Antibodies to *Candida albicans* in hospital patients with and without spinal injury and in normal men and women

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SYNOPSIS The presence of serum agglutinins and precipitins to *Candida albicans* in men and women who had impaired bladder function following spinal injury is compared with that in other patients in hospital and in apparently fit men and women. Antibody levels which have commonly been regarded as significant were found in 35 of 76 patients who had urinary candidiasis; in 10 of 52 patients who had spinal injury without urinary candidiasis; in eight of 57 patients who had neither of these conditions, and in nine of 191 normal men and women. Observations are made on the antigens involved in these reactions and on the need for standardization of both reagents and techniques.

It is common for patients to develop urinary tract infections following spinal injury and for them to be treated with antibiotics. As a complication of this, *Candida albicans* sometimes colonizes the bladder where it is found usually as a transitory, but occasionally as a persistent, organism in the urine, with or without the presence of pus, and commonly without any obvious systemic disturbances in the patient. No treatment is necessary unless there is some evidence of impending invasive candidiasis. One measure of systemic involvement is the presence of circulating antibodies. Although Winner (1955) regarded agglutinins to *C. albicans* as of little or no diagnostic significance, Stallybrass (1964) found that precipitating antibodies to *C. albicans* were produced in response to systemic but not to superficial infection. The present study was undertaken to compare the levels of antibody to *C. albicans* in patients with impaired neurophysiological function of the bladder (resulting from spinal injury) with those found in patients with postoperative urinary candidiasis but no spinal injury and in normal persons.

Materials

Human sera were collected from 376 persons; they can be divided into four groups:

1. A 'normal' population of 191 persons: 83 men (water works employees) and 108 women of child-

bearing age (family planning clinic patients). A vaginal swab was also taken from the women.

2. Seventy-nine patients in a spinal injuries unit: 56 men and 23 women. Thirteen of these men and 14 of the women were known to have urinary candidiasis.

3. Fifty-seven orthopaedic patients (29 men and 28 women) without spinal injuries but of a comparable age range to the previous group.

4. Forty-nine patients in a general hospital who developed urinary candidiasis (25 males and 24 females).

Sera were not inactivated and were stored at -20°C .

C. albicans (A) precipitating antigen, obtained in the lyophilized state from the Mycological Reference Laboratory of the Public Health Laboratory Service, was dissolved in phosphate-buffered saline (Dulbecco solution A) to give a solution containing 2.5 mg per ml. Sodium azide (to 1 g/litre) was then added.

A preparation of mannan derived from *C. albicans* was obtained from the same source and treated in the same manner. (This was not available until towards the end of the investigation.)

BORATE AGAR GEL pH 8.6 (IONIC STRENGTH 0.05)

This has the composition:

Boric acid 6.7 g

Sodium borate (decahydrate) .. 13.4 g
 Distilled water 1000 ml
 Oxoid agar no. 1 10 g
 Ethylene diamine tetraacetic acid disodium salt 0.25 g
 The agar is dissolved in half the water by heating; the other reagents and the remainder of the water are then added.

BARBITONE AGAR GEL PH 8.6 (IONIC STRENGTH 0.05)

This has the composition:

Sodium diethyl barbiturate 10.3 g
 Diethyl barbituric acid 1.84 g
 Sodium azide 1 g
 Oxoid agar no. 1 10 g
 Distilled water 1000 ml

It is prepared in the same manner as is borate agar gel.

COOMASSIE BRILLIANT BLUE R STAIN

This has the composition:

Coomassie Brilliant Blue R (Searle

Diagnostic) 0.2 g
 Methanol 45 ml
 Acetic acid 10 ml
 Distilled water 45 ml

SHEEP ANTI-*C. albicans* SERUM (BURROUGHS WELLCOME)

This had no reference number but was dated February 1971. It was used as a positive control serum in both the double diffusion and agglutination tests.

Methods

AGGLUTINATION TESTS

Preparation of the suspension

Roux bottles containing glucose peptone agar were inoculated with an 18-hour culture of a stock strain of *C. albicans* A. After incubation for not more than 32 hours the growth was washed off in phosphate-buffered saline (PBS). The harvested cells were washed twice in PBS containing 1 g/litre of sodium azide, packed by centrifugation, and the packed cell volume of the suspension was measured. This suspension was distributed in convenient volumes into a series of screw-capped bottles and stored at -20°C . Before use in agglutination tests sufficient PBS containing 1 in 1000 sodium azide was added to each bottle to yield a 2% suspension of yeast cells.

Performance of the test

Sera were diluted with PBS and azide in a WHO tray to give a range of doubling dilutions commencing

at 1/8, each dilution being in a final volume of 0.4 ml. One drop (0.02 ml) of a 2% *Candida* suspension was then added to each well, the whole mixed, and then left at room temperature for 24 hours. The deposit was resuspended by gently shaking and the results were read under oblique illumination against a black background. Under these conditions the Burroughs Wellcome sheep anti-*C. albicans* serum agglutinated the suspension to a dilution of 1/64 whereas no agglutination was observed in PBS alone.

DOUBLE DIFFUSION TESTS

Molten borate agar was distributed in 2.5 ml volumes per 5 cm petri dish on a level surface. When cool, patterns were cut with the use of the templates according to the plan below, using a no. 4 cork borer and a 2 mm diameter needle.

A and A' were filled with the appropriate antigen (2.5 mg/ml). The sera under investigation, together with positive controls, were placed in the peripheral cups 1-4, these again being just filled. (Each unknown serum was therefore tested at two ratios against the antigen, the volume of the large antigen cup A to that of the small antigen cup A' being approximately 9:1) (fig 1).

The plates were incubated at room temperature for four days, read, washed in azide saline for two days, dried, stained with Coomassie Brilliant Blue for 30 minutes, decolourized in a mixture of the solvents without the stain, dried and read again.

If precipitin lines were produced the sera were retested to elicit a reaction of identity with the Burroughs Wellcome control serum. Selected sera were also tested in a similar way against *C. albicans* mannan and, after absorption with *C. albicans* mannan, against *C. albicans* antigen. The procedure for absorption was as follows:

Two drops (0.04 ml) of each serum under test were placed in adjacent peripheral cups: in one of these cups was placed one drop of 2.5% mannan

Fig 1

Fig 1 Routine screening of patients' serum (peripheral cups 1-4) against *Candida albicans* antigen (CAA) in the small central cup. The rather diffuse line produced by sera 1, 3, and 4 was shown in subsequent experiments to result from antibody to the mannan component.

Fig 2

Fig 2 Identification of *Candida* antibodies in human sera by simple absorption with mannan. Cup 108 contains serum from patient 108. Cup 108 (M) contains the same serum absorbed with mannan. Cup BW contains sheep antiserum to CAA and cup BW(M) the same antiserum after absorption. The central cup contains *C. albicans* antigen. Serum 108 is shown to contain three antibodies.

solution. The central cup was filled with *C. albicans* antigen (fig 2).

IMMUNOELECTROPHORESIS TESTS

These were carried out in a milieu either of barbitone or of borate buffers. The gel was poured to a depth of 2 mm on a plate of 10 × 10 cm and after cutting a suitable pattern of troughs and holes, electrophoresis was carried out at 'constant current'. With a current of 25 mA flowing through each plate of the above dimensions a satisfactory separation was obtained in three to four hours.

CULTURES

Standard microbiological methods were used for examining urine samples from hospital patients and high vaginal swabs from the family planning clinic patients. Urine samples were catheter specimens and vaginal swabs were transported in Stuart's medium.

Results

AGGLUTININS TO *C. albicans* IN HUMAN SERA

Agglutinin titres of 1/64 or more were found in two

of 83 apparently healthy men. Such titres were, however, much more commonly found in male hospital patients with a spinal injury (7/56) or in general hospital patients with urinary candidiasis (11/25). In male hospital patients who had neither spinal injury nor urinary candidiasis, agglutinins at a level of 1/64 or more were not found in any of the 29 sera examined. With women the pattern of serum agglutinins followed that of men with the exception that women in hospital with neither spinal injury nor urinary candidiasis showed high titres slightly more frequently than did normal women (table I).

Some patients' sera showed a prozone and some gave a coarse and others a fine agglutination reaction.

PRECIPITINS TO *C. albicans* IN HUMAN SERA

Fifty-one persons, including some apparently healthy men and women, were found to have precipitins reacting with the *C. albicans* antigen. The presence of precipitins was not always associated with agglutinins (table II) though it was more commonly so in patients who had urinary candidiasis (tables III and IV).

Population	Men					Women				
	Reciprocal of Agglutinin Titre				Total Patients	Reciprocal of Agglutinin Titre				Total Patients
	16 or less	32	64	128		16 or less	32	64	128	
Normal	80	1	2	0	83	103	2	3	0	108
Spinal injury	42	7	6	1	56	13	7	2	1	23
Other orthopaedic	28	1	0	0	29	23	3	0	2	28
Urinary candidiasis (general hospital)	11	3	10	1	25	12	3	6	3	24

Table I Agglutinins to *C. albicans*

Population	Men					Proportion of Total Patients	Women					Proportion of Total Patients
	Reciprocal of Agglutinin Titre				16 or less		Reciprocal of Agglutinin Titre				16 or less	
	32	64	128	32			64	128				
Normal	1	0	1	0	2/83	1	2	2	0	5/108		
Spinal injury	2	3	1	1	7/56	3	1	1	1	6/23		
Other orthopaedic	1	0	0	0	1/29	3	2	0	2	7/28		
Urinary candidiasis (general hospital)	2	2	9	1	14/25	0	2	5	2	9/24		

Table II Agglutinins in 51 patients with precipitins to *C. albicans*

Population	1	2	3	Total of Columns 1 and 3	Total Patients in Population Group
	Number with an Agglutinin Titre of 1/64 or More	Number with Precipitins	Number with Precipitins and Agglutinin Titre of 1/32 or less		
Normal	2	2	1	3	83
Spinal injury with or without coexisting urinary candidiasis	7	7	5	12	56
Other orthopaedic	0	1	1	1	29
Urinary candidiasis (general hospital)	11	14	4	15	25
Spinal injury and coexisting urinary candidiasis	3	2	1	4	13

Table III Precipitins and 'significant' agglutinins to *C. albicans* in men

Population	1	2	3	4	5
	Number with an Agglutinin Titre of 1/64 or More	Number with Precipitins	Number with Precipitins and Agglutinin Titre of 1/32 or less	Total of Columns 1 and 3	Total Patients in Population Group
Normal females	3	5	3	6	108
Spinal injury with or without coexisting urinary candidiasis	3	6	4	7	23
Other orthopaedic	2	7	5	7	28
Urinary candidiasis (general hospital)	9	9	2	11	24
Spinal injury and coexisting urinary candidiasis	2	5	3	5	14
Normal females with positive vaginal swabs for <i>C. albicans</i>	1	2	1	2	13

Table IV Precipitins and 'significant' agglutinins to *C. albicans* in women

Tables III and IV also show the number in each group studied who had either an agglutinin titre of 1/64 or more, or precipitins, or both. These two tests taken together in this way confirm the conclusions stated above for the agglutinin tests alone.

IMMUNOCHEMICAL ANALYSIS OF THE ANTIGENS USED IN THE DOUBLE DIFFUSION TESTS

Simple double diffusion tests with sheep anti-*C. albicans* serum against the *C. albicans* antigen showed it to be a complex mixture. On immunoelectrophoresis in barbitone agar no fewer than seven separate components could be recognized (fig 3). These were characterized in terms of their electrophoretic mobility using human serum proteins as a reference. In this way there was distinguished one major component of pre-albumin mobility and lesser components of α_1 , α_2 , β , and γ mobility. Electrophoresing in borate agar (fig 4) gave a similar pattern but the mobility of the γ component increased to that of β .

In the same way analysis of the mannan preparation showed it to contain a fraction having γ mobility in barbiturate agar and β mobility in borate agar (figs 3 and 4).

The *C. albicans* antigen had been thought to be relatively free from *C. albicans* mannan: these experiments showed that it contained quite enough to give a good precipitin line provided that the antigen-antibody ratios were correctly adjusted for the system. Equally the sheep anti-*C. albicans* serum contained a small amount of anti-mannan. With experience it was found that precipitin lines of the mannan-anti-mannan system were often recognizable, being softer in outline and less dense than those produced with other antigen-antibody systems.

That a line was or was not due to mannan/anti-mannan was confirmed by the absorption experiments described above (fig 2) or by double diffusion tests in which the mannan was employed as antigen (fig 1). All sera from the control groups, viz, normal males, normal females, and 'other orthopaedic' patients which gave positive precipitin tests with the *C. albicans* antigen were further examined in this way: no antibody other than that to *C. albicans* mannan could be demonstrated.

Discussion

High agglutinin titres were not diagnostic of urinary candidiasis nor did their absence exclude the condi-

Fig 3

Fig 3 Immunoelectrophoretic analysis in barbitone agar of *Candida albicans* antigen (CAA) and mannan antigen employing two human sera and the Burroughs Wellcome sheep antiserum to CAA. A sample of normal human serum was analysed simultaneously on the same plate against anti human serum: 'A' indicates the centre of the albumin arc so produced.

Fig 4

Fig 4 As fig 3 but the analysis is carried out in borate agar. Note the increased mobility of the mannan fraction in both the CAA and mannan antigens.

tion. High agglutinin titres were found in only 14 of 38 men and 11 of 38 women with genitourinary candidiasis whereas comparable titres were found in about 2.5% of healthy men and women.

Damage to the bladder nerve supply caused by spinal injury can reduce bladder tone. We found no evidence that *C. albicans* in the bladder is more invasive (as judged by the presence of antibodies) in patients with a spinal injury than in other patients who have urinary candidiasis but no spinal injury.

It has been suggested that agglutinins to *C. albicans* are directed against the mannan component of the cell wall. Although our precipitin tests regularly identified precipitins to mannan, agglutinins and precipitins were not always both detectable in the same serum even if the agglutinating titre were high and it seems likely that some agglutinins are directed at components of the cell wall other than mannan.

Immunoelectrophoretic analysis of the *C. albicans* antigen used in our precipitin tests with the sheep anti-*C. albicans* serum identified seven components. The number of antigens identified in any mixture depends on the number of homologous antibodies in the polyvalent serum used for the analysis as well as the sensitivity of the particular technique employed. Using the two-dimensional immunoelectric method of Clarke and Freeman (1966), 78 water-soluble antigens have been identified in an homogenate of *C. albicans* (Axelson, 1973). The increased electrophoretic mobility of the mannan component when borate buffer is used instead of barbitone buffer is a property common to all polysaccharides having cis-hydroxyl groups.

Our precipitin tests were carried out at two antigen-antibody ratios: had we tested at more ratios with smaller intervals the number of positive results could probably have been increased as would have been the case if the sera had been concentrated. Even so, if we had relied only on precipitin lines visible in the gel before staining many positive results would have been missed. Coomassie Brilliant Blue is an excellent stain for weak precipitin lines and is preferable to Amido Black for this purpose.

Confusion exists in the literature because many of the tests employed by different observers, although bearing the same name, have varied in their performance, in the materials employed, in the results they have given and the conclusions drawn from them. There is a real danger of serological tests for candidiasis falling into disrepute unless more standardized methods are accepted. Thus as recently as 1973, Jones, Brennan, and Kundsins reported that while the precipitin test did not differentiate local from systemic invasion with *C. albicans*, nonetheless it was of value because it did not give positive results with any of their blood donor control sera. Dolan and Stried

in the same year found no precipitins in 50 blood bank donor sera. Both studies employed S antigen prepared by sonication followed by removal of cell walls by centrifugation. However, as can be seen from the experimental results reported by Taschdjian, Kozinn, Cuesta, and Toni (1972) it does contain cell wall mannan as a minor component. Preisler, Hasenclever, Leviton, and Henderson (1969) found precipitating antibody in the antemortem sera from only four of 14 persons dying of acute leukaemia in whom evidence of visceral candidiasis was found at necropsy. In none of these four patients was precipitating antibody directed solely against S antigen or solely against mannan. Taschdjian and her associates in an examination of sera from 'high risk' patients, found no precipitins in nine patients, shown at necropsy to be free of candida infection, and positive precipitin tests in 21 out of 24 patients, in whom systemic candidiasis was proved either in life or at necropsy. Chew and Theus (1967), by a combination of concentration of serum globulins and the choice of a sensitive technique, concluded that precipitating antibodies could be demonstrated in the sera of all healthy adults. Goldstein and Hoeprich (1972), recommending comparative titrations of agglutinating antibody as a means of identifying patients developing systemic candidiasis, said of the precipitin test that 'while the discrepancies may reflect variations in methods of testing the significance of precipitins is uncertain at present'. Some workers appear to have tested sera and antigens at only one ratio, a common cause of false negative reactions in any gel diffusion test and perhaps particularly so with *C. albicans*, as has been suggested by Remington, Gains, and Gilmer (1972). For these reasons, we considered it necessary to define as carefully as we could the source of our materials and the techniques used, although the techniques are, with minor modifications, based on those described by Murray, Buckley, and Turner (1969).

Our results, particularly those with sera from our control groups, support the view that the presence of antibody to *C. albicans* mannan is of minor importance in the diagnosis of systemic candidiasis. Present hopes are that antibody production to other (protein) antigens of *C. albicans* might be a better indicator of systemic candidiasis. Certainly antibodies to these other antigens do not appear so liberally in our sera as do those to mannan and they are demonstrated more commonly in patients with candidiasis than in controls. Such differences may be quantitative rather than absolute and whatever test is devised for the future it is essential to ensure its standardization and to fix its level of sensitivity. Until such time as *C. albicans* antigen can be fractionated and defined, gel diffusion tests when used

alone have no advantage over the more easily quantifiable agglutination test.

Probably in the present state of development of these tests, the most important contribution which any routine microbiological laboratory can make, presented with the patient at high risk from systemic candidiasis, is to retain at -15°C a baseline serum specimen so that it can, if necessary, be examined with subsequent sera by a laboratory which specializes in this work (Kozinn, Mackenzie, Hasenclever, Protzman and Seelig, 1972.)

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footnote

¹Since this work was completed we have been informed that a batch of *C. albicans* precipitating antigen free of mannan has been prepared by the Mycological Reference Laboratory.

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