Antibody response to the 45 kDa *Candida albicans* antigen in an animal model and potential role of the antigen in adherence

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The *Candida* antigen CR3-RP (complement receptor 3-related protein) is supposed to be a ‘mimicry’ protein because of its ability to bind antibody directed against the α subunit of the mammalian CR3 (CD11b/CD18). This study aimed to (i) investigate the specific humoral isotypic response to immunization with CR3-RP in vivo in a rabbit animal model, and (ii) determine the role of CR3-RP in the adherence of *Candida albicans* in vitro using the model systems of buccal epithelial cells (BECs) and biofilm formation. The synthetic *C. albicans* peptide DINGGGATLPQ corresponding to 11 amino-acids of the CR3-RP sequence DINGGGATLPQALXQITGVIT, determined by N-terminal sequencing, was used for immunization of rabbits to obtain polyclonal anti-CR3-PR serum and for subsequent characterization of the humoral isotypic response of rabbits. A significant increase of IgG, IgA and IgM anti-CR3-RP specific antibodies was observed after the third (P<0.01) and the fourth (P<0.001) immunization doses. The elevation of IgA levels suggested peptide immunomodulation of the IgA1 subclass, presumably in coincidence with *Candida* epithelial adherence. Blocking CR3-RP with polyclonal anti-CR3-RP serum reduced the ability of *Candida* to adhere to BECs, in comparison with the control, by up to 35% (P<0.001), and reduced biofilm formation by 28% (P<0.001), including changes in biofilm thickness and integrity detected by confocal laser scanning microscopy. These properties of CR3-RP suggest that it has potential for future vaccine development.

INTRODUCTION

*Candida albicans* is the most frequently isolated fungal pathogen associated with infection of immunocompromised patients in hospital settings. The high propensity to cause disease is due to the expression of many virulence factors including highly immunogenic cell surface proteins (Alberti-Segui *et al.*, 2004; Jeng *et al.*, 2005; Pietrella *et al.*, 2006) able to trigger cellular and humoral response (Viudes *et al.*, 2001; López-Ribot *et al.*, 2004; Omaetxebarria *et al.*, 2005; Fukuizumi *et al.*, 2006). Additionally, a variety of cell surface molecules expressed by *Candida* help it to evade the host immune response by mimicry of host receptors (Gustafson *et al.*, 1991; Phan *et al.*, 2007). The receptor MAC-1 (CD11b/CD18) on lymphocytes is the surface β2 integrin that mediates lymphocyte adhesion to *C. albicans* (Forsyth & Mathews, 2002). However, *Candida* is not only the target for MAC-1, but also CR3-RP identified on the yeast surface might be classified as a member of the integrin family due to its antigenic, structural and functional relation to the α subunit of the mammalian neutrophil receptor CD11b/CD18 (Gilmore *et al.*, 1988;
Hostetter et al., 1990; Hostetter, 1996). Binding the human complement fragment iC3b to this receptor results in inhibition of opsonization and partial blocking of phagocytosis (Hostetter et al., 1984; Heidenreich & Dierich, 1985). Several putative fragments of CR3-RP have been reported: these include a 165 kDa fragment (Hostetter et al., 1990), 66, 55 and 42 kDa fragments (Alaei et al., 1993), and 42 and 32 kDa fragments (Bujdáková et al., 1999). Furthermore, a gene encoding a 188 kDa integrin with 18 % homology to the human CR3 has also been described in C. albicans (Gale et al., 1996). Recently, the effect of oestrogen on CR3-RP expression was studied (Warolin et al., 2005). Others reported that the transcription of CR3-RP was found to be dependent on Ca²⁺ ions and the yeast-mycecellial transformation; its expression being higher in germ tubes and in the mycelial form (Spötä et al., 1994; Bujdáková et al., 1997).

C. albicans is able to bind to collagen, laminin or fibronectin via special surface molecules (Makihiira et al., 2002; Cateau et al., 2007), and CR3-RP has been reported to participate in adhesion to endothelial cells (Würzner et al., 1996). Adhesion of C. albicans to host cells is a crucial step in establishment of candidiasis. Moreover, it is also critical for an early phase in biofilm formation, the phenomenon associated with the new classes of diseases correlated with using medical devices (Kojic & Darouiche, 2002; Cateau et al., 2002). Various fragments of CR3-RP have been reported (Hostetter et al., 1993). Several putative fragments of CR3-RP have been isolated from vaginal candidiasis, was selected based on the high expression of CR3-RP (Bujdáková et al., 1996). Several genes that code for proteins that enhance the adherence capacity of C. albicans have been reported (Ibrahim et al., 2005; Nails et al., 2006; Nobile et al., 2006). However, anti-Candida antibodies can reduce Candida binding to different surfaces (Rodier et al., 2003; Elguezabal et al., 2004; López-Ribot et al., 2004). To that end, the aim of this study was to (i) investigate the specific humoral isotypic response to immunization with CR3-RP in vivo in a rabbit animal model, and (ii) determine the role of CR3-RP in adherence of C. albicans in vitro using the model systems of buccal epithelial cells (BECs) and biofilm formation.

**METHODS**

**Purification and analysis of the 45 kDa protein.** The strain C. albicans K2 (CCY 29-3-162 from the Culture Collection of Yeasts (CCY), Institute of Chemistry, Slovak Academy of Sciences), originally isolated from vaginal candidiasis, was selected based on the high expression of CR3-RP (Bujdáková et al., 1997). Total protein extract (100 ml) was obtained from 6 l germ tube suspension (10⁷ germ tubes ml⁻¹) by breakage with glass beads (diameter 2 mm; Sigma-Aldrich) in breaking buffer (20 %, v/v, glycercol; 0.1 M Tris/HCl, pH 8.0; 1 mM DTT; 1 mM PMSF; all from Sigma-Aldrich). Germ-tube formation was induced as described previously (Bujdáková et al., 1997). Briefly, an overnight yeast culture of C. albicans in yeast form was transformed to germ tube form. The germ tubes were collected by centrifugation (5000 g, 10 min) and the sediment was resuspended in 40 ml breaking buffer. Then the suspension was divided into 1 ml aliquots in 2 ml Eppendorf tubes and an approximately equal volume of glass beads was added to every tube. Cells were disrupted by vortexing 40 times at high speed for 1 min. Between vortexing steps, the tubes were cooled in an ice bath for 15 s. Then broken cells were centrifuged again (10000 g, 4 °C, 10 min) and the supernatant containing protein extract was collected and dialysed in 20 mM Tris/HCl (pH 7.5) overnight. Then the extract was transferred into a new Falcon tube and 1 mM PMSF was added. An aliquot (4 ml) of the protein extract was stored at −20 °C until needed and the remaining sample was concentrated by filtration through an Amicon device (Millipore) to 4 ml. Partial purification of the 45 kDa protein (Bujdáková et al., 1999) from the protein extract concentrate was performed by FPLC with Superose 12 P (Amersham) column (2 × 40 cm) equilibrated with 0.02 M Na₂HPO₄/KH₂PO₄, 0.1 M Tris/HCl, pH 8.0; 1 mM DTT; 1 mM PMSF; 100 mM NaCl supplemented with 5 % (v/v) non-fat dry milk, the membrane was incubated with the OKM1 antibody diluted 1:40 in TBS for 1 h at room temperature (RT). After three washing steps in TBS, 0.05 % (v/v) Tween 20 (Merck) (TBST), anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1:30 000 in TBS was added. The membrane was incubated for 1 h at RT. After four washes in TBST, the membrane was developed using Sigma Fast BCIP/NBT tablet (Sigma-Aldrich) dissolved in 10 ml deionized water. The colour reaction was stopped with deionized water.

The PVDF membrane was stained with amido black solution (Applichem) and subjected to N-terminal sequencing in two laboratories independently: at Innsbruck Medical University, where 11 amino acids were sequenced, and at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, where 21 amino acids were identified by Edman degradation on an ABI 491 protein sequencer.

**Preparation of polyclonal antibody to CR3-RP, ELISA and Western blotting.** A synthetic peptide with >90 % purity, as revealed by HPLC and MS analysis, was generated by KJ Ross-Petersen, Denmark. Its sequence corresponded to the first 11 amino acid residues (DINGGGATLPQ) at the N-terminus of the CR3-RP protein. This peptide was used to raise polyclonal anti-CR3-RP serum by immunization of rabbits (female, 8-weeks-old, 2250 g weight, variety Hyla; Research Institute of Animal Production, Nitra, Slovakia). The rabbits were split in two groups of each three. Preliminary serum samples were collected as a negative control before the experiment. The two groups were immunized by: (i) an intramuscular injection of 40 μg CR3-RP mixed with complete Freund’s adjuvant (Sigma-Aldrich) at day 0, boosted with another four doses in incomplete Freund’s adjuvant (80 μg, 160 μg, 300 μg, 300 μg) 2 weeks apart (five immunizations in total); or (ii) heat inactivated Candida yeast-form cell suspension in sterile saline (10⁷ ml⁻¹) administered intravenously in a marginal ear vein three times, 2 weeks apart. Both groups of rabbits were bled 2 weeks after the last injection. Antibody titres were determined by ELISA in 96-well plates

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Production of CR3-RP specific IgG, IgM and IgA antibodies in a rabbit model, and ELISA. The CR3-RP specific rabbit IgG, IgM and IgA isotope antibodies induced by immunization with either CR3-RP in Freund's adjuvant or whole Candida cells were detected using a modified ELISA. Briefly, 10 μg CR3-RP in carbonate/bicarbonate buffer pH 9.6 (100 μl per well) was applied onto Immulon 4 HBX microplates (Dynex) for 18 h at 4 °C, and the plates were coated with 2% (w/v) non-fat milk (KPL) in PBST pH 7.2. Sequential titrations of rabbit antisera were performed stepwise with the pre-coated CR3-RP overnight at 4 °C. Immune complexes were tested for alkaline phosphatase-labelled protein A (Calbiochem) for IgG, and with alkaline phosphatase-labelled goat anti-rabbit IgA and IgM (Bethyl Laboratories) for antisera IgA and IgM levels, respectively. The enzyme reaction was developed with the BluePhos microwell phosphatase substrate system (KPL) and absorbance measured at 630 nm (MRX II; Dynex). The results were expressed as arbitrary units, defined as the amount of the anti-CR3-RP specific IgG, IgA and IgM present in 1:1000 dilution of the reference whole cell antiserum, and were calculated as a percentage of the reference whole cell antiserum (100%) and presented as the mean ± SEM of three rabbits per group.

Computational and statistical analysis. Results from in vitro experiments were calculated as mean values ± SEM. Similarly, in vivo results were expressed as the mean ± SEM for each examined group. Normality of distribution was evaluated according to the Shapiro–Wilk test at the 0.05 level of significance. Statistical comparison between groups was performed using one-way analysis of variance (ANOVA) and post-hoc Bonferroni and Tukey tests. The results were significant if the difference between the analysed groups equaled or exceeded the 95% confidence level (P<0.05). Analyses were performed using Origin 7.5 Pro software (OriginLab).

Adherence to BECs. Adherence of Candida cells, with and without pre-incubation with the polyclonal anti-CR3-RP serum, to BECs was performed according to Moragues et al. (2003) with some modifications. Briefly, human BECs were collected from six healthy volunteers by gently scraping cheek mucosa with sterile wooden spatulas. After centrifugation (1000 g, 10 min, RT), the cells were washed three times with PBS and suspended at 10^6 cells ml^-1 in PBS. C. albicans cells from an overnight culture in YPD were washed with PBS three times and diluted in PBS to a density of 10^8 cells ml^-1. For testing the polyclonal anti-CR3-RP serum, 100 μl Candida suspension was harvested by centrifugation at 2000 g for 5 min at 4 °C, and washed gently three times with 500 μl blocking solution (3% gelatin in PBS). Then, 50 μl polyclonal anti-CR3-RP serum diluted 1:100 in PBS was mixed with Candida cells and incubated on ice for 1 h. This suspension was washed three times with PBST, collected and used directly for incubation with BECs. In the control experiment, Candida cells were treated only with PBS instead of polyclonal anti-CR3-RP serum. The 500 μl BEC suspension was mixed with 50 μl Candida suspension treated with polyclonal anti-CR3-RP serum or PBS and incubated for 2 h with gentle agitation (100 r.p.m.) at 37 °C. The mixtures were centrifuged (1000 g, 10 min, RT), the supernatants discarded and the pellets resuspended in 50 μl PBS by gentle rotary mixing. The cells were then counted with a haemocytometer. A minimum of 100 BECs were analyzed using light microscopy at 400 x magnification to determine the number of adherent Candida cells. The number of adherent yeast upon pretreatment with specific antibody and pre-immune serum was calculated as a percentage of those pretreated with PBS in the parallel control experiment (100%). The final percentage of adherent cells was determined as the difference between the percentage of Candida cells treated with polyclonal anti-CR3 serum and those treated with pre-immune serum. For capturing an image, the suspension of BECs with Candida cells was dropped onto a glass slide, dried at RT, and then fixed with methanol for 10 s, covered with 5 μl crystal violet and dried again at RT (Fig. 1).

Determination of cell surface hydrophobicity (CSH) and biofilm formation. Yeast C. albicans K2 used for both hydrophobicity and biofilm assays was cultivated on Sabouraud dextrose agar (Biomark Laboratories) at 28 °C for 24 h. A large loop of cells was transferred into yeast nitrogen base with amino acids (YNB) broth (Sigma-Aldrich) supplemented with 0.9% D-glucose (Applichem), and after overnight incubation at 37 °C, the cells were centrifuged at 4000 g for 5 min and washed twice with 0.5 ml PBS. The cells were resuspended in 1.5 ml YNB broth and the suspension was adjusted to OD_600 1.0. CSH was measured according to the method of Klotz et al. (1985) using the biphasic separation method with n-octan (Merck) overlay.

![Fig. 1. Binding of C. albicans yeast cells to human BECs visualized by crystal violet staining. Arrows indicate adherent, as well as non-adherent C. albicans and BECs.](image-url)
Characterization of Candida albicans surface antigen

The relative percentage of CSH was calculated as follows: [\(\frac{\text{OD}_{600 \text{ nm}} \text{ of the control} - \text{OD}_{600 \text{ nm}} \text{ after octane overlay}}{\text{OD}_{600 \text{ nm}} \text{ of the control}}\) × 100.

Biofilm was formed using polystyrene 96-well plates (Sarstedt) in 100 µl YNB medium supplemented with 0.9 % d-glucose at 37 °C and quantified by its ability to reduce XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] sodium salt to water soluble formazan (Sigma-Aldrich) as described by Li et al. (2003). Parallel experiments were performed with pre-incubation of C. albicans cells with polyclonal anti-CR3-RP serum and pre-immune serum. The yeast suspension prepared as described above, was centrifuged, the cell pellet treated by three washes in 3 % (w/v) gelatin (Oxoid) dissolved in PBS, and then incubated with 50 µl polyclonal anti-CR3-RP serum or pre-immune serum, both diluted 1:100 in PBS, for 1 h on ice. Serum was removed by centrifugation and three washes in PBST and the yeast cells were used for the hydrophobicity and biofilm assays.

Confocal laser scanning microscopy (CLSM). Mature biofilms formed on polyethylene coverslips in Petri dishes (both from Sarstedt), prepared according to Li et al. (2003), were transferred to new Petri dishes and 20 µl tetramethyl rhodamine methyl ester perchlorate (TMRM; excitation wavelength 549 nm, emission wavelength 573 nm; Invitrogen), diluted in distilled deionized water to final concentration of 5 μM, was applied to each coverslip. Stained biofilms were observed with a LSM 510 META confocal laser scanning microscope head, mounted on an Axiovert 200 M inverted microscope (both from Carl Zeiss, Germany). In fluorescence mode, the 543 nm line of the He–Ne laser excited the sample and a 545 nm dichroic mirror with 565–615 nm band-pass emission filter were used for fluorescence detection. In transmission mode the laser light at 543 nm passing through the sample was detected by a photodiode attached to the microscope condenser. A 20×0.75 PlanApochromat dry objective was used, with the confocal pinhole opening corresponding to 1 Airy unit. The half-width of the estimated point-spread function in Z direction was 1.8–2 μm (in the 500–600 nm wavelength range), therefore we used 0.9 μm Z-axis sampling for three-dimensional (3D) data recording. For each image, 153 × 153 μm area was scanned with a resolution of 512 × 512 pixels, 16 × line averaging and 8-bit quantization. The images were further processed with LSM Image Examiner software. Biofilm images were either displayed individually as two-dimensional plots, or reconstructed in 3-D projections. Vertical (xz) sections or side views of the 3D reconstructed images were used to determine biofilm thickness and architecture. The thickness was estimated from the outer edges of the area, where TMRM signal gain intensity was above half of its maximum.

RESULTS AND DISCUSSION

Purification of CR3-RP, and preparation and evaluation of polyclonal anti-CR3-RP serum

The human CR3, also known as MAC-1 (CD11b/CD18), expressed on leukocytes is a principal adhesion receptor for binding iC3b-opsonized pathogens. The existence of integrin-like proteins in C. albicans with affinity for complement conversion product iC3b has been suggested by several authors (Eigentler et al., 1989; Hostetter et al., 1990; Gustafson et al., 1991; Alaei et al., 1993; Spötl et al., 1994; Hostetter, 1996; Bujdákova et al., 1999); however, information about its contribution to Candida virulence is very limited. C. albicans expressing CR3-RP was able to form rosettes consisting of iC3b-coated sheep erythrocytes bound to germ tubes or mycelial forms. Furthermore, immunofluorescence, as well as ELISA results, revealed CR3-RP cross-reactivity with anti-human CR3 mAb named OKM1 (Eigentler et al., 1989; Bujdáková et al., 1997, 1999), which is able to block the function of the lectin domain of CD11b. CR3-RP was purified as described in Methods and the molecular mass was estimated to be around 45 kDa (Fig. 2). N-terminal sequencing of the putative CR3-RP protein revealed two amino acid motifs with sequences DINGGGATLPQ and DINGGGATLPQLXQITGVIT (the latter sequence was submitted to the UniProt Knowledgebase, under UniProt accession number P85437, entitled CR3-RP in C. albicans). The first 11 amino acids exhibited 100 % homology between the protein samples sequenced independently in two different laboratories. This identical sequence was used to search the Swiss-Protein database using BLAST available online, but revealed no homology to any Candida proteins or other fungal proteins already published. The sequence corresponding to the first 11 amino acids was synthesized and this peptide was used for immunization of rabbits as described in Methods.

Immunization of rabbits with CR3-RP and characterization of the humoral response

We hypothesized that CR3-RP, as further characterized in this study, belongs to the group of surface molecules adopted by pathogenic microorganisms to improve entry into the host (Sturtevant & Calderone, 1997; Taborda & Casadevall, 2002; Nosanchuk et al., 2003). These antigens are usually mannoproteins able to elicit a response from the host immune system. Successful anti-Candida defence requires tight cooperation of the different mechanisms of innate and adaptive immune responses (Roeder et al., 2004; Netea et al., 2006). In spite of fact that cell-mediated

Fig. 2. (a) SDS-PAGE separation of Candida protein extract after liquid chromatography visualized by silver staining assay. (b) The interaction of the CR3-RP blotted to nitrocellulose membrane with polyclonal anti-CR3-RP serum visualized by alkaline phosphatase assay. M, Protein size marker; S, sample.
immunity is important during the first contact with fungal pathogen, the contribution of the specific humoral response has also been demonstrated in recent years (López-Ribot et al., 2004; Cutler, 2005; Paulovičová et al., 2005). The induction of an antigen-specific humoral response was evaluated by IgG, IgM and IgA anti-CR3-RP specific sera levels in immunized rabbits (Fig. 3). While IgM is the first antibody produced in the humoral response, mediating opsonization of micro-organisms by activation of the classical complement pathway, the IgG class has a protective role, including reduction of the C. albicans binding capacity to host and medical devices (Kondori et al., 2003; Rodier et al., 2003), and serum IgA, mainly its subclass unit antibody, is an important part of mucosal immunity initiating numerous host defence activities, such as phagocytosis, cytotoxicity and respiratory burst (Morton et al., 1996; van Spriel et al., 2002). The experimental sera were evaluated by ELISA and the isotypic distribution induced by CR3-RP alone and/or heat-inactivated whole Candida cells was determined. The prime-boost immunization strategy enhanced, in particular, polyclonal production of CR3-RP–specific IgG, IgA and IgM isotype antibodies. CR3-RP administration induced the highest increase of anti-CR3-RP IgG and IgA levels after the third (P<0.01) and later (P<0.001) immunization doses. Comparison of induced specific anti-CR3-RP-isotypes with the results from whole Candida cells, especially after the second booster, demonstrated a higher immunogenicity of CR3-RP. While induction of IgM and IgG antibodies has been described for many Candida antigens (Rodier et al., 2003; Torosantucci et al., 2005), the marked elevation of IgA levels in serum suggested CR3-RP peptide immunomodulation of the IgA1 subclass, presumably in coincidence with Candida epithelial adherence.

**Role of CR3-RP in adherence to BECs and biofilm formation, determination of CSH, and CLSM**

To test the hypothesis about participation of CR3-RP in adherence, we coated fungal adherence receptors with an antibody that can reduce the adherence of C. albicans cells (Moragues et al., 2003; López-Ribot et al., 2004). Upon treatment with the polyclonal anti-CR3-PR serum, the binding of Candida cells to BECs was reduced up to 35% in comparison with the control sample (P<0.001). This suggested that the polyclonal anti-CRP-PR serum might also affect biofilm formation – a complex process in which adherence is a determining factor (Blanco et al., 2006; Blankenship & Mitchell, 2006). Indeed, we observed a reduction in biofilm formation, most likely due to a decreased adherence capacity mediated by blocking CR3-RP, by about 28% in comparison with the control (P<0.001) (Fig. 4). As hydrophobicity of the yeast cell wall is an important factor affecting adherence to different substrates (Hazen et al., 2001; Singleton et al., 2005) and is directly correlated with the surface protein composition, we investigated how it is affected by CR3-RP. The percentage of hydrophobic cells decreased after the pre-

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**Fig. 3.** Specific humoral immune response induced by CR3-RP immunization. The experimental data are expressed as geometric means ± SEM. The normality of the distribution of each data group was evaluated at a 0.05 level of significance. The results are expressed as arbitrary units (AU), which are defined as the amount of anti-CR3-RP specific IgG, IgA and IgM present in 1:1000 dilution of the reference whole cell antiserum, calculated as a percentage of reference whole cell antiserum (100%). Comparisons of all examined groups with pre-immune baseline results were performed by ANOVA. Levels of significance: ***, 0.000<P<0.001; **, 0.001<P<0.01, *, 0.01<P<0.05. Differences were considered significant where 0.01<P<0.05.

**Fig. 4.** Effect of pretreatment of C. albicans cells with polyclonal anti-CR3-RP serum on biofilm formation. The biofilm was quantified by XTT reduction assay as described in Methods. The experimental data are expressed as geometric means ± SEM.
change in the ratio of hydrophobic/hydrophilic cells in a Candida suspension can indirectly affect the capacity of C. albicans to adhere to BECs or to polystyrene microtitre plates in the biofilm model. Using CLSM, a corresponding reduction in biofilm thickness was observed, from 14.5 μm in the control to 9 μm in the sample pre-incubated with the polyclonal anti-CR3-RP serum (P<0.05). Moreover, a loss of biofilm integrity after pre-incubation with polyclonal anti-CR3-RP serum was observed (Fig. 5). Taken together our results provide evidence for the strong immunomodulatory efficiency of CR3-RP and its contribution to the adherence of C. albicans. These properties suggest that CR3-RP is a fungal antigen with a potential for vaccine development.

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