis, and might be used diagnostically (18, 24). In these studies only the height of the titer of antibody was influenced by the patient's immunological status. In our immunocompromised patient group, an increase in anti-mannan antibody titers was observed in the first two weeks after the first clinical signs of invasive candidiasis had appeared. In contrast, anti-mannan antibody titers in sera of immunocompromised patients that were only colonized with *C. albicans* were significantly lower and tended to decrease. The fact that anti-mannan antibody levels increase so quickly in patients with invasive disease is compatible with a secondary immune response to an antigen against which antibodies are already present in normal human sera (15). The follow-up of anti-mannan antibody titers for detection of *Candida* infection was recently confirmed in a study of children with malignant disease and invasive candidiasis (17). However, anti-mannan antibodies in this particular study appeared slowly since the childrens antibody response is a primary immunoresponse and not a secondary one, as is the case with adult patients.

The moderate sensitivity of the HA assay might be caused by two phenomena, in the first place anti-mannan antibodies might not be detected due to a decrease in titer as a result of anti-mannan antibodies interacting with mannan or with whole *C. albicans* cells released during invasive infection or secondly by a too early or too late blood sampling during the infectious process. This however can partly be overcome by serial sampling as shown in the present study.

The antibody response toward cytoplasmic antigens of *C. albicans* in our patients was poor. This was not the result of the use of frozen serum samples since the anti-mannan antibody titers as determined by M(+)CIE were about the same in frozen serum samples compared to titer values in the same sera prior to freezing. The immune response toward *C. albicans* cytoplasmic components is poor, probably because it constitutes a primary immune response (8). In immunocompetent surgical patients, however, we previously demonstrated that the M(-)CIE assay discriminated well between those with invasive candidiasis and those with colonization alone (23). Thus, different

serological assays may be needed for the diagnosis of invasive candidiasis in patients with different types of underlying diseases.

The antigen titers determined by the Cand-Tec assay remained stable over time in immunocompromised patients with proven invasive candidiasis, while in patients that were only colonized antigen levels decreased in time. Although, antigen titers were generally low in these patients, their persistence during the course of hospitalization could further support the diagnosis of invasive infection.

We conclude, that the anti-mannan antibody assays are of clinical value in immunocompromised patients. They may help to confirm the clinical diagnosis of an invasive *Candida* infection. Documenting increases in antibody titer during the progression of infection is recommended since it avoids the limitations of interpreting the test results on a single serum sample. Measuring changes in the level of anti-mannan antibodies seems to be more sensitive than the detection of cytoplasmic antigens or antibodies directed against cytoplasmic antigens as indicator for invasive candidiasis in immunocompromised patients.

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CHAPTER 5

Improved detection of *Candida albicans* by PCR in blood of Neutropenic mice with systemic candidiasis

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ABSTRACT

A PCR using primers aimed at the multicopy gene coding for the small subunit rRNA and resulting in the synthesis of a 180-bp fragment was evaluated for its use in diagnosing invasive candidiasis in comparison with blood culture. With the use of a *C. albicans*-specific probe, ± 10 to 15 *C. albicans* cells are detected in 100 µl of whole blood by Southern analysis. A DNase pretreatment was critical in the purification process of yeast DNA from whole blood. Omission of the DNase pretreatment decreased assay sensitivity 10-fold. PCR analysis of blood-specimens collected from mice with invasive candidiasis is more sensitive than blood culture (100 versus 67 %, respectively) at 72 h after intravenous (i.v.) inoculation with *C. albicans*. Furthermore, the intensity of the hybridization signals increased with the progression of infection. In contrast, multiple blood samples from gastrointestinally colonized mice were all negative by PCR, indicating that the PCR assay is also specific and may, therefore, have a positive contribution to the detection and follow-up of invasive candidiasis.

INTRODUCTION

The opportunistic pathogen *Candida albicans* is able to cause disseminated infections in immunocompromised patients. To reduce mortality in patients with invasive candidiasis, early initiation of antifungal drug therapy is critical (1). The clinical diagnosis is complicated because *C. albicans* commonly colonizes or causes mild infection of the mucous membranes or skin. It is, therefore, often difficult to judge the clinical significance of the finding of *C. albicans* in secretions or on the body surfaces when systemic infection is suspected in case of occult fever or for other reasons. Microbiological evidence for the diagnosis of invasive candidiasis requires the demonstration of *C. albicans* in sterile body fluids, biopsies or in multiple consecutive blood cultures (7). There are, however, many problems inherent in these tests (12). Berenguer et al. (2), for instance, recently demonstrated that even by application of the lysis-centrifugation technique, a sensitivity of only 43% was established in autopsy-proven cases of invasive candidiasis. They also demonstrated that the sensitivity of the lysis-centrifugation blood culture technique correlated with the number of infected tissue sites, and therefore, these investigators stressed the need for more sensitive methods for detection of invasive candidiasis.

Since PCR has proven to be a powerful tool in the early diagnosis of several infectious diseases, it might also be a more sensitive alternative assay in the diagnosis of invasive candidiasis. Several PCR methods for the detection of *Candida* spp. in patient materials have been published (3, 6, 8, 10). However, the diagnostic usefulness of PCR remains to be established because of the reported limitations of the technique when whole blood is used (6, 8), the lack of verification with a species-specific probe for the amplified product (3) and the inability to detect medically important *Candida* spp. other than *C. albicans* (11).

The application of PCR in the early diagnosis of systemic candidiasis has to be ascertained in comparison with that of the blood culture technique, and furthermore, PCR results must be negative in patients who are merely colonized with *C. albicans*. In the present study the diagnostic usefulness of PCR was studied by detecting *C. albicans* in whole blood of systemically infected mice and in gastrointestinally colonized mice in order to determine the sensitivity and accuracy of the PCR assay for discriminating between colonization and invasive candidiasis.

MATERIALS AND METHODS

Animals and *Candida* strain. Specific-pathogen-free, 11- to 13 weeks-old female BALB/c mice weighing ± 22 g were used (Iffa Credo, L'Arbresle, France). *C. albicans* (ATCC 44858) was used throughout all the experiments. The yeast was stored at -80°C in Todd-Hewitt broth (Difco

Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol. Stationary-growth-phase cultures were obtained after incubation of the yeast in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. Yeasts were washed in phosphate-buffered saline (PBS), counted in a hemocytometer, and adjusted to 5 x 10^4 cells per ml in PBS.

Systemic infection in neutropenic mice. Neutropenia was induced by intraperitoneal administration of cyclophosphamide (Sigma) at 100 mg/kg of body weight 4 days prior to inoculation with *C. albicans*, followed by additional doses of 75 mg/kg on the day of inoculation and at 3-day intervals during the course of infection (14). This treatment resulted in persistent granulocytopenia of < 100 granulocytes per μl of blood from the time of inoculation with *C. albicans* up to termination of the studies. In each experiment 21 neutropenic mice were infected by inoculation of 200 μl of 5 x 10^4 CFU of *C. albicans* per ml in the tail vein.

At 1, 6, 24, 48, 72, 96, and 144 h after inoculation with *C. albicans*, the mice were sacrificed, using three mice for each time point. At the above-indicated time intervals, blood was collected in EDTA-coated tubes by cardiac puncture. Of each blood sample, 100 μl was directly cultured on Sabouraud dextrose agar (SDA) plates (Oxoïd, Basingstoke, England) and incubated for 5 days at 37°C. Another 100 μl of blood was used for DNA extraction.

The kidneys, spleen, liver, and lungs were removed from each mouse and separately homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (VirTis Co. Inc., Gardiner, N.Y.). Two hundred microliters of the serial twofold dilutions of each homogenate was plated on SDA plates. The remainder of the homogenate, together with an equal volume of double-strength SDA, was mixed and poured into petri dishes. All plates were incubated for 48 h at 37°C.

Gastrointestinal colonization in immunocompetent mice. Gastrointestinal colonization was established by pretreating mice for five days with cefradine (1 g/liter, Velosef 250; Bristol Myers-Squibb, Woerden, The Netherlands). Cefradine was added to their drinking water throughout the experiment. Inoculation with 1.10^7 CFU of *C. albicans* was performed by gastric intubation. Feces were collected and cultured, at 2-day intervals. The mice were sacrificed 8 days after inoculation with *C. albicans*. Blood was sampled by cardiac puncture and collected into EDTA-coated tubes. One hundred microliters of blood was directly subcultured on SDA plates, and 100 μl of whole blood was used for DNA extraction. Kidneys, lungs, liver, and spleen as well as stomach and gastrointestinal tract were removed, homogenized, and cultured as described above.

PCR sample preparation. For *Candida* DNA extraction from blood we used a method modified from Buchman et al. (3). Briefly, 0.1 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100) was added to 0.1 ml of whole blood. After lysis, cell debris and *Candida* yeast cells were pelleted by centrifugation (Eppendorf; Merck, Amsterdam, The Netherlands) at 16,000 x *g* for 5 min. The pellet was resuspended in 0.2 ml of lysis buffer. To remove free, non-yeast cell DNA, 7 μl of DNase (10 mg/ml, Boehringer GmbH, Mannheim, Germany) was added and the samples were incubated at 37°C for 1 h. After centrifugation at 16,000 x *g* for 5 min, pellets were resuspended in 0.2 ml of TEG buffer (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing 1.5 μl Lyticase (900 U/ml; Sigma Chemical Co., St. Louise. Mo.) and incubated for a further 1 h at 37°C. Subsequently, 3.0 μl of pronase (15 mg/ml; Boehringer GmbH) and 10 μl of 10% sodium dodecyl sulfate (SDS) were added and further incubated for 1 h at 37°C. DNA extraction and purification was realized by using Prep-A-gene DNA Matrix according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). The matrix was eluted with 40 μl of TE buffer (10 mM Tris-HCl [pH 8.0], and 1 mM EDTA).

Filter tips (Biozym, Landgraaf, The Netherlands) were used throughout the whole isolation procedure to avoid contamination.

Primers and PCR protocol. PCR was performed on 10 μl of the DNA samples, which corresponded to 25 μl of the original blood sample. The following primer set, generating a 180-bp fragment spanning the V4 region of the small subunit rRNA gene, was used: 532 (5'-TATTAAAGTTGTTGCAG-3') and 651 (5'-CCTGCTTTGAACACTCTAATTT-3') (13). PCR was performed in 100 μl of PCR solution containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM of (each) dNTP, 50 pmol of each primer, 0.1 mg of gelatin per ml, 1 U of *Taq* DNA polymerase (Promega, Leiden, the Netherlands) and 10 μl sample specimen. The reaction mixture was overlaid with 100 μl of mineral oil (Sigma) to prevent evaporation and was preincubated for 5 min at 94°C for DNA denaturation. Forty cycles of amplification were performed with a PCR processor (Biomed Gmbh, Theres, Germany). Each cycle consisted of a denaturation step at 94°C for 1 min, a primer-annealing step at 52°C for 1.5 min and a chain-elongation step at 74°C for 1 min. After 40 cycles, a temperature-delay step of 5 min at 74 °C completed elongation. Thirty microliters of the amplified PCR product was analyzed by agarose (1.5% wt/vol) gel electrophoresis.

Southern blot analysis of PCR products. DNA was transferred from agarose to Hybond Plus nylon filters (Amersham International, plc, Amersham, United Kingdom) by overnight diffusion blotting in 0.4 N NaOH. The PCR products were analyzed with the *C. albicans*-specific probe (5'-GTAGCCATTTATGGCGAACC-3') (13). The membranes were preincubated at 37°C in a hybridization solution (5x SSC [1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1x Denhardt's solution) for 1 h. Hybridization occurred overnight at 37°C with 20 pmol of T4 polynucleotide kinase (Life Technologies, Ltd., Renfrewshire, Scotland) [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham)-labelled oligonucleotide probe. The filters were washed once for 15 min at 37°C and once for 15 min at 55°C with 2 x SSC, 0.1% (wt/vol) SDS. Autoradiography was performed with Kodak Royal X-omat film and a 3.5-h exposure time.

RESULTS.

PCR sensitivity. In order to develop a test with a better sensitivity than blood culture, special effort was put into determining the method of DNA extraction and into creating optimal storage conditions for the blood samples. As a guideline for the DNA extraction, the method described by Buchman et al. (3) was used. An essential step in the DNA extraction procedure proved to be the digestion of non-yeast DNA by DNase pretreatment. Figure 1 shows the results of making a 10-fold dilution series of *C. albicans* in whole sheep blood and performing the DNA extraction with and without DNase pretreatment. By application of DNase pretreatment we were able to detect 10 to 15 cells of *C. albicans* per 100 μl of whole blood. Leaving out the DNase treatment, only 150 to 200 cells of *C. albicans* per 100 μl could be detected, thus reducing the sensitivity of the PCR assay at least 10-fold.

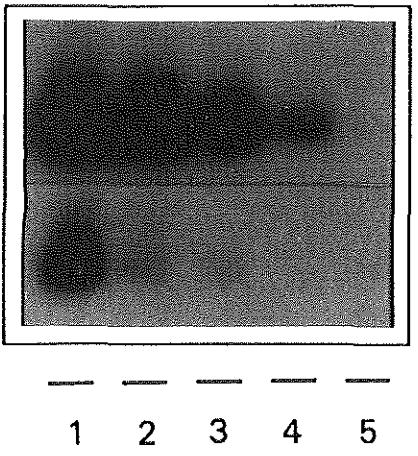

Figure 1. *Southern blot analysis of C. albicans PCR products in whole sheep blood with (A) and without (B) DNase pretreatment. Lanes: 1 through 5, C. albicans cells spiked at 2,300, 230, 115, 23, and 0 (negative control) per 100 µl of whole blood, respectively.*

Before starting the in vivo experiments, several conditions were studied with regards to the storage of blood samples for PCR analysis. Several blood samples were spiked with a 10-fold dilution series of *C. albicans* in whole sheep blood, and the following three procedures were tested: (i) direct extraction by the standard procedure, (ii) partial processing of the samples up to the pronase treatment step and subsequently completion of the rest of the standard protocol 1 week later after the samples had been kept at -20°C, and (iii) freezing the samples at -20°C for 1 week and subsequent extracting DNA by the standard protocol. We found that direct extraction or partial processing incurs no loss in reproducibility or sensitivity. However, freezing the blood samples at -20°C for 1 week prior to DNA processing was associated with some loss of reproducibility and sensitivity of the PCR assay (Fig. 2). We therefore decided to routinely process the blood samples obtained in the in vivo experiments with the two-stage extraction procedure, i.e., procedure (ii), so that all samples belonging to one in vivo experiment, could be processed in a single PCR.

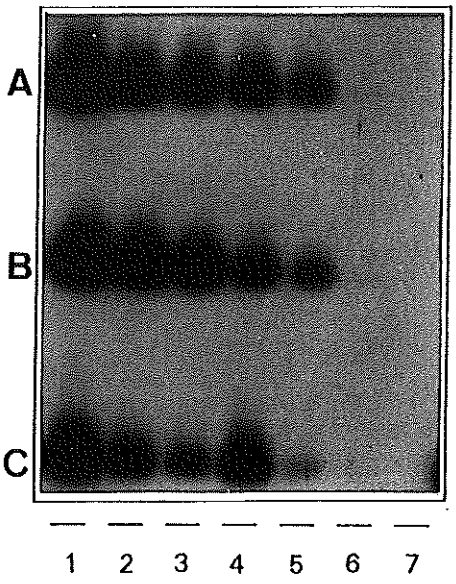

Figure 2. *Sensitivity and reproducibility results of the C. albicans PCR assay conducted with whole sheep blood processed by three different procedures: direct testing after DNA extraction by the standard procedure (A), partial processing of the samples up to the pronase treatment step and subsequent completion of the rest of the standard protocol 1 week later after the samples had been kept at -20°C (B), and freezing the samples at -20°C for 1 week and subsequent extraction and testing according to the standard protocol (C). Lanes: 1 through 5, C. albicans DNA from 3,000, 300, 150, 30, and 10 cells per 100 µl of whole blood, respectively; lanes 6 and 7 are negative controls. Lane 6, 100 µl of processed blood sample; lane 7, 100 µl of sterile PBS (both lanes without spiked Candida cells).*

Systemically infected mice. To test the sensitivity and in vivo accuracy of the *Candida* PCR assay, we used an induced systemic *C. albicans* infection in mice with cyclophosphamide-induced neutropenia. Since cyclophosphamide is metabolized in the liver into metabolites which actively cross-link DNA chains, control experiments were performed to check whether blood containing active cyclophosphamide metabolites would interfere with the PCR. Therefore, purified *C. albicans* DNA was added to blood, collected from mice 1 and 6 hours after they had received intraperitoneal cyclophosphamide, and incubated at 37°C. At 15, 30 and 60 min of incubation, samples were taken and the DNA was extracted and assayed by PCR. From these experiments it was concluded that active cyclophosphamide metabolites did not interfere with the

Candida PCR assay on whole blood (data not shown).

After the mice were i.v. challenged with 10^4 CFU of *C. albicans* the mean number of CFU per organ increased (kidney) or remained more or less constant (lung, liver, and spleen) over the course of the experiment (Fig. 3). The infection model is reproducible since the number of CFU per organ remained in the same order of magnitude (Fig. 3).

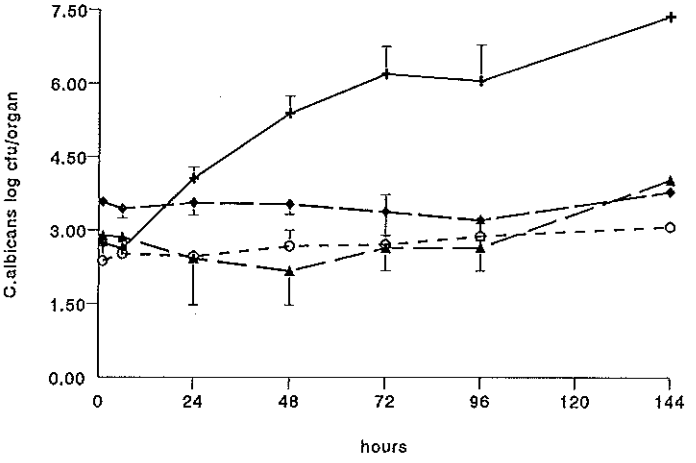

Figure 3. *Progression of systemic infection in neutropenic mice, expressed as the mean number of CFU of C. albicans in the kidney (+), spleen (O), lung (▲) and liver (♦), obtained from three separate in vivo experiments. In each experiment, 21 mice were inoculated with 10^4 CFU of C. albicans and three mice were sacrificed at 1, 6, 24, 48, 72, 96, and 144 h after i.v. inoculation. Each symbol represents the geometric mean, the standard deviation, for three experiments using three mice each (n = 9).*

The results of the blood cultures and PCR are given in Table 1. Blood culture results varied considerably and showed CFU counts ranging from 0 to 45 CFU per 100 μl of blood. Early after the onset of the infection as well as at 72 h into the infection, blood cultures tended to yield higher numbers of CFU. At 24 and 48 h, the CFU counts were often lower than at 1 and 72 h. In Figure 4 PCR results of the blood samples, from each mouse, from experiment 1 and 3, indicate that with progression of the infection more DNA targets became available for amplification. However, this increase in PCR signal did not correlate with the number of CFU of *C. albicans* cultured from the same blood sample (Table 1). This difference in the sensitivity of the PCR versus blood culture was scored at each sampling moment (Table 1). PCR was more often positive at 48 and 72 h and has, therefore, a better sensitivity than blood culture. In only one blood sample *C. albicans* was cultured (1 CFU per 100 μl) but no PCR signal could be obtained (experiment 2; 24 h).

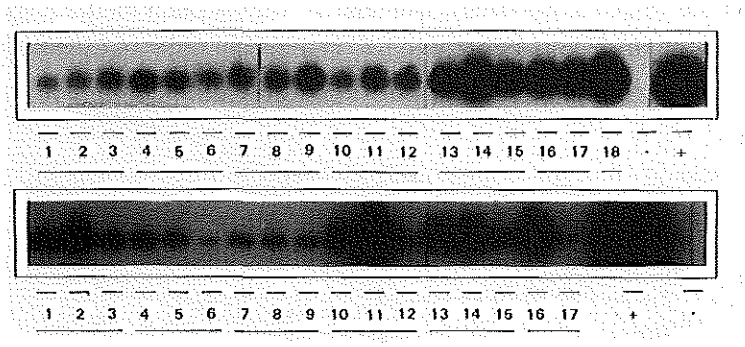

Figure 4. *Southern blot analysis of Candida PCR amplificates from the blood of each mouse with systemic candidiasis sacrificed at the indicated time intervals. Lanes: 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 15, 16 and 17, and 18 represents mice sacrificed at 1, 6, 24, 48, 72, 96, and 144 h after i.v. inoculation, respectively. +, positive controls; -, negative controls (processed blood of uninfected mice). The top gel corresponds to experiment 1, and the bottom gel corresponds to experiment 3 (Table 1).*

Gastrointestinal colonization of mice. From the previous results we concluded that the PCR assay may be more sensitive than blood culture for the detection of systemic *C. albicans* infection. To gain insight into the ability of the PCR assay to accurately discriminate between colonization and invasive candidiasis, a model for gastrointestinal-tract *C. albicans* colonization in mice was established.

After inoculation of six mice with 10^7 CFU of *C. albicans*, feces were cultured from the animals at days 2, 4 and 6. Feces cultures grew *C. albicans* in a yield of 14 to 1,500 CFU per mg of feces. In contrast, feces samples of cefradine-treated mice before inoculation were all negative for *C. albicans*.

Eight days after inoculation with *C. albicans* mice were sacrificed and several organs were cultured. The stomach and gastrointestinal tract grew *C. albicans* in each case. In contrast kidneys, liver, lung, and spleen were always sterile. Cultures of blood samples obtained at day 8 via cardiac puncture were all culture negative as were the results of PCR on the same blood samples (data not shown).

Table 1. *Individual blood culture and PCR results obtained from neutropenic mice sacrificed at the indicated times after i.v. inoculation with C. albicans, in each of three separate experiments*

Time (h)	Results[a] for expt: 1		2		3		Sensitivity (%)[b]	
	No. of CFU[c]	PCR[d]	No. of CFU	PCR	No. of CFU	PCR	Culture	PCR
1	39, 7, 6	+, +, +	9, 12, 2	+, +, +	6, 3, 5	+, +, +	100	100
6	6, 1, 3	+, +, +	3, 3, 4	+, +, +	0, 3, 0	+, +, +	78	100
24	1, 0, 0	+, +, +	3, 1, 1	+, -, +	8, 4, 0	+, +, +	67	89
48	2, 0, 0	+, +, +	0, 0, 0	+, +, -	20, 7, 1	+, +, +	44	89
72	0, 0, 0	+, +, +	27, 15, 13	+, +, +	34, 45, 14	+, +, +	67	100
96	0, 0, #[e]	+, +, #	#, #, #	#, #, #	5, 1, #	+, +, #	50	100
144	1, #, #	+, #, #	#, #, #	#, #, #	#, #, #	#, #, #	100	100

[a] Each result is for a single mouse (three mice per experiment).

[b] Sensitivities were determined presuming all samples should be culture and PCR positive.

[c] Per 100 μl of blood collected in EDTA-coated tubes.

[d] Using 25 μl of pretreated blood.

[e] Mouse died prematurely.

DISCUSSION.

Several previous studies have described the development of specific PCR techniques for the detection of *C. albicans* and assessed its sensitivity in vitro and in vivo. Buchman et al. (3), Miyakawa et al. (10) and Holmes et al. (5) were able to detect ±10 *Candida* cells per 100 µl of 1 ml of blood. Buchman et al. (3), Kahn et al. (8) and Crampin and Matthews (4) were able to detect *C. albicans* not only in blood samples but also in other clinical specimens including sputum and urine. However, since these specimens were usually positive by conventional culture as well, the PCR did not contribute much to the actual diagnostic sensitivity. Furthermore, most assays described were too specific, in the sense that they were designed to detect *C. albicans* only.

The PCR method described in this paper uses primers aimed at the multicopy gene coding for the small subunit rRNA and results in a 180-bp fragment. The advantage is that with the use of a species-specific probe both yeast genus- and species-specific information can be obtained by a single PCR (13). To study the merits of this PCR method, its sensitivity and specificity were tested in neutropenic mice with an induced systemic infection and in immunocompetent mice with gastrointestinal colonization only. The sensitivity with this PCR is on the order of 10 to 15 *C. albicans* cells per 100 µl of spiked blood, which is comparable with the results of other methods (3, 5, 10). We also demonstrated that the digestion of non-yeast DNA is essential for obtaining such levels of sensitivity. Omitting the DNase pretreatment procedure reduces the sensitivity by at least a factor of 10 and may also result in aspecific amplificates, as was shown in the paper of Crampin and Matthews (4), although they used another primer set aimed at the HSP 90 protein. Other parameters for increasing the sensitivity, like concentrations of $MgCl_2$, dNTP's and primers, have been optimized as described previously (13).

Furthermore, it has been demonstrated that the storage conditions of blood samples influences the sensitivity and reproducibility of the PCR assay. We did not succeed in clarifying the negative effect of freezing, since by microscopy *Candida* cells looked intact and were still viable when checked by culture. Possibly, the *Candida* cells become fragile during freezing and thawing such that the DNase, during the pretreatment step, can reach some of the essential *Candida* target DNA, thereby reducing the sensitivity and reproducibility of the PCR assay.

From the neutropenic mice with a systemic *Candida* infection, a higher percentage of positive test results was obtained by PCR than was obtained by conventional blood culture. Kahn (8) also demonstrated positive PCR results in a candidemic murine model. However, in that study, blood samples obtained from five mice were pooled and were always positive by culture. In contrast, we demonstrated positive PCR results from blood samples of individual mice, some of which had sterile blood cultures at the same time. Positive PCR results may be due to the amplification of circulating nonviable, *Candida* cells, e.g., those within phagocytic cells. It is this aspect of the PCR technique which

makes the method more sensitive than blood culture. Makimura et al. (9) also obtained positive PCR results from culture-positive as well as from culture-negative blood samples of mice infected by *C. albicans*. However, for DNA extraction they used a volume of blood which was twice that used for culture. Since the sensitivity of an assay is influenced by the volume tested, their results should be interpreted with great care. In our study we actually applied only 25 μl of blood for PCR compared to 100 μl for blood culture. Thus, the sensitivity of our PCR may be further increased by using more target DNA in the final reaction. Despite the sensitivity of the PCR, PCR results obtained from blood samples of gastrointestinally colonized mice were always negative. This particular aspect makes this PCR useful since it is able to discriminate between invasive candidiasis and colonization. The negative findings with the colonized model also underline the target specificity of this PCR method, in that no false positive results were obtained because of existing normal mouse bacterial flora.

Although the PCR assay of blood samples of mice with systemic candidiasis was not designed to give quantitative information, the intensity of the hybridization signal clearly increased with progression of the infection. These findings suggest a correlation between the PCR results and the degree of dissemination of infection and make the technique possibly valuable as a clinical monitoring assay.

In conclusion, PCR with primers directed against the small subunit rRNA gene in combination with the *C. albicans*-specific probe provided a sensitive and specific assay for detecting *Candida* DNA in whole blood. This PCR method promises to be more sensitive than blood cultures and may be an adequate technique in the early detection and follow-up of invasive candidiasis. Besides being sensitive, this PCR is specific, since PCR results obtained from blood samples of gastrointestinally colonized mice were negative; therefore, this PCR is diagnostically useful. Another positive diagnostic contribution is the relative speed of the PCR, since results are achieved within two days, whereas conventional blood cultures take 1 to 7 days or even longer. The true clinical relevance and utility of this test has to be proven in a prospective clinical study in which conventional blood cultures are evaluated in relation to PCR.

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CHAPTER 6

PCR-monitoring of response to liposomal Amphotericin B treatment of systemic candidiasis in neutropenic mice

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ABSTRACT

When a diagnosis invasive candidiasis has been made, treatment with toxic fungicidal agents is inevitable. The crucial decision of when to stop such treatment is difficult to make, because cultures are often negative despite ongoing invasive candidiasis and can therefore not be used as a reliable parameter of effective therapy. In the present study, the use of PCR in monitoring the therapeutic efficacy of antifungal treatment with liposomal amphotericin B was evaluated by using neutropenic mice with systemic candidiasis. Blood cultures of infected mice treated with different doses of liposomal amphotericin B were only positive at the early onset of the infection process and became sterile within 3 days; this was true even with mice treated with 1 mg of liposomal amphotericin B per kg of bodyweight that experienced a relapsed infection 14 days later. A significant correlation between presence of *C. albicans* CFU in the kidneys and PCR results obtained with blood was demonstrated. Thus PCR results obtained with blood samples correlated well with the therapeutic efficacy of antifungal treatment.

INTRODUCTION

Invasive candidiasis is an important cause of mortality and morbidity, particularly in immunocompromised patients. Unfortunately, efficient diagnosis is hampered by the lack of clinical parameters that can be used to recognize invasive candidiasis. A combination of microbiological, histological, and serological results may sometimes contribute to the diagnosis, but these tests are not 100% sensitive and specific (3). Recently, non-culture-dependent assays such as the polymerase chain reaction (PCR) have gained increasing importance in the rapid diagnosis of infections in patients at risk (10). The contribution of PCR assays in the diagnosis of fungal infections has now been demonstrated with a wide variety of clinical specimens including blood, serum, respiratory secretions, urine, peritoneal fluid, and cerebrospinal fluid (2, 4-7, 9, 12). It has been demonstrated that PCR has a better sensitivity than blood cultures in detecting *Candida albicans* infection in persistently neutropenic mice (14). Apart from helping to diagnose invasive candidiasis, an ideal assay should also help in monitoring the response to treatment and thus in determining the duration of therapy. Since toxic fungicidal agents are used, achieving accurate estimates of the time at which treatment can be stopped is clinically highly relevant. In the present study, we evaluated the use of PCR in monitoring the response to antifungal treatment with a liposomal formulation of amphotericin B (AmB) (AmBisome®) in neutropenic mice with systemic candidiasis. PCR results were compared with blood culture results at several time points during the course of the infection.

MATERIALS AND METHODS

Animals and *C. albicans* strain. Specific-pathogen-free, 11- to 13-week-old female BALB/c mice weighing 22 ± 1.3 g were used (Iffa Credo, L'Arbresle, France). *C. albicans* (ATCC 44858) was used throughout all the experiments. The yeast was stored at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol. Stationary-growth-phase cultures were obtained after incubation of *C. albicans* in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. Yeast cells were washed in phosphate-buffered saline (PBS), counted in a hemocytometer, and adjusted to 5×10^4 cells per ml in PBS.

Antifungal agent. AmBisome®, AmB, encapsulated in small unilamellar vesicles consisting of hydrogenated soybean phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol in a molar ratio of 2:1:0.8, was kindly provided by Vestar Inc. (San Pimas, Calif., USA) as a lyophilized preparation. The powder was reconstituted with distilled water at 65°C according to the manufacturer's instructions to give a liposomal suspension containing 4 g of AmB per liter and 35 g of lipid per liter.

Systemic infection in neutropenic mice. A model of invasive candidiasis in persistently neutropenic mice as previously described, was used (15). Briefly, neutropenia was induced by intraperitoneal administration of cyclophosphamide (Sigma Chemical Co., St. Louis, Mo.) at 100

mg/kg of body weight 4 days prior to inoculation with *C. albicans*, followed by additional doses of 75 mg/kg on the day of inoculation and at 3-day intervals during the course of infection (15). This treatment resulted in persistent granulocytopenia of < 100 granulocytes per μl of blood from the time of inoculation with *C. albicans* up to termination of the studies.

In each experiment, 19 neutropenic mice were infected by inoculation of 200 μl of 5 x 10^4 *C. albicans* CFU/ml in the tail vein. AmB was administered intravenously (i.v.) for 4 consecutive days at dose of 1, 7, or 14 mg/kg of body weight. At 1 h, or 3, 6, 14, 21, and 35 days after inoculation with *C. albicans*, groups of 3 to 4 mice were sacrificed and blood was collected in EDTA-coated tubes by cardiac puncture. Of each blood sample, 100 μl was directly cultured on Sabouraud dextrose agar (SDA) plates (Oxoid, Basingstoke, England) and incubated for 5 days at 37°C. Another 100 μl of blood was used for DNA extraction.

The kidneys, spleen, liver, and lungs were removed from each mouse and separately homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (VirTis Co. Inc., Gardiner, N.Y.). Residual AmB was eliminated from the homogenates by washing the sediment after centrifugation three times with PBS. Two hundred microliters of the serial 10-fold dilutions of each homogenate was plated on SDA plates. Two milliliters of the homogenate and the remainder of the homogenate, together with 18 ml and an equal volume of double-strength SDA respectively, were mixed and poured into petri dishes. All plates were incubated for 48 h at 37°C.

PCR sample preparation. For *Candida* DNA extraction from blood we used a method modified from that of Buchman et al. (2, 14). Briefly, 0.1 ml of lysis buffer (0.32 M Sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100) was added to 0.1 ml of whole blood. After lysis, cell debris and *Candida* cells were pelleted by centrifugation (Eppendorf centrifuge; Merck, Amsterdam, The Netherlands) at 16.000 x g for 5 min. The pellet was resuspended in 0.2 ml of lysis buffer. To remove free, non-yeast cell DNA, 7 μl of DNase (10 mg/ml, Boehringer Mannheim Gmbh, Mannheim, Germany) was added and the samples were incubated at 37°C for 1 h. After centrifugation at 16.000 x g for 5 min, pellets were resuspended in 0.2 ml of TEG buffer (50 mM Glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) containing 1.5 μl of Lyticase (900 U/ml, Sigma Chemical Co.) and incubated for a further 1 h at 37°C. Subsequently 3.0 μl of pronase (15 mg/ml, Boehringer Mannheim) and 10 μl of 10% sodium dodecyl sulfate (SDS) were added and incubation was continued for 1 h at 37°C. DNA extraction and purification were realized by using Prep-A-Gene DNA Matrix according to the instructions of the manufacturer (Bio-Rad laboratories, Richmond, Calif., USA). The matrix was eluted with 40 μl of TE buffer (10 mM Tris-HCl [pH 8.0], and 1 mM EDTA).

Filter tips (Biozym, Landgraaf, The Netherlands) were used throughout the whole isolation procedure to avoid contamination.

Primers and PCR protocol. PCR was performed in a 100 μl volume under standard conditions with 5 and 10 μl of the DNA samples. The following primer set, generating a 180-bp fragment spanning the V4 region of the ssu-rRNA gene, was used: 532 (5'-TATTAAAGTTGTTGCAG-3') and 651 (5'-CCTGCTTTGAACACTCTAATTT-3') (10). PCR was performed in 100 μl of PCR solution, containing 50 mM KCl, 10 mM Tris HCl [pH 8.3], 2.5 mM $MgCl_2$, 200 μM of each desoxynucleoside triphosphate, 50 pmol of each primer, 0.1 mg of gelatin per ml, 1 unit of *Taq* DNA polymerase (Promega, Leiden, the Netherlands) and 5 or 10 μl sample specimen. The reaction mixture was overlaid with 100 μl of mineral oil (Sigma) to prevent evaporation and was preincubated for 5 min at 94°C for DNA denaturation. Forty cycles of amplification were performed with a PCR processor (Biomed Gmbh, Theres, Germany). Each cycle consisted of a denaturation step at 94°C for 1 min, a primer annealing step at 52°C for 1.5

min, and a chain elongation step at 74°C for 1 min. After 40 cycles a temperature delay step of 5 min at 74°C completed elongation. A 30-μl volume of the amplified PCR product was analyzed by agarose (1.5% wt/vol) gel electrophoresis.

Southern blot analysis of PCR products. DNA was transferred from agarose to Hybond Plus nylon filters (Amersham International, plc, Amersham, United Kingdom) by overnight diffusion blotting in 0.4 N NaOH. The PCR products were analyzed with the *C. albicans* specific probe (5'-GTAGCCATTTATGGCGAACC-3') (10). Membranes were pre-incubated at 37°C in a hybridization solution (5 x SSC [1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate], 1 x Denhardt's solution) for 1 h. Hybridization occurred overnight at 37°C with 20 pmol of T_4 polynucleotide kinase (Bethesda Research Laboratories Inc.) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Internationl plc.)-labelled oligonucleotide probe. The filters were washed 1 x 15 min at 37°C and 1 x 15 min at 55°C with 2 x SSC, 0.1% (wt/vol) SDS. Autoradiography was performed with Kodak Royal X-Omat film and a 3.5-h exposure time.

RESULTS

Optimization of *Candida* PCR. To rule out an inhibitory effect of residual AmB on the PCR assay, blood of mice pretreated with AmB at 17 mg/kg for 4 days was spiked with a 10-fold dilution series of 2,000 to 2 cells of *C. albicans* and incubated for 30 min at 37°C, and tested in the PCR assay. No difference in the lower limit of detection was found between blood of treated and untreated mice (data not shown). In a previous study we used 10 μl of DNA sample in the final PCR (10). In the present study, we tried to improve the sensitivity of the assay by adding several volumes of extracted DNA obtained from blood samples spiked with different numbers of yeast cells. No significant differences were obtained by using 5 or 10 μl of extracted DNA sample in this in vitro experiment. However, the sensitivity of the PCR decreased 100-fold if 15 μl of the same DNA sample was used (Fig. 1). We thus decided to use 5 μl as well as 10 μl of DNA samples in the current study.

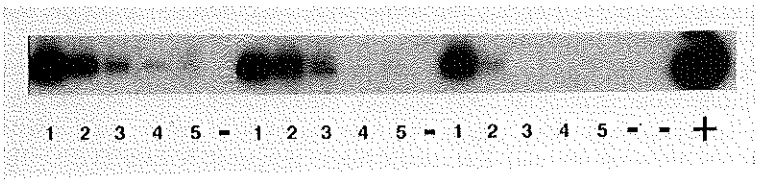

Figure 1. *Southern blot analysis of C. albicans PCR products obtained by amplification of different volumes of processed blood samples spiked with 2,000, 200, 20, 10, 2, and 0 (negative control [-]) C. albicans cells per 100 μl of blood. Lanes 1 - 5; represent the dilution series obtained by amplification of 5, 10, and 15 μl of processed blood (lane sets from left to right); +, positive control (whole blood spiked with C. albicans cells).*

PCR monitoring of invasive candidiasis. After challenge with *C. albicans*, AmB was administered for 4 consecutive days at dose of 1, 7, or 14 mg/kg of body weight. In mice treated with 1 mg of AmB per kg an initial drop in CFU per organ in lung, liver, and spleen was observed, and then there was an increasing number of CFU per organ from 14 to 21 days after inoculation (Fig. 2). Number of CFU in the kidneys, however remained stable and increased from 10^4 at day 14 to 10^6 at day 35.

Cultures from lungs, livers, and spleens of mice treated with 7 and 14 mg of AmB per kg became sterile within three days of treatment (Fig. 2). In contrast, the number of CFU in the kidneys of mice treated with 7 and 14 mg of AmB per kg decreased only slowly and cultures became sterile between days 14 and 21 (Fig. 2).

Eight of nine blood samples taken 1 h after the challenge grew *C. albicans* (Table 1). Of the mice treated with 1 mg of AmB per kg, only one had a *C. albicans* positive blood culture for the sample taken 3 days after the challenge. All other blood samples taken later in the infection process in all three treatment regimens failed to grow *C. albicans*.

The PCR results obtained by amplification of 5 μl of target DNA (Fig. 3) of blood samples of mice treated with 1 mg of AmB per kg show an initial decrease in PCR hybridization signals, which were absent on day 14. However, at day 21 PCR gave positive results again (Fig. 3; Table 1). PCR with 10 μl of target DNA actually demonstrated that a constant level of target DNA was present in the blood; however, the intensity of the hybridization signals of samples taken on day 35 was higher (Fig. 4).

Mice treated with 7 mg of AmB per kg showed positive PCR results only at 1 h and 35 days if 5 μl of target DNA was used. PCR with 10 μl showed a similar profile. However, more often a positive PCR result was found between 1 h and 35 days (Fig. 3 and 4; Table 1). Samples from mice treated with 14 mg of AmB per kg gave several positive PCR signals over time if 5 μl was used; however, 5 μl target DNA samples were all negative at day 35. If 10 μl was used only negative PCR signals were obtained (data not shown).

A positive correlation between the PCR results obtained by using 10 μl of processed sample for all three treatment regimens and the positive kidney cultures of the mice was demonstrated ($P = 0.04$; Fisher exact test). No such correlation could be demonstrated with the data from PCR with 5 μl of processed blood sample.

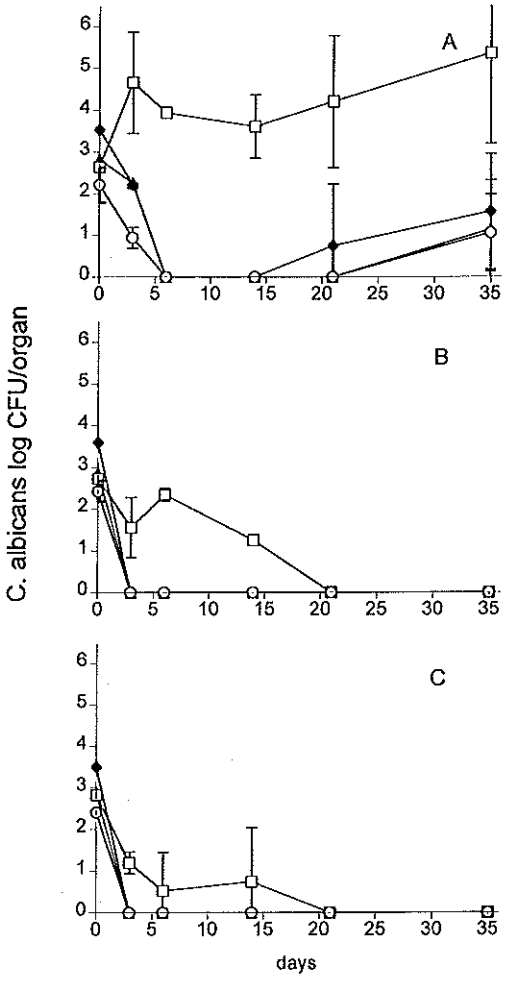

Figure 2. *Progression of systemic infection in neutropenic mice treated with 4 daily doses of 1 mg of AmB per kg (A); 7 mg of AmB per kg (B); 14 mg of AmB per kg of body weight (C). Data are expressed as the mean number ± standard error (error bars) of CFU of C. albicans in the kidney (□), spleen (○), lung (▲), and liver (♦). Data are based on three to four mice.*

Table 1. *Blood culture and PCR hybridization results in neutropenic mice after i.v. inoculation with C. albicans.*

Time	Results with AmB regimen[a]								
	1 mg Amb/kg			7 mg AmB/kg			14 mg AmB/kg		
	No. of CFU[b]	PCR[5c]	PCR[10d]	No. of CFU	PCR[5]	PCR[10]	No. of CFU	PCR[5]	PCR[10]
1 h	0,6,4	+,+,+	+,+,+	1,1,7	-,+,+	+,+,+	8,6,6	+,+,+	-,-,-
3 d	0,93,0	-,+,-	+,+,+	0,0,0	-,-,-	-,-,-	0,0,0	-,-,-	-,-,-
6 d	0,0,0	-,+,+	+,+,+	0,0,0	-,+,-	+,+,+	0,0,0	-,-,-	-,-,-
14 d	0,0,0	-,-,	-,+,+,+	0,0,0	+,-,-	+,+,-	0,0,0	+,+,-	-,-,-
21 d	0,0,0,0	-,+,+,+	+,-,-,+	0,0,0,0	+,+,-,-	+,+,+,+	0,0,0	+,+,+	-,-,-
35 d	0,0,0	+,+,+	+,+,+	0,0,0	+,+,-	+,+,-	0,0,0	-,-,-	-,-,-

[a] Mice were treated with respectively 1, 7, and 14 mg of AmB per kg for 4 days, and sacrificed at indicated interval; data are from individual animals in each of three separate experiments.

[b] Data are per 100 μL of blood.

[c] PCR[5] hybridization signals obtained by using 5 μl of processed blood for PCR, + = positive signal; - = negative signal.

[d] PCR[10] hybridization signals obtained by using 10 μl of processed blood for PCR, + = positive signal; - = negative signal.

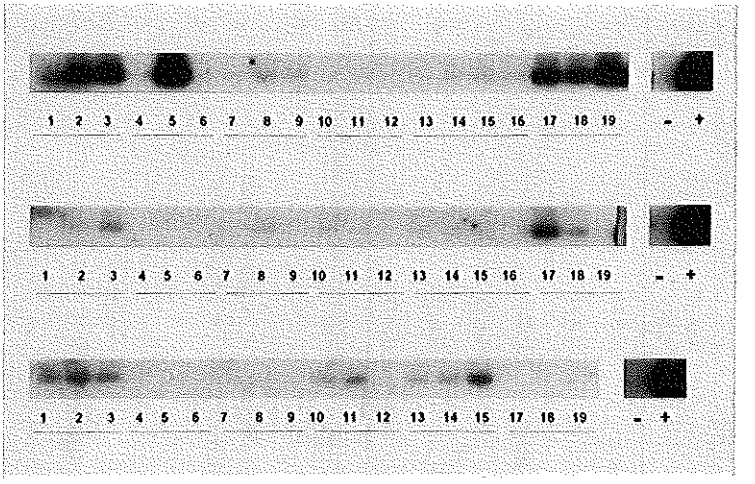

Figure 3. *Southern blot analysis of 5 µl of C. albicans PCR products obtained from blood samples from mice treated with 1 (top), 7 (middle), 14 (bottom) mg of AmB per kg. Lanes 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 16, and 17 to 19 represent mice sacrificed at 3 h, and 3, 6, 14, 21, and 35 days after i.v. inoculation, respectively. +, positive controls (whole blood spiked with C. albicans cells); -, negative controls (= whole blood of uninfected control mice)*

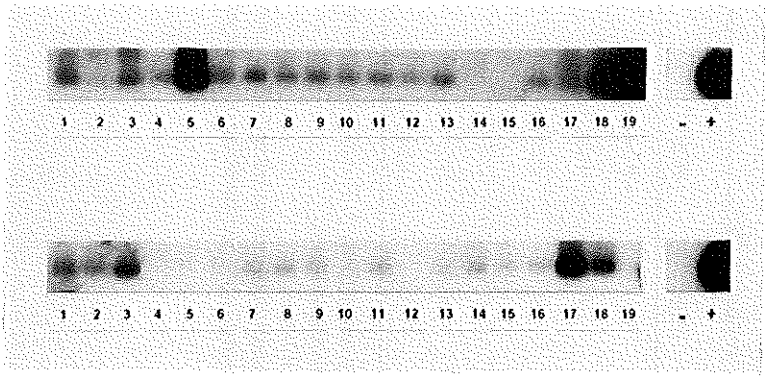

Figure 4. *Southern blot analysis of 10 µl of C. albicans PCR products obtained with blood samples of mice treated with 1 (top) and 7 (bottom) mg of AmB per kg. Lanes 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 16, and 17 to 19 represent mice sacrificed at 3 h, and 3, 6, 14, 21, and 35 days after i.v. inoculation, respectively. +, positive controls (whole blood spiked with C. albicans cells); -, negative controls (whole blood of uninfected control mice).*

DISCUSSION

Generally, it takes 2 to 7 days for *Candida* spp. to grow from patients' specimens. Therefore, microbiological culture cannot be regarded as an early diagnostic tool. Furthermore, even the best blood culture technique often gives negative results despite evidence of systemic infection (1). A breakthrough in diagnostic microbiology has been the introduction of the PCR technique for the detection of *Candida* spp. in several clinical specimens (8, 11). In a previous study, we demonstrated that PCR was more sensitive than culture in detecting *C. albicans* in blood from neutropenic mice with invasive candidiasis (14).

Once the diagnosis of invasive candidiasis has been made treatment with toxic fungicidal agents is inevitable. The decision of when to stop treatment is even more difficult to make, since negative cultures are not always indicative of a therapeutic response. Therefore, the aim of the present study was to examine the usefulness of PCR as an assay to monitor the response to AmB in neutropenic mice with invasive candidiasis.

Berenguer et al. (1) demonstrated in a clinical setting the existence of a correlation between the number of organs infected by *Candida* spp. and the number of positive blood cultures. Our results showed that blood cultures of infected mice treated with three different doses of AmB were positive only at the beginning of the treatment and became sterile within 3 days, even for mice treated with 1 mg of AmB per kg that experienced a relapse of infection after 14 days. On the other hand, a significant correlation between the number of CFU in the kidneys and the PCR results was demonstrated. Likewise, hybridization signals of the PCR increased with increasing numbers of *C. albicans* CFU in the kidneys of untreated mice (14). This correlation between the number of CFU in the kidneys and positivity of the PCR can only be explained by the fact that intact *Candida* cells are still present and detectable by PCR in the peripheral circulation. Since in the DNA extraction procedure a DNase treatment step is incorporated before the actual lysis of the *Candida* cells, the possibility that we are detecting circulating *C. albicans* DNA only is ruled out. Blood culture results for the same samples were negative, however, probably because of the low level or complete absence of metabolic activity of the *Candida* cells or the inhibitory presence of residual AmB.

Strong hybridization signals were observed at day 35 with mice that had been treated with a regimen of 7 mg of AmB per kg for 4 days, suggesting a relapse of the infection despite negative cultures from the kidneys, livers, lungs, and spleens at that moment. This hypothesis could not be further analyzed since we did not evaluate the progression of infection beyond day 35. Samples obtained with a regimen of 14 mg of AmB per kg showed negative PCR results at day 35. This regimen might thus be more effective than 7 mg of AmB per kg implicating that mice treated with the 7 mg of AmB per kg had not completely cleared the *Candida* cells. On the other hand, false-positive

signals are not a problem with this PCR since multiple blood samples from gastrointestinally colonized mice (14) and normal control mice were all negative by PCR.

PCR with 5 μl of processed target DNA was less sensitive than PCR with 10 μl of target DNA of blood samples in the in vivo experiment for mice treated with 1 or 7 mg of AmB per kg, indicating that more target DNA increases the sensitivity of the PCR assay. Application of 15 μl of processed target DNA in the PCR, however, caused false negative results, probably due to inhibitory factors present in the blood samples. For the same reason no hybridization signals were obtained by using 10 μl of target DNA of blood samples of mice treated with 14 mg of AmB per kg, whereas the same samples were PCR positive if 5 μl was used. It indicates the narrow balance between target DNA and inhibitory factors influencing the outcome of the PCR. Therefore, to exclude false-negative PCR results it may be necessary to use routinely several concentrations of target DNA in the PCR assay. This limitation in sensitivity of the PCR might be overcome in the near future if rRNA instead of DNA is used, since there is a high copy number of rRNA present in each cell. Another advantage of rRNA is that it can be amplified in a commercial available nucleic acid sequence-based amplification system, which is faster than PCR (13) and whose enzymes are less sensitive to inhibitory factors. The advantage of commercial systems is that they are easy to perform and detection formats are less laborious, which makes the introduction of these techniques into all microbiology laboratories possible.

We conclude that in this neutropenic murine model of invasive candidiasis, PCR results correlated well with the response to the therapy administered. PCR is much better in this respect than routine blood cultures. However, before this assay can be used as universal monitoring tool, PCR needs to be further evaluated in this or other animal models and in a clinical setting as well.

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CHAPTER 7

Anti-enolase antibodies protective against candidiasis in mice.

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Submitted for publication.

ABSTRACT

Passive transfer of heterologous rabbit antibodies (IRS) directed against a crude *Candida albicans* extract and homologous mice anti-enolase antibodies (IMS) were administered in a non-acute but lethal systemic *Candida* model in mice. Protection could only be demonstrated by repeated administration of homologous as well as heterologous immunesera. The protective effect was not due to non-specific, heat-labile, serum factors since fractionated anti-cytoplasmic rabbit immunoglobulins gave also a decreased mortality rate. These findings could not be ascribed to enhanced opsonization as observed in in vitro opsonization experiments using peritoneal macrophage monolayers. In conclusion anti-enolase antibodies are protective against lethal *C. albicans* infections. The protection was not due to enhanced initial clearance of viable *C. albicans* from the internal organs, but was presumably the result of as yet unknown effects of antibodies occurring later in the infectious process.

INTRODUCTION

Candidiasis represents a spectrum of diseases ranging from colonization of the skin or mucous membranes to severe and often fatal disseminated disease. Consequently, it

is to be expected that a variety of host immune mechanisms interact in the response to *Candida albicans*. There is now a body of evidence showing that cellular elements including the neutrophil, the helper T-lymphocyte and the monocyte-macrophage play an important role in the defense against fungal infections (1, 5, 6, 8, 28). In contrast, evidence pertaining to antibody-mediated defense against candidiasis is contradictory and, consequently, its role in the pathogenesis of *Candida* infections is poorly understood. Maiti et al., (12) gathered indirect evidence supporting a role for specific antibodies in protection against *Candida* infections in mice. Several authors have failed to demonstrate a protective role of antibodies in passive immunization experiments (2, 7), whereas others succeeded in demonstrating direct protective effects by transfer of hyperimmune serum in mice (13, 17, 19). Some authors have found a protective effect only after vaccination with viable *C. albicans* cells (4, 29).

No overall conclusion can, thus, be drawn at this time about specific antibody protection against candidiasis partly because different treatment schedules of immune sera directed toward various antigenic determinants have been used. Also, experiments were performed in different animal models of invasive infection.

In the present study, we evaluated the protective capacities of antibodies directed against the immunodominant enolase antigen of *C. albicans*, and compared its activity to that of antibodies directed against a crude *C. albicans* antigen extract. A non-acute but lethal systemic candidiasis mouse model was chosen because it was deemed to be more relevant clinically compared to previously published, more acute, lethal infection models. Furthermore, in vitro phagocytosis and killing of *C. albicans* by macrophage monolayers were studied.

MATERIALS AND METHODS.

***Candida* strains.** Throughout all experiments strain *C. albicans* ATCC 44858 was used. For preparation of the crude antigen extract and of the purified enolase antigen *C. albicans* CBS 5982 serotype A (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands) was used.

Preparation of anti-cytoplasmic immunoglobulins. Antisera were obtained by immunizing New Zealand White rabbits, with a crude *C. albicans* antigen extract, prepared as described elsewhere (9). The animals were injected subcutaneously with 12 mg of crude antigen in Ribi adjuvant (Ribi Immunochem, Research, Inc, Montana USA). The antibodies directed against the crude antigen determinants were monitored by ELISA using the crude antigen. This polyclonal polyspecific antiserum was pooled and kept frozen at -80°C. Antibodies directed toward mannan were evaluated by counterimmunoelectrophoresis (CIE) and crossimmunoelectrophoresis (XIE), techniques which have previously been described (24).

Immunoglobulin fractionation. Fractionation of immunoglobulins from rabbit sera was performed by ammonium sulphate precipitation at 4°C. Ten ml of serum, diluted with an equal volume of 10 mM Tris in 0.9% NaCl (pH 7.6), was brought to 50% saturation by adding a saturated solution of $(NH_4)_2SO_4$. After 30 min at room temperature the suspension was centrifuged

at 1,700 x g for 15 min. The pellet was dissolved in 0.9% NaCl and brought to 40% saturation by adding a saturated solution of $(NH_4)_2SO_4$. Again the solution was left at room temperature for 30 min and centrifuged at 1,700 x g for 15 min. This procedure was repeated for 33% saturation. The pellet was dissolved in 5 ml sterile phosphate-buffered saline (PBS) and dialysed against sterile PBS overnight. The titer of anti-*Candida* antibodies in this immunoglobulin fraction was equal to the titer of anti-*Candida* antibodies of the initial serum, as determined by ELISA. The immunoglobulin fraction of immune rabbit serum (IRS) will be designated as IgIRS and the immunoglobulin fraction of normal rabbit serum (NRS) as IgNRS.

Preparation of anti-enolase immunoglobulins. Antisera to enolase were raised in BALB/c mice (Iffa Credo, L'Arbresle, France) by repeated immunization with purified enolase. Purification of *C. albicans* enolase has been described previously (25). Mice were repeatedly injected subcutaneously with 50 μg of purified enolase in Ribi adjuvant (Ribi Immunochem). Anti-enolase antibody levels were monitored by ELISA (25) using enolase as the antigen. This polyclonal monospecific anti-enolase antiserum from several mice was pooled and stored at -80°C. Specificity of the anti-enolase antibodies was determined by Western blotting using the crude antigen extract of *C. albicans*. Procedures for gel electrophoresis and immunoblotting were similar to those used in previous studies (21).

Preparation of a monoclonal antibody (MAB) against mannan. Immunization of 14-week-old BALB/c mice (Iffa Credo) was performed with 500 μg of crude antigen emulsified in an equal amount of complete Freund's adjuvant (0.1 ml) (Life Technologies B.V., Breda, The Netherlands) as described by Mirza et al. (15). For MAB production the draining lymph nodes were removed and pooled for fusion with the mouse myeloma cell line SP 2/0, as described by Ollert et al. (18). From one clone ascites was produced and characterized by immunoblotting, CIE, and XIE. Ascites was dialysed against sterile PBS and diluted four times in sterile PBS before use. The MAB is an IgG class antibody, and will be denoted as 18C11.

ELISA to determine anti-crude antigen antibodies. Highly activated polystyrene microtiter plates (Nunc Immunoplate Maxisorp F96) were coated with 0.3 μg of crude antigen in 100 μl coating buffer (0.13 M sodium carbonate and 0.03 M hydrogenated sodium carbonate [pH 9.6]) per well and were subsequently incubated overnight at 4°C. The plates were washed twice with 200 μl of TBS-block (0.01 M Tris, 0.15 M NaCl [pH 7.6], 0.1% gelatin) per well and were then incubated with 200 μl of TBS block per well at 37°C for 2 h. The plates were then drained. Immune sera or the immunoglobulin fractions were diluted twofold in TBS (0.01 M Tris, 0.15 M NaCl [pH 7.6]) starting at a dilution of 1:50. A total of 50 μl of each serum or immunoglobulin fraction was added to each well, and plates were subsequently incubated at 37°C for 60 min. After three cycles of washing with TBSt (TBS with 0.04% [wt/vol] gelatin and 0.05% [vol/vol] Tween 20), 50 μl of an alkaline phosphatase-labelled goat anti-rabbit IgG conjugate (diluted 1:1,000 in TBSt [Sigma Chemical Co., St. Louis, Mo.]) was added to each well; this was followed by incubation for 1 h at 37°C. A 100 μl volume of acid enzyme substrate solution (9.7% diethanolamine, 3 mM NaN_3, 5mM $MgCl_2.6H_2O$, 1 mg/ml *p*-nitrophenylphosphate [Sigma]) was added after three cycles of washing with TBSt, and the plates were incubated at 37°C in the dark. Color development was stopped after 30 min of incubation by adding 100 μl of 3 M NaOH to each well. The optical densities at 405 nm were determined by using an automated plate reader (Bio-Tek Instruments, Inc, Winooski, Vt.).

Protective role of various immune sera and Ig fractions in mice. Four hours before mice were infected by intravenous (i.v.) inoculation of 3 x 10^5 CFU of *C. albicans* into the tail vein (27), mice were intraperitoneal (i.p.) inoculated with 0.4 ml immune rabbit serum (IRS) or immune mouse serum (IMS) or with IgIRS. Additional doses of 0.3 ml of sera were given at 6,

12 and 18 days after infection. Normal rabbit serum (NRS) or normal mouse serum (NMS) or MAB 18C11 or PBS served as negative controls, as indicated.

At 50 days after infection, surviving mice were sacrificed, and their kidneys, lungs, liver, and spleen quantitatively cultured for *C. albicans* (27) on Sabouraud dextrose agar (Oxoïd, Basingstoke, England).

Macrophage monolayers. For macrophages monolayers a modified method of van Etten et al., (26) was used. Briefly, macrophages were obtained from peritoneal cavities of 12- to 13-week-old specific-pathogen-free BALB/c mice (Iffa Credo). The macrophages were washed twice in Dulbecco modified Eagle medium (Flow Laboratories, Irvine, Scotland) supplemented with 1% glutamine. Monolayers of peritoneal macrophages were cultured at 37°C on chamber slides (Lab-Tek, Bayer, The Netherlands) under a fully humidified atmosphere of 7.5% CO_2 in air in culture medium containing Dulbecco modified Eagle medium with 5% inactivated fetal bovine serum (FBS; Hy-Clone, Logan, Utah). After the first 2 h of incubation, fresh culture medium was added to the monolayer, and the macrophages were incubated for 24 h. After this incubation period, preopsonized *C. albicans* cells were added to the monolayers, resulting in a *C. albicans*-to-macrophage ratio of 10:1. *C. albicans* was preopsonized by incubation of a washed stationary-growth-phase suspension with 10% of indicated sera or immunoglobulin fractions under continuous rotation at 8 rpm for 15 min at 37°C, and subsequent washing the yeast twice in PBS.

After a 5 or 10 min uptake period, the non-ingested yeasts were removed by washing the monolayer three times with cold PBS. The monolayer was fixed in methanol and stained with May-Grünwald-Giemsa, after removal of the plastic chamber from the slide. The uptake of *C. albicans* was determined microscopically, and expressed as percentage of phagocytosing macrophages and the average number of *Candida* cells per macrophage. Intracellular killing was performed as described by van Etten et al. (26), and determined after a 5 and 10 min uptake period.

Statistical analysis. Statistical evaluation of the differences in the survival curves between serum-treated, immunoglobulin-treated and PBS-treated groups was performed by using the log rank test. This test examines the decrease in survival with time as well as the eventual percentage of survival. The Kruskal-Wallis test was used for evaluation of the differences in the percentage of phagocytosis of the in vitro assays between the various opsonized *C. albicans* cells.

RESULTS

Characterization of raised immune sera. Characterization of the immune sera was performed by Western blotting, CIE, and XIE techniques. The IMS reacted as a single band in the immunoblot, and IRS reacted with multiple antigenic determinants of 20 to 90 kDa including the enolase antigen (Fig. 1). IRS did not react with mannan as was found by CIE and XIE by using the purified mannan as antigen (data not shown). The MAB 18C11 did not recognize any protein by immunoblotting, it only reacted with mannan as demonstrated by XIE (data not shown). Titration of the MAB 18C11 by CIE resulted in an anti-mannan titer of 1:256. NRS or NMS did not react with *Candida* antigens as determined by immunoblotting (Fig. 1), ELISA or CIE (data not shown).

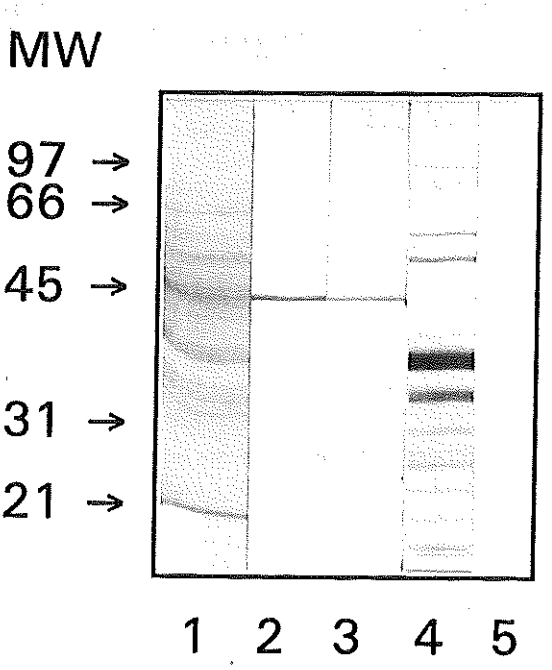

Figure 1. *Western blotting analysis of IMS and IRS using the crude C. albicans antigen as antigen extract. Lanes: 1, Coomassie stained crude antigen; 2, and 3, IMS; 4,IRS; 5, NMS. MW, molecular weight markers are indicated on the left.*

Protective role of IRS. Serial blood samples were collected from mice given IRS, in order to study the length of time that the anti-*C. albicans* antibodies remained detectable in the blood after immunotransfer. Antibody titers against the crude *C. albicans* antigen and purified enolase were determined (Table 1). It was found that after administration of 0.4 ml of IRS to uninfected mice, antibodies directed towards the crude antigen decreased over 14 days but did not completely disappear. In sera of mice with systemic candidiasis, however, anti-enolase antibodies and anti-crude antigen antibodies decreased much more rapidly. Fourteen days after administration, titers decreased under the detection limits of the respective assays. In order to maintain a constant level of antibodies it was, therefore, decided to give IRS repeatedly at intervals of 6 days.

Table 1. Antibody titers, expressed in OD_{405} values, against the crude antigen and enolase in sera of mice after a single transfer of IRS, monitored during 14 days in infected and uninfected mice.

	Crude *C. albicans* antigen [a]		enolase antigen[a]
time after immunotransfer	uninfected mice	infected mice	infected mice
1 hour	0.605[b]	0.700	0.540
1 day	0.545	0.654	0.442
3 days	0.583	0.513	0.058
7 days	0.544	0.472	0.028
14 days	0.482	0.0	0.0

[a] antigen used in ELISA in determining anti-*C. albicans* antibodies.
[b] optical densities at 405 nm at a serum dilution of 1:50.

In subsequent challenge experiments mice treated with the IRS showed a significant delay in mortality (Fig. 2; $P=0.003$). In contrast, mice treated with NRS or PBS all died within three weeks, most within two weeks (Fig. 2). No significant differences were found in survival between the PBS-treated and NRS-treated mice ($P=0.25$). The three IRS-treated mice still alive at day 50 were sacrificed to obtain quantitative cultures of the organs. Lungs, liver and spleen were sterile whereas cultures of the kidneys revealed 1.7, 5.1 and 3.1 log CFU of *C. albicans*. Sera determined for two of the surviving mice, had high titers of autologous antibodies against the enolase and lower antibody titers against the crude antigen (Table 2). Please note that the sera of mice surviving at day 50 did not contain heterologous rabbit anti-*Candida* antibodies anymore.

Protective role of IgIRS fraction. To rule out that the observed protective effect of IRS was due to nonspecific serum factors the IRS was fractionated to obtain purified rabbit immunoglobulins. Again the IgIRS showed a significant delay in mortality compared to the PBS (Fig. 2; $P=0.003$). The IgIRS provided protection equal to that of complete IRS ($P=0.77$). The three IgIRS-treated mice, still alive at day 50 were sacrificed. Of one mouse all internal organs were sterile. From the other two mice *Candida* was cultured from the kidneys (respectively 3.5 and 4.8 log CFU of *Candida*/two kidneys). Again, sera determined for two of the surviving mice revealed high titers of autologous anti-enolase antibodies and less anti-crude antigen antibodies (Table 2).

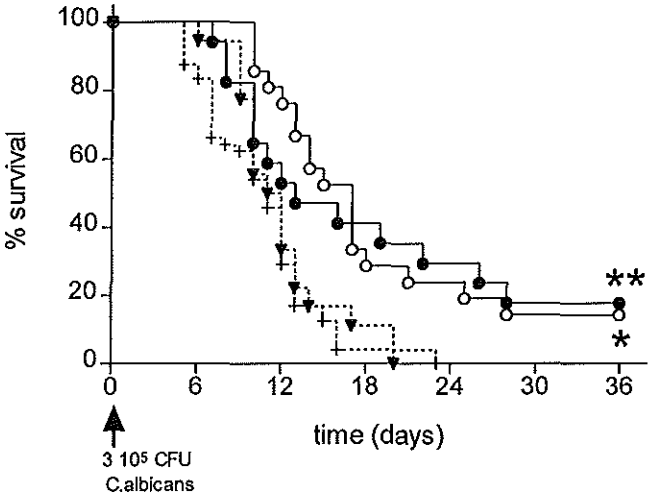

Figure 2. *Effect of repeated administration of IRS or IgIRS on survival of immunocompetent mice with candidiasis. Mice were intraperitoneal treated with 0.4 ml IRS (-o-) or IgIRS (-●-) 4 h before i.v. inoculation with 3 x 10^5 CFU C. albicans and treated with 0.3 ml IRS or IgIRS at 6, 12 and 18 days after Candida inoculation. Control mice were likewise treated with NRS (-▾-) or PBS (-+-). (number of mice treated was: IRS, n = 21; IgIRS, n = 17; NRS, n = 22; PBS, n = 27). *P = 0.003 and *P < 0.001 versus NRS or PBS treated mice respectively, **P = 0.003 versus PBS treated mice.*

Table 2. *Antibodies against enolase and against crude antigen in IRS-, IgIRS-, and IMS-treated mice surviving C. albicans i.v. challenge for 50 days.*

mouse transferred with	anti-enolase antibodies[a]	anti-crude antigen antibodies[a]
IRS	0.479[b]	0.118
IRS	0.764	0.098
IgIRS	2.107	0.046
IgIRS	1.079	0.070
IMS	1.217	0.281
IMS	0.266	0.070

[a] determined by using anti-mouse IgG conjugate.
[b] optical densities at 405 nm at a serum dilution of 1:400
IRS, immune rabbit serum; IgIRS, immunoglobulin fraction of immune rabbit serum; IMS, immune mouse serum.

Protective role of IMS. From the above described experiments it was not clear which antibody or antibodies were responsible for the protective effect observed. The IMS administered, targeted specifically toward the enolase antigen, induced a significant delay in mortality in comparison to NMS or PBS (Fig. 3; P=0.005 and P=0.015, respectively). NMS did not provide protection, the mortality rate was similar to the PBS-treated controls. Two of the 15 IMS-treated mice, still alive at day 50 were sacrificed. *C. albicans* was only cultured from the kidneys (respectively 1.7 and 5.2 log CFU of *C. albicans*/two kidneys) the other internal organs were sterile. Both mice had higher titers of autologous anti-enolase antibodies than antibodies to the crude antigen extract (Table 2). The specificity of the protective effect of the anti-enolase IMS was also confirmed by treating the mice with an anti-mannan monoclonal antibody which did not show any protective effect (fig. 3).

To gain more insight into the potential mechanisms of the protective effect of the IMS, mice were sacrificed 1, 2, and 3 days after the onset of infection. Control mice treated with NMS and PBS were sacrificed at the same intervals. Quantitative cultures of their internal organs showed that administration of the IMS had no modulating effect on the number of *C. albicans* cells per organ during the initial 3 days after initiation of infection, as compared to the mice treated with NMS or PBS (Table 3). Similar results were found when the inoculum size was reduced to 4 x 10^4 CFU (data not shown).

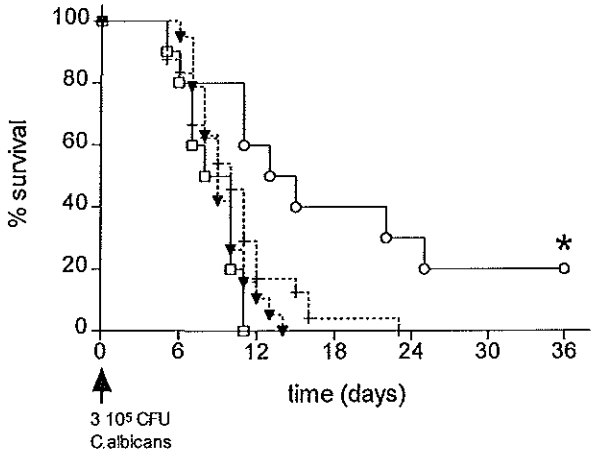

Figure 3. *Effect of repeated administration of IMS or MAB 18C11 on survival of immunocompetent mice with candidiasis. Mice were intraperitoneal treated with 0.4 ml IMS (-o-) or MAB 18C11 (-▾-) 4 h before i.v. inoculation with 3 x 10^5 CFU C. albicans and treated with 0.3 ml IMS or MAB 18C11 at 6, 12 and 18 days after Candida inoculation. Control mice were likewise treated with NMS (-□-) and PBS (-+-). (number of mice treated was: IMS, n=15; MAB 18C11, n=19; NMS, n=10; PBS, n=27). *P≤0.005 versus NMS treated mice *P=0.015 versus PBS treated mice.*

Table 3. *CFU of C. albicans per indicated organ at day 1, 2, and 3 after inoculation with 3 x 10^5 CFU C. albicans of mice given IMS, NMS or PBS 4 hr before.*

organ	day 1 after i.o.[a]			day 2 after i.o.			day 3 after i.o.		
	IMS	NMS	PBS	IMS	NMS	PBS	IMS	NMS	PBS
spleen	4.0[b]	3.9	4.3	3.6	3.8	4.0	4.16	3.8	3.8
kidneys	5.0	4.9	5.1	5.4	5.9	6.5	6.9	6.4	6.6
lung	3.0	2.8	3.0	2.9	2.7	3.3	3.6	2.9	3.1
liver	4.6	4.5	4.7	4.2	4.3	4.5	4.7	4.4	4.3

[a] i.o., inoculation.
[b] log CFU of C. *albicans* (mean of 2 mice).

In-vitro phagocytosis experiments. To study the influence of antisera against enolase on the phagocytosis and killing of *C. albicans* by macrophages, in vitro opsonization experiments were performed using peritoneal macrophage monolayers as indicator cells. Phagocytosis was determined after a 5 and 10 min incubation period since after 30 min *Candida* already started to form germtubes which influenced the reproducibility of the assay. From the data in Table 4 it can be seen that after 5 min of incubation differences in percentages of *C. albicans* phagocytosis were not significant between cells preopsonized with IRS, NRS and their respective immunoglobulin fractions or their heat-inactivated form. However, at 10 min of incubation macrophages had taken up significantly higher numbers of IRS- and NRS- preopsonized *C. albicans* cells compared to cells preopsonized with IgIRS, IgNRS, heat-inactivated IRS or NRS. Also, the percentage of macrophages participating in the phagocytic process was significantly higher ($P<0.05$; Kruskal-Wallis test) in these cases. Phagocytosis of IRS-opsonized *Candida* at 10 min of incubation did not significantly differ from that of the NRS-opsonized yeast cells (Table 4).

Similar results were observed for fresh IMS compared to heat-inactivated IMS and fresh NMS to inactivated NMS (Table 5). Already after 5 min of incubation phagocytosis of *C. albicans* opsonized with fresh anti-enolase serum was significantly higher than that of yeast opsonized with heat-inactivated anti-enolase serum. Phagocytosis of IMS-opsonized *Candida* at 5 min of incubation did not significantly differ from that of NMS-opsonized yeast cells (Table 5). A lower percentage of phagocytosing macrophages as well as a lower number of *C. albicans* cells per macrophage was observed if heat-inactivated IMS were used to opsonize the *Candida* cells. Although the MAB 18C11 bound well to the cell wall of *C. albicans*, as determined by immunofluorescence, it did not promote phagocytosis. To the contrary, phagocytosis rates of 18C11 opsonized cells was similar to the PBS controls (Table 5). We were not able to detect killing of *C.*

albicans by the peritoneal macrophages regardless of the opsonins used to pretreat the yeast cells.

Table 4. *Effect of antiserum and purified antibodies against crude antigen on phagocytosis of C. albicans*

	C. albicans cells per macrophage		% phagocytosing macrophages	
opsonin	5 min	10 min	5 min	10 min
IRS	2.4 ±0.54	4.23 ±0.6[a]	59 ±6	86 ±4[a]
NRS	2.7 ±0.8	4.17 ±0.9[b]	56 ±6	83 ±3[b]
$IRS^{inactivated}$	1.64 ±0.11	2.25 ±0.04	34 ±12	66 ±2
$NRS^{inactivated}$	2.09 ±0.11	2.27 ±0.04	52 ±5	59 ±1
IgIRS	2.19 ±0.44	3.19 ±0.5	48 ±7	66 ±6
IgNRS	1.58 ±0.48	2.55 ±0.23	51 ±5	70 ±6

[a] IRS significantly different from $IRS^{inactivated}$ and IgIRS ($P < 0.05$; Kruskal-Wallis test)
[b] NRS significantly different from $NRS^{inactivated}$ and IgNRS ($P < 0.05$; Kruskal-Wallis test)
IRS, IgIRS; anti-crude antigen antibody rabbit sera or immunoglobulin fraction
NRS, IgNRS; normal rabbit serum or immunoglobulin fraction.

Table 5. *Effect of anti-enolase antibodies antigen antisera on phagocytosis of C. albicans*

	C.albicans cells per macrophage		% phagocytosing macrophages	
opsonin	5 min	10 min	5 min	10 min
IMS	2.65 ±0.7[a]	3.33±0.78[a]	59 ±6[a]	70 ±6[c]
NMS	2.3 ±0.11[b]	2.84±0.51[b]	53 ±7[b]	44 ±12
$IMS^{inactivated}$	1.67 ±0.02	1.88 ±0.17	22 ±3	47 ±7
$NMS^{inactivated}$	1.29 ±0.12	1.73 ±0.03	5 ±1	30 ±5
18C11	1.83 ±0.46	2.69 ±0.78	16 ±3	39 ±10
PBS	2.14 ±0.26	2.39 ±0.17	10 ±5	32 ±7

[a] IMS significantly different from $IMS^{inactivated}$, $NMS^{inactivated}$, 18C11, and PBS ($P<0.05$; Kruskal-Wallis test).
[b] NMS significantly different from $NMS^{inactivated}$ ($P<0.05$; Kruskal-Wallis test)
[c] IMS significantly different from all other opsonins ($P<0.01$; Kruskall-Wallis test)
IMS, anti-enolase antigen mouse serum; NMS, normal mouse serum; 18C11, monoclonal anti-mannan antibody.

DISCUSSION.

Several studies have described delayed mortality after the administration of specific antibodies in animal models of deep-seated candidiasis (12, 13, 17, 19). Antibodies used were mostly polyclonal and raised against a variety of undefined *Candida* antigens. Matthews et al. (13), however, demonstrated protection which was achieved by the administration of antibodies specifically directed toward a 47 kDa immunodominant antigen.

In the present study we evaluated the effect on mortality in a model of chronic, but ultimately fatal candidiasis, of antibodies raised against a variety of undefined antigens (IRS) and against the specific enolase antigen (IMS). The reason for studying anti-enolase antibodies for its protective capacities is the fact that enolase has been shown to be an immunodominant antigen in patients with invasive candidiasis (25). In this respect enolase is comparable to the immunodominant 47 kDa break down product of the 90 kDa heat shock protein (13). As Mourad et al., (17) reported that the mortality rate became similar to that of controls if the administration of antibodies is prematurely stopped, the design of a study aimed at demonstrating protection should assure a sufficient and constant antibody level in the blood of the experimental animal. Fulfilment of these conditions was achieved in our study by repeated injections of the antisera with an interval of 6 days. Experiments in which antiserum was administered only once 4 hours before initiation of infection failed to show any protective effect (data not shown). These findings might also explain the results of Kagaya et al., (10) who reported lack of effectiveness of specific immunoglobulins administered only once every two weeks.

A protective effect in our model could only be shown in mice which had received three repeated doses of IRS, IgIRS or IMS. All three regimens had similar protective effects. The experiment in which mice were treated with IgIRS indicate that the protective effect is largely mediated by the antibodies present in the immune sera and not due to non-specific heat-labile serum factors. The IMS we used is a well defined polyclonal antiserum, monospecific for enolase. In contrast, IRS was directed toward various undefined antigenic determinants present in the crude *Candida* extract. The results obtained with the IMS correlate well with the findings of Matthews et al., (13) who also demonstrated a protective effect with an antiserum directed specifically against another (the 47 kDa) immunodominant antigen. The specificity of the protective effect of anti-enolase antibodies was underlined by the results of MAB 18C11.

Another finding which underlines the potential protective effect of anti-enolase antibodies is the fact that mice surviving for 50 days after the *Candida* challenge have high levels of circulating anti-enolase antibodies; this would indicate that these mice have, by that time, developed antibodies against the enolase that may have helped to extend their survival. These antibodies had to be of mouse origin as indirectly proven in animals given IRS or IgIRS who had high proportions of autologous mouse anti-enolase

antibodies.

To gain insight into the mechanism(s) of decreased mortality observed with immune-sera, the lungs, liver, spleen, and kidneys were cultured of mice sacrificed during the first three days. No significant effect on the number of *C. albicans* cultured in these organs was observed after IMS treatment. From this it was concluded that the protective effect is not the result of an early decrease in the number of viable CFU's per organ. Presumably effects of immune sera occur later in the infection process, as suggested by others (16, 22).

The effect of immune sera (IMS and IRS) could not simply be explained by enhancement of the phagocytic process by leukocytes or macrophages through an opsonic mechanism. In in vitro experiments increased uptake was seen with the IMS- or IRS-opsonized *C. albicans* cells; however, in the control experiments using non-immune sera similar increases in *Candida* uptake were observed. From these opsonophagocytosis experiments it became clear that the opsonic effect of immune serum is to a significant degree dependent upon complement-activation since heat-treated sera were much less opsonic. Although complement promoted the rate of phagocytosis it did not enhance the intracellular killing of *C. albicans* by peritoneal macrophages as was postulated by Pereira and Hosking (20). Surprisingly, monoclonal anti-mannan antibodies did not enhance phagocytosis although the antibody clearly bound to the surface of the *Candida* cells. Lack of opsonic activity of this IgG class antibody is not expected and in any case points to an inability to interact with FC-receptors on the macrophage.

Another somewhat puzzling finding is the fact that most of the internal organs of the mice surviving at day 50 were sterile except for the kidneys. This failure to clear *Candida* from the kidneys might imply that the anti-*Candida* activity of antibodies is anatomically restricted. Sohnle et al., (23) reported that the infiltration of monocytes into infectious loci is enhanced in the presence of antibodies. However, histopathological examinations, even in immunized mice, showed infiltration of granulocytes predominantly and less mononuclear cells in the kidney, which slowly changes at varying times after challenge with *C. albicans* (22). This slow infiltration of mononuclear cells may help explain the unique susceptibility of the kidney to *Candida* infections (22), which would restrict antibody-mediated protection through mononuclear cells to only the later stages of *Candida* infection. Immunotherapy with specific antibody, therefore, seems to protect predominantly the organs of the reticuloendothelial system whereas other interactions are needed to enhance resistance against renal candidiasis (22).

There are still a number of remaining questions as to the mechanism involved of these antibody protective effects. Probably, antibody dependent antigen presentation to T-cell may have been involved, allowing earlier recognition of *C. albicans* cells, probably combined with enhancement of cell mediated mechanisms, which might have resulted in increased survival. Therefore, we conclude, as have others, that protective immunity against systemic candidiasis involves a combination of innate defenses, humoral and

cellular immunity (11).

In conclusion, we have shown that enolase-specific antibodies provide partial protection against lethal *C. albicans* infections, an observation that warrants their further evaluation in future studies.

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CHAPTER 8

GENERAL DISCUSSION

C. albicans is an opportunistic pathogen of the human gastrointestinal tract that takes advantages of factors which decrease the normal host resistance and increase the concentration of yeast in the gut such that it can invade across the mucosa of the gastrointestinal tract and disseminate (1, 2, 3). Haematogenous dissemination of *C. albicans* can lead to infection in virtually every organ or tissue of the human body. These infections are among the most important causes of morbidity and mortality among patients with cancer and those that are otherwise severely immunocompromised (1). Despite awareness of the seriousness of the *Candida* infections, diagnosis of invasive candidiasis remains difficult (4).

The aim of this study was to develop diagnostic techniques which are easy to apply and have a high sensitivity and specificity for diagnosing invasive candidiasis. Diagnostic tests can be divided into culture dependent and non-culture dependent techniques. By microbiological techniques the yeast is demonstrated in patient specimens by culture or microscopy. Non-culture techniques include the detection of anti-*Candida* antibodies, antigens, metabolic products of *Candida*, and more recently the demonstration of species-specific DNA or RNA sequences, in blood or other body fluids.

Anti-*Candida* antibodies were studied extensively in the 1960s and 1970s (5-9). The major drawback of these early studies is that crude undefined non-standardized antigen preparations were used for antibody detection (10, 11). Also, for evaluation of the assays often not properly characterized patient populations were used; immunodeficient patients and immunocompetent patients were combined within one group or patients with oesophagitis were classified as having invasive candidiasis (12). The specificity of the assays was often related to undefined control groups (13, 14). We describe the diagnostic use of serological assays by making use of well-characterized antigens for antibody detection. Assays were evaluated in sera of immunodeficient and immunocompetent patients with invasive candidiasis and in sera of matched control groups of *C. albicans* colonized patients. We showed that the diagnostic accuracy of antibody detection by a counter immunoelectrophoresis technique in a group of surgical patients could be improved by using cytoplasmic antigens depleted of mannan and glycoproteins. The specificity of the counter immunoelectrophoresis assay increased from 28.6% to 78.6% if the cytoplasmic antigen was depleted of mannan; the sensitivity slightly decreased from 80 to 70%.

Since cytoplasmic antigens are released during invasive infection, it is conceivable that such antigens when applied in an antibody detection assay, could discriminate between invasive infection and colonization (10). Matthews et al. (15) and Porsius et al.

(16) analyzed the antibody response by Western blotting and demonstrated that antibodies directed against an immunodominant antigen of *C. albicans* with a molecular mass of 47 kDa correlated with invasive infection. However, several immunodominant antigens with different molecular weight have been identified (17, 18, 19, 20, 21). Probably all these immunodominant cytoplasmic antigens are either identical or closely related.

The immunodominant antigen was characterized by Western blot analysis and tandem crossed immunoelectrophoresis as the glycolytic enolase enzyme with a molecular weight of 45 kDa. By using the enzyme-linked immunosorbent assay (ELISA), the diagnostic sensitivity and specificity of antibodies directed against this purified enolase antigen was evaluated. The anti-enolase antibody response was measured in sera of immunocompetent and immunocompromised patients with either invasive candidiasis or *C. albicans* colonization. The diagnostic specificity of the enolase ELISA was high up to 86% for the immunocompetent patient group. However, even if multiple serum samples were used the gain in specificity was counteracted by a loss of sensitivity, i.e. a decrease from 80 to 50% compared with that of the counter immunoelectrophoresis with crude cytoplasmic antigen. This loss in sensitivity probably cannot be amended by using more sensitive detection assays, but rather calls for a combination of tests with other antibody, antigen, or metabolite detection assays. We further showed that by using healthy donors as a control group the specificity reached 100%, indicating the importance of a matched control group in determining the specificity of a diagnostic assay. For the immunodeficient patient group the anti-enolase ELISA showed significantly higher titers in the sera of patients with invasive candidiasis than in the sera of patients only colonized by *C. albicans*, however these patients generally showed lower anti-enolase antibody titers than those found in the sera of immunocompetent patients. In extremes, patients with leukemia and invasive candidiasis did not have detectable anti-enolase antibodies at all.

In the immunodeficient patient group the use of a crude antigen for antibody detection had a higher sensitivity than the same antigen depleted of mannan. Therefore, an assay with an acceptable sensitivity might be obtained in the immunocompromised patient by detecting anti-mannan antibodies. Since anti-mannan antibodies are universally present in sera of patients with either invasive candidiasis, *Candida* colonization or healthy individuals (22), our test assay was standardized and a cut off value was applied in order to obtain an acceptable specificity. Furthermore, to gain insight into the antibody kinetics of anti-mannan and anti-cytoplasmic antibodies, patients were followed serologically in time. Serial evaluation of test results is important because serological test results of a single serum sample are heavily biased by the moment of blood sampling in relation to the usually unknown stage of the *Candida* infection. Thus, improper sampling may give rise to false negative results. The increase in anti-mannan antibody levels was demonstrated to be an early indicator for invasive candidiasis. This rapid rise in anti-mannan antibodies is probably due to a secondary immuneresponse. We therefore,

recommend to search for increases in anti-mannan antibody titers in several sera of immunocompromised patients during the progression of infection since this avoids the limitations of interpreting the test results of a single serum sample (23, 24, 25). The clinical usefulness of follow-up of anti-mannan antibody titers for detection of *Candida* infections was recently confirmed in a study of children with malignant disease and invasive candidiasis (26). However, anti-mannan antibodies in this particular study were not early indicators for invasive candidiasis since the children's antibody response is a primary and not a secondary immuneresponse as is the case with adult patients. Similarly determining increases in anti-enolase antibodies in several sera of immunocompetent patients will be very useful in confirming a diagnosis of invasive infection in such categories of patients.

Interestingly, antigen titers in sera of immunocompromised patients, although discriminating between invasive candidiasis and *C. albicans* colonization, did not correlate with the pathogenesis of the *Candida* infection process. We demonstrated that antigen detection in immunocompromised patients is usually not the test of choice. Probably, the processing of yeast cells by phagocytic cells is necessary for the release of sufficient amounts of antigens into the circulation (27). In an attempt to improve the predictive value of the assay with respect to invasive candidiasis the results of the antibody assays were combined with the antigen titers obtained by the Cand-Tec assay. However, no significant increase in predictive power was obtained by this combination as determined by multivariable analysis. Research for the detection of new antigens from yeast cells or hyphae of *Candida* spp. related to invasive candidiasis is ongoing. In this respect, *Candida* metabolites are believed to be very important clues that can be used for diagnosis. Especially, the detection of D-arabinitol and 1,3-ß-D-glucan (28, 29, 30, 31, 32). Unfortunately, there is yet no commercial test for these metabolites available that can be used in clinical laboratories.

Efforts to further improve the diagnosis of invasive candidiasis by serological assays are still going on, but it is highly questionable whether the sensitivity and specificity of one single test can be optimalized to become clinically relevant. The combined use of the anti-mannan and anti-enolase antibody detection assays in relation to the underlying disease of the patient would probably improve the accuracy of a diagnosis of invasive infection. Further improvements are possibly accomplished by detecting specific IgG, IgM and/or IgA antibodies directed towards these mannan and enolase antigens.

Since the sensitivity of a serological assay is influenced by the immunestatus of the patient and the moment of sampling during the infection process, we evaluated the PCR as a diagnostic tool in the detection of invasive candidiasis. Our PCR makes use of primers aimed at the multicopy gene coding for the small subunit rRNA resulting in the synthesis of a 180 bp fragment. With the use of a *C. albicans* specific probe, 10 to 15 *C. albicans* cells could be detected in 100 μl of whole blood by Southern analysis. PCR analysis of blood specimens collected from mice with invasive candidiasis was more sensitive than blood cultures (100% versus 67%, respectively). Furthermore, the intensity

of the hybridization signals increased with the progression of infection. Negative results by PCR were found for mice gastrointestinally colonized only. This observation underlines the specificity of this PCR method. However, the lower limit of *Candida* cells that could be detected in blood samples could not further be improved by using more target DNA or other sample preparations. Improvements in PCR methodology, by using other strategies for *Candida* cell isolation from clinical specimens or by using rRNA may in the future lead to acceptable levels of sensitivity. The amplification of rRNA by nucleic acid sequence-based amplification is also faster than PCR (33), and its amplification enzymes potentially are less sensitive to inhibitory factors in blood which makes it an interesting technique to be compared to PCR.

Apart from helping to diagnose invasive candidiasis, an ideal assay should also help in monitoring the response to treatment and, thus, in determining the duration of therapy. We used PCR in monitoring the therapeutic efficacy to antifungal treatment. This prognostic aspect of the PCR assay was evaluated in a model of lethal *C. albicans* infection in neutropenic mice, who were treated with the antifungal agent AmBisome® at doses of 1, 7 and 14 mg AmB/kg body weight, respectively. Blood cultures of infected mice treated with three different doses of AmBisome® were only positive at the early onset of the infection process and became sterile after three days even in those mice that had a relapse of infection 14 days later. However, this relapse of infection as expressed in the number of CFU in the kidney, correlated well with the hybridization signals of the PCR. PCR data correlated well with the therapeutic response to antifungal treatment. However, to establish its clinical value this PCR warrants further evaluation in patients at risk for candidiasis. Further improvements in PCR technology involve, among others, reducing the number of steps in the DNA extraction procedure.

Besides improving the diagnosis of invasive candidiasis by developing sensitive and specific assays, we also studied the mechanism of host defense against *C. albicans* infections. The mechanism of host defense against *C. albicans* infections has been the subject of great interest and controversy for many years (34, 35, 36, 37, 38, 39). Generally, the humoral response is thought to be less important in host resistance to *Candida* spp. than cellular immune factors; however, several studies have suggested that anti-*Candida* antibodies may protect against invasive candidiasis. We evaluated the protective capacities of antibodies directed against the immunodominant enolase antigen of *C. albicans*, and compared its activity to that of antibodies directed against a crude *C. albicans* antigen extract and that of a monoclonal antibody directed against mannan. Passive transfer of anti-enolase antibodies and of antibodies directed against a crude *C. albicans* extract provided significant protection against lethal *C. albicans* infections in mice. In contrast, no protection was obtained with the monoclonal antibody against mannan. The immunoprotection was not due to better initial clearance of *C. albicans* from the internal organs but was presumably the result of an as yet unknown effect of antibodies occurring later in the infection process. The in vivo results could also not be ascribed to enhanced opsonization of the yeast cells as observed in in vitro opsonization

experiments. The protective effect observed with anti-enolase antibodies can be due to enhanced T- and B-lymphocyte functions, the stimulation of cytotoxic activity of peripheral blood mononuclear cells and natural killer cells or they could assists in the chemotaxis of leucocytes to the site of infection and enhance phagocytosis of *Candida* cells. Undoubtly none of these reactions occur independently but indicate that humoral factors and cellular factors may cooperate in the defense against invasive candidiasis. One may speculate that the clinical application of enolase-specific antibodies may retard or delay the infectious process, and increase the effectiveness of antifungal therapy. However, this hypothesis warrants further evaluation in future studies.

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SUMMARY

During the past decades the frequency of invasive *Candida* infections has increased significantly. This is primarily due to a growing group of immunocompromised patients, who due to new kinds of medical treatment are kept alive for prolonged periods of time. However, these patients carry several risk factors for obtaining invasive candidiasis. These risk factors include the use of cytostatic and immunosuppressive agents, the use of broad-spectrum antibiotics, and long periods of central vein catheters, or the presence of other accessories to the tissue.

The greater part of the invasive infections is still caused by *Candida albicans*. However, other *Candida* species are isolated with increased frequency from infected patients. These yeasts are opportunistic pathogens and belong to the normal human gastrointestinal system and live under normal circumstances in balance with the cells of the skin or mucous membranes of the host. Disturbance of this balance due to the previous mentioned risk factors, can lead to overgrow of these yeasts resulting in superficial infections of the mucous membranes or invasive infections of virtually every organ of the body.

Superficial infections are relatively easy to recognize and can usually be treated with topical agents. In contrast, invasive infections have no specific clinical picture and require systemic treatment. Because systemic antifungal agents are toxic it is important to distinguish superficial infections from invasive infections.

The diagnosis of invasive candidiasis is difficult: biopsies cannot be performed routinely, and blood cultures often remain negative. On the other hand isolation of *C. albicans* from sputum, urine, or feces is no proof of invasive candidiasis. A correct diagnosis is very important since mortality is high in patients with invasive candidiasis who are not promptly treated systemically with toxic antifungal agents. Therefore, serodiagnostic results are of growing importance in diagnosing invasive candidiasis. Serodiagnosis is currently not fully reliable due to the commensal character of the yeasts, which can cause false-positive results, or can lead to false-negative results due to a lack of an immuneresponse in immunocompromised patients.

It is furthermore known that antibodies directed against the cell wall mannan, are universally present in patients with invasive candidiasis, in patients with superficial infections, and in healthy individuals. Therefore, antigens related to the invasive form of infection have been used for the detection of antibodies directed against *Candida* species.

In this thesis the usefulness of antibodies directed against cytoplasmic antigens in the diagnosis of invasive candidiasis in immunocompetent patients was investigated (Chapter 2). The antibody response directed against mannan-free cytoplasmic antigens [M(-)] was compared with the antibody response directed against a crude *C. albicans*

extract including cell wall mannan [M(+)], by using the counterimmunoelectrophoresis technique (CIE). Both antibody responses were evaluated in sera of surgical patients with either invasive or superficial candidiasis. These results showed an increased diagnostic specificity by using M(-) CIE (78.6%) compared to M(+) CIE (28.6%), with a minimum loss in diagnostic sensitivity from 80 to 70% which makes it a more reliable diagnostic assay for this particular patient group. It was further shown that during the first two weeks after the clinical signs of invasive candidiasis the amount of positive antibody titers increased from 50 to 67%. This work provided further evidence that the use of cytoplasmic antigens of *C. albicans* for the detection of antibodies improved the serodiagnosis of invasive candidiasis for the immunocompetent patients.

To further improve serodiagnosis of invasive candidiasis we purified an immunodominant antigen, a glycolytic enolase enzyme, in order to detect specific antibodies. The anti-enolase antibody response was evaluated in sera of immunocompetent and immunocompromised patients with either invasive candidiasis or *C. albicans* colonization (Chapter 3). The enzyme-linked immunosorbent assay (ELISA) was used in order to develop a quantitative and sensitive antibody detection assay. The diagnostic specificity of the enolase ELISA was high up to 86% for the immunocompetent patient group. Despite the use of multiple serum samples, the increase in specificity was counteracted by a loss of sensitivity, i.e. a decrease from 80 to 50% compared with that of the M(+)CIE. However, the good specificity of the enolase ELISA may well support the clinician in making a decision allowing to or withhold toxic antifungal agents. For the immunodeficient patient group, the enolase ELISA showed significantly higher titers in the sera of patients with invasive candidiasis than in the sera of patients only colonized by *C. albicans*. However, these patients generally showed lower anti-enolase antibody titers compared to immunocompetent patients. Patients with leukemia and invasive candidiasis did not have detectable anti-enolase antibodies at all (Chapter 3). The detection of anti-mannan antibodies would probably be more accurate for serodiagnosis of invasive candidiasis in immunodeficient patients.

Serial determination of antibody responses against cytoplasmic antigens and a crude *Candida* extract as well as antigen detection was evaluated in immunocompromised patients (Chapter 4). The increase in anti-mannan antibody levels was demonstrated to be an early indicator for invasive candidiasis in these patients. This study illustrated that the anti-mannan antibody determination is of value in immunocompromised patients to confirm the diagnosis of *Candida* infection and that it can be used to follow the progression or resolution of systemic candidiasis. Measuring the kinetics of the antibody titers during the pathogenesis of a systemic *Candida* infection also overcomes the limitation of interpreting the test results of a single serum sample. Titers of antigen, although discriminating between invasive candidiasis and *C. albicans* colonization, did not correlate well with the infectious process of candidiasis. Therefore, antigen detection does not seem to be the test of choice in immunocompromised

patients. It was considered that different serological criteria are necessary for the diagnosis of invasive candidiasis in patients with different kinds of underlying diseases, e.g. leukemic patients and patients undergoing surgery.

The diagnosis of invasive candidiasis could possibly be improved by the use of molecular techniques. Small quantities of cells can then be detected in patient specimens. These methods are also faster than the long incubation periods needed in classical culture techniques. The polymerase chain reaction (PCR) was used for the amplification of a small subunit ribosomal RNA gene that is conserved among many fungi. With the use of a *C. albicans*-specific probe, 10 to 15 *C. albicans* cells were detected in 100 μl of whole blood by Southern analysis (Chapter 5). The diagnostic value of this PCR was evaluated in gastrointestinally colonized mice and neutropenic mice with invasive candidiasis. Negative results by the PCR were found for gastrointestinally colonized mice which underlines the specificity of this PCR method. PCR analysis of blood specimens collected from mice with invasive candidiasis were more often positive than blood culture, 100% compared to 67% determined at 72 hours after the onset of infection (Chapter 5). Furthermore, the intensity of the hybridization signals increased with the progression of infection. This is what might make the PCR assay useful in the follow-up of invasive candidiasis during treatment.

This prognostic aspect of the PCR assay was evaluated in a model of a lethal *C. albicans* infection in neutropenic mice, who were treated with different doses of liposomal Amphotericin B (AmBisome) (1, 7, and 14 mg of AmB per kg of bodyweight) in order to establish different courses of the infectious process (Chapter 6). Mice treated with 4 daily doses of 7 or 14 mg of AmB per kg were culture sterile at 35 days after inoculation with 10^4 CFU *C. albicans*, whereas the lungs, livers, and spleens of mice treated for 4 days with 1 mg of AmB per kg experienced a relapse of infection 21 days after inoculation with 10^4 CFU *C. albicans*. This relapse of infection correlated with an increased intensity of the hybridization signals of the PCR, whereas blood cultures of the same blood samples remained culture sterile. Again the PCR proved to be more sensitive than blood culture and correlated with the course of infection during antifungal therapy.

Besides the diagnostic use of antibodies directed against several antigens of *C. albicans*, their role in the mechanism of host defense against invasive candidiasis has been the subject of great interest and controversy for many years. In Chapter 7 we evaluated the protective capacities of antibodies directed against the immunodominant enolase antigen of *C. albicans*, and compared its activity to that of antibodies directed against a crude *C. albicans* antigen extract and a monoclonal antibody directed against mannan (MAB 18C11). Passive transfer of anti-enolase antibodies, raised in mice (IMS) and antibodies directed against a crude *C. albicans* extract, raised in rabbits (IRS) when repeatedly administered, provided partial protection against lethal *C. albicans* infections in mice, whereas no protection was obtained with the MAB 18C11. The immunoprotection was not due to an initial clearance of *C. albicans* from the internal

organs but was presumably the result of as yet unknown effects of antibodies occurring later in the infection process. The in vivo results could not be ascribed to enhanced opsonization of the yeast cells as observed in in vitro opsonization experiments using peritoneal macrophage monolayers. The use however of this observed protection of enolase-specific antibodies against lethal *C. albicans* infection may first permit a delay of the infectious process which may be very important in the effective treatment with antifungal agents, but warrants further evaluation in future studies.

SAMENVATTING

De laatste decennia is het aantal invasieve *Candida* infecties toegenomen. Dit heeft met name te maken met een groeiende groep van immuun gecompromiteerde patiënten die dankzij nieuwe behandelingsmethoden lange tijd in leven kunnen worden gehouden. Deze patiënten dragen echter één of meerdere risicofactoren voor het krijgen van een invasieve *Candida* infectie met zich mee. Onder deze risicofactoren verstaan we het gebruik van cytostatica en immuunsuppresiva, het gebruik van breedspectrum antibiotica en het langdurig in situ verblijf van intraveneuze catheters.

Het merendeel van de invasieve infecties wordt nog altijd veroorzaakt door *Candida albicans*. Er worden echter steeds vaker andere *Candida* soorten bij geïnfecteerde patiënten geïsoleerd. Deze gisten zijn opportunisten en behoren dan ook tot de normale flora van het maagdarm-kanaal en leven daar onder normale omstandigheden in evenwicht met de cellen van de huid en slijmvliezen van de gastheer. Verstoring van deze balans als gevolg van bovengenoemde risicofactoren, kan leiden tot uitgroei van deze gisten hetgeen kan resulteren in oppervlakkige infecties van de slijmvliezen of in gedissemineerde infecties waarbij via het bloed vrijwel elk orgaan van het lichaam geïnfecteerd kan raken.

Oppervlakkige infecties zijn gemakkelijk herkenbaar en kunnen in het algemeen behandeld worden met lokale middelen, terwijl gedissemineerde infecties geen karakteristiek beeld tonen en altijd systemisch behandeld moeten worden. Het is dus van belang om deze vormen van infectie van elkaar te kunnen onderscheiden in verband met de toxiciteit van de huidige antifungale middelen die gebruikt worden bij systemische infecties.

De diagnostiek van invasieve *Candida* infecties is erg moeilijk; het nemen van biopten is meestal niet mogelijk en bloedkweken zijn ondanks het gebruik van de gevoelige lysiscentrifuge methode nog vaak negatief. Anderzijds is het aantonen van *C. albicans* in sputum, urine en feces geen bewijs voor invasieve candidiasis. De correcte diagnose is echter van groot belang voor de patiënt, gezien het vaak fatale verloop van invasieve infecties, met name wanneer er niet behandeld wordt. Men is dan ook steeds meer aangewezen op de uitslagen van serodiagnostisch onderzoek. De betrouwbaarheid van serologisch onderzoek laat echter te wensen over. Dit heeft te maken met: a, het commensale karakter van verschillende gistsoorten hetgeen kan leiden tot fout-positieve uitslagen en b, het niet optreden van een immuunrespons bij immuun gecompromiteerde patiënten hetgeen nogal eens fout-negatieve uitslagen geeft.

Het is bekend dat antilichamen gericht tegen de buitenkant van de gist, het celwand-mannan, algemeen voorkomen bij patiënten met een invasieve candidiasis, patiënten met een *Candida* kolonisatie of bij gezonde personen. Voor het aantonen van antilichamen gericht tegen *Candida*-soorten is gezocht naar antigenen die gerelateerd zijn

aan de invasieve vorm van infectie.

In ons onderzoek is gekeken naar de bruikbaarheid van antilichamen gericht tegen cytoplasmatische antigenen voor de diagnostiek van invasieve candidiasis (Hoofdstuk 2). Met behulp van de counterimmunoelectrophorese (CIE), een immunodiffusie techiek, zijn zowel de antilichamen gericht tegen cytoplasmatische antigenen [M(-)CIE] bepaald als ook tegen een ruw *Candida* extract [M(+)CIE] welke nog het celwand-mannan bevat. Beide antilichaam titers zijn bepaald in sera van immunocompetente patiënten met een *Candida* kolonisatie of met een invasieve candidiasis. De specificiteit van de M(-)CIE (78.6%) was hoger in vergelijking met de specificiteit van de M(+)CIE (28.6%), hetgeen gepaard ging met een beperkt verlies in gevoeligheid van 80% voor de M(+)CIE naar 70% voor de M(-)CIE. Het gebruik van cytoplasmatische antigenen ontdaan van mannan voor de detectie van antilichamen verbetert de serodiagnostiek van invasieve candidiasis in immunocompetente patiënten.

Het gebruik van cytoplasmatische antigenen werd verder uitgebouwd door gebruik te maken van een compleet gezuiverd immunodominant antigeen, het glycolytisch enolase enzym, voor de detectie van antilichamen in sera van immunocompetente en immunodeficiente patiënten met invasieve candidiasis of een *C. albicans* kolonisatie (Hoofdstuk 3). Om eventueel verlies in gevoeligheid van de assay te beperken, werd gebruik gemaakt van een gevoelige antilichaam detectiemethode, namelijk de enzyme-linked immunosorbent assay (ELISA). Desondanks bleek de enolase ELISA, bij immunocompetente patiënten, een minder gevoelige diagnostische methode (50%) dan de M(+)CIE (80%). De goede specificiteit van de enolase ELISA (86%) ondersteunt de diagnose invasieve candidiasis in tegenstelling tot de M(+)CIE. Bij immunodeficiente patiënten, met name leukemie patiënten met een invasieve candidiasis heeft de enolase ELISA een beperkte waarde. Bij deze patiënten konden namelijk geen anti-enolase antilichamen worden aangetoond. Voor hen is waarschijnlijk antigeen detectie of de anti-mannan antilichaam detectie diagnostisch waardevoller.

In een drietal immunodeficiente patiënten met invasieve candidiasis werd de produktie van antilichamen gericht tegen cytoplasmatische antigenen en tegen een ruw *Candida* extract als ook de antigeen vorming gedurende lange tijd bestudeerd (Hoofdstuk 4). Bij deze immuun gecompromiteerde patiënten bleek dat antilichamen gericht tegen het celwand-mannan en de detectie van antigenen een belangrijke rol spelen bij het stellen van de diagnose invasieve candidiasis. De toename in de concentratie van anti-mannan antilichamen is bruikbaar als een vroege indicator voor een actief infectie-proces. Deze waarneming werd bevestigd bij bestudering van een grotere groep immunodeficiente patiënten met invasieve candidiasis (Hoofdstuk 4). In deze studie bleek verder dat de antigeen-detectie niet correleerde met het infectie proces en daardoor niet automatisch als optimale serologische test bij immuun gecompromiteerde beschouwd mag worden. Het gebruik van serologische testen is dus afhankelijk van het onderliggend lijden van de patiënt en uitslagen van serologische testen bij bijvoorbeeld leukemie of

chirurgische patiënten eisen dan ook een andere interpretatie.

Moleculair biologische technieken zijn waardevol gebleken bij het aantonen van verschillende bacteriële infecties en zouden tevens de diagnostiek van invasive *Candida* infecties kunnen verbeteren. Deze techniek biedt namelijk de mogelijkheid om kleine hoeveelheden cellen in klinisch materiaal aan te tonen. De methode is vaak sneller dan bloedkweken, omdat deze langdurig moeten worden geïncubeerd voordat zij als negatief kunnen worden beschouwd. In dit onderzoek is gebruik gemaakt van de enzymatische amplificatie van het gen coderend voor het small subunit ribosomale 18S RNA met behulp van de polymerase chain reaction (PCR). Het geamplificeerde produkt is niet specifiek voor *C. albicans* maar is geconserveerd over verschillende eukaryoten. Met gebruikmaking van species-specifieke probes en hybridisatie technieken kon het amplificaat van 10 tot 15 *C. albicans* cellen in 100 μl bloed specifiek worden aangetoond (Hoofdstuk 6).

De diagnostische waarde van de PCR is onderzocht in een muizenmodel met een maagdarm-kolonisatie en in een muizenmodel met invasieve candidiasis. Negatieve hybridisatiesignalen en negatieve bloedkweekresultaten werden verkregen van bloed afgenomen bij muizen met een maagdarm-kolonisatie met *C. albicans*. In muizen met een invasieve candidiasis bleek de PCR vaker positief dan de bloedkweek. Tweeënzeventig uur na het aanbrengen van de infectie bleek 100% van de muizen een positieve PCR reactie te hebben terwijl slechts 67% positieve bloedkweken had (Hoofdstuk 6). Daarnaast bleek de intensiteit van het hybridisatie-signaal toe te nemen naarmate het infectie proces vorderde. Het hybridisatie-signaal leek gecorreleerd te zijn aan de intensiteit van de *Candida* infectie. De PCR-techniek zou dus gebruikt kunnen worden in het vervolgen van de *Candida* infectie tijdens bijvoorbeeld antifungale behandeling.

Dit prognostische aspect van de PCR werd bestudeerd in muizen met een lethale *C. albicans* infectie die effectief of partieel behandeld werden met verschillende concentraties liposomaal Amphotericine B (Ambisome®) (1, 7 en 14 mg AmB/kg). Na 4 dagelijkse behandelingen met 1 mg AmB/kg groeide de *Candida* infectie bij deze muizen weer uit terwijl 4 behandelingen met 7 of 14 mg AmB/kg steriele muizen op basis van kweekresultaten gaf. Het hybridisatie-signaal van de PCR uitgevoerd op bloed bleek te correleren met de mate van infectie. Dit was niet het geval met de bloedkweken die meestal negatief waren. Dit betekent dat de PCR gebruikt kan worden voor het vervolgen van een *Candida* infectie tijdens antifungale behandeling. Voor het vaststellen van de bruikbaarheid van de PCR als diagnostische en prognostische test is verder onderzoek bij patiënten noodzakelijk.

Naast de diagnostische waarde van antilichamen gericht tegen verschillende antigenen van *C. albicans*, staat hun rol in het afweermechanisme tegen een invasieve *Candida* infectie al geruime tijd in de belangstelling. Het belang van antilichamen in de afweer tegen candidiasis werd bestudeerd in een muizenmodel, waarbij immunotransfer

werd uitgevoerd met verschillende antilichamen gericht tegen *C. albicans*. Deze antilichamen zijn gericht tegen het immunodominante enolase, tegen een ruw *C. albicans* extract en tegen het celwand-mannan (Hoofdstuk 5). Het anti-enolase antiserum (IMS) en het anti-mannan antiserum (MAB 18C11), beide opgewekt in muizen en het anti-ruw *C. albicans* antiserum opgewekt in konijnen (IRS), werden herhaaldelijk ingespoten in muizen met een dodelijk verlopende invasieve *Candida* infectie. Het IMS en IRS maar niet het MAB 18C11, gaven een gedeeltelijke bescherming. Dit beschermend effect kon niet worden verklaard door een verbeterde opruiming van de gistcellen in de verschillende organen, of door een andere verdeling over de verschillende organen, in een vroeg stadium van het infectieproces. Eveneens kon er geen verschil worden aangetoond in de mate waarin de met verschillende immuunsera en normaal sera geopsoniseerde *C. albicans* cellen werden gefagocyteerd door peritoneaal macrofagen in in vitro fagocytose experimenten. Een verklaring voor het beschermend effect van anti-enolase antilichamen kan nog niet gegeven worden verder onderzoek naar de werkingsmechanisme zal nodig zijn.

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