

Enhanced IgE response to *Candida albicans* in postoperative invasive candidiasis

J. SAVOLAINEN, A. RANTALA*, M. NERMES†, L. LEHTONEN‡ and M. VIANDER‡

Departments of Clinical Allergology, *Surgery, †Paediatrics and ‡Medical Microbiology, MediCity Research Laboratory, University of Turku, Turku, Finland

Summary

Background Invasive candidiasis is a life-threatening complication problem in post-operative and immunocompromized patients, e.g. those treated by intensive care. *Candida* is frequently cultured from the mucous membranes of hospital patients and fungal cultures offer little diagnostic help. Other diagnostic methods, such as blood cultures, serology and diagnostic imaging techniques produce results too late and, if positive, low sensitivity.

Objective To study the value of *Candida*-specific antibodies, especially those of IgE class, in diagnosing invasive *Candida* infection.

Methods The immunoglobulins IgE, IgG and IgM responses to antigens of *Candida albicans* in the sera of 14 patients with culture, biopsy and/or autopsy proven postoperative invasive candidiasis and of 11 colonized and 19 non-colonized operated patients were studied by mannan radioallergosorbent test (RAST), mannan enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

Results Detection of IgE antibodies to *C. albicans* polysaccharide (mannan) and protein antigens proved specific and sensitive in diagnostics of invasive candidiasis after major abdominal surgery. IgE rose early in the course of the infection and the method made a clear distinction between invasive infection and mucous colonization. Immunoblotting for protein antibodies was most sensitive while nitrocellulose-RAST for mannan antibodies was most specific. The combined use of immunoblotting and RAST increased the sensitivity and the specificity. Determinations of anti-*Candida* IgG and IgM antibodies had low sensitivity and specificity.

Conclusion Critically ill patients with invasive candidiasis develop IgE antibodies to *Candida* antigens probably because of disturbed TH₁/TH₂ responses. Determination of specific IgE antibodies can be used as a diagnostic aid in the early stage of invasive *Candida* infection.

Keywords: *Candida albicans*, IgE, invasive candidiasis

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Introduction

Invasive fungal infections are a major therapeutic problem in intensive care patients, especially if they are immunocompromised [1]. The diagnosis of deep mycosis is often delayed because clinical signs may be subtle and there are no rapid and reliable diagnostic methods. Currently, the diagnosis of

invasive candidiasis is based, in addition to clinical suspicion, on blood culture, diagnostic imaging and tissue biopsies [2]. The sensitivity of *C. albicans*-specific serological markers and circulating antigens, like mannan [3] or enolase [4], or metabolites, like D-arabinitol [5] is usually low and positive results are usually obtained only late, when the disease is advanced [6].

Although phagocytic cells are important effectors of the defence against fungi, impaired cell-mediated immunity is the main cause for susceptibility to fungal infection. In

Correspondence: Dr J. Savolainen, Department of Clinical Allergology, Medicity, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland.

experimental murine models of invasive *C. albicans* infection, protection against fatal infection was associated with the development of T helper (TH₁) responses in contrast to TH₂ responses which were associated with susceptibility to infections [7–9]. Thus, the outcome of the disease seems, at least from this perspective, to result from a balance between host-protective TH₁ and disease-promoting TH₂ responses.

Determination of specific anti-*Candida* IgA, IgG and IgM antibodies cannot generally be used for diagnosing invasive *Candida* infections, since these antibodies are common also in normal subjects [10,11] and may be absent in severely immunocompromized subjects. *Candida*-specific IgE antibodies occur in atopic subjects and are associated with severe atopic dermatitis and asthma [10,12]. This IgE response has been attributed to a predominance of TH₂ over TH₁ cells [13]. Since fatal invasive candidiasis in mice is associated with a TH₂ response [7–9] the present study was undertaken to investigate the role of IgE-antibodies in human invasive candidiasis.

Patients, materials and methods

Patients

Forty-four patients who underwent abdominal surgery at the Department of Surgery, University of Turku, Finland, participated in the study. All of the patients had a leucocyte count $> 2.0 \times 10^9/L$. Serial serum samples were collected during the hospital stay and stored at -70°C . The severity of the fungal infection was determined retrospectively on the basis of the clinical course and on clinical, microbiological and autopsy records. All patient samples were analysed retrospectively, and the results had no effect on the therapeutic decisions. Samples were taken when signs of septic infection or a superficial infection seen during the first postoperative week. Fourteen patients (mean age 56.2, range 35–88, 11 male/three female) had postoperative invasive candidiasis and 39 samples from them were analyzed. Eleven patients (mean age 52.6, range 27–80, seven male/four female) had superficial *Candida* colonization (16 samples) and 19 patients (mean age 56.5, range 24–82, 14 male/six female) were non-colonized operated, hospitalized controls (24 samples).

The criteria for including patients in the group of invasive candidiasis were any one of the following: positive histology or culture from deep organ of *Candida* at autopsy; more than one blood culture positive for *Candida* and clinical signs of septicemia; culture of *Candida* positive in normally sterile body site (other than urine); culture of *Candida* positive from central catheter tip and from cutaneous lesions with clinical signs of septicemia; or *Candida* endophthalmitis by fundoscopy together with clinical signs of septicemia. Patients whose urine was positive for *Candida* were

excluded because of the difficulty to interpret the severity of infection. Multiple criteria were accepted only if the same species of *Candida* grew in culture. Patients with colonization were defined as those patients who were hospitalized for over a week after the operation and who had *Candida* isolated from clinical specimens other than blood, visceral organs, deep abscess or urine and had no signs of septicemia. The control group consisted of postoperative patients from whom no *Candida* was isolated, but from whom at least two samples were available. According to the patient files of the Turku University Central Hospital two patients had a history of atopic diseases, i.e. asthma, allergic rhinoconjunctivitis or atopic dermatitis. These patients belonged to the group of colonized controls.

Immunoassays

IgE-, IgM- and IgG-antibodies against the protein antigens were analysed by immunoblotting and IgE-antibodies against a polysaccharide (mannan) by nitrocellulose RAST, and IgM- and IgG-antibodies against mannan with ELISA. For immunoblotting studies a purified protein fraction of a crude cytoplasmic extract was prepared, while chemically purified mannan was used for nitrocellulose RAST and ELISA.

Preparation of antigens The *C. albicans* strain 72024 was supplied and typed by the Mycological Laboratory of the Department of Dermatology, University of Turku and grown in a fermentor in peptone-supplemented medium as previously described [14]. A crude cytoplasmic extract was prepared by disruption of the yeast cells with a Manton-Gaulin homogenizer after extensive washing as previously described [14]. The purified protein extract was prepared in a carbohydrate-free form by Concanavalin A (ConA) affinity chromatography purification as previously described [15,16]. Mannan was prepared according to the Peat method by degradation under alkaline conditions and separation by Fehling's precipitation [17]. The mannan fraction with a molecular weight >100 kDa was separated by Amicon ultrafiltration (Amicon Co, Danvers, Mass., USA) and used for RAST and ELISA [18].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The cytoplasmic antigen and molecular weight markers (Electrophoresis Low Molecular Weight Calibration Kit, Pharmacia-LKB Biotechnology, Uppsala, Sweden) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 5–17.5% gradient gel as described [19] and transferred to nitrocellulose sheets (Millipore 0.22 μm , Millipore Co, Bedford, Mass, USA) in a Transphor apparatus (LKB, Bromma, Sweden) as described [20]. The specific IgE-antibodies bound to allergens on nitrocellulose were detected with a validated

method [21] with beta-galactosidase-labelled anti-human IgE antibodies (Phadezym RAST, Pharmacia Diagnostics, Uppsala, Sweden). The IgM- and IgG-antibodies bound to the *C. albicans* antigens were detected with Horse radish peroxidase (HRP)-conjugated rabbit anti-human IgM and anti-human IgG antibodies (DAKO Immunoglobulins, Denmark) as previously described [22].

Mannan-RAST A modification [18] of the previously described method by Walsh *et al.* [23] and ^{125} I-labelled anti-IgE antibodies (Pharmacia, Uppsala, Sweden) were used. Discs of a diameter of 5.5 mm were cut from nitrocellulose sheets (Millipore, pore size 0.22 μ m, Millipore Co., Bedford, Mass., USA). The optimum concentration of mannan solution to be used for coupling the discs was sought from the concentration range from 15.6 μ g/mL to 4 mg/mL, and a concentration 0.1 mg/mL was found appropriate and was used for coupling the nitrocellulose discs. The discs were washed, air dried and stored at room temperature. Before use the discs were rehydrated and then blocked with phosphate buffered saline (PBS)-0.5% Tween 20 in a shaker at 37°C for 1 h. After three washes with 0.9% NaCl Tween 20, the discs were set in RAST tubes, one disc in each tube. 50 μ L of serum were applied into the tubes and the discs were incubated in serum at 37°C for 2 h. After three washes with 0.9% NaCl Tween 20, the ^{125} I-labelled anti-IgE was added for incubation at room temperature overnight. Finally the discs were washed three times after which the remaining bound radioactivity was detected by a gammacounter. The results were expressed as a mannan RAST index:

$$\frac{(\text{sample (cpm)} - \text{buffer (cpm)}) \times 10^2}{\text{total activity (cpm)} - \text{buffer (cpm)}}$$

The variation coefficients within and between the assays were determined by using sera of low, medium and high anti-mannan IgE responses; the coefficients ranged from 8% to 10.2%. Reference mannan RAST index values were determined by analysing samples from a non-atopic population. The mean index value of 102 non-atopic patients was 0.0817 (SD 0.12). Thus, 0.32 (mean + 2 SD) was considered as the limit for a positive mannan-RAST index value.

Mannan-ELISA The mannan-specific IgG and IgM antibodies were measured with an ELISA-technique as previously described [24]. Polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 50 μ L of the mannan antigen (0.5 mg/mL) in PBS, pH 7.3, and kept at +37°C overnight. After three washings with PBS the wells were saturated with 100 μ L of 1% normal sheep serum (NSS) in PBS at +37°C for 2 h. After the washings, 100 μ L of 1:200 diluted sera in 1% NSS in PBS were added and incubated at +37°C for 2 h. The wells were washed as previously and incubated with 75 μ L of a 1:300 dilution of

alkaline phosphatase-conjugated goat anti-human IgG, anti-human IgA and anti-human IgM (Orion Diagnostica, Helsinki, Finland) in 1% NSS in PBS at +37°C for 2 h. After additional washings, 75 μ L of *p*-nitrophenylphosphate in diethanolamine-MgCl₂ buffer (1 mg/mL; Orion Diagnostica, Helsinki, Finland) was added and incubated at +37°C for 30 min. The enzyme reaction was stopped with 120 μ L of 1 M NaOH. The absorbances were measured with a Multiscan Photometer (Labsystems, Helsinki, Finland) at a wavelength of 405 nm. All the tests were made in duplicates.

Serum arabinitol determination

Serum arabinitol levels were measured by gas chromatography-mass spectrometry (GC-MS) as previously described [25]. For derivatization, a modification of the procedure described by Roboz *et al.* [26] was used. HP 5890A gas chromatograph (Hewlett-Packard) with a capillary column (25 m long, 0.32 mm internal diameter, coated with Noribond SE-54 liquid stationary phase layer with a thickness of 0.1 μ m (Nordion Instruments, Finland) coupled directly to a VG TRIO-1 mass spectrometer (VG, UK) equipped with a Lab-Base Data System was used for gas GC-MS. The standard calibration plot for arabinitol was prepared by adding increasing amounts of authentic arabinitol to normal serum. Reference arabinitol levels were determined from 30 patients in the reference group. Arabinitol values were graded as strongly elevated (> mean + 4 SD) or slightly elevated (> mean + 2 SD < mean + 4 SD). Thus, arabinitol levels >1.23 μ g/mL were considered to be strongly elevated and concentrations 0.77–1.23 μ g/mL slightly elevated. When the coinciding serum creatinine was over 200 μ mol/L, arabinitol/creatinine ratios were used as modified from Gold *et al.* [27]. Ratios over 6.15×10^{-3} were considered strongly elevated and ratios over 3.85×10^{-3} slightly elevated.

Statistical analyses

Differences of means of normally distributed data were analysed by Student's *t*-test. The sensitivity of the assay was calculated as TP/(TP + FN) [4], and the specificity TN/(TN + FP) [4]. (TP = true positive, TN = true negative, FP = false positive and FN = false negative).

Results

Table 1 shows the patient data and the IgE-assay results. The first sample was taken as a representative in Table 1, Figs 1 and 2. Follow-up is presented in Fig. 3 when follow-up was sufficiently long (>2 weeks, samples available).

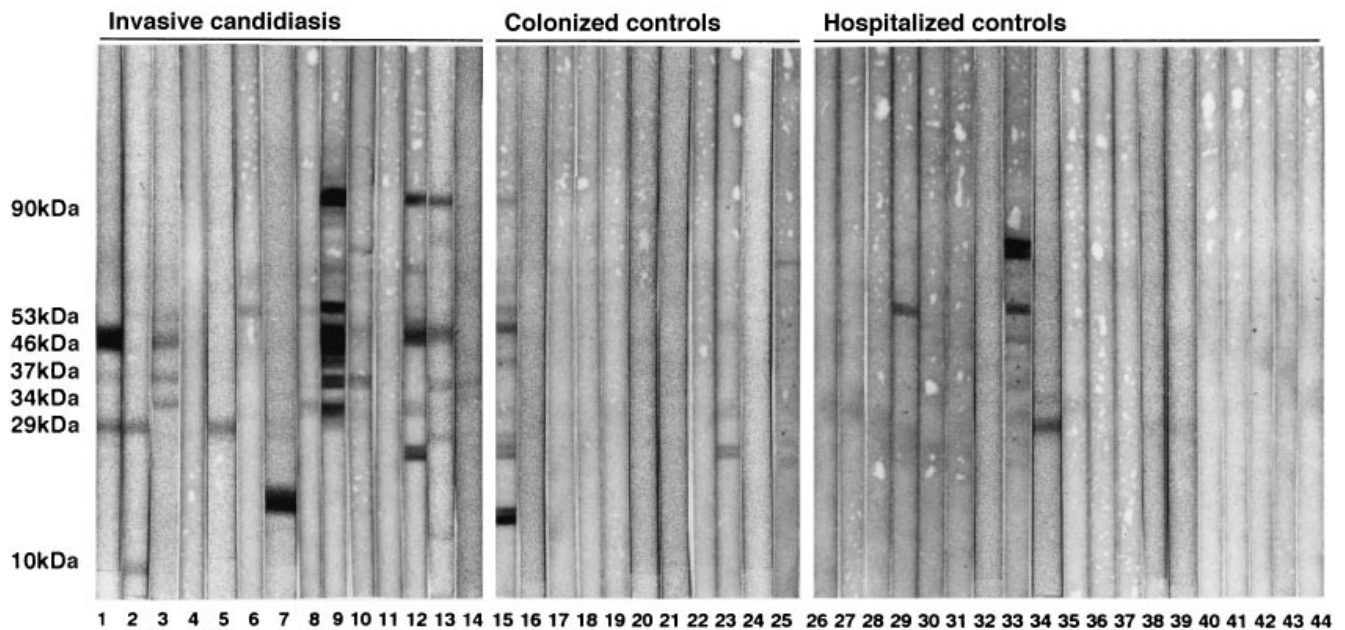


Fig. 1. IgE-immunoblotting of a purified protein preparation of *Candida albicans* allergen extract with patient sera. The most important allergenic peptides are indicated on the left.

IgE assays

IgE-Immunoblotting Immunoblotting was performed with the purified protein antigen devoid of mannan which made it also possible to analyse the high molecular weight protein bands. The IgE responses in different study groups are

presented in Fig. 1. IgE responses against protein bands were most common in the invasive candidiasis group (12/14) compared with colonized (3/11) and hospitalized (3/19) controls. The IgE-responses are directed against 12 different bands and the most important bands in the invasive candidiasis

Table 1. Patients with invasive candidiasis and their anti-*Candida albicans* IgE responses

	Age/Sex	Disease	Diagnostic criteria	Mannan-RAST*	IgE-binding bands§
1.	51/M	Enterocutaneous fistulae	Autopsy (Heart, lung, kidney)	0.981	46,29
2.	50/F	Colitis ulcerosa	Autopsy (Heart, lung), blood	0.338	29,10
3.	86/F	Intestinal strangulation	Autopsy (Liver, lung, kidney, heart)	0.468	46,37
4.	87/M	Intestinal haemorrhage	Autopsy (Lung, kidney), EO-itis	0.424	–
5.	51/M	Hepatic abscess	Bile cytology and culture	0.299	29
6.	60/M	Stricture of CBD	Bile culture	0.120	53
7.	33/M	Traumatic pancreas rupture	Abscess culture, bile culture	0.397	18
8.	35/M	Haemorrhagic pancreatitis	Abscess culture	0.565 Ú	53,34
9.	59/M	Cecal perforation	Abscess culture, EO-itis	9.487 Ú	90,53,46,41,37,33
10.	41/M	Crohn's disease, abscess	Blood cultures, EO-itis	0.555 Ú	37
11.	46/F	Perineal fascitis	Blood cultures, EO-itis	0.244	–
12.	54/M	Multiple burns	Blood cultures, EO-itis	1.005	90,46,33,25
13.	66/M	Duodenal ulcer, haemorrhage	Blood cultures	0.360	90,46,37,27
14.	62/M	Ischaemic colitis	Blood cultures	0.125	37

*Positive values in bold. §Molecular weight (kDa). M. Male. F. Female. EO-itis. Endophthalmitis. CBD. Common bile duct. ÚFollow-up presented in Fig. 3.

group are the 46 kDa and 37 kDa bands against which 36% (5/14) of the invasive candidiasis patients responded. There were no responses against the 46 kDa and 37 kDa bands in the control groups.

Mannan-RAST IgE-antibodies against *C. albicans* mannan in different patient groups are presented in Fig. 2. The levels are significantly higher in the invasive candidiasis group compared with both hospitalized ($P < 0.0005$) and colonized ($P < 0.005$) controls. Both atopic patients were negative for IgE immunoblotting and for mannan RAST.

Follow-up Figure 3 (a, b, c) present the follow-up of the mannan-RAST values in three different patients with invasive candidiasis in whom the disease history was longer than 2 weeks and follow-up sera were available. The

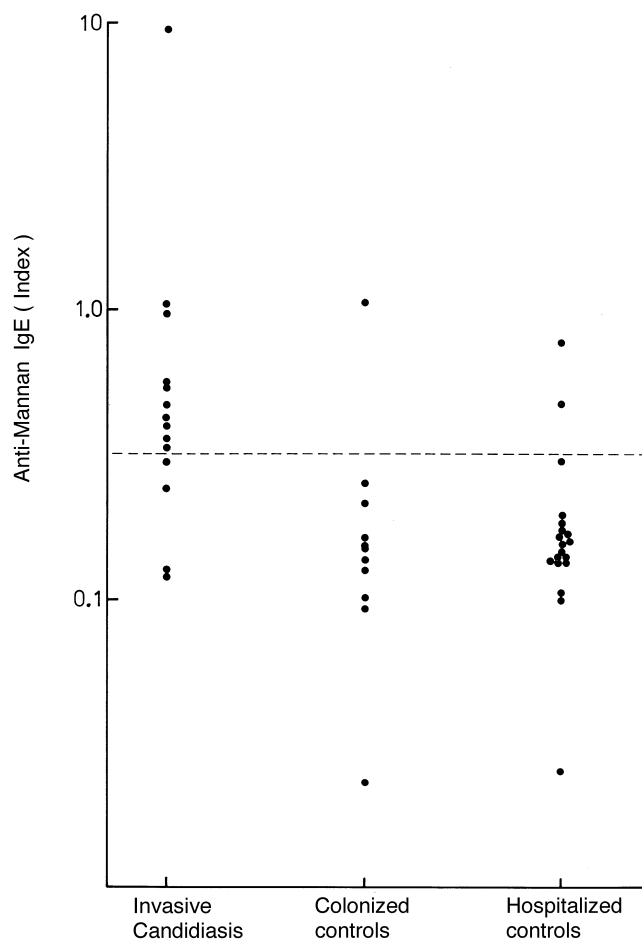


Fig. 2. Nitrocellulose-RAST for anti-mannan IgE determination. Mannan RAST index values are given. Limit for positivity = 0.32 (Student's *t*-test)/vs colonized controls: $P < 0.005$ vs operated controls: $P < 0.0005$.

C. albicans-specific IgE-level was markedly raised soon at the start of the disease history in all patients and the IgE level changed in concordance with the disease history. The IgE level rose earlier than serum arabinitol.

IgE immunoblotting and mannan-RAST in diagnosis of invasive candidiasis IgE-immunoblotting was more sensitive in the diagnosis of invasive candidiasis than mannan-RAST (0.86, and 0.71, respectively) while the specificity was better with mannan-RAST (0.09, and 0.80, respectively). The combination of either a positive result by IgE-immunoblotting or by mannan-RAST raised the

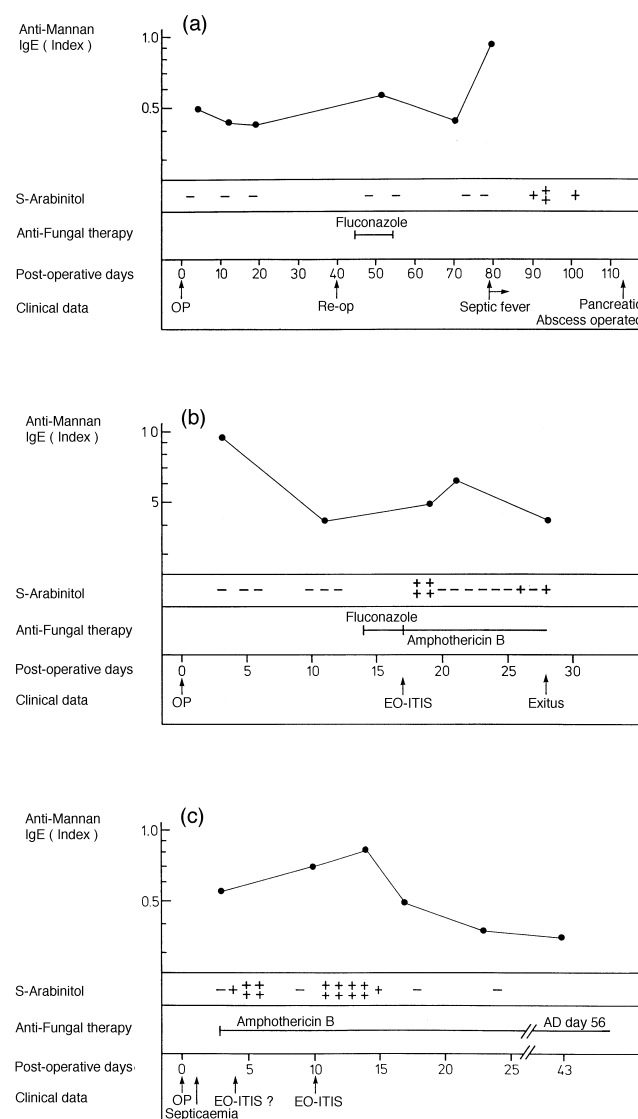


Fig. 3. Follow-up of mannan-RAST values in three patients with invasive candidiasis (patients 8, 9, 10) presented with clinical data and serum arabinitol results. (a) Patient 8. (b) Patient 9. (c) Patient 10.

Table 2. *Candida albicans* specific IgE assays in diagnosis of invasive candidiasis

		Patient groups			Specificity	Sensitivity	Predictive value	
		Invasive candidiasis	Colonized controls	Operated controls			Positive	Negative
IgE-Immunoblotting	+	12	3	3	0.80	0.86	0.67	0.92
	–	2	8	16				
Mannan-RAST	+	10	1	2	0.90	0.71	0.77	0.87
	–	4	10	17				
Immunoblotting or RAST	+	13	3	5	0.73	0.93	0.62	0.96
	–	1	8	14				
Immunoblotting and RAST	+	9	1	0	0.97	0.64	0.90	0.85
	–	5	10	19				

sensitivity to 0.93; the specificity was 0.73. A positive result by IgE-immunoblotting and by mannan-RAST raised the specificity to 0.97 at the expense of sensitivity (0.64) (Table 2).

IgG and IgM assays

IgG- and IgM-immunoblotting All subjects in all groups had positive responses to protein antigens of *C. albicans* by IgG and IgM immunoblotting. There were no clear differences in the intensity of the staining or staining patterns of the IgG and IgM immunoblots between the groups. The IgG responses were directed against 28 different protein bands; of these 25 were common to all groups. There were no significant differences between the study groups with regard to the binding patterns. The most important target antigens for IgG were a 76 kDa band (50% of the patients in the invasive candidiasis group, 45% in the colonized group and 60% in the hospitalized group), a 85 kDa band (57%, 64% and 42%, respectively), 27 kDa (57%, 37%, 53%) and a 46 kDa band (50%, 55%, 21%).

The IgM responses were directed against 29 protein bands, and of these 21 were common to all groups. Again there were no significant differences in most of the binding patterns between the study groups. However, there was an IgM-positive 22 kDa band that was identified in 14/19 of the hospitalized control group but in only 2/14 of the invasive candidiasis group and in only 3/11 of the colonized group. The most important target antigens for IgM were a 46 kDa band (36% of the patients in the invasive candidiasis group, 18% in the colonized group and 68% in the hospitalized group), a 34 kDa band (64%, 45%, 53%, respectively) and a 20 kDa band (43%, 55%, 53%).

IgG and IgM ELISA

IgG ELISA against mannan had overall poor specificity (0.64) and sensitivity (0.63), although the means of the IgG ELISA absorbances were significantly different when the invasive candidiasis patients were compared to hospitalized ($P < 0.005$) and colonized ($P < 0.05$) patients. IgM ELISA against mannan was also not very specific (0.71) and sensitive (0.50) although there was, again, a statistically significant difference between invasive candidiasis and hospitalized patients ($P < 0.01$) but not between invasive candidiasis and colonized ($P = 0.15$) patients. The IgM ELISA values were highest in the colonized group, significantly higher than in the hospitalized group ($P < 0.001$).

Discussion

Invasive fungal infections are a diagnostic and therapeutic challenge in intensive care patients and in immunocompromized hosts. The outcome is often fatal, since early diagnosis is usually not possible [28]. All current diagnostic methods are not sensitive and the results of tests are slow to come. What is required is a sensitive and rapid method that may guide therapeutic decisions.

Blood cultures are often negative in invasive candidiasis [29,30] as in five out of 14 patients in this study. Conventional IgG, IgA and IgM serology in diagnosis of invasive *Candida* infection has three major disadvantages: antibodies to *C. albicans*, especially mannan, are common in normal healthy individuals [10,11], immunocompromized hosts do not raise a strong antibody response [31,32], and it takes an inappropriately long time for a positive response to develop [33]. Only repeated samples and titre changes may give information that may guide

therapeutic decisions, but still these approaches do not help early diagnosis [6].

In immunoblotting an immunodominant 47 kDa protein antigen of *C. albicans* to IgG antibodies has been recognized as enolase [34–37]. Another important antigen with a molecular weight of 47 kDa has been identified as a split-down product of a fungal heat shock protein 90 [38]. The most important protein antigen of all immunoglobulin classes in our study was the 46 kDa band. This protein is apparently the most important allergen of *C. albicans*, in some studies a major allergen [10,39]. This major allergen has simultaneous IgE binding and amino acid homology with *S. cerevisiae* enolase and is likely to be enolase [39,40]. However, the IgG and IgM assays are usually late indicators of invasive candidiasis. Furthermore, anti-enolase IgG and IgM antibodies were also present in colonized (50%) and hospitalized (20%) controls in our analysis.

Antigen detection techniques have been based on detection of single important antigens, like mannan or enolase, or mixtures of antigens in serum samples. Detection of circulating mannan antigen is highly specific and moderately sensitive and the usefulness of this test can be increased by multiple sampling [41,42]. Indeed, repeated determinations of circulating mannan antigen is currently the most sensitive method in serology in both animal and human studies [43,44]. Enolase in only one sample is not sensitive and requires repeated samples if adequate sensitivity is to be gained [4]. Thus, daily sampling is suggested, but the impact on therapy of this approach is not known.

Commercial antigen detection methods with uncharacterized antigens have low sensitivity as compared to mannan [41,45]. The detection of arabinitol, a metabolite of *Candida*, in serum samples is specific but has low sensitivity, which can be increased with multiple samples [25]. The problem in all serological and antigen detection methods is their low sensitivity and that seropositivity comes late. The *C. albicans*-specific IgE-assays in the present study provide by far the most promising serological tool to diagnose invasive candidiasis.

A direct comparison of positive blood cultures with the IgE-determinations was not possible, because the samples were not taken simultaneously. However, in all five patients whose diagnoses were established by biopsies or autopsies and who remained negative by repeated blood cultures, IgE-determinations were positive. During follow-up (Fig. 3) the patients were clearly mannan-RAST positive from the start and patient no 8 already 4 months before the diagnosis was established. The anti-*C. albicans* IgE-antibodies were elevated in these patients already in the early phase of the infection. The early IgE-response thus is not only an early infection marker but it may indicate that there is

an immunological defect, probably in the balance of the T-helper cells, making the individual susceptible to the infection. Therefore the IgE assays might even serve as prognostic tests.

Whether the IgE assays work in immunocompromized patients remains to be studied. AIDS patients are able to raise IgE antibodies to *Pneumocystis* and *C. albicans* [46]. This may be an indicator of disturbed balance of the T-helper cells which leads to an infection by these agents common in AIDS patients [47]. On the other hand, all immunocompromized patients with T-helper dysfunction may produce IgE and an IgE assay might be usable as a diagnostic tool when measurements of other immunoglobulins do not work.

Although phagocytic cells are important effectors in the defence against fungi, impaired cell-mediated immunity is regarded as the main cause for susceptibility to these lethal infections. In experimental invasive *Candida albicans* infection in the mouse, protection against the infection was associated with the development of a T-helper 1 (TH₁) response while in susceptible mouse strains a TH₂ response was observed [7–9]. Thus, the outcome of the disease could result from a balance between host-protective TH₁ and disease-promoting TH₂ responses. This was corroborated by injecting neutralizing anti-interferon gamma (IFN γ) antibodies to the resistant mice: down-regulation of IFN γ production turned the mice susceptible for infection and a *Candida*-specific TH₂ response developed which was characterized by the production of IL-4, eosinophilia, and a strong rise in serum levels of *Candida*-specific antibodies of the IgA and IgE isotypes. Human invasive candidiasis is associated with enhanced IgE-production against *C. albicans*, especially against the mannan antigen. In accordance with the mouse model IgE production seems to provide no protection in contrast to the cell mediated response. The strong delayed type hypersensitivity response to *C. albicans* is common in the normal population and the 'candidin' reaction is often used as a marker of intact cellular immunity. The enhanced TH₂ response occurs in association with an enhanced IgE-production and it is possible that individuals with a weak TH₁ response are susceptible to invasive *Candida* infection.

Further studies are needed to assess the cytokine profiles of the *C. albicans* specific cells in patients with invasive candidiasis and healthy individuals. In case of disadvantageous T-helper cell balance therapy with cytokines used in other diseases with TH₂ predominance like atopic dermatitis and asthma, e.g. IFN γ , could be justified [48,49].

Our study shows that measurement of *C. albicans*-specific IgE-antibodies (especially mannan) may provide a useful tool in the diagnosis of invasive candidiasis.

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