Production and characterisation of a monoclonal antibody to a cell-surface, glucomannoprotein constituent of *Candida albicans* and other pathogenic *Candida* species

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Summary. A murine monoclonal antibody (MAB AF-1) class IgM was raised to a soluble glucomannoprotein extract (GMP) of *Candida albicans*. Agglutination and indirect immunofluorescence assays with purified MAB showed that AF-1 was directed against a cell-surface epitope shared by *C. albicans* serotypes A and B, *C. tropicalis*, *C. guilliermondii* and *C. viswanathii*, but not by *C. krusei*, *C. parapsilosis*, *C. kefyr* (*pseudotropicalis*) and *T. glabrata*. Treatment with heat, protease or periodate-treated GMP and with other cell-wall extracts of *C. albicans* provided evidence that the epitope recognised by MAB AF-1 is carried by the polysaccharide moiety of cell-surface glucomannoprotein molecule(s).

Introduction

By their unique feature of recognising single or iterative epitopes on complex antigenic structures, monoclonal antibodies (MABs) are widely used to dissect molecular structures and identify their biological functions.

Although MABs raised against different cell-wall antigens of *Candida* spp. have been obtained and used for diagnostic (Miyakawa *et al.*, 1986) or physiological (Brawner and Cutler, 1984, 1986; Hopwood *et al.*, 1986) studies, these reagents were not produced by immunisation with a soluble glucomannoprotein extract. The low intrinsic anti-mannoprotein humoral immune response in mice has been observed repeatedly, and Domer *et al.* (1986) have demonstrated that some mannoprotein from the cell wall of *C. albicans* may suppress the B-lymphocyte response. A MAB has been obtained by immunisation with a previously characterised glucomannoprotein-rich antigenic extract (GMP) from the cell wall of *C. albicans* (Ausiello *et al.*, 1986; Scaringi *et al.*, 1988). In this paper we describe some of the distinctive features of this new MAB.

Materials and methods

Organism and growth conditions

C. albicans, strain BP, serotype A, was from the collection of the Istituto Superiore di Sanità, Rome. Strains CA2 and A12 of *C. albicans* were aegerminative variants, the origin of which has been reported elsewhere (Mattia *et al.*, 1982). All other strains or species employed throughout the studies were isolated from patients with vaginitis (Mondello *et al.*, 1986). The organisms were routinely maintained on Malt-extract Agar (Difco) at 28°C. Liquid cultures were in low-glucose Winge medium, as described by Mattia and Cassone (1979). Unless otherwise stated, the experimental results refer to *C. albicans* strain BP.

Cell-wall fractions

A cell-wall mannan fraction (M) was extracted and purified as reported by Peat *et al.* (1961). A glucomannoprotein complex (GMP), an alkali-extracted mannan preparation (M-alk) and an alkali-acid insoluble glucan ("glucan ghosts", GG) were prepared as described by Ausiello *et al.* (1986) and Cassone *et al.* (1981). The crude chemical composition of these fractions has been reported by Ausiello *et al.* (1986) and Scaringi *et al.* (1988). In particular, polysaccharide composition was determined by gas-liquid chromatography according to the method of Albersheim *et al.* (1967).

Treatments of GMP

Protease treatment. A solution of GMP 2 mg/ml in 0.1M phosphate-buffered saline (PBS), pH 7.2, was treated overnight at 37°C with Pronase (Calbiochem-Behring, La Jolla, CA) dissolved at a final concentration of 0.1 mg/ml in the same buffer. The enzyme was precipitated by heating for 5 min at 100°C and the supernate dialysed against water and lyophilised. Control samples were heated in PBS only. The protein content in the samples was determined before and after the treatment by the method of Lowry *et al.* (1951).

Periodate oxidation. GMP was dissolved at a concentration of 1 mg/ml in 0.05M acetate buffer, pH 4.5, containing 0.2M NaIO₄ and incubated for 18 h at 4°C; at the end of the incubation period, excess periodate in the mixture was blocked by adding 1 ml of ethylene glycol, and then the samples were extensively dialysed against water and lyophilised.

Immunisation and hybridoma production

Two female BALB/c mice (Charles River, Calco, Italy) were given subcutaneous injections at weekly intervals of 0.1 mg of GMP (three times as a suspension in 0.25 ml of complete Freund's adjuvant and the fourth time dissolved in 0.25 ml of saline). The final boosting dose (0.025 mg in saline) was given intravenously 3 days before the fusion. Spleen cells of GMP-immunised mice were fused at a 1:3 ratio with myeloma cells of the murine line X63-Ag8 653 and hybrids selected and maintained as reported by Malavasi *et al.* (1984). Culture supernates were screened for antibody production by an ELISA technique with the immunising antigen as coating, as described by Ausiello *et al.* (1986). An irrelevant IgM MAB was used as a negative control and a human anti-*Candida* serum (HA titre 640) was the positive control for the ELISA tests (see also below). The stable, selected hybridoma was cloned twice by limiting dilution and subsequently grown *in vivo* in pristane-treated BALB/c mice. The ascitic fluids thus obtained were used as a massive source for MAB purification.

Monoclonal antibody purification

(i) **Ammonium sulphate precipitation.** MAB was precipitated by adding (NH₄)₂SO₄ 50% w/v to PBS-diluted ascitic fluids. The precipitates were collected by centrifugation and dialysed extensively against PBS.

(ii) **Affinity chromatography.** The MAB from ascitic fluids was purified on a 6.5 cm × 0.5 cm column with Affi-Prep protein A support matrix, equilibrated with MAPS II buffers (BioRad, Richmond, USA). This support, according to the manufacturer's specifications and the personal reports of others (Mariani and Cianfriglia, I.S.S., Rome), when used in conjunction with optimised MAPS buffers, is highly efficient at binding a wide range of immunoglobulins, including murine IgM. Briefly, the column was pre-equilibrated with MAPS II buffer, pH 9.0, then loaded with 0.5–2.0 ml of ascitic fluid,

diluted 1 in 5 in buffer, at a flow rate of 2 ml/h. After washing with buffer to eliminate unbound proteins, the immunoglobulin fraction was eluted at pH 5 and pH 3 with 0.1M citrate buffer containing 0.15M NaCl. Fractions corresponding to protein peaks were dialysed against PBS and tested by ELISA. The yield of MAB AF-1 after this purification was around 70%.

Antibody titres of ammonium sulphate-precipitated and chromatographically-purified batches of the MAB were determined as the highest dilution of the antibody that gave at least twice the absorbance value obtained in the negative control.

Determination of the immunoglobulin class

Ig class attribution of the MAB was inferred from reactivity with conjugated goat anti-mouse μ or γ chain, and from the results of the Outchlerlony test performed on affinity-purified MAB with goat anti- μ chain or anti- γ chain specific antiserum in 0.1M veronal buffer, pH 8.1, in 1% purified agar. Sensitivity to 2-mercaptoethanol (2-ME) was determined by treating the MAB for 2 h at 37°C with 0.1M 2-ME and assaying the reactivity of the treated antibody by the ELISA technique. Finally, the purified MAB was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970).

Immunofluorescence assay

Drops of freshly grown, washed yeast cells were heat-fixed on microscope slides at 60°C for 30 min and then allowed to react for 30 min at 37°C with affinity-purified MAB (ELISA titre 3000), diluted 1 in 5 with PBS. The smears were washed twice in PBS and the bound MAB was shown by treating for 30 min at 37°C with a 1 in 50 dilution of FITC-conjugated goat anti-mouse IgM (Sigma). Slides were mounted in 0.2M carbonate buffer, pH 9.6, containing 90% glycerol, and examined by fluorescence microscopy (SM-LUX; Leitz, Wetzlar, Germany).

Agglutination tests

Reactivity of the MAB with different species of *Candida* was assessed by a slide agglutination test with either glutaraldehyde-inactivated or viable cells. The standard test was performed with yeast cells grown for 24 h in Winge medium at 28°C, washed and then adjusted to a concentration of 3×10^7 cells/ml in saline; 25 μ l of cell suspensions were mixed with 25 μ l of the MAB at the appropriate dilution on an agglutination slide and the results assessed visually were recorded after gentle rotation of the samples for 10 min. For absorption assays, 5 μ l of a suitable dilution of purified MAB was added to 100 μ l of a suspension containing approximately 3×10^7 glutaraldehyde-inactivated cells of different *Candida* species. The suspensions were incubated overnight at room temperature and the undiluted supernates were tested for agglutination of the *Candida* species as

tested for agglutination of the *Candida* species as described above. No appreciable differences were observed between the agglutination of live or chemically-inactivated cells.

ELISA-inhibition tests

Cell-wall fractions were assayed in an ELISA-inhibition test essentially as described by Ausiello *et al.* (1986) and Scaringi *et al.* (1988). Briefly, each fraction was added, at suitable concentration, to an appropriate MAB dilution and incubated overnight at room temperature. The mixtures were then added to antigen (GMP)-coated wells and the immunosorbent assay performed as previously reported (Ausiello *et al.*, 1986). The percentage inhibition by the cell-wall fraction was calculated from the formula $A_{405}T - A_{405}I / A_{405}T - A_{405}B$, where $A_{405}I$ was the reading from the wells containing the putative inhibitor fraction and $A_{405}T$ and $A_{405}B$ the readings from the wells without inhibitor or with the fraction and the irrelevant MAB (negative control), respectively.

The ELISA tests were read with a Titertek Multiscan (Flow Laboratories, Milan, Italy) set at 405 nm and the blank set against air. Tests were performed in triplicate and the A_{405} of the well with the irrelevant MAB (usually 0.14–0.16) was taken as the negative background reading. A positive test was one with at least twice the absorbance (A_{405}) of the mean background reading. The SD values of the readings were always <0.01% and have been omitted from the results.

Results

Hybridoma production, MAB secretion and purification

Splenocytes from a mouse immunised with *C. albicans* GMP were fused with cells of the murine

myeloma line X63-Ag8.653. After 14 days, 2 wells out of 384 in which cells had grown gave positive results in the ELISA tests with GMP as solid-phase antigen. One of the two selected primary cultures gave rise to a stable hybridoma which, after cloning and propagation into pristane-treated mice, was used as the source of the MAB (hereafter referred to as MAB AF-1). The MAB from the ascitic fluid was partially purified by ammonium-sulphate precipitation and affinity chromatography. The MAB was sensitive to 2-mercaptoethanol treatment. Moreover, indirect ELISA binding with anti-mouse IgM, Ouchterlony tests, and SDS-PAGE showed that MAB AF-1 belonged to the IgM class (data not shown).

Slide agglutination assay with glutaraldehyde-inactivated or viable yeast cells showed that MAB AF-1 had strong agglutination properties (table I), and agglutinated all the isolates of *C. albicans* serotypes A and B, *C. tropicalis*, *C. guilliermondii* and *C. viswanathii* that were tested but did not agglutinate *T. glabrata*, *C. krusei*, *C. parapsilosis* or *C. kefyr*.

Two agerminative isolates of *C. albicans* were also agglutinated by MAB AF-1 to an extent not visibly different from that of the germinative isolates. Table I also shows the antigenic formula of the species tested for agglutination with polyclonal sera (Tsuchiya *et al.*, 1984). The pattern of cell agglutination by MAB AF-1 does not indicate an obvious sharing of any of the antigenic factors considered in the formulation. Absorption of MAB AF-1 with cells of any of the agglutinated species completely abolished the reactivity with all of them,

Table I. Slide agglutination reactivity of *C. albicans* and related species with MAB AF-1*

Species	Number of isolates tested	Agglutination (number positive/number tested)† with		
		Ammonium sulphate-precipitated MAB	Affinity chromatography-purified MAB	Antigenic formula‡
<i>C. albicans</i> serotype A	9	4/4	5/5	1, 4, 5, 6
<i>C. albicans</i> serotype A (agerminative isolates)	2	N.D.	2/2	1, 4, 5, 6
<i>C. albicans</i> serotype B	5	3/3	2/2	1, 4, 5, 13b
<i>C. tropicalis</i>	10	4/4	6/6	1, 4, 5, 6
<i>C. parapsilosis</i>	9	0/3	0/6	1, 5, 13, 13b
<i>C. guilliermondii</i>	5	1/1	4/4	1, 4, 9
<i>C. glabrata</i>	10	0/4	0/6	1, 4, 6, 34
<i>C. kefyr</i> (pseudotropicalis)	7	0/4	0/3	1, 8
<i>C. krusei</i>	10	0/4	0/6	1, 5, 11
<i>C. viswanathii</i>	1	N.D.	1/1	1, 4, 6, 13b

* A standard slide agglutination assay with freshly prepared suspension of live cells and a 1 in 25 dilution of a MAB solution with an ELISA titre of 32 000.

† Different isolates were used for each MAB preparation to reach the total number of isolates reported in the second column.

‡ According to Tsuchiya *et al.* (1984).

whereas repeated absorptions of the MAB with cells of each of the non-agglutinated strains did not reduce significantly the extent of clumping of each agglutinable species.

Only those species that were agglutinated by MAB AF-1 gave positive results in an indirect immunofluorescence assay performed with purified MAB and FITC-labelled goat anti-mouse IgM serum. Fig. 1 shows an example of this reaction.

Specificity of MAB AF-1

To determine the chemical nature of the epitope recognised by MAB AF-1, the reactivity of the MAB with different *C. albicans* cell-wall-based fractions was investigated with two distinct ELISA tests that were specially devised. The first was based

on a direct binding of the MAB with each fraction used as coating antigen; in the second, an inhibition ELISA test was performed with GMP antigen as the solid-phase reagent and each tested fraction as a free-phase, MAB AF-1 ligand in a competition reaction with GMP. This double approach was necessary because of the potential inability of some of the fractions to adsorb effectively to the plastic of the well. Data in table II show that MAB AF-1 reacted specifically with all mannan-based fractions from *C. albicans*, but not with mannan extracts from *S. cerevisiae*. The highest reactivity was apparent with a low-protein mannan extracted by the method of Peat *et al.* (1961), whereas the weakest reaction was with an alkali-extracted cell-wall mannan (M-alk). Table II also shows that periodate oxidation abolished the reactivity of GMP with MAB AF-1, whereas GMP deproteinisation by pronase or heat treatment did not greatly affect the structure of the epitope targeted by MAB AF-1. It should be noted that pronase-treated GMP was not effective as a coating antigen but its reaction with the MAB was easily evidenced in the ELISA-inhibition assay. Finally, the MAB did not react with a β -glucan preparation (GG).

The results of the ELISA tests were fully confirmed by immuno-diffusion which showed that MAB AF-1 formed readily visible precipitin lines of identity with the mannan-rich fractions of *C. albicans* but not with glucan or periodate-treated antigen (fig. 2).

Discussion

In this study, an agglutinating and precipitating murine monoclonal antibody (MAB AF-1) of the IgM class was obtained after immunisation with a glucomannoprotein extract from the cell wall of *C. albicans* (Ausiello *et al.*, 1986). MABs directed against cell-wall antigens of this micro-organism have been reported but, generally, undefined extracts, cell walls, or heat-killed or otherwise inactivated whole cells were used as immunogens (Brawner and Cutler, 1984; Chardès *et al.*, 1986; Miyakawa *et al.*, 1986; Calderone *et al.*, 1988). In one case (Hopwood *et al.*, 1986), the MAB was obtained from an interspecific fusion between mouse myeloma cells and splenocytes of *Candida*-infected rats. Because it belongs to the IgM class as well as having precipitating and agglutinating properties, MAB AF-1 resembles most of the aforementioned MABs to cell surface determinants of *C. albicans*. However, MAB AF-1 shows a distinctive specificity. In fact, the panel of the species agglutinated by MAB AF-1, including *C. albicans*

Fig. 1. Indirect immunofluorescence staining of *C. albicans* (strain BP, panel A and strain CA2, panel B) with MAB AF-1. A dilution of 1 in 5 from a batch of affinity-purified MAB (ELISA titre 3000) was used for both tests. Bar = 10 μ m.

Table II. Indirect ELISA and ELISA-inhibition tests with MAB AF-1 and cell-wall extracts of *C. albicans*

Fraction	Main sugar component* (percentage of dry weight)	A ₄₀₅ in ELISA tests†			
		A. Fraction used as coating antigen at a MAB dilution of		B. Fraction used as inhibitor at a concentration of	
		10 000	20 000	0.5 µg/ml	0.1 µg/ml
None	—	0.10	0.10	0.65	
GMP	mannose (83.7)	0.59	0.29	0.23 (89.4)	0.50 (31.9)
M	mannose (95.8)	0.92	0.52	N.D.	
M-alk	mannose (66.6)	0.32	0.24	0.46 (39.6)	0.59 (12.5)
GG	glucose (93.1)	0.14	0.11	0.65 (0)	N.D.
Pronase and heat-treated GMP	mannose (61.9)	0.10	0.10	0.34 (63.3)	0.50 (30.6)
Heat-treated GMP	mannose (72.0)	0.49	0.27	0.38 (57.4)	0.58 (14.9)
Periodate-treated GMP	N.D.	0.11	0.10	0.64 (2.1)	0.65 (0)

N.D. = not done. * Determined by gas-liquid chromatography.

† Tests A and B were performed independently. In A, each fraction was used as coating (solid phase) antigen at a concentration of 5 µg/ml. In the inhibition test B, MAB AF-1 was used at a dilution (1 in 10 000) giving the indicated absorbance reading in the uninhibited control (first line). Values in brackets represent the percentage inhibition. Three experiments were performed, with similar results; the ELISA values reported are those from one of these experiments. For other details, see *Materials and methods*.

serotypes A and B, *C. tropicalis* and *C. guilliermondii*, does not correspond to any of the major *Candida* groups in the serological classification scheme of Tsuchiya *et al.* (1984). On the contrary, MABS previously reported as anti-cell-wall reagents reacted with epitopes largely overlapping with major determinants (e.g., factors 4 and 5) of Tsuchiya's

classification. Moreover, the epitope recognised by MAB AF-1 was also present in *C. viswanathii*, a pathogenic *Candida* species that was not tested for its reactivity with previous MABs.

The experiments involving cell-wall fractions and modified antigens of *C. albicans* consistently demonstrated that the determinant recognised by MAB AF-1 is part of a cell-wall polysaccharide-protein component, probably a mannoprotein, with the specific epitope being a piece of the polysaccharide moiety. Apart from its reactivity with the mannoprotein-rich extract (GMP) used for immunisation and antibody screening, MAB AF-1 also reacted with all other *C. albicans* mannan fractions tested, one of which (M) was extracted by a procedure yielding a mannan with very little protein. The MAB reacted poorly with an alkali-extracted mannoprotein constituent (M-alk) but

Fig. 2. Ouchterlony tests with affinity-purified MAB AF-1 in central wells (ELISA titre 16 000, 20 µl) and antigenic fractions in the peripheral wells. Each fraction was used as a concentration of 2 mg/ml in a volume of 20 µl/well. Panels A and B show unstimulated preparations, panels C and D show tests performed as above but stained with Coomassie blue (0.1% in methanol 50% and acetic acid 10%). A and C: 1, 2 and 6 represent GMP from three independent batches; 3, pronase and heat-treated GMP; 4, heat-treated GMP; 5, M. B: 1, GMP; 2, GG; 3 and 4, periodate-treated GMP (two independent preparations); 5, GMP from *S. cerevisiae*; 6, M-alk. D: 1, GMP; 2, M-alk; 3 and 4, periodate treated GMP (two independent preparations); 5, GMP from *C. cerevisiae*; 6, GG.

did not react at all with an insoluble glucan constituent of the cell wall (GG). GMP ability to bind MAB AF-1 was not lost after heat or pronase-plus-heat treatment whereas it was highly sensitive to periodate oxidation, as expected of a carbohydrate epitope.

Previous studies (Ausiello *et al.*, 1986; Scaringi *et al.*, 1988) have shown that the polysaccharide component of GMP contained mannose and glucose (in a ratio of about 30:1). The cell-surface antigens of *C. albicans* identified by MABs H9 and C6 of Brawner and Cutler (1984, 1986) were also

polysaccharide in nature but with a much lower mannose: glucose ratio.

The overall evidence obtained so far suggests that MAB AF-1 identifies a new polysaccharide epitope of the cell wall of some pathogenic *Candida* species.

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