Electric and chemical fusions for the production of monoclonal antibodies reacting with the in-vivo growth phase of *Candida albicans*

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Summary. To obtain monoclonal antibodies (MAbs) directed preferentially against the pathogenic phase of *Candida albicans*, mice were immunised with germ tubes of *C. albicans* serotype A, strain VW.32, killed by exposure to ultraviolet (UV) irradiation. Fusions were performed either by the standard chemical procedure with polyethylene glycol, or by electric discharge following linkage of the myeloma and lymphocyte cells with a Concanavalin A-mannoprotein bridge. The preliminary characteristics of one MAb obtained from each of these fusions are described. An IgM antibody (3B7) obtained from the chemical fusion reacted with a polysaccharide antigen that was heterogeneously distributed on both in-vitro and in-vivo forms of *C. albicans*. This MAb agglutinated different strains of *C. albicans* irrespective of their serotype. An IgG₁ antibody (3G6) that had been obtained from the electric fusion was found to react *in vitro* with a proteinaceous antigen located only on the germ tubes of strain VW.32. However, MAb 3G6 displayed strong reactivity against all growth forms of *C. albicans* *in vivo* and reactivity extended to other strains.

Introduction

There have been numerous reports describing monoclonal antibodies (MAbs) produced against the pathogenic yeast *Candida albicans* (Brawner and Cutler, 1984; Strockbine *et al.*, 1984; Barbier *et al.*, 1985; Chardes *et al.*, 1986; Hopwood *et al.*, 1986; Miyakawa *et al.*, 1986; Polonelli and Morace, 1986; Reiss *et al.*, 1986). These MAbs were produced following immunisation of mice with heat-killed or formol-inactivated whole blastospores (Brawner and Cutler, 1984; Chardes *et al.*, 1986), or protein (Strockbine *et al.*, 1984), or polysaccharide (Miyakawa *et al.*, 1986; Reiss *et al.*, 1986) antigens extracted from blastospores. Taking into account the antigenic variability of *C. albicans* and the possible relationship between antigen expression and pathogenicity (Poulain *et al.*, 1983), attempts have also been made to produce MAbs against the pathogenic phase of *C. albicans* (Hopwood *et al.*, 1986). Because of the difficulty of inducing an immune response in mice following experimental infection (Winterrowd and Cutler,

1983; Hopwood *et al.*, 1986), in this study mice were immunised with growth forms of *C. albicans* obtained *in vitro* that were closely related morphologically to the pathogenic phase. Non-viable germ tubes were used as the immunogen because this growth form more closely resembles that found *in vivo* than blastospores (Odds, 1985). Germ tubes have also been shown to be more reactive than blastospores against sera from patients infected with *C. albicans* (Poulain *et al.*, 1983) and to possess specific antigens (Smail and Jones, 1984; Sundstrom and Kenny, 1984). To preserve the integrity of thermolabile antigens and to minimise antigen denaturation following aldehyde fixation, the germ tubes were killed by UV irradiation.

The major antigenic components of *Candida* spp. are mannoproteins (Ballou, 1976; Poulain *et al.*, 1985). Concanavalin A (Con A) binds to the polysaccharide components of these molecules (Ballou, 1976) and has also been shown to bind to myelocyte membranes (Monsigny *et al.*, 1978). In this study we favoured fusing myeloma cells with selected B lymphocytes secreting antibodies against *C. albicans* by linking the two cell types with Con A before exposure to an electrical discharge. The lymphocytes were preselected by their ability to bind purified mannoproteins.

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The electric and chemical methods employed to fuse the cells resulted in 12 hybridomas secreting antibodies to *Candida* spp. We describe the preliminary characteristics of one MAb from each of these fusions, that reacted strongly with the in-vivo growth phase of *C. albicans*.

Materials and methods

Strains and culture conditions

For antibody production we used a cloned strain of *C. albicans* serotype A (VW.32) that had been previously employed to produce MAbs (Hopwood *et al.*, 1986). This strain was selected for its high reactivity against sera from patients and animals infected with *C. albicans* and was previously defined as being rich in P antigens (quantitatively and/or qualitatively variable antigens expressed preferentially by the pathogenic phase) (Poulain *et al.*, 1983). The reference strains used for studying the distribution of the antigens recognised by the MAbs consisted of five *C. albicans* strains, serotype A or B, and seven *Candida* spp. other than *C. albicans* (tables I and II). The origin of these strains and their serological reactivities against polyclonal and monoclonal antibodies were described by Poulain *et al.* (1983) and Hopwood *et al.* (1986).

Blastospores for screening were obtained by culture on Sabouraud Dextrose Agar (SDA) for 24 h at 37°C. *C. albicans* blastospores were washed twice in phosphate-buffered saline (PBS), pH 7.2, and incubated in tissue-culture medium 199 (Eurotroph; Eurobio, Paris, France) for 2.5 h at 37°C, with continual stirring, for the production of germ tubes.

Immunisation of animals

Eight-week-old BALB/c mice (Iffa Credo, France) were inoculated intraperitoneally six times a week with 0.4 ml of a suspension of UV-killed *C. albicans* strain VW.32 germ tubes (10^7 /ml in physiological saline) for 6 weeks. The absence of viable germ tubes was determined by culture on SDA for 48 h at 37°C. Mice were bled weekly from the orbital sinus, and the serum was assayed for antibody production by immunofluorescence (IF) (see below). At the end of the sixth week, mice producing antibodies were rested for 7 days and then given a booster injection of 4×10^6 germ tubes intravenously. Spleens were removed for fusion three days after the booster injection.

Hybridoma production

Murine myeloma cell line SP2/0 was cultured in Dulbecco Modified Eagle's Medium (DMEM) (Seromed, Strasbourg, France) supplemented with fetal calf serum 10%, non-essential amino acids, glutamine, pyruvate and gentamicin (Galfre *et al.*, 1977) (medium A).

Fusion of spleen cells from immunised animals with

myeloma cells was performed either by the standard chemical procedure of Galfre *et al.* (1977) with polyethylene glycol (PEG) or by a modification of the electric-fusion procedure described by Lo *et al.* (1984). Both fusion techniques were used for cells from the same spleen.

Electric fusion. The principle of this fusion procedure is schematised in fig. 1. Preparation of myeloma and spleen cells was performed at 4°C. Myeloma cells were washed twice in PBS, pH 7.4, supplemented with 1 mM CaCl_2 and 1 mM MgSO_4 (PBS-CM). The cells (10^6 /ml) were then suspended in a solution of Con A 0.1 $\mu\text{g}/\text{ml}$ (IBF, Villeneuve la Garenne, France) in PBS-CM, incubated for 1 h and washed twice in PBS-CM.

A suspension of spleen cells was prepared by gentle homogenisation of the spleen in PBS-CM and the splenocytes were then washed twice in PBS-CM. The cells were next incubated for 1 h in PBS-CM containing mannoproteins prepared from *C. albicans*, 10 $\mu\text{g}/\text{ml}$. Mannoproteins were prepared by heating blastospores for 2 h at 134°C in 19 mM citrate buffer (Peat *et al.*, 1961). The extract was then made up to molar concentration

Fig. 1. Hypothesis for the possible mechanism of linkage between B lymphocytes (Lc) and myeloma cells (Myel) via Con A—mannan bridges established before electric fusion. Mannoproteins extracted from the cell wall (CW) of *C. albicans* react via their polysaccharide side chains, namely the mannans (M), with both Con A and surface bound anti-candida immunoglobulins. Linkage with the lymphocytes may be established via either the polysaccharide side chains or the protein moiety (P) depending on the specificity of the surface-bound antibodies (to simplify the scheme only a few of the mannan side chains are represented in the figure).

with acetic acid, neutralised with 6 M NaOH, precipitated with ethanol, redissolved in water, dialysed and lyophilised. Protein content (Pierce BCA protein assay reagent) of the extract was 400 mg of protein/g dried weight. After washing the spleen cells twice in PBS-CM, the myeloma and spleen cells were mixed in a ratio of 1:1, centrifuged for 10 min at 700 *g*, and the pellet was resuspended in 272 mM sucrose in 7 mM PBS, pH 7.2, containing 1 mM MgSO₄ (Teissie *et al.*, 1982) at a density of 4×10^7 cells/ml. The electric fusion was performed in a sterile chamber made by fixing two stainless-steel razor blades parallel to each other, 3 mm apart (Teissie *et al.*, 1982); 200 μ l of cell suspension were deposited between the two blades and three electric pulses were then generated with 4-s delay between pulses, by discharge of a 1 μ F condenser into the blades so that the field strength was 4 kV/cm at the beginning of discharge (half charge duration 20 μ s). At the end of electric pulsing the cells were gently aspirated, suspended in medium A at 37°C and dispensed into the wells of 96-well microtitration plates (7×10^4 cells/well) containing 3×10^3 peritoneal cells/well as a feeder layer.

After incubation at 37°C for 18 h, hypoxanthine, aminopterin and thymidine were added to select for hybrid cells (Galfre *et al.*, 1977). Positive hybrids were subcloned twice by limiting dilution.

Chemical fusion. Fusion was performed by the method of Galfre *et al.* (1977). Briefly, spleen cells (7×10^7) were fused with myeloma cells (1.4×10^7) with polyethylene glycol 1500 (PEG) 35% in DMEM. The cell suspension in medium A was seeded in microtitration plate wells (3×10^4 cells/well) containing 3×10^3 peritoneal cells as a feeder layer. After 18 h, hypoxanthine, aminopterin and thymidine were added to select for hybrid cells. Positive hybrids were subcloned twice by limiting dilution.

Screening for MAb reactivity

Preliminary screening for antibody secretion was performed by immunofluorescence (IF), with strain VW.32 germ tubes as the antigen, as described previously (Hopwood *et al.*, 1986). Commercial fluorescein conjugated anti-mouse immunoglobulins (Zymed, Biosoft, Paris, France; Nordic, Tebu, Versailles, France) were used at dilutions ranging from 1 in 40 to 1 in 100. Supernates from wells that gave positive results in the screening were tested further with a range of candida antigens to determine the distribution of the epitope recognised, and for preliminary characterisation of the antigenic molecules.

Determination of the MAb reactivity

Immunofluorescence (IF) reactions were performed with treated or untreated in-vitro and in-vivo growth forms of the reference yeast species. In-vitro blastospores and germ tubes were obtained from the strains listed in tables I and II as described above for strain VW.32.

The germ tubes of strain VW.32 were subjected to various physico-chemical treatments for antigen extrac-

tion or denaturation. These methods were described in detail by Hopwood *et al.* (1986). Briefly, cells were heat treated for various periods of time—56°C for 30 min, 80°C for 10 min, or 134°C for 2 h. Enzymic treatment consisted of incubation in PBS with 2.5 mg/ml of the following enzymes; pronase, trypsin, or chymotrypsin, for periods ranging from 3 h to 24 h.

IF with in-vivo growth forms was performed on cryostat sections prepared from kidneys of rabbits infected with strain VW.32 or strain 996B as described previously (Hopwood *et al.*, 1986).

Direct agglutination was performed by mixing 20 μ l of hybridoma supernate with 20 μ l of a suspension of glutaraldehyde-fixed blastospores (10^8 cells/ml) (Hopwood *et al.*, 1986). Agglutination was recorded macroscopically after agitation for 2 min on a Kline agitator.

Precipitating activity of the MAbs was determined by counterimmunoelectrophoresis (CIE) on cellulose acetate membranes against a somatic antigen of strain VW.32 as described by Poulain *et al.* (1986).

Nature of MAbs and production of ascites

Isotypes were determined with class and sub-class specific antibodies in IF reactions and confirmed by immunodiffusion with class and sub-class specific antibodies (Zymed, Biosoft, Paris, France; Nordic, Tebu, Versailles, France).

Ascitic fluid was prepared from BALB/c mice pretreated with pristane (0.5 ml) and inoculated with 10^6 hybridoma cells.

Results

Electric fusion

Twenty five hybridomas were obtained of which six secreted antibodies reacting with *C. albicans*. As the reactivity of these six clones against the various *Candida* strains appeared to be identical, we have chosen to describe only one of them, named 3G6, here.

This mouse IgG₁ MAb reacted in IF with the homologous antigen producing a fluorescent reaction located on the germ tubes. Mother blastospores were unlabelled (fig. 2a). None of the germ tubes or blastospores produced *in vitro* by the other strains of *C. albicans* tested was labelled (fig. 2b); neither were blastospores of other yeast species (table I).

When IF reactions were performed on in-vivo growth forms of *C. albicans*, the homologous strain VW.32 and also strain 996B, which gave completely negative results *in vitro*, exhibited strong reactivity (fig. 2c). This reactivity was located on both the blastospores and hyphal forms. Physico-chemical treatment demonstrated the heat lability of the

Fig. 2. Reaction of MAb 3G6 against *C. albicans* by indirect immunofluorescence. **a.** Germ tubes of strain VW.32 obtained in medium 199. The fluorescence is distributed over the whole surface of the germ tube whereas the mother blastospore is completely unlabelled. **b.** Germ tubes of strain 996B obtained in medium 199. Both the germ tubes and mother blastospore are unlabelled. **c.** In-vivo growth form of strain 996B in sections from infected rabbit kidneys. Strong fluorescence is apparent on all fungal elements.

antigens on VW.32 germ tubes, recognised by the reactions with MAb 3G6 as well as by their susceptibility to proteolytic digestion (table I). Under the experimental conditions described here we were unable to demonstrate any agglutinating or precipitating activity of this MAb.

Chemical fusion

The chemical fusion resulted in six hybridomas which secreted antibodies reacting with *C. albicans*.

However, there was variation in the reactivity of these antibodies against the various *Candida* strains and growth forms in IF reactions. We report here the properties of one of these antibodies, an IgM (κ chain), referred to as 3B7. The reactivity of MAb 3B7 against the different candida antigens is summarised in table II.

MAb 3B7 reacted in IF with cells of the homologous strain VW.32 with fluorescence usually distributed on the germ tubes and on mother blastospores. However, a proportion of the blastospores was unlabelled. This is referred to as a mosaic reaction. The proportion of unlabelled blastospores was greater when they were grown on SDA than when they were incubated in medium 199. Mosaic reactions were observed on either the germ tubes or on the blastospores of the other *C. albicans* strains tested. One strain of *C. albicans* serotype A (995A) was completely devoid of reactivity; this absence of reactivity was confirmed by direct agglutination (table II). The in-vivo growth forms of the two strains tested (VW.32 and 996B) were highly reactive.

The antigen recognised by MAb 3B7 was resistant to all of the physico-chemical treatments used, except for autoclaving. The antigen was also present in a somatic antigen extract prepared from blastospores of strain VW.32 as demonstrated by the precipitin line observed when MAb 3B7 was allowed to react with the extract in CIE.

Discussion

Our ability to produce hybridomas which secrete antibodies to *C. albicans* following electric fusion demonstrates the success of this procedure and its potential as an alternative method to PEG for the production of MAbs. Prior incubation of the lymphocytes from the immunised mice with *C. albicans* mannoproteins contributed to the establishment of linkages between the lymphocytes and the myeloma cells preincubated in Con A, although the possibility of a direct linkage between myeloma and lymphocyte membranes with Con A cannot be excluded (Monsigny *et al.*, 1978).

However, the fact that all of the six MAbs obtained had the same specificity suggests that the former unique procedure for selecting the lymphocytes with mannoprotein had indeed occurred. A possible mechanism for linkage with the mannan portion of mannoproteins is schematised in fig. 1. The apparent specificity of the reactions of MAb 3G6 against protein antigens, however, does not seem to be compatible with the theory that linkage

Table I. Reactivity of MAb 3G6 against in-vitro and in-vivo growth forms of *Candida* spp. by IF, direct agglutination and CIE

Yeast antigens			Immunofluorescence reaction				
			in-vitro form		in-vivo form	Reaction by	
			Blastoconidia*	germ-tubes	blastoconidia and hyphae		
			S	199			direct agglutination
<i>C. albicans</i>							
strain VW.32 (untreated)			—	—	++	+++	—
treated	{	30 min 56°C	—	—	—	...	...
		10 min 80°C	—	—	—	...	...
		2 h 134°C	—	—	—	...	...
		Pronase 3h/24 h	—	—	—	...	...
		Trypsin 3 h/24 h	—	—	+ / ±	...	...
		Chymotrypsin 3 h/24 h	—	—	+ / ±	...	...
strain 995 A			—	—	—	...	
strain 3153 A			—	—	—	...	
strain 996 B			—	—	—	+++	—
strain L(B)			—	—	—	...	—
<i>C. tropicalis</i>							
strain Ct 1.7			—	—	—	...	—
strain Ct 2			—	—	...	...	—
strain Ct V			—	—	...	...	—
<i>C. glabrata</i>			—	—	...	...	—
<i>C. pseudotropicalis</i>			—	—	...	...	—
<i>C. parapsilosis</i>			—	—	...	...	—
<i>C. krusei</i>			—	—	...	...	—

* Blastospores were obtained either on SDA (S) or after incubation in medium 199 (199).

Fluorescence reaction: — = negative; + / ± = faint; ++ = moderate; +++ = strong; ... = experiment not performed.

takes place via mannans. Lymphocyte-bound surface antibodies may also react with the protein moieties of mannoproteins thereby establishing linkages with the myelocyte, bound via Con A to the mannan oligosaccharide side chains.

The specificity of MAb 3G6, which is limited *in vitro* to germ tubes of the homologous strain VW.32, extends *in vivo* to all of the growth forms of the homologous strain and also to the growth forms of other strains that gave completely negative results *in vitro*. The expression of the antigens recognised by MAb 3G6, which appear to be preferentially associated with the pathogenic phase, agrees with the concept of P antigens established earlier (Poulain *et al.*, 1983). It would appear from its sensitivity to heat and pronase that the epitope itself is proteinaceous in nature or is carried by a protein.

MAb 3B7, which was chosen as representative of the MAbs obtained from the chemical fusion on the other hand, seems to recognise epitopes which are polysaccharide in nature as demonstrated by

their thermostability at 80°C, their extraction by the method of Peat *et al.* (1961) and their resistance to proteolytic enzymes.

This MAb shares a number of characteristics in common with some of the other MAbs to *C. albicans* described previously: it is an agglutinating IgM class immunoglobulin (Brawner and Cutler, 1984; Barbier *et al.*, 1985; Chardes *et al.*, 1986; Miyakawa *et al.*, 1986; Reiss *et al.*, 1986) that recognises cell-surface determinants which are heterogeneous in distribution (Brawner and Cutler, 1984; Miyakawa *et al.*, 1986). The characteristics of MAb 3B7 lie in its strong reactivity against in-vivo growth forms and its ability to differentiate strains of *C. albicans* independently of serotype. However, previous experience with MAbs has demonstrated the complexity and inter-strain variability in antigen expression by *Candida* spp. during the cell cycle (Brawner and Cutler, 1984; Hopwood *et al.*, 1986). Further work is necessary, therefore, before it can be concluded that any of the antigens recognised are related to pathogenicity. Nevertheless, the

Table II. Reactivity of MAb 3B7 against in-vitro and in-vivo growth forms of *Candida* spp. by IF, direct agglutination and CIE

Yeast antigens		Immunofluorescence reaction					
		in-vitro form			in-vivo form	Reaction by	
		Blastoconidia*		germ-tubes	blastoconidia and hyphae		
		S	199			direct agglutination	precipitation (CIE)
<i>C. albicans</i> strain VW.32 (untreated)		+/- M	++ M	+++	+++	+++	1 PL
treated	30 min 56°C	...	++ M	+++	...	...	...
	10 min 80°C	...	++ M	+++	...	...	...
	2 h 134°C	...	—	—	...	...	...
	Pronase 24 h	...	++ M	+++	...	...	...
	Trypsin 24 h	...	++ M	+++	...	...	...
	Chymotrypsin 24 h	...	++ M	+++	...	...	...
strain 995 A		—	—	—	...	—	...
strain 3153 A		+/- M	+ M	+++	...	+	...
strain 996 B		+/- M	++ M	+++	+++	+	...
strain L(B)		—	—	++ M	...	—	...
<i>C. tropicalis</i> strain Ct 1-7		—	—	—	...	...	...
strain Ct 2		—	...	...	...	...	...
strain Ct V		+ M	...	...	...	...	...
<i>C. glabrata</i>		—	...	...	...	...	...
<i>C. pseudotropicalis</i>		—	...	...	...	...	...
<i>C. parapsilosis</i>		—	...	...	...	...	...
<i>C. krusei</i>		—	...	...	...	...	...

* Blastospores were obtained either on SDA (S) or after incubation in medium 199 (199).
Fluorescence reaction: - = negative; +/- = equivocal; + = weak; ++ = moderate; +++ = strong; M = mosaic reaction (a proportion of cells unlabelled); PL = precipitin line; ... = experiment not performed.

production of MAbs reacting preferentially with the pathogenic phase of *C. albicans* provides us with a probe for studying such a relationship.

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