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Enzyme-Linked Immunosorbent Assay Measurement of Fluctuations in Antibody Titer and Antigenemia in Cancer Patients with and without Candidiasis

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Antibody titers against purified sulfate-soluble fraction (PSSF) obtained from cytoplasmic extracts of *Candida albicans* were determined retrospectively over a 2-year period for 123 cancer patients by enzyme-linked immunosorbent assay. Antibody against cell wall mannan (CWM) was also measured by the hemagglutination test and the production of precipitins by a serum interacting with a yeast cell homogenate by immunodiffusion. Invasive candidiasis determined by histological evidence at autopsy was present in 10 patients. Fourfold or greater rises in anti-CWM and anti-PSSF antibodies were detected for eight of the patients with invasive candidiasis at 14 to 22 days after the onset of fever. The immunodiffusion test was positive for four patients with invasive candidiasis. For patients with no evidence of candidiasis, significant rises in anti-CWM and anti-PSSF antibodies were observed at a frequency of 20 and 10%, respectively. The concentrations of serum mannan were sequentially measured by the enzyme-linked immunosorbent assay. Antigenemia (≥3 ng/ml) was found in 9 of the 10 patients with invasive candidiasis and in 2 of the 4 patients with thrush, whereas the serum of 1 of the 36 patients with no evidence of candidiasis was positive for antigen. The first antigenemia antedated significant rises in antibody levels against *Candida* species by 6 to 23 days.

Systemic candidiasis has been recognized as a important infectious complication in immunocompromised patients (17). For several reasons, however, an antemortem diagnosis of invasive candidiasis is difficult to establish. The clinical presentation is usually nonspecific, and a microbiologic diagnosis is often difficult to obtain. Conventional quantitative serologic tests are agglutination of *Candida* species blastoconidia, immunodiffusion (ID), and latex agglutination. But interpretation of the data obtained with these tests is difficult, because the procedures do not allow precise quantitation of antibody levels. On the other hand, the radioimmunoassay (15) and the enzyme-linked immunosorbent assay (ELISA) (16) have been reported to be sensitive and quantitative methods for the determination of antibody titers against *Candida* species. When these methods are used, major overlapping in antibody levels are found in healthy, colonized, and infected individuals. These results indicated that no single antibody level can be used to diagnose candidiasis.

A recent report (20) dealt with the detection of *Candida* antigens by a variety of methods, such as hemagglutination (HA) inhibition, the radioimmunoassay, the ELISA, and ELISA inhibition. Most recent studies have been directed toward the detection of serum mannan in humans and infected animal model systems. However, only 47 to 70% of disseminated candidiasis cases in humans have been positive by the radioimmunoassay or ELISA, which are more sensitive than other methods. Moreover, in a retrospective study of leukemic patients, Meckstroth et al. (16) reported that antigenemia was detected in the sera of 7 of 10 patients with disseminated candidiasis; for the remaining 3 patients, sera were drawn too early to be of diagnostic value.

Previous results indicated that sera must be obtained at frequent intervals for the successful application of immunological methods to diagnose systemic candidiasis. For these reasons, the fluctuations in antibody titer and serum mannan concentration were examined by the ELISA in sera from cancer patients with and without candidiasis. The results of the ELISA for antibody titer were also compared with those of the ID and the HA tests.

MATERIALS AND METHODS

Patients and controls. During the 2-year period of February 1983 to February 1985, 988 samples were obtained from 123 hospital inpatients at least once a week, and all sera were frozen at −80°C until use. Included in the study were 62 patients with leukemia, 33 patients with solid tumors, 20 patients with malignant lymphoma, and 8 patients with multiple myeloma. Invasive candidiasis and other fungal infections were determined by histological evidence at autopsy. In 38 autopsy cases, systemic candidiasis was shown in seven patients, and invasive gastrointestinal candidiasis was shown in three patients. Four patients had oral candidiasis. The underlying disorders of patients with candidiasis were leukemia (11 cases), malignant lymphoma (2 cases), and gastric cancer (1 case). Other fungal infections included aspergillosis (5 patients) and mucormycosis (one patient). Of 109 patients with no evidence of candidiasis, 81 were discharged and 28 died. Autopsy was performed on all these patients who died, and no evidence of candidiasis was

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found. Urine, stool, and throat culture swabs were taken at admission and once weekly thereafter. Diagnostic culture specimens, such as blood, sputum, catheter tips, and so on, were taken for suspected infection when indicated. Blood samples were inoculated into each bottle (Hoffman-La Roche & Co. Ltd., Basel, Switzerland) as follows. About 5 ml of blood was inoculated into a bottle containing 70 ml of thioglycolate broth with 0.025% sodium polyanetholsulfonate, and about 5 ml was inoculated into a bottle containing 70 ml of tryptic soy broth with 0.025% sodium polyanetholsulfonate. The Hoffman-La Roche Septi-Chek slide chamber was attached to the tryptic soy broth bottle in accordance with manufacturer guidelines. Cultural and serological data were obtained from patients over periods from 16 days to 15 months, with the mean testing time being slightly over 2.5 months. The healthy controls (38 females and 15 males), who were devoid of any acute illness or chronic skin condition, were selected from hospital staff.

Antigen. The identification of yeasts isolated from clinical materials was performed by using the API 20C Auxanogram kit (API System S.A., Montalieu, Vercieu, France). The production of germ tubes and chlamydospores by *Candida* species was examined by standard techniques (24a). Five *Candida albicans* strains isolated from blood cultures were used for preparing antigens. After cultivation in glucose-peptose-yeast extract broth for 48 h at 35°C, the yeast cells were washed three times with phosphate-buffered saline (PBS; pH 7.2). Next, the collected cells were suspended in distilled water so as to give an A_{640} of 40. Glass beads (0.17 mm; 40 g) were added to each 30 ml of the cell suspension, which was then placed in a Braun disintegrator (Braun, Melsungen, Federal Republic of Germany) for 5 min with continuous CO_2 cooling. The solution was centrifuged at 3,000 × *g* for 30 min. The resultant clear supernatant solution, which was called whole cell antigen (WCA), was centrifuged at 100,000 × *g* for 90 min to remove cell membrane and lipids (9). The supernatant solution was dialyzed against distilled water (containing 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride; Sigma Chemical Co., St. Louis, Mo.) and lyophilized. The sulfate-soluble fraction (SSF) of the cytoplasmic extract was obtained by the method described by Jones (9) as follows. The lyophilized material was dissolved (10 mg/ml) in 0.03 M $NaBO_3$–0.15 M NaCl (pH 8.2), and an equal volume of saturated ammonium sulfate solution was added. The precipitate was removed by centrifugation at 12,000 × *g* for 20 min. The supernatant was dialyzed against PBS. The absorption of mannan by concanavalin A was achieved by combining an equal volume of SSF (0.75 mg of protein and 0.35 mg of carbohydrate per ml) and concanavalin A Sepharose 4B (Pharmacia, Uppsala, Sweden) (13). Mixtures were incubated at room temperature with agitation for 40 min and centrifuged at 1,000 × *g* for 10 min. The supernatant was removed, and an equal volume of concanavalin A Sepharose 4B was added. After incubation and centrifugation, the supernatant (0.35 mg of protein and 0.02 mg of carbohydrate per ml), which was called purified SSF (PSSF), was removed. Further adsorption with concanavalin A could hardly have reduced the mannan content of the PSSF. Cell wall mannan (CWM) was prepared by the method of Peat et al. (18). The protein content of the WCA, PSSF, and CWM was determined by the method of Lowry et al. (14). Carbohydrate was measured by the orcinol reaction (10), using D-mannose as a standard. Paper chromatography of CWM was done by the method of Summers et al. (25).

Antibodies against *C. albicans*. The ID test was performed by the standard method (12). The WCA was adjusted to a total protein concentration of 1 g/100 ml. It was used undiluted and at a 1:5 aqueous dilution. The sera showing precipitins to WCA were examined to identify the precipitins by the ID test with CWM (5 mg/ml) and PSSF (5 mg/ml). The test was considered positive when the study of serial serum specimens demonstrated serological conversion from negative to positive tests or an increase in the number of precipitins.

Anti-CWM antibody titer was determined by the HA test. Formalinized and lyophilized sheep erythrocytes coated with and without cell wall antigens of *C. albicans* were obtained (commercially available) from Hoffman-La Roche. Microtiter V plates (Toyoshima Industry Co. Ltd., Tokyo, Japan) were used in the test. Briefly, 0.05 ml of the patient's serum was added to 0.55 ml of the 20% sheep erythrocyte absorption suspension. The mixture was incubated for 30 min at 50°C in a water bath and centrifuged for 10 min at 3,000 × *g*. Serial twofold dilutions with 0.025 ml were made, starting with the serum diluted 1:40. Finally, 0.025 ml of the 1% antigen suspension was added to every dilution. To check for complete agglutination absorption, 0.025 ml of the sheep erythrocyte control suspension was also added to 0.025 ml of the initial 1:40 dilution. This serum preparation was mixed by rotation and incubated for 2 h at 37°C. The final interpretation was made on the day after incubation at 4°C.

Antibodies against the PSSF were measured by the ELISA. Wells of polystyrene microtitration plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 0.1 ml of PSSF (4 μg of protein per ml in carbonate buffer [pH 9.6]) by passive adsorption overnight at 4°C. Coated plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Serial twofold dilutions of the sera were then made in the wells. Plates were incubated for 2 h at 4°C and washed with PBST. Peroxidase-conjugated anti-human immunoglobulin G (Miles Laboratories, Rehovot, Israel) diluted 1:4,000 in PBST containing 0.2% bovine serum albumin was added to each well, and the plates were incubated for 2 h at 4°C. After washing, substrate solution (1 mM phosphate-citrate buffer [pH 4.0]) containing 2.5 mM ABTS (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma) and 5 mM hydrogen peroxidase was added at 0.1 ml per well (1). The enzyme-substrate reaction was allowed to proceed at room temperature for 1 h, and the A_{405} was measured in a spectrophotometer (Dynatech) using PBST as the blank. Sera from healthy adults that were negative (<1:40) in the HA test were pooled and served as the negative control. A serum sample that was obtained a month after the clinical onset of systemic candidiasis and that was positive in both the HA test (1:10,240) and the ID test served as the positive control. The reported antibody titer was the highest dilution that resulted in an absorbance at least twice the average of that of nine wells containing the serial dilutions of negative sera.

Antiserum preparation. For the most part, the method described by Scheld et al. (22) was used for preparing the antisera against *C. albicans*. Five *C. albicans* strains grown on Sabouraud plates were harvested by washing with PBS. Five Japanese White rabbits weighing 2 to 3 kg were inoculated biweekly subcutaneously with a 1:1 emulsion (1 ml) of a 20% saline suspension of heat-killed (80°C for 1 h) *C. albicans* and Freund complete adjuvant. After 6 weeks of injections, booster immunizations of 1×10^8 to 3×10^8 heat-killed cells were given intravenously, and the rabbits were tested for antibody responses with the HA test at 4-day

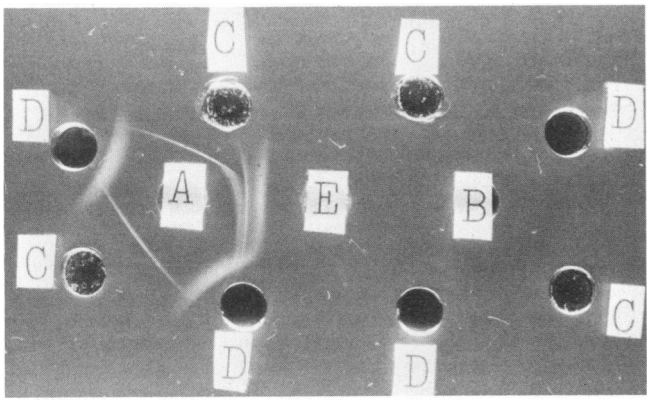

FIG. 1. Characterization by ID of *Candida* antigens. A, Serum from patient with systemic candidiasis; B, pooled sera from normal adults; C, purified sulfate-soluble antigen; D, CWM; E, WCA.

intervals thereafter. When an HA titer of 1:2,560 or more was obtained, blood was collected, and sera were stored at −80°C until use.

Conjugation of peroxidase to Fab'. Horseradish peroxidase type VI (Sigma) was conjugated to Fab' by the method of Yoshitake et al. (28). Briefly, peroxidase (2 mg) was dissolved in 0.3 ml of 0.1 M sodium phosphate buffer (pH 7.0) and incubated with 1.6 mg of the *N*-hydrosuccinimide ester of *N*-(4-carboxycyclohexylmethyl)maleimide (Zieben Chemical Co., Tokyo, Japan) in 0.02 ml of *N*,*N*-dimethylformamide (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) at 30°C for 1 h with continuous stirring. The maleimideperoxidase (0.9 mg/0.2 ml) was incubated with rabbit Fab' (1.0 mg/0.2 ml) in 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA at 4°C for 20 h. After incubation, the mixture was subjected to gel filtration with Ultrogel AcA 44 (LKB-Produkter AB, Bromma, Sweden). The A_{280} and A_{405} of the fractions containing the conjugate were 0.22 and 0.16, respectively. The specificity of peroxidase, examined by the method described by Yoshitake et al. (28), was 90 to 95% of the original level on the basis of A_{405}. The conjugate was stored at −80°C. For continuous use, the conjugate was kept at 4°C in the presence of 0.1% bovine serum albumin and 0.01% of thimerosal. The reagent was stable for at least 1 month at 4°C.

ELISA for *Candida* mannan. A double-antibody sandwich ELISA was used to detect antigenemia (19). Polystyrene microtiter plates (Immulon II; Dynatech) were coated with 0.1 ml of the immunoglobulin G fraction of antiserum (8 μg/ml). Coating was done at 4°C overnight, followed by washing with PBST. The sera to be tested were treated by a method almost exactly as described by Reiss et al. (19). Briefly, 0.16 ml of the PBST containing 0.1 M EDTA was added to 0.4 ml of serum in a 10-ml polycarbonate tube (Tomy Seiko Co. Ltd., Tokyo, Japan). The mixture was boiled in a water bath for 3 min and then centrifuged at 8,000 × *g* for 20 min. A 0.1-ml sample of supernatant was added to the wells and incubated at 4°C for 3 h. Each well was washed, and 0.1 ml of diluted conjugate (10 μg/ml in PBST containing 0.2% bovine serum albumin) was added, and incubation took place at 4°C for 3 h. After washing as described above, substrate was added, and the A_{405} was measured as described above. If the samples produced an absorbance >2 standard deviations (SD) above the mean absorbance of five mannan-free standards, they were reassayed with and without the addition of antibody against *Candida* species; to 0.15 ml of boiled sample was added 0.025 ml of PBST, and to the other 0.15 ml of the same sample, 0.025 ml of immunoglobulin G fractionated from immune rabbit serum (final immunoglobulin G concentration, 0.05 mg/ml) was added. The standard curve in pooled serum prepared with known concentrations of mannan diluted in human serum was generated each time the ELISA was performed. The concentrations of mannan in test sera were extrapolated from the standard curve.

RESULTS

Throat swab cultures grew *Candida* species for 11 (79%) patients with candidiasis and 41 (38%) patients with no evidence of candidiasis. From 5 patients with systemic candidiasis and 20 (18%) patients with no evidence of candidiasis, *Candidia* species were isolated with both throat swabs and urine or feces. For 3 of 10 patients with invasive candidiasis, antemortem blood cultures were positive for *Candida* species. Amphotericin B (300 mg daily) was given by mouth to most patients to protect them from candidal colonization during their hospital stay, whereas only three patients with invasive candidiasis were treated with antimycotics, two patients were treated with intravenous amphotericin B, and one patient was treated with 5-fluorocytosine. The remaining seven patients did not receive antimycotics, and antemortem diagnoses of candidiasis were not made. On the other hand, 5-fluorocytosine was successfully given to all patients with oral thrush.

WCA and PSSF preparations contained about 2.2 and 0.06 mg of carbohydrate per mg of protein, respectively. CWM contained 95% carbohydrate and 1% protein by weight and

TABLE 1. Distribution of reciprocal antibody titers against *C. albicans*

Antibody	Group no.[a] (no. of cases)	No. of cases with the following titer[b]:									
		<40	40	80	160	320	640	1,280	2,560	5,120	>10,240
Anti-CWM	1 (14)					1	6	2	1 (1)	3 (3)	1 (1)
	2 (109)	7	10	19	20	18	13 (1)	14 (5)	7 (3)	1 (1)	
	3 (53)	5	13	13	12	8	2				
Anti-PSSF	1 (14)			4	3 (1)	3 (2)	2 (1)	1			1 (1)
	2 (109)	48	16	12 (2)	16 (1)	7 (3)	8 (3)	1	1 (1)		
	3 (53)	17	20	9	6	1					

[a] Groups: 1, patients with candidiasis; 2, patients with no evidence of candidiasis; 3, healthy adults.

[b] For hospital inpatients, the highest reciprocal titer obtained at any time during the study is shown. The number of patients whose sera showed a precipitate in the ID test is shown in parentheses.

TABLE 2. Changes in antibody levels against *C. albicans* in cancer patients with and without candidiasis

Diagnosis or group	No. of cases	No. (%) of sera positive in the ID test		No. (%) of cases with the following rises in titer:					
				Anti-CWM			Anti-PSSF		
		CWM	PSSF	4	8	>16	4	8	>16
Systemic candidiasis	7	3	3	1	1	5	2	2	3
Invasive gastrointestinal candidiasis	3	1		1			1		
Oral candidiasis	4			1	2	1		1	
Total		4 (29)	3 (21)		12 (86)			9 (64)	
Aspergillosis	5	1				1			1
Mucormycosis	1								
Inpatients without fungal infections	103	6	1	10	6	5	3	4	3
Total		7 (5)	1 (1)		22 (20)			11 (10)	

showed only one spot with the mobility of mannose. ID characterization of *Candida* antigens is shown in Fig. 1. The results showed that the WCA contained both the PSSF and CWM and that the PSSF and CWM were different antigens. Rabbit antisera produced against whole *C. albicans* cells had an anti-CWM antibody titer of 1:2,560 and an anti-PSSF antibody titer of 1:40. The positive control serum (anti-CWM antibody titer, 1:10,240; anti-PSSF antibody titer, 1:5,120) and pooled sera from healthy adults were tested in the ID, HA, and ELISA tests 25 times on different days. Generally, the maximal variation in titers was not greater than fourfold in our tests. Because the absorbance of negative samples was 0.105 ± 0.018 (mean ± SD) in the ELISA, we set standard cutoff values of 0.210 for the assay of anti-PSSF antibody titers.

The results of the ID test and the distribution of reciprocal antibody titers according to patient group are shown in Table 1. Among the healthy adults, two patients (4%) had an anti-CWM antibody titer of 1:640 or more, and one patient had an anti-PSSF antibody titer of 1:320 or more. For 109 patients with no evidence of candidiasis, the sera of 35 (32%) were positive for anti-CWM antibody (≥1:640), and the sera of 17 (16%) were positive for anti-PSSF antibody (≥1:320). For the 14 patients with candidiasis, high antibody titers were recovered more frequently than for patients with no evidence of candidiasis (chi-square test; $P < 0.01$). Sera that produced precipitins against WCA also formed precipitins against CWM. Whereas the sera of three patients with systemic candidiasis and one patient with no evidence of candidiasis formed precipitins against both CWM and the PSSF, no sera from healthy adults showed precipitins in the ID test.

The numbers of patients with seroconversion detected by the ID and HA tests and the ELISA are shown in Table 2. The ID test had a sensitivity and specificity of 29 and 94%, respectively. The HA test (fourfold or greater rises in antibody titer) had a sensitivity and specificity of 86 and 80%, respectively. The ELISA results (fourfold or greater rises in antibody titer) were between those of the ID and HA tests, with a sensitivity and specificity of 64 and 90%, respectively. The predictive value that a positive result (significant antibody response) was a true-positive result was 36% by ID, 35% by HA, and 45% by the ELISA. The predictive values of negative results by ID, HA, and the ELISA were 90, 98, and 95%, respectively.

The absorbance in pooled serum and PBST containing various concentrations of *Candida* mannan is shown in

TABLE 3. Absorbance in pooled serum and PBS containing various concentrations of mannan

Mannan concn (ng/ml) in:	A_{405}[a]	CV (%)[b]
PBST		
3.13	0.135 ± 0.008	4.8
25	0.392 ± 0.026	5.2
Pooled serum[c]		
0	0.040 ± 0.005	4.8
1.5	0.064 ± 0.005	4.6
3.13	0.108 ± 0.012	5.5
6.25	0.158 ± 0.011	6.0
12.5	0.234 ± 0.015	6.8
25	0.353 ± 0.022	6.5
50	0.489 ± 0.039	7.1
100	0.672 ± 0.055	7.5
Pooled serum[d]		
3.13	0.104 ± 0.012	6.0
25	0.340 ± 0.025	6.6

[a] Mean ± SD from 10 experiments.
[b] CV, Coefficient of variation.
[c] Pooled from five normals. The solutions were treated at 100°C for 3 min.
[d] Pooled from five normals. The solutions were treated at 121°C for 5 min.

TABLE 4. Mannan antigenemia in cancer patients and healthy adults

Diagnosis or group	No. of cases (total no. of sera)	Range (mean) of A_{405}	No. of cases (total no. of sera) positive for mannan (>3 ng/ml)
Systemic candidiasis	7 (38)	0.030–0.731 (0.153)	7 (29)
Invasive gastrointestinal candidiasis	3 (12)	0.028–0.332 (0.132)	2 (4)
Oral candidiasis	4 (20)	0.034–0.292 (0.093)	2 (3)
Other fungal infections	6 (38)	0.030–0.059 (0.041)	0 (0)
Inpatients without fungal infections	30 (158)	0.032–0.158 (0.050)	1 (2)
Normal healthy adults	30 (30)	0.031–0.061 (0.040)	0 (0)

TABLE 5. Clinical, microbiological, and serological data for seven patients with systemic candidiasis

Patient no., age (yr), and sex	Primary disease	Organs found infected	Day after onset (outcome)	AMPH[a] (intravenous)	*Candida* isolation				ID test		Anti-CWM titer	Anti-PSSF titer	Mannan concn (ng/ml)
					Blood	Oral	Urine	Feces	CWM	PSSF			
1, 46, Female	Acute myelogenous leukemia	Bowel, liver, spleen, lung, heart, kidney	4			−	−	−	−	−	80	<40	10
			6						−	−	80	<40	15
			11			+	−	−	−	−	80	<40	<2
			15						−	−	80	80	9
			22			+	−	+	+	+	1,280	10,240	8.5
			23		+								
			25						+	+	5,120	10,240	<2
			27			+	−	+	+	+	5,120	10,240	6.5
			28						+	+	5,120	10,240	12.5
			29 (patient died)										
2, 34, Male	Chronic myelogenous leukemia	Bowel, liver, spleen, kidney	2			+	−	−	−	−	160	<40	6
			7			+	−	−	−	−	160	<40	<2
			11						−	−	160	<40	24
			14			+	−	−	−	−	160	<40	6
			20						−	−	160	<40	5
			25			+	−	−	−	−	640	<40	3.5
			28			+	+	−	−	−	1,280	40	17
			31 (patient died)										
3, 55, Female	Acute myelomonocytic leukemia	Bowel, kidney	8			+	−	+	−	−	160	<40	<2
			15						−	−	40	<40	<2
			16			+	+	+	−	−	40	<40	30
			19						−	−	80	<40	40
			22			+	−	+	−	−	320	<40	<2
			25						−	−	1,280	80	
			27			+	−	+	−	−	1,280	80	<2
			29 (patient died)										
4, 36, Female	Non-Hodgkin's lymphoma	Bowel, spleen, bladder	1						−	−	160	<40	8
			5			−	−	+	−	−	160	<40	4
			15						−	−	80	<40	10
			19			+	+	+	−	−	160	<40	4
			22 (patient died)						−	−	640	80	<2
5, 39, Male	Adult T-cell leukemia lymphoma	Esophagus, spleen, heart	2			−	−	−	−	−	40	40	48
			10			−	−	−	−	−	40	40	<2
			13						−	−	320	160	3
			16			−	−	−	−	−	640	160	<2
			17 (patient died)										
6, 69, Male	Gastric cancer	Brain, kidney	5						−	−	80	<40	45
			10			−	−	−	−	−	80	<40	26
			15			−	−	−	−	−	160	<40	<2
			17		+								
			18		+								
			23	+	+	−	−	−	+	+	1,280	320	28
			28	+		−	−	−	+	+	10,240	2,560	17
			38	+		−	−	−	+	+	10,240	5,120	40
			41 (patient died)	+							10,240	5,120	
7, 19, Female	Acute lymphocytic leukemia	Bowel, liver, spleen, lymph nodes	1			+	−	−	−	−	320	<40	<2
			5						−	−	320	<40	3
			11		+	+	−	−	−	−	640	<40	<2
			15		+	+	−	+	+	+	2,560	40	4
			24	+					+	+	5,120	80	4
			36	+		+	+	−	+	+	5,120	320	<2
			51	+		+	+	+	+	+	5,120	320	16
			55 (patient died)	+					+	+	2,560	160	

[a] Amphotericin B.

Table 3. When 3.13 and 25 ng of mannan per ml were added to normal sera instead of to PBST, a 10 to 20% reduction in absorbance was obtained. The mean absorbance value of >2 SD above the mean absorbance of five zero-mannan sera was 0.051, whereas pooled sera containing 1.5 ng of mannan per ml showed absorbance values of 0.064 ± 0.005. We therefore considered that the ELISA could detect antigen concentrations to a lower limit of 1 ng/ml. The absorbance of the mean plus 2 SD in sera from patients with no evidence of candidiasis was 0.088 (mannan concentration, about 2 ng/ml). With this as the upper limit, 5 of 197 sera from 36 patients with no evidence of candidiasis showed mannan concentrations of >2 ng/ml; 2 sera from 1 patient with chronic myelogenous leukemia were antigenemic (4.5 and 6 ng/ml), and sera obtained at 18 to 20 days after the first antigenemia showed significant rises in anti-CWM and anti-PSSF antibody levels. At autopsy, no infection by *Candida* cells was seen in any organs. The other three sera from two patients with no evidence of candidiasis had mannan concentrations ranging from 2 to 2.5 ng/ml. Sera obtained from these two patients during their hospital stay showed no rises in antibody levels. For patients with candidiasis, mannan antigenemia was detected in seven patients with systemic candidiasis (3 to 120 ng/ml) and in two patients with invasive gastrointestinal candidiasis (6 to 24 ng/ml; Table 4). In addition, three sera from two patients with thrush were positive for serum mannan (3 to 14 ng/ml), two of eight sera from one patient and one of four sera from the other. These two patients were successfully treated with 5-fluorocystosine for 5 to 7 days and discharged. In healthy adults, none of the sera had mannan concentrations of 2 ng/ml or greater. Clinical evaluation of the detection of mannan (>3 ng/ml) for 10 patients with invasive candidiasis was made, and the sensitivity and specificity of serum mannan were 90 and 96%, respectively.

The antibody titers and concentrations of mannan in serum obtained for seven patients with systemic candidiasis are shown in Table 5. Two patients had renal insufficiency (creatinine concentrations in serum, >1.5 mg/dl). The ranges of creatinine concentration in serum for patients with renal insufficiency were as follows: patient 2, 0.5 to 3.5 mg/dl, and patient 7, 1.5 to 4.4 mg/dl. *C. albicans* was isolated from blood obtained from patient 6 at 17, 18, 23, and 41 days after the onset of fever and from patient 7 at 11 and 15 days after the onset of fever. A blood culture obtained from patient 1 at 23 days after the onset of fever grew *Candida tropicalis*. Fourfold or greater rises in anti-CWM and anti-PSSF antibody levels occurred at almost the same time as the serologic conversion from negative to positive in the ID test. These serologic responses were detected at 14 to 22 days after the onset of fever, probably due to candidal infections. Moreover, antigenemia antedated the antibody responses against *C. albicans* by 6 to 23 days, and it antedated the positive blood cultures by 6 to 19 days. In most patients, despite the progression of infection, it showed little tendency to increase the antigen levels, which varied strikingly in and between patients. In addition, no correlation was observed between mannan concentration and creatinine level.

DISCUSSION

A variety of methods for the premortem diagnosis of invasive candidiasis have been reported (20). Although fungal surveillance cultures may help to predict such infection (21), their value is limited because *Candida* species are frequently isolated from clinical materials (throat swabs, urine, and feces) obtained from patients with and without candidiasis. In this study, premortem diagnosis of candidiasis was made in only 3 of 10 patients with invasive candidiasis proven by autopsy. Because of the saprophytic nature of *Candida* species, almost everyone encounters them, which means that antibodies to the organisms are always present in the sera of healthy humans. The antigens recognized by these serum antibodies have been reported to be the CWM of *Candida* species. Taschdjian et al. (26) emphasized the necessity of using cytoplasmic extract as the antigen in the ID test and suggested that the precipitating antibody against the antigens may be more specific for visceral candidiasis than agglutinating, complement-fixing, and indirect fluorescent antibody. However, other investigators (4, 7) concluded that the detection of the cytoplasmic antigens is not useful diagnostically because of the high incidence of false-negative responses (27 to 50%) in immunosuppressed patients. Moreover, precipitating antibodies against cytoplasmic antigens have been shown to occur often in the absence of overt systemic infection (6). Although the ID method was simpler to perform and more specific than other procedures for anti-CWM and anti-PSSF, the sera of only 4 of 10 patients with invasive candidiasis were positive in the ID test. In 1980, Jones (9) showed that cytoplasmic antigens contain large amounts of mannan and antigens from the cytoplasm of *Candida* species. Recently, Karwowska et al. (11) reported that antibodies against the SSF which was passed through a concanavalin A Sepharose 4B column reflected the degree of *C. albicans* invasion. However, the sera of 16% of patients with no evidence of candidiasis were positive for antibody against the SSF in our study.

Some investigators (3, 15) recommended that monitoring the dynamic evolution of the titer is very important; a single measurement is, for the most part, of little value. Our study showed that all patients with systemic candidiasis had fourfold or greater rises in antibody titers against mannan and PSSF. However, significant rises in these antibody titers also occurred in patients with no evidence of candidiasis (10 to 20%). With regard to the length of time of the patient's illness in days at the time of testing, a measurable change in antibody levels has been detected by week 2 after the diagnosis of fungal septicemia (8). In our study, at least 14 days passed after the onset of fever before significant antibody responses could be detected. In addition, five of the seven patients with systemic candidiasis died within one week after the recognition of rises in antibody levels. In conclusion, antibody measurement seemed to be of little value in diagnosing *Candida* infections in cancer patients.

As rising antibody titers generally take at least 14 days to develop, the detection of circulating antigens or yeast products has emerged as a promising aid in the diagnosis of systemic candidiasis as early as possible. It is apparent that at least the mannan polysaccharide antigen does not always occur free in serum but may be bound to antibodies as soluble immune complexes presenting a barrier to its detection. Segal et al. (24) and Meckstroth et al. (16) treated sera with NaOH at 56°C for 2 h to dissociate the complexes. In addition to the alkali method, the boiling method (100°C for 3 min; 19) and the heat-extraction method (121°C for 5 min; 13) have been reported to facilitate the detection of mannan in experimental and human infections. When serum standards containing mannan were treated at 121 or 100°C, 10 to 20% reductions in absorbance were found, compared with those obtained with PBST standards. Because it has been reported that *Candida* mannan is heat

stable (13), the reduction in absorbance after boiling or autoclaving may have resulted from the trapping of antigen within coagulum.

Using the RIA technique, Weiner and Coats-Stephen (27) detected antigenemia in 5 of 11 patients with systemic candidiasis. Lew et al. (13) described an ELISA which detected antigenemia in 8 of 15 patients with documented and suspected candidiasis. Kaufman and Reiss (12) observed that mannan concentrations greater than 2 ng/ml are presumptive evidence of infection, and sera from 60% of the patients with systemic candidiasis in their study were positive for antigen. All patients with systemic candidiasis had 3 ng/ml or greater concentrations of serum mannan in our study, whereas 35 of 36 patients with no evidence of candidiasis had antigen concentrations of 2.5 ng/ml or less. Only one patient, who had no evidence of candidal infection at autopsy, was positive for antigen followed by a significant rise in antibody level against *C. albicans* and was treated with intravenous amphotericin B for suspected mycotic infections. The serologic results obtained from this patient may reflect unrecognized foci of infection of mucosae. In this study, we believe that the detection of 3 ng/ml or more mannan in serum is important in the diagnosis of invasive candidiasis.

It is an important consideration that antigenemia can be transient. In our study, 66% of sera from patients with invasive candidiasis were antigenemic. These results and others (12, 16) showed that sera should be collected at frequent intervals from patients at risk for invasive candidiasis. Although Schreiber et al. (23) and Harding et al. (5) reported that *Candida* antigen detection might be useful in the follow-up of patients with disseminated candidiasis, there was no firm correlation between the severity of infection and antigen concentration. A similar observation was made by Bailey et al. (2). This study showed that positive antigenemia was strongly suggestive of candidiasis and frequent antigenemia with and without positive blood culture might indicate systemic infection.

Further studies should be done prospectively to elucidate whether detection of serum mannan can bring about prompter treatment and increased patient survival. Using the ELISA, we are now measuring serum mannan and *Aspergillus* antigen sequentially in patients with leukemia.

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