Antibodies to germinating and yeast cells of *Candida albicans* in human and rabbit sera

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SUMMARY Two major antigenic components, I and II, were detected by double immunodiffusion in sonic extracts of the germinating (G) or yeast (Y) cells of the dimorphic organism, *Candida albicans* group A. Component I may be a heterogeneous mixture of antigens which are stable to heating and phenol. Component II is more homogeneous but is labile to heat and phenol.

Rabbit antisera, showing only precipitin to component II or certain human sera at high dilution, were found to react with G cells to give an immunofluorescence which was confined to the germ tubes. This suggested that component II is localised on the germ tubes, whereas no immunofluorescent reaction against the yeast cells could be detected under the same conditions although component II was as readily extracted from these cells as from G cells. This suggested that component II might exist in a cryptic state in the Y cells. In support of the latter contention it was shown that live Y cells did not absorb precipitin to component II nor were they capable of providing these antibodies in rabbits.

Using both human and rabbit sera, it was shown that the antigenic specificity of the immunofluorescence assay where Y cells were used was related to component I and that where G cells were used it was related to both components I and II.

Opportunistic infection with *Candida albicans* is becoming increasingly prevalent, particularly among patients who are compromised immunologically or otherwise (Bodey, 1966; Hart *et al.*, 1969; Ashcraft and Leape, 1970; Ho *et al.*, 1977). An efficient method for its diagnosis is still lacking, however. In an earlier report (Ho *et al.*, 1976) we obtained sera from healthy subjects and patients who showed clinical and mycological evidence of candidiasis. The sera were tested for indirect immunofluorescence (IF) against the yeast (Y) and germinating (G) cells of the dimorphic organism, *C. albicans* group A, which are known to differ with respect to their antigens and chemical composition (Chattaway *et al.*, 1968; Evans *et al.*, 1973; Syverson *et al.*, 1975). It was found that the patients generally had significantly higher serum antibody titres than the healthy subjects and that the IF reaction against G cells observed with many of the patients' sera at high dilution was confined to the germ tubes. These findings are compatible with an extensive mycelial infiltration observed in sections of tissue infected

with this organism (Winner, 1969) and suggest that the immunofluorescence assay might be a useful diagnostic aid in candidiasis.

We report the results of further studies in which we sought to define the antigenic specificity of the immunofluorescence assays by comparing the IF titres with precipitating antibodies to *C. albicans* using human sera and rabbit antisera produced against Y and G cells of *C. albicans* group A.

Material and methods

PATIENTS

Serum specimens were obtained from 33 patients among whom were 10 with carcinoma of the oesophagus, two each with carcinoma of the stomach, colon, and liver respectively, one each with carcinoma of the bladder, lung, and bronchus, and two patients with leukaemia. Nine of the patients with carcinoma of the oesophagus and the two with carcinoma of the colon were on hyperalimentation. Two additional patients were on hyperalimentation, one with intestinal obstruction and one with a choledochal cyst. The remaining

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patients included those with status asthmaticus, neurogenic bladder, malformation of the urinary tract, lung abscess, hepatosplenomegaly, monilia infection of the perineum, and burns. These patients had been routinely investigated mycologically and serologically (see Appendix, p. 404).

HUMAN SERA

Serum specimens were obtained from the 33 patients within two weeks of the positive culture of *C. albicans* group A. These, along with sera collected from 25 healthy subjects, were stored at -20°C until used.

ORGANISMS

C. albicans group A was inoculated onto Sabouraud agar. After 48 hours' incubation at 37°C the growth was scraped off, washed, and resuspended in distilled water. Under these cultural conditions, the growth was made up predominantly of the Y form.

To obtain G cells, the organism was inoculated into 1% mycological peptone (Difco, US) supplemented with 0.2% glucose, according to Evans *et al.* (1975). Cultures were shaken at 40°C for 2 to 3 hours. The cultures were harvested by centrifugation and the resulting G cells were washed with and resuspended in saline.

IMMUNOFLUORESCENCE ASSAY

IF assays were performed as described by Ho *et al.* (1976). Serially diluted sera were allowed to react with acetone-fixed smears of G cells or Y cells for one hour. The smears were washed and incubated for one additional hour with fluorescein conjugated (FITC) anti-human immunoglobulin (1:16 diluted, Hyland, US) or FITC anti-rabbit immunoglobulin (1:16 diluted, Wellcome, UK). The titre IF-G or IF-Y was expressed as the reciprocal of the maximum serum dilution that gave a positive IF reaction.

IMMUNISATION

Suspensions of live G or Y cells containing 1×10^6 organisms per ml were given intravenously to New Zealand white rabbits at weekly intervals. Animals were test-bled on the third day after the fourth injection by the IF assays. Larger quantities of blood were obtained by heart puncture. Heat-killed suspensions of Y and G cells, prepared by heating live suspensions at 65°C for 6 hours, were used to immunise rabbits as well.

EXTRACTS

Y or G cells washed and packed before or after heat treatment were suspended in an equal volume of saline for extraction. To prepare sonic extracts, the suspensions were subjected to ultrasonication

with a Branson sonifier at maximum output for 35 minutes. Sonication was carried out at 2-minute intervals with rests at 4°C . The sonicates (Y and G) were clarified by centrifugation at $12\,000\text{ g}$ for 30 minutes.

Phenol extracts (Yp and Gp) were prepared by mixing a suspension of *C. albicans* with equal volumes of phenol. The mixtures were vigorously shaken for 30 minutes at room temperature and centrifuged, and the aqueous layers were aspirated and dialysed extensively against distilled water and saline. The extracts were then clarified by centrifugation at $12\,000\text{ g}$ for 30 minutes.

DOUBLE IMMUNODIFFUSION

Double immunodiffusion was performed at room temperature in 1% agarose made up in 0.1 M NaCl.

AGGLUTINATION TEST

Serially diluted aliquots of sera were mixed with an equal volume of a yeast cell suspension containing 3×10^6 cells per ml of saline. The mixtures were incubated at 37°C for 2 hours and then allowed to stand at 4°C overnight. Agglutinin titre is the reciprocal of the maximum serum dilution giving a positive agglutination.

Results

PRECIPITATING ANTIBODIES IN RABBIT AND HUMAN SERA

Rabbit antisera were produced against live G or Y cells of *C. albicans*. These antisera, anti-G and anti-Y, were tested by double immunodiffusion against a sonic extract of G cells (Fig. 1). Both antisera

Fig. 1 Double immunodiffusion patterns of rabbit sera against a sonic extract of live G cells (G). The antisera included anti-Y (AY), anti-G (AG), and the same antisera which had been exhaustively absorbed with Y cells (AY-Y; AG-Y) or with Y cells followed by G cells (AY-Y-G; AG-Y-G).

reacted to give a diffused zone of precipitate probably due to a heterogeneous mixture of antigens referred to hereafter as component I. In addition, anti-G, but not anti-Y, gave a well-defined line of precipitate, and the corresponding antigen is referred to as component II. A third antigenic component (component III) was detected using anti-Y, but this antigen was not consistently resolved in subsequent analysis.

The rabbit antisera were exhaustively absorbed with live Y or G cells and then similarly tested. It was shown that precipitin to component I may be absorbed with either type of cells while absorption of precipitin to component II could be effected with live G cells only. The precipitation line observed between the antisera wells in Fig. 1 was probably

Fig. 2 Double immunodiffusion patterns of rabbit antiserum to live G cells (AG) against sonic extracts of live or heat-killed G cells (G and Gh) or of Y cells (Y and Yh).

Fig. 3 Double immunodiffusion patterns of rabbit antiserum to live G cells (AG) against sonic or phenol extracts of live G cells (G and Gp) or live Y cells (Y and Yp).

Fig. 4 Double immunodiffusion patterns of rabbit sera against a sonic extract of live G cells (G). The antisera were; AG, antisera against heat-killed G cells (AGh) and Y cells (AYh).

due to residual antigens present in the absorbed sera.

Figures 2 and 3 show the immunodiffusion patterns obtained by reacting anti-G against different extracts of Y and G cells. Component I was detected in all extracts tested, whereas component II, which appeared to be labile to heating and phenol, was detected only in the sonic extracts of live G and Y cells.

In Fig. 4, rabbit antisera produced against live or heat-killed organisms were tested against a sonic extract of G cells. All the antisera tested were reactive against component I but only anti-G contained antibodies to component II. It seems that component II, although extractable from Y cells (see Fig. 2), might exist in a cryptic state in these cells, and, as such, Y cells are neither capable of producing antibodies to component II nor of absorbing these antibodies (see Fig. 1). Thus component II is distinct from the mycelial specific antigens observed by other investigators (Syverson *et al.*, 1975), and, probably because of limited resolution of the present technique, phase specific antigens were not observed.

Thirty-three patients were investigated serologically and mycologically. The results of these investigations and the primary clinical symptoms of these patients are shown in the Appendix. Precipitating antibodies to *C. albicans* present in these sera were tested by double immunodiffusion and compared with those formed with rabbit anti-G. Based on the occurrence of the precipitating antibodies, the sera may be divided into four groups, one of which, as exemplified by serum P₁, reacted with a sonic extract of live G cells to give a line of identity to the reaction between component II and the rabbit anti-G. The second group of sera, an example of which is P₂, contained precipitin to

component I only. The third group of sera, for example P₃, showed precipitin to both components I and II, and the fourth, P₄, did not show precipitating antibodies to *C. albicans* (Fig. 5).

CORRELATION BETWEEN IF REACTIVITIES AND PRECIPITATING ANTIBODIES TO *C. albicans*

Table 1 shows IF titres and the occurrence of precipitin to *C. albicans* in different rabbit antisera. Precipitin to component I and IF reactivity to Y cells present in anti-G and anti-Y were concomitantly absorbed with Y cells. The residual activities of anti-G after exhaustive absorption with Y cells included precipitin to component II and IF reactivity against G cells, which in turn were concomitantly absorbed with G cells. It should be noted also that the ratio of IF titre as determined against G cells to that against Y cells (IF-G/IF-Y) was higher for anti-G than for anti-Y or for the

antisera produced against heat-killed Y or G cells. A further increase in the ratio was observed after absorption of anti-G with Y cells. It seems from these results that the antigenic specificity of the IF assay where Y cells were used is related to component I while that of the IF assay using G cells is related to both components I and II.

The above conclusion was corroborated by results obtained with human sera. It was shown that the ratio of geometric mean IF titre, as determined against G cells, to that against Y cells (IF-G/IF-Y) was the highest for the group of sera with precipitin to component II only. This was followed by those sera with precipitins to both components I and II. The groups of sera having either precipitin to component I only or no detectable precipitating antibodies also had low values of IF-G/IF-Y (Table 2).

Discussion

It may be concluded from results obtained with rabbit and human sera that the antigenic specificity of the IF assay using Y cells is related to component I. As judged by the immunoprecipitation pattern, component I probably consisted of a heterogeneous mixture of antigens while their apparent stability to heating and phenol extraction suggests that they are probably polysaccharides. These antigens seem to be shared partly or wholly by both the G and Y cells but we did not attempt further to analyse them by immunological techniques of higher resolution.

The antigenic specificity of the IF assay using G cells is related to both components I and II. As indicated by the well-defined line of precipitate, component II is more homogeneous than component I. The fact that this antigen is labile to heating and phenol extraction suggests that it is probably of a proteinic nature.

Fig. 5 Double immunodiffusion patterns of patients' sera (P₁, P₂ and P₃) and rabbit serum (AG) against a sonic extract of live G cells (G).

Table 1 Reactivities of rabbit sera against *C. albicans*

Sera*	Reactivities					
	Precipitin†			Immunofluorescence‡ (titre)		IF-G/IF-Y
	I	II	III	IF-Y	IF-G	
Anti-G	—	—	—	2560	5120	2
Anti-G (-Y)	—	—	—	< 20	320	> 16
Anti-G (-Y, -G)	—	—	—	< 20	< 20	—
Anti-Y	—	—	—	5120	2560	0.5
Anti-Y (-Y)	—	—	—	< 20	< 20	—
Anti-Y (-Y, -G)	—	—	—	< 20	< 20	—
Anti-Gh	—	—	—	2560	2560	1.0
Anti-Yh	—	—	—	2560	320	0.1

*Hyperimmune rabbit sera were produced against live or heat-killed G cells (anti-G, anti-Gh) or Y cells (anti-Y, anti-Yh). Anti-G and anti-Y were exhaustively absorbed first against Y cells (anti-G (-Y), anti-Y (-Y)) followed by G cells (anti-G (-Y-G), anti-Y (-Y-G)).

†Precipitating activities of sera were tested by double immunodiffusion against a sonic extract of live G cells.

‡IF-Y and IF-G titres of sera were assayed as described in Material and methods.

Table 2 Seroreactivities against *C. albicans* group A in patients and healthy subjects

Sera		Precipitating antibodies†	GMT‡ (range)		IF-G/IF-Y
Source	Number		IF-G	IF-Y	
Patients*	7	I	1560 (2635-924)	640 (1706-240)	2.44
	8	I + II	2560 (4863-1348)	830 (2537-292)	3.08
	5	II	1470 (2626-823)	279 (380-204)	5.27
	13	Negative	712 (1573-322)	245 (477-126)	2.91
Healthy subjects	25	Negative	47 (309-7)	59 (334-10)	0.80

*Patients as described in Appendix.

†Patients' sera were grouped according to their precipitin against component I complexes and II detected by double immunodiffusion against the sonic extract of germinating cells of *C. albicans* group A.

‡Indirect immunofluorescence titres against acetone-fixed germinating cell (IF-G) and yeast cells (IF-Y) of *C. albicans* group A observed in individuals in each group were shown as geometric mean titres and ranges.

Table 3 Antibodies to *C. albicans* group A* in sera obtained from patients and healthy subjects

Sera		Geometric mean titres*			No. of sera* with precipitins to:	
Subjects	No.	IF-G	IF-Y	Agg	I	II
Patients with repeated positive blood and/or urine cultures	17	2174 (3869-1222)	694 (1787-270)	188 (510-70)	13	12
Other patients†	16	761 (1592-364)	258 (497-134)	104 (192-56)	2	1
Healthy subjects	25	47 (309-7)	59 (334-10)	49 (112-21)	0	0

*Serum specimens were tested by indirect immunofluorescence against G cells (IF-G) and Y cells (IF-Y), by agglutination using Y cells (Agg) and by immunodiffusion against components I and II (I and II) present in a sonic extract of G cells.

†The 33 patients described in the Appendix were divided into 17 who gave repeated positive blood and/or urine cultures of *C. albicans* and 16 who did not.

Neither component I nor component II is a growth phase specific antigen, as both could be extracted by sonic disruption of Y or G cells. However, after exhaustive reaction of anti-G with Y cells, the absorbed serum, which contained precipitin to component II, gave an IF reaction confined to the germ tubes of G cells. Immunofluorescence confined to the germ tubes was likewise observed with some human sera at high dilution (Ho *et al.*, 1976). It seems that component II is probably localised on the germ tubes. On the other hand, this antigen seems to exist in a cryptic state in Y cells because these cells were not capable of producing antibodies to component II in rabbits, nor were they able to absorb these antibodies despite the fact that component II was as readily extracted from Y cells as from G cells.

If infection with *C. albicans* involves mycelial infiltration, as is supported by histological evidence (Winner, 1969), it may be anticipated that antibody response to component II may be a distinguishing feature of infection. This is supported by our earlier observations that patients who showed mycological and clinical evidence of candidiasis

sustained significantly higher titres of IF antibodies to *C. albicans* than healthy subjects. To evaluate further the diagnostic application of antibodies to *C. albicans* the patients shown in the Appendix were divided into two groups, as in Table 3. All these patients had primary clinical symptoms that are known to predispose them to infection with *C. albicans* (Bodey, 1966; Hart *et al.*, 1969; Ashcraft and Leape, 1970; Ho *et al.*, 1977) but only patients in one of the groups had repeated positive blood and/or urine cultures of *C. albicans*. Although none of those patients is a confirmed case of candidiasis because mycological evidence alone is insufficient to differentiate active infection from the transient presence of these organisms in the blood and/or urine, it seems reasonable to assume that the group of patients who gave repeated positive blood and/or urine cultures were more likely to be infected with *C. albicans* than the other group of patients. It was of interest therefore that precipitin to components I and II was detected more frequently in the sera from the former group of patients who also showed significantly higher geometric mean titres of anti-

bodies to this organism. Also shown in this table were results obtained from 25 healthy subjects, among whom none showed precipitating antibodies, and their mean serum antibody titres were lower than those observed among the patients. The healthy subjects are also the least likely of the three groups to sustain an infection with *C. albicans*.

Thus, in the three groups of subjects, the serological findings seem to be related to the relative susceptibility to infection with *C. albicans*. It should be noted that these groups of subjects were better separated on the basis of the frequency of precipitating antibodies to *C. albicans* and by their geometric mean titres of serum antibodies, as determined by immunofluorescence against G cells (IF-G). It seems that antibodies to component II may indeed be of diagnostic value in candidiasis.

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Appendix

Clinical, mycological, and serological data on 33 patients

Case	Sex/ Age	Primary clinical conditions	Positive cultures of <i>C. albicans</i> group A		Precipitins to:		Antibody titres		
			Specimen	No. of positive cultures	Component I	Component II	IF-G	IF-Y	Agglutination
1	F62	Carcinoma oesophagus	Mid-stream urine	2	+	+	1280	1280	160
2	F54	Status asthmaticus	Catheterised urine	2	+	+	5120	5120	160
3	FAd	Cirrhosis of liver	Sputum	2	+	+	5120	320	320
4	M14	Neurogenic bladder	Mid-stream urine	4	+	+	5120	2560	1280
5	F76	Carcinoma oesophagus (on hyper- alimentation)	Catheterised urine	5	+	+	2560	640	80
6	M75	Carcinoma bladder	Rectal swab	3	+	+	2560	320	40
7	F56	Intestinal obstruction (on hyper- alimentation)	Blood	3	+	+	1280	640	160
8	F13	Choledochal cyst (on hyper- alimentation)	Intravenous catheter tip	2	+	+	2560	640	160
9	FAd	Carcinoma bronchus	Mid-stream urine	4	+	+	1280	320	160

contd.

Appendix—continued

Case	Sex/ Age	Primary clinical conditions	Positive cultures of <i>C. albicans</i> group A		Precipitins to:		Antibody titres		
			Specimen	No. of positive cultures	Component I	Component II	IF-G	IF-Y	Agglutination
10	M70	Carcinoma colon, intestinal fistula (on hyper-alimentation)	Throat swab	3	+	—	2560	2560	1280
			Rectal swab	3					
			Mid-stream urine	5					
			Blood	1					
			Wound swab	4					
			Nutritive solution from IV drip	2					
11	F62	Chronic myeloid leukaemia, lung abscess	Sputum	5	+	—	2560	320	160
			Mid-stream urine	2					
12	FAd	Carcinoma lung	Sputum	6	+	—	1280	160	40
13	MAd	Carcinoma oesophagus (on hyper-alimentation)	Nasal swab	7	+	—	1280	1280	160
			Throat swab	2					
			Rectal swab	4					
			Mid-stream urine	2					
			Blood	1					
			Wound swab	5					
			Nutritive solution from IV drip	6					
14	MAd	Carcinoma oesophagus (on hyperalimentation)	Wound swab	1	+	—	640	640	160
15	MAd	Carcinoma oesophagus (on hyperalimentation)	Mid-stream urine	3	+	—	2560	1280	640
			Nutritive solution from IV drip	4					
16	M62	Drug addict, lung abscess	Sputum	6	—	+	1280	320	160
17	M2m	Malformation of urinary tract (posturethral valve)	Catheterised urine	8	—	+	640	160	320
			Catheterised urine from R and L ureter	1					
			Mid-stream urine	4					
			Wound swab	2					
18	M49	Carcinoma stomach	Rectal swab	2	—	+	2560	320	80
			Wound swab	4					
			Mid-stream urine	5					
19	MAd	Carcinoma oesophagus (on hyper-alimentation)	Rectal swab	5	—	+	2560	320	320
			Blood	2					
			Wound swab	2					
			Drip site swab	1					
			Intravenous catheter tip	1					
			Nutritive solution from IV drip	1					
20	M58	Carcinoma liver	Peritoneal swab	1	—	+	1280	320	160
21	M45	Lung abscess	Sputum	4	—	—	320	80	80
22	M50	Carcinoma stomach	Mid-stream urine	1	—	—	1280	160	80
23	M29	Burn	Wound swab	2	—	—	1280	320	160
24	F13	Hepato-splenomegaly	Wound swab	3	—	—	1280	320	80
25	M68	Carcinoma oesophagus (on hyperalimentation)	Intravenous catheter tip	2	—	—	2560	640	40
			Rectal swab	2					
26	F75	Carcinoma colon (on hyper-alimentation)	Throat swab	4	—	—	640	160	80
			Rectal swab	4					
			Mid-stream urine	1					
			Wound swab	4					
27	F76	Carcinoma oesophagus (on hyper-alimentation)	Throat swab	5	—	—	1280	640	160
			Rectal swab	1					
			Wound swab	3					
28	MAd	Carcinoma liver	Skin swab	1	—	—	640	320	160
29	F76	Carcinoma oesophagus (on hyperalimentation)	Rectal swab	1	—	—	320	160	160
			Nutritive solution from IV drip	1					
30	M69	Carcinoma oesophagus (on hyper-alimentation)	Throat swab	2	—	—	160	80	40
			Rectal swab	2					
			Wound swab	2					
31	FAd	Carcinoma oesophagus	Nasal swab	1	—	—	1280	320	80
			Wound swab	1					
32	F30	Monilia infection of perineum	Swab from perineum	2	—	—	320	320	320
33	M60	Faecal fistula	Nasal swab	1	—	—	640	320	160
			Throat swab	1					
			Skin swab	1					