

Antibodies mediating type I skin test reactions to polysaccharide and protein antigens of *Candida albicans*

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Summary

Immediate, type I, prick test reactions in man to purified fractions of *Candida albicans* group A were mediated by different classes of antibody. The allergens were the cell-wall polysaccharide, mannan, and a 'purified' cytoplasmic protein fraction, free of mannan, prepared by passage through Sepharose-concanavalin-A. Passive transfer tests in the monkey gave immediate reactions, (1) to the purified, cytoplasmic, protein mediated by heat-labile long-term sensitizing antibody, and (2) to the polysaccharide, mannan, mediated by heat-stable short-term sensitizing antibody.

Introduction

Most of the preparations of antigens from *Candida albicans* in use at present for skin, serological and other tests are crude mixtures containing proteins and polysaccharides. The main polysaccharide is the cell wall mannan against which precipitins are demonstrable in the sera of most normal subjects if sensitive enough methods are used (Chew & Theus, 1967).

Furthermore, when the mannan is used for skin tests it can elicit both type I, immediate and type III (Arthus) reactions and acts as a potent stimulant of specific precipitating antibodies (Pepys *et al.*, 1968). The protein components are responsible for type IV allergic reactions but can also elicit type I and type III-like, reactions (personal observation). The purpose of the present investigation was to prepare cytoplasmic proteins of *C. albicans* in a mannan-free form, for comparison with purified mannan preparations first prepared by Hasenclever & Mitchell (1964), and to provide such a preparation for general use as a reference 'antigen'.

Materials and methods

C. albicans group A cells

A non-antigenic growth medium was used containing 1% casamino acids (Difco

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Technical), 0.04 M NaCl, 0.05 M CH_3COONa . This was sterilized by heat or filtration, and then a solution of d-glucose, sterilized separately, was added to give a final concentration of 2% glucose, pH being corrected to 5.5 aseptically.

A 400 l batch of this medium was inoculated with a shaken seeding culture of *C. albicans* group A strain B311, and grown with vigorous aeration at pH 5.2–6.2 for 48 hr at 37°C to produce a culture of cells in the blastospore form. The cells were centrifuged free of supernatant, washed twice with physiological saline containing 0.05% sodium azide and stored as a thick paste (approximately 4 kg) in aliquots of 250 g at -160°C . The 400 l batch was grown for us at the Microbiological Research Establishment, Porton.

Preparation of the crude cytoplasmic extract

An aliquot of cell paste was thawed, and diluted 1:1 with physiological saline. Twenty-five millilitre quantities were transferred to the chamber of an 'X-press' (A.B.Biox, Nacka, Sweden), re-frozen to -20°C and disrupted by pressure three times. This resulted in substantially complete destruction of the cells. The material was separated by differential centrifugation at 3000, 10,000 and 40,000 rev/min (105,000 g) to remove the walls, fragmented walls and cytoplasmic organelles respectively. The final supernatant was termed the crude cytoplasmic extract, and the total solids of this extract contained approximately 15% mannan.

Purification of the crude extract by affinity

chromatography on Sepharose-concanavalin-A (Con A)

The crude extract was first dialysed for 24 hr at 4°C against several changes of separation buffer consisting of 0.1 M CH_3COONa , 0.4 M NaCl and 10^{-3} M Ca, Mg and Mn chlorides, pH 6.5. After membrane filtration through a 0.45 μm Millipore membrane, 15 ml of the extract was applied to Sepharose Con A column 20 cm $\times$ 2 cm diameter at 4°C (Pharmacia). This had been repeatedly washed with the separation buffer just before use. The concanavalin-A has a high affinity for mannans and it was used to provide a mannan-free preparation of the cytoplasmic proteins. The fractions obtained from the column were scanned at 280 nm, and those containing the protein were bulked, dialysed against 0.02 M NH_4HCO_3 for 24 hr and lyophilized. The pilot batch of purified cytoplasmic proteins was obtained from two columns.

The large batch was produced by bulking together the protein-containing fractions of many columns.

Tests of purity of cytoplasmic protein fraction

Two preparations of this material were examined, the first a small amount from a pilot preparation and the second the large remainder. These materials were tested for their content of (a) mannan and (b) concanavalin-A, the latter possibly derived from the separation process.

(a) *Mannan content.* The mannan gives a precipitation reaction in agar gel double diffusion tests against solutions of concanavalin-A (Pharmacia).

In tests against concanavalin-A at 1 mg/ml, purified mannan could be detected by the precipitation reaction down to a concentration of 0.075 mg/ml. No precipitation was given by the purified cytoplasmic protein fractions from both batches when tested at 20 mg/ml, implying that its mannan content, if any, would be less than

0.00375%. Tests with the crude cytoplasmic extract before purification gave precipitation reactions down to 0.625 mg/ml, indicating that it contained 12% of mannan.

(b) *Concanavalin-A* content. This was tested in various ways:

Rabbit anti-concanavalin-A antiserum. Two antisera were used, one provided commercially by Miles-Yeda and the other prepared in our own laboratories.

Immuno-electrophoretic tests were made using the method of Longbottom & Pepys (1964). The concanavalin-A was electrophoresed and tested against the antisera in the trough and gave specific precipitation reactions down to 0.05 mg/ml of concanavalin-A. No reactions were given by the purified cytoplasmic protein fractions from either the pilot or the main batch, tested at 20 mg/ml.

In immuno-electrophoretic tests in which the antisera were electrophoresed and tested against the purified cytoplasmic proteins at 20 mg/ml, a precipitation reaction was obtained in the gamma-globulin region with the material from the main but not the pilot batch. This reaction corresponded to that given by a concentration of 0.01 mg/ml of a concanavalin-A solution. This reaction to the purified protein fraction was inhibited by absorption with rabbit anti-concanavalin-A antiserum but not with normal rabbit serum. This indicates a possible content of concanavalin-A of 0.5 µg per mg of the main batch of 'purified' cytoplasmic protein.

Latex particle test. Latex particles sensitized with rabbit antiserum were agglutinated by concentrations of concanavalin-A down to 10^{-5} mg/ml. Tests with 1.0 mg/ml of the pilot batch of 'purified' cytoplasmic protein failed to give agglutination, showing that its content, if any, of concanavalin-A as judged by these tests to be less than 0.001%.

Sheep erythrocyte agglutination test. These cells are agglutinated by concanavalin-A, and this can be inhibited by absorption with the rabbit anti-concanavalin-A antiserum before the addition of the erythrocytes. The test was made with 1 mg/ml stock solutions of concanavalin-A and of the main batch of 'purified' cytoplasmic protein. The rabbit antiserum was used at 1/100 and 1/10 dilution in phosphate buffered saline. For the test, 0.25 ml of each dilution were placed in a well of a Lenbro-MRC-96 test plate, to which 0.02 ml of a 3% suspension of washed sheep erythrocytes in Dulbecco saline was added. The plates were incubated at 37°C for 1 hr and then read.

The concanavalin-A caused agglutination to a dilution of 1/8192, i.e. a concentration of 0.12 µg/ml. When the concanavalin-A was absorbed with the 1/100 dilution of the rabbit antiserum, the titre of erythrocyte agglutination fell to 1/256 and absorption with the 1/10 antiserum led to a titre of 1/32, thus confirming the specificity of the agglutination by the concanavalin-A.

In tests with the 'purified' cytoplasmic protein, no agglutination was seen, starting with concentrations of 1 mg/ml, indicating that content of concanavalin-A by this test to be less than 0.12 µg/ml.

Comparison of 'purified' cytoplasmic proteins and concanavalin-A in skin tests in the guinea-pig. The two preparations were examined for their capacity to elicit skin test reactions in a normal Hartley strain guinea-pig (weight 500 g). Intracutaneous injections were given of 0.05 volumes of serial tenfold dilutions of each preparation. The animal was observed for 30 min for immediate type weal and flare reactions and none were seen. After periodical observation up to 48 hr, an intracardiac injection was given of 1.8 ml of a 10% eosin solution in phosphate buffered saline and the injection sites were examined under ordinary and long-wave ultraviolet light.

The concanavalin-A injections showed small areas of vasodilation down to an absolute skin test dose of $0.005 \mu\text{g}$. No reaction was seen with a skin test dose of $500 \mu\text{g}$ of the 'purified' cytoplasmic protein, the postulated concanavalin-A content of which, in the light of the tests reported above, would have been $2.5 \mu\text{g}$ concanavalin-A.

2-dimensional immunoelectrophoresis

This was modified from that of Laurel (1965) using 1% agarose gel in barbiturate buffer, pH 8.6, ionic strength 0.02μ , thickness 1 mm on an $8 \times 8 \text{ cm}$ glass slide.

In the first dimension $3.4 \mu\text{l}$ of a 20 mg/ml dilution of antigen was run for 1 hr with a voltage drop of 4.5 V/cm . For the second dimension a 'window' of $5 \times 3.5 \text{ cm}$ was removed from the gel and refilled with a 2.4 ml agarose containing 0.2 ml of rabbit anti-*Candida albicans* antiserum (Dako, Denmark) and run for 20 hr at a voltage drop of 1.4 V/cm . (Fig. 1).

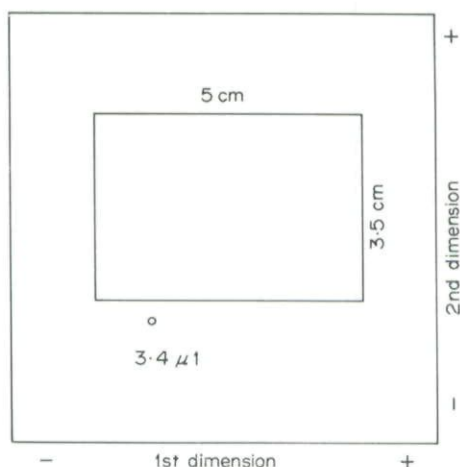


Fig. 1. Procedure for 2-dimensional electrophoresis.

Preparation of mannan A

This was prepared from the washed, disrupted cell wall debris by heat extraction and precipitation as copper complexes by the method used by Peat *et al.* (1961) for the preparation of mannan from *Saccharomyces*.

It was purified further by ion exchange chromatography on DEAE cellulose in 0.25 M tris- 0.015 M HCl buffer, pH 8.4. The first, non-absorbed fractions were collected, bulked and lyophilized.

Skin tests in allergic patients

Skin prick tests were carried out on patients with the mannan and purified cytoplasmic protein antigens from the pilot batch at 10 and 1 mg/ml , and the sera of twelve patients were then used for passive transfer tests in monkeys.

Skin tests in monkeys

Passive cutaneous anaphylaxis (PCA) and Prauznitz-Kustner (PK) tests were performed on 2-year-old male Rhesus monkeys, using unheated and heated sera from the ten human patients who had shown immediate type skin sensitivity by prick test to the

'purified' cytoplasmic protein (pilot batch), the mannan or to both antigens, and also two control sera from patients giving negative skin tests to both. The monkeys were anaesthetized by intramuscular injection of 2 ml of Sernylan (Bio-Centric Labs, St. Joseph, U.S.A.) diluted 1:10. The hair was removed from the thorax and abdomen with clippers, and 24 hr before challenge with antigen 0.1 ml quantities of the unheated test sera and controls were injected intracutaneously.

The monkeys (A and B) were allowed to recover and 20 hr later were anaesthetized again and 0.1 ml quantities of the test sera and controls, heated at 56°C for 1 hr, were injected intracutaneously into new sites. Two hours later, i.e. 2 hr before challenge, further 0.1 ml quantities of heated test and control sera were injected into a third set of sites. At the time of challenge, 24 hr after the first injections, 5 ml of a 5% solution of Evans Blue dye (Gurr, London), was injected into the saphenous vein of the hind leg. After this had circulated, the sites were inspected to check for vascular leakage before challenge with allergen. No reactions were apparent. Intravenous injections were then given by the same route of 5 mg protein into one monkey (A) and 5 mg mannan into the other (B). After 20 min the sites were inspected for areas of vascular leakage, shown by areas of blueing and the sites were photographed. The monkey (B) which had been challenged with the mannan was later challenged with the 'purified' cytoplasmic protein.

In a third monkey (C) the skin sites which had been passively sensitized by injection with heated and unheated sera, neat and diluted 1/10 were tested directly by intracutaneous injection of 0.05 ml of the appropriate antigen, after the intravenous injection of the dye.

Results

Characteristics of the extracts

'Purified' cytoplasmic protein. The purified protein from the pilot batch consisted of 96% protein, and two such preparations gave the amino acid composition in Table 1.

On electrophoresis in polyacrylamide gel the material showed seventeen protein staining bands, and on 2-dimensional immunoelectrophoresis using a rabbit antiserum to cytoplasmic *C. albicans* (Dako, Denmark) it showed at least twenty-five distinct antigen/antibody peaks (Fig. 2). On comparison with the crude cytoplasmic extract tested at the same concentration, the 'purified' cytoplasmic protein shows a considerable increase in content of each antigenic component judged by the increase in the height and therefore the area under the peaks, since the area varies with concentration of antigen where concentration of antibody is constant.

Mannan A. The mannan had a N₂ content of 0.02% by ninhydrin assay, and 6% amino acids of composition (Table 1). It can be seen that this composition differs from that of the protein in that there were relatively high amounts of glycine, alanine and aspartic acid which were not a feature of the protein profiles.

In vivo tests

The results are summarized in Table 2.

Monkey (A) challenged with 'purified' cytoplasmic protein. Only sites sensitized 24 hr

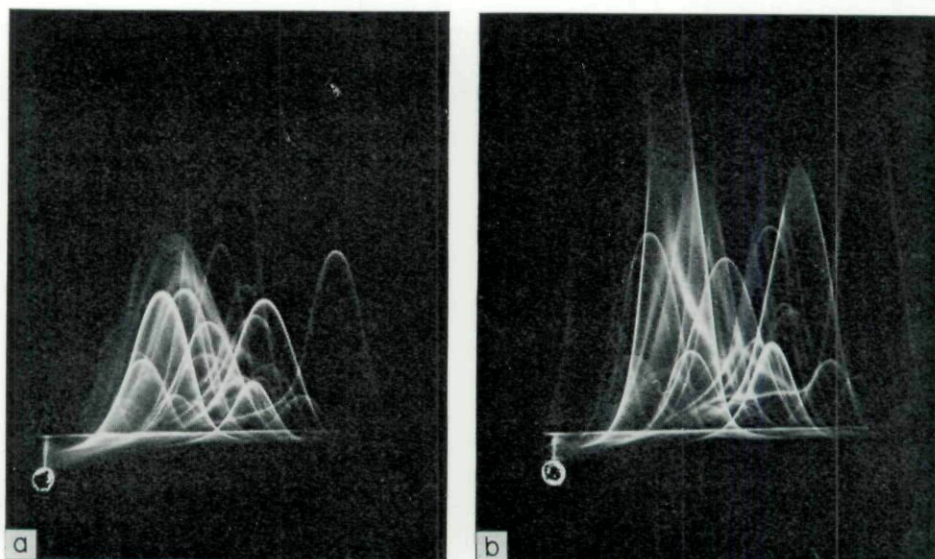


Fig. 2. Two-dimensional immunoelectrophoresis patterns of A, crude cytoplasmic extract; B, purified protein solution; $3.4 \mu\text{l}$ of a $20 \text{ ng}/\mu\text{l}$ of each solution electrophoresed in the first dimension, and then by cross-electrophoresis in the second dimension, into the rabbit anti-*Candida* containing gel ($11.5 \mu\text{l}/\text{cm}^2$).

Table 1. Amino acid analysis of two preparations of *C. albicans* protein and one of *C. albicans* mannan

Amino acid	Protein I (mol. %)	Protein II (mol. %)	Mannan I
His	2.8	2.7	3.5
Lys	10.0	11.3	5.2
NH ₄	1.0	1.2	4.1
Arg	4.7	4.4	2.2
Asp	8.7	9.6	12.7
Thr	6.0	5.0	7.2
Ser	6.0	6.0	3.3
Glut ^c	14.3	12.0	9.5
Pro	3.1	3.1	1.3
Gly	5.7	5.2	17.8
Ala	8.2	8.0	10.8
Val	7.4	9.0	7.3
Met	Trace	Trace	—
Ileu	6.5	6.8	2.4
Leu	7.9	8.5	2.4
Tyr	2.8	2.6	0.5
Ph-ala	5.0	4.7	1.1
OH-Pro	—	—	8.5

Table 2. Monkey PCA tests with sera from twelve patients

	Prick test allergens			
	+ ve Mannan	+ ve Protein	+ ve Mannan and protein	- ve Mannan and protein
Prick test results	2	4	4	2
Monkey PCA-protein tests (I.V.)				
24 hr sensitization (unheated serum)	0	2	2	0
2 and 4 hr sensitization (heated serum)	0	0	0	0
Monkey PCA-mannan tests (I.V.)				
24 hr sensitization (unheated serum)	0	0	0	0
2 and 4 hr sensitization (heated serum)	1	1*	0	1

* This was not one of the 2 sera which were positive in the 24 hr sensitization test.

before challenge with unheated serum showed intense blueing typical of IgE antibody mediated reactions. Such blueing reactions were given by the sera of four out of eight of the patients giving positive immediate skin reactions to the prick test with the protein antigen. None of the sera from patients with negative skin tests to this antigen showed a blueing reaction. There was no detectable blueing or other reaction at any of the sites sensitized with heated sera 2 and 4 hr before challenge with this allergen.

Monkey (C) challenged with mannan. The blueing reactions elicited were of quite a different character from those described above, in that they were slower to develop. In the monkeys challenged by intravenous injection of the allergen, the reactions appeared only around the periphery of the sites which had been sensitized 2 and 4 hr beforehand.

Monkey B challenged with 'purified' cytoplasmic protein after challenge with mannan. The same four sensitized sites which had reacted to the 'purified' cytoplasmic protein in the first monkey (A) reacted as strongly in this monkey (B).

Monkey (C) challenged by the PK method with mannan. Positive results were obtained at the 2-4 hr sites sensitized with heated sera. The strongest reactions, as measured by the diameter of the blueing reaction, occurred with the serum samples diluted to 1/100 and to an antigen concentration of 1 mg/ml.

Discussion

The preparation from *Candida albicans* group A of a purified cytoplasmic protein fraction, free of the mannan cell-wall polysaccharide, in addition to purified mannan free of protein, has made it possible to show that both antigenic preparations, which are present together in currently available test materials, are capable of eliciting type I, immediate, allergic skin test reactions in man and on passive transfer in the monkey.

These reactions to the different antigens were, however, mediated by different classes of antibody. Thus, the reactions to the purified cytoplasmic protein were mediated by heat-labile, long-term sensitizing antibody, presumably IgE antibody, whereas the reactions to the mannan were mediated by heat stable, short-term sensitizing antibody, presumably in the IgG class as described by Parish (1970, 1973).

A variety of polysaccharide antigens have been found to elicit type I reactions, such as blood group substances (Holborow & Loewi, 1962), and preparations such as glucans from the *Trichophyton* spp. (Barker *et al.*, 1962; How *et al.*, 1972). How *et al.* (1972) report that glycopeptide fractions from the *Trichophyton* spp. gave stronger immediate reactions than the glucan and in addition gave type IV reactions. The *C. albicans* mannan can give not only type I but type III skin test reactions for which precipitins are demonstrable, but not type IV reactions (Pepys *et al.*, 1968). We have also observed types I and IV reactions to the purified cytoplasmic proteins and in addition, a reaction with the features of a type III reaction in the absence of demonstrable precipitins. The emphasis in the present report is on type I reactivity, but it is clear that purer antigenic fractions will help in the analysis of the other forms of reaction which can be elicited with *C. albicans* antigens.

The type I skin prick test reactions in man to the protein and polysaccharide antigens of *C. albicans* were indistinguishable in appearance. In the passive transfer tests to the monkey, there were, however, differences. The blueing reactions in response to intravenous injection of the mannan developed slowly and were more evident at the periphery of the reacting sites. In response to the direct skin test challenge, the blueing appeared at the point of injection and spread slowly outwards. The reactions were less intense than those to the purified cytoplasmic protein, which developed rapidly and showed a more even distribution of the blueing. The need to test with a range of dilutions of serum and of the antigens is shown by the reactions to the mannan at sites sensitized with heated serum, that is with short-term sensitizing antibodies. The strongest reactions were given at sites sensitized with 1/100 dilution of serum and in response to tests with mannan at 1 mg/ml.

Of the twelve patients' sera tested, seven gave positive passive transfer reactions to the monkey, four to the purified cytoplasmic protein and three to the mannan, and none gave reactions to both antigenic preparations.

Our findings emphasize the need for chemically purified and characterized antigens for immunopathological analysis of the skin test and other reactions. The tests made show that the purified cytoplasmic protein is free of mannan as a result of the passage of the crude cytoplasmic extract through the Sepharose-concanavalin-A column. The various tests for the presence of concanavalin-A in the purified cytoplasmic extract were negative for the small pilot batch, and were also negative in most of the tests on the large main batch, with the exception of the immuno-electrophoretic test, which gave reactions compatible with the presence of 0.5 µg/mg of concanavalin-A. To exclude a possible contribution by the concanavalin-A to test reactions to this purified cytoplasmic protein extract, a preparation of concanavalin-A containing 0.5 µg/mg should be used as a control.

References

- BARKER, S.A., CRUICKSHANK, C.N.D., MORRIS, J.H. & WOOD, S.R. (1962) The isolation of *Trichophyton* glycopeptide and its structure in relation to the immediate and delayed reactions. *Immunology*, **5**, 627.

- CHEW, W.H. & THEUS, T.L. (1967) *Candida* precipitins. *Journal of Immunology*, **98**, 220.
- HASENCLEVER, H.F. & MITCHELL, W.O. (1964) Immunochemical studies on polysaccharides of yeasts. *Journal of Immunology*, **93**, 763.
- HOLBOROW, E.J. & LOEWI, G. (1962) The immune response to blood-group substances. *Immunology*, **5**, 278.
- HOW, M.J., WHITNALL, M.T. & CRUICKSHANK, C.N.D. (1972) Allergenic glucans from Dermato-phytes. I. Isolation, purification and biological properties. *Carbohydrate Research*, **25**, 341.
- LAUREL, C.B. (1965) Antigen-antibody crossed electrophoresis. *Analytical Biochemistry*, **10**, 358.
- Longbottom, J.L. & PEPYS, J. (1964) Pulmonary aspergillosis: diagnostic and immunological significance of antigens and C-substance in *Aspergillus fumigatus*. *Journal of Pathology and Bacteriology*, **88**, 141.
- PARISH, W.E. (1970) Short-term anaphylactic IgG antibodies in human sera. *Lancet*, **ii**, 591.
- PARISH, W.E. (1973) A human heat-stable anaphylactic or anaphylactoid antibody which may participate in pulmonary disorders. In: *Asthma, Physiology, Immunopharmacology and Treatment* (Ed. by K. F. Austen and L. M. Lichtenstein), p. 71. Academic Press, New York.
- PEAT, S., WHELAN, W.J. & EDWARDS, T.E. (1961) Polysaccharides of Baker's yeast. IV. Mannan. *Journal of the Chemical Society*, 29.
- PEPYS, J., FAUX, J.A., Longbottom, J.L., MCCARTHY, D.S. & HARGREAVE, F.E. (1968) *Candida albicans* precipitins in respiratory disease in man. *Journal of Allergy*, **41**, 305.

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