Growth Inhibition of *Candida albicans* in the Presence of Antiserum Elicited in Rabbits by Mannan-Protein Conjugate

Eva Machová* and Slavomír Bystrický

Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovak Republic. Fax: + 421-2-5941022. E-mail: chememch@savba.sk

* Author for correspondence and reprint requests

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Antifungal properties of rabbit antiserum prepared by immunization are reported. The immunization was done by a chemically prepared conjugate consisting of *Candida albicans* (serotype A) surface mannan and human serum albumin. Addition of rabbit antiserum to D-glucose medium inoculated with *C. albicans* effectively inhibited its growth. Moreover, *C. albicans* cells treated with rabbit antiserum revealed the entire loss of viability (expressed as decreased mitochondrial dehydrogenase activity). No growth of treated cells on an agar plate was observed. The results confirmed that the mannan-protein conjugate could be considered as an effective component of perspective vaccine.

Key words: C. albicans Mannan-Protein Conjugate, Rabbit Antiserum, Growth Inhibition

Introduction

Candida albicans surface mannan conjugated to a protein carrier is immunogenic in rabbits and elicits booster response of IgG serum level (Han *et al*., 1999; Bystrický *et al*., 2003). In our previous paper, the antifungal quality of the antisera was documented by the *C. albicans* growing inhibition test (Bystrický *et al*., 2003). The classical view of antibody-mediated immunity is attributed to their indirect functions, such as opsonic and complement-activating properties (Casadevall and Pirofski, 2004). In the past years, the direct antimicrobial activities of antibodies were also published. IgM and IgG antibodies against *Borrelia burgdorferi* surface proteins damage the proteins and the impairment of the bacterial coat leads to its death (Connolly *et al.,* 2004). It is believed that serum antibodies play a key role in the protection against candidiasis (Brena *et al*., 2007). 40 years ago, Mourad and Friedman (1968) examined the effect of passive immunization against intravenously applied *C. albicans* on mice. The experiments indicated that passive immunization confers demonstrable protection, 33% of the animals survived the entire period of observation. Casanova *et al.* (1990) found that *Fab* fragments from a monoclonal antibody against a germ-tube mannoprotein block the transition of yeast to mycelium in *C. albicans*. Moragues *et al*. (2003) described a monoclonal antibody directed against *C. albicans* cell wall mannoprotein which exerted an antifungal activity through the three mechanisms – interference with adherence, inhibition of germination, and direct candidacidal activity. The comprehensive role of antibodies against yeasts is still not clear.

Here we try to shed light on the quality of antiserum from rabbits immunized with conjugate consisting of *C. albicans* surface mannan and human serum albumin.

Experimental

Microorganism

The strain *C. albicans* CCY 29-3-32 (serotype A) was from Culture Collection of Yeasts (CCY), Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

Preparation of conjugate and immunization

C. albicans mannan-human serum albumin (mannan-HSA) conjugate was prepared and characterized as described elsewhere (Bystrický *et al*., 2000, 2003). Immunization of rabbits with mannan-human serum conjugate as well as with mannan alone was proceeded intravenously (i. v.) five times in intervals of one week, no adjuvant was used. Antisera were stored at –20 °C. Inactivation of complement in rabbit antisera was realized at 56 °C during 30 min as recommended by Knudtson and Fetters (1990).

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Cultivation of C. albicans in 2% D-glucose medium

C. albicans grown on slant agar was used for the preparation of a stock *C. albicans* suspension ($3.02 \cdot 10^6$ cells in 1 mL of sterile water). The composition of 2% D-glucose medium was as follows: 2 g D-glucose, 0.05 g KH_2PO_4, 0.1 g $MgSO_4$, 0.3 g $(NH_4)_2SO_4$, 0.5 g yeast autolysate and 10 μL of microelement solution (2.4 g $FeCl_3 \cdot 6H_2O$, 2.0 g Na_2MoO_4, 1.2 g $ZnSO_4$, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.05 g KI, 0.4 g $CuSO_4$, 1.2 g H_3BO_3, filled to 100 mL with distilled water). The yeast cultivation was performed in L-shaped tubes containing 85 μL of stock *C. albicans* suspension ($2.57 \cdot 10^5$ cells) and 7 mL of 2% D-glucose medium on a reciprocal shaker (120 oscillations per min) at 27 °C in triplicate.

Growth protocols

1) 100 μL of rabbit antiserum obtained after the 5th immunization (after complement inactivation) were added to 2% D-glucose medium with 85 μL of stock *C. albicans* suspension. 2) 300 μL of stock *C. albicans* suspension with 250 μL of complement inactivated rabbit antiserum were shaken during 3 h at room temperature and stored overnight at 4 °C. The suspension was centrifuged at $1500 \times g$, and treated *C. albicans* cells were twice washed with PBS buffer and inoculated to 2% D-glucose medium. 3) 1st negative control: 100 μL of rabbit preimmune serum (after complement inactivation) were added to 2% D-glucose medium with 85 μL of stock *C. albicans* suspension. 4) 2nd negative control: 100 μL of rabbit antiserum (after complement inactivation) were added to 2% D-glucose medium with 85 μL of stock *C. albicans* suspension. 5) Positive control: 85 μL of stock *C. albicans* suspension were added to 2% D-glucose medium. In all experiments the growth of *C. albicans* was monitored by measuring the absorbance at 660 nm.

Viability test

The colorimetric assay of cellular viability utilizes XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide} (Sigma) which is reduced by mitochondrial dehydrogenases of metabolically active yeast cells to a dark blue formazan product (Kuhn *et al*., 2003). The different volumes of *C. albicans* suspensions (0.1 mL, 0.3 mL, 0.5 mL) with 0.3 mL of rabbit antisera were incubated on a reciprocal shaker for 1 h at room temperature. The treated *C. albicans* cells were collected at $1500 \times g$ for 10 min, sediments were twice washed with PBS buffer and diluted to final volumes of 1.2 mL. The colorimetric reagent, 0.2 mg of XTT in 200 μL PBS and 0.02 mg of phenazine methosulfate (PMS, Sigma) in 100 μL PBS, was added and the reaction mixture was incubated for 1.5 h at 37 °C. The suspension was centrifuged at $4000 \times g$ (supernatant 1). Formazan retained in cell pellets was released by 100% dimethylsulfoxide and the suspension was again centrifuged (supernatant 2). After collection of both supernatants, the produced formazan was observed at 492 nm (Shimadzu 1240 UV-mini spectrophotometer). Autoclaved *C. albicans* cells with damaged metabolic functions were used as control killed yeast sample. The assays were repeated in four biologically independent samples.

Results and Discussion

Growth of C. albicans in the presence of rabbit antiserum

Nowadays, the main trend in the preparation of vaccines against pathogenic bacteria and yeasts is chemical conjugation of surface polysaccharide antigens with carrier protein (Lucas *et al.,* 2005). Such conjugates overcome the immunological deficiency of the saccharide antigen alone and greatly extend the vaccine efficacy. Concerning *Candida* pathogens, mannan polysaccharide is the most exposed surface antigen determining their immunological properties (Cutler, 2005).

In our previous paper, *C. albicans* antiserum prepared by i.v. immunization of the rabbit with the prepared mannan-HSA conjugate exhibited growth inhibition zones on agar plates inoculated with *C. albicans.* Besides this antiserum contained significantly higher levels of anti-mannan IgG antibodies compared with hyperimmune serum. No adjuvant was used in the immunization experiments (Bystrický *et al*., 2003).

Here, addition of 100 μL of rabbit antiserum (after complement inactivation) completely in hibited the growth of *C. albicans* in 2% D-glucose medium during 72 h of cultivation (Fig. 1). Also, *C. albicans* cells were incubated for 3 h with 250 μL of rabbit antiserum and then inoculated in 2% D-glucose medium (Fig. 1) as well as plated in agar, and no growth of *C. albicans* was observed. Contrary to these results, 100 μL of preimmune rabbit

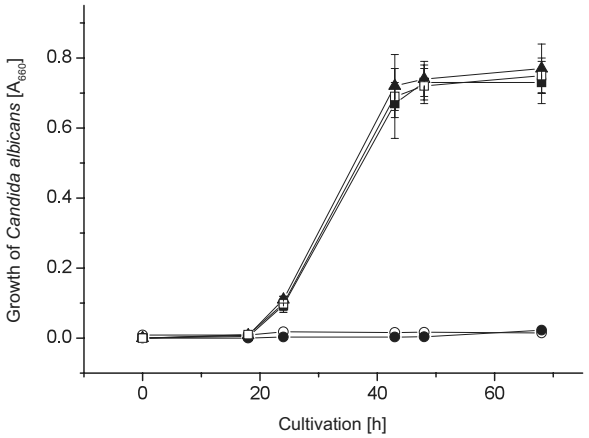

Fig. 1. Growth curves of *C. albicans* in 2% D-glucose medium during 72 h. (●) Addition of 100 μL rabbit antiserum to *C. albicans* inoculated in the growth medium; (○) *C. albicans* cells treated with 250 μL rabbit antiserum for 3 h and then inoculated into the growth medium; (■) addition of rabbit preimmune serum to *C. albicans* inoculated in the growth medium as 1st negative control; (▲) addition of rabbit antiserum to *C. albicans* inoculated in the growth medium as 2nd negative control; (□) native *C. albicans* inoculated in the growth medium as positive control. Error bars represent standard deviation of the mean value (n = 3).

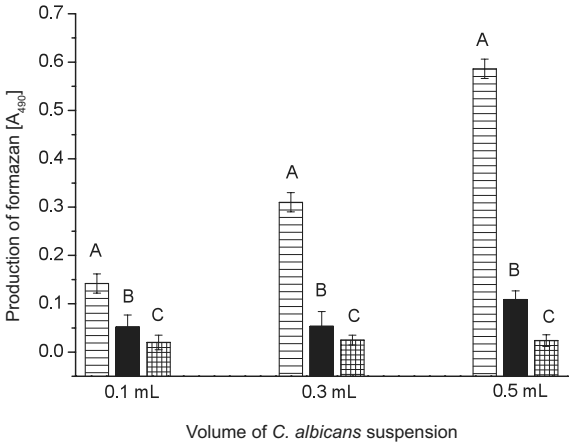

Fig. 2. Viability of *C. albicans* as a function of different amounts of yeast: 0.1, 0.3 and 0.5 mL of *C. albicans* suspension. (A) Native *C. albicans*; (B) treated *C. albicans* cells – 3 h incubation with rabbit antiserum; (C) negative control – *C. albicans* autoclaved 15 min at 120 °C. Error bars represent standard deviation of the mean value (n = 4).

serum as well as 100 μL of rabbit antiserum used as negative controls did not influence the growth of *C. albicans* (Fig. 1). Native *C. albicans* cells were used as positive control of growth (Fig. 1).

Viability of C. albicans in the presence of rabbit antiserum

The viability of *C. albicans* cells was monitored using the XTT colorimetric assay: the production of formazan was evident in native *C. albicans* cells having functional mitochondrial dehydrogenases. The amount of produced formazan was proportional to the increasing amounts of native *C. albicans* (0.1–0.5 mL) (Fig. 2, columns A). In contrast, *C. albicans* treated with rabbit antiserum showed markedly decreased mitochondrial activity (Fig. 2, columns B). Autoclaved *C. albicans* (15 min at 120 °C) used as killed cells did not show mitochondrial dehydrogenase activity (Fig. 2, columns C).

Microscopic observation of *C. albicans* treated 3 h with rabbit antiserum showed the formation of clusters of yeast cells typical for a behaviour under stress conditions.

Evidently, the observed antifungal activity of rabbit antiserum is related to an increased level of the protective anti-mannan IgG antibodies in the serum (Bystrický *et al*., 2003). It is known that antibodies against *Candida* antigens are commonly present in the sera due to the colonization of mucosa with *Candida* sp. or subclinical disease occurrence. Cutler (2005) supposed that *C. albicans* induces highly complex pools of heterogeneous polyclonal antibody responses. Within the pool, the protective antibodies may not be present in sufficient titer to effect antifungal protection. We propose that immunization with the synthetically prepared mannan-protein conjugate would elicit a high level of protective antibodies. Based on the presented observations we presume that the protective effect of antibodies induced by the mannan-protein conjugate is rendered by direct blocking of vital functions of *C. albicans* rather than any complement-dependent lysis. The presented results support the notion that the *Candida* surface polysaccharide-based conjugate would be a perspective candidate for preventive immunomodulation treatment (Cassone *et al*., 2007).

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