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Expression of the C3d-Binding Protein (CR2) from *Candida albicans* during Experimental Candidiasis as Measured by Lymphoblastogenesis

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The complement C3d-binding protein (CR2) of *Candida albicans* has been purified by immunoaffinity chromatography, and its specificity has been characterized by immunoblotting with monoclonal antibodies to the *C. albicans* CR2 and the mammalian CR2. Recent studies with immunoelectron microscopy indicated that the CR2 was expressed during a systemic infection in a murine model of candidiasis. As a continuation of these observations, the immunogenicity of the *C. albicans* CR2 was investigated in a lymphoblastogenesis assay. Lymph node cells as well as splenic lymphocytes from mice infected subcutaneously with viable blastoconidia of *C. albicans* reacted to the *C. albicans* CR2 to a significantly greater extent than did lymphocytes from uninfected mice ($P < 0.01$). The maximum stimulation of splenic lymphocytes by the purified receptor occurred at a concentration of 0.54 μg of protein per ml after 72 h of incubation of lymphocytes and receptor. Also, splenocytes from infected or CR2-immunized mice exhibited significantly reduced responses to the T-cell-dependent mitogen phytohemagglutinin ($P < 0.01$). These data indicate that lymphocytes from infected mice respond to the *C. albicans* CR2 in a lymphoproliferation assay to a greater extent than do lymphocytes from uninfected mice, indicating that the CR2 is expressed in vivo.

Candida albicans can bind the complement C3 conversion product C3bi or C3d by using cell surface mannoproteins as receptors (1, 7, 8, 10, 11, 13, 17, 27). These activities are similar to those of a variety of mammalian cells which have specific complement receptors (CR2, which binds C3d, or CR3, which binds C3bi) (26). Some degree of homology exists between the *C. albicans* CR3 and the mammalian CR3, since anti-mammalian CR3 antibodies cross-react with *C. albicans* CR3-like proteins (7, 8, 10, 11).

The *C. albicans* C3d-binding protein (CR2) has been purified from hyphal extracts of the organism by affinity chromatography with either C3d ligand or monoclonal antibody (MAb) CA-A (1, 17, 27). The purified receptor consists of a doublet of 60 and 66 to 68 kDa, although biological activity, i.e., the inhibition of C3d binding to whole cells, seems to be associated with the 60-kDa component (17). The function of the *C. albicans* CR2, other than its affinity for C3d, has not been established, although Tronchin et al. (29) have shown that proteins with similar molecular masses also bind fibrinogen and laminin and may be important in the attachment of the organism to plastic devices (30).

Recently, we have begun studies to determine whether the *C. albicans* CR2 is expressed in kidney tissue or peritoneal lavage fluid from mice infected with *C. albicans* (14). Using a monospecific, polyclonal rabbit antiserum to the purified *C. albicans* CR2, we observed by immunoelectron microscopy the expression of the CR2 on both the hyphal and blastoconidial forms of the organism (14). Immunoreactivity was predominantly associated with the surface of hyphae but appeared as a subsurface component of blastoconidia, most often associated with the plasma membrane.

Recent data have pointed to the immunoregulatory activities of cellular components from *C. albicans* which can induce suppression either in vivo or in vitro (4, 5, 24). Although the nature of the inhibitory component is not entirely clear, the most commonly suggested etiology is a circulating cell surface antigen, in particular, some form of mannan or mannoprotein (9, 21).

In this study, we have examined the immunogenicity of the *C. albicans* CR2. Both lymph node and splenic cells from noninfected and infected animals were used in lymphoblastogenic assays with either a dithiothreitol (DTT) extract or the purified *C. albicans* CR2. In similarly treated animals, the effect of the *C. albicans* CR2 on the mitogen-induced responses of splenic cells was also investigated.

MATERIALS AND METHODS

Organism and culture conditions. *C. albicans* 4918 was used in all experiments and has been described previously (1, 19). Cultivation of hyphal cells was performed with Lee's medium (16) as described previously (31). In brief, blastoconidia were grown to the stationary phase for 20 h at 24°C and subcultured in Lee's medium at 37°C for 16 h on a shaker (150 rpm).

Preparation of DTT extracts. Hyphal cells grown as described above were collected by centrifugation, washed, and extracted with DTT (Sigma Chemical Co., St. Louis, Mo.) by a previously described method (28). Hyphae were suspended in 10 volumes of 0.05 M Tris-HCl (pH 7.5) containing 0.8 M mannitol–12 mM DTT and incubated at 37°C for 4 h with shaking. After the addition of 0.05 M iodoacetamide (Sigma) in 0.05 M Tris–0.8 M mannitol buffer (pH 7.5) to a final concentration of 17 mM, the cells were incubated at 24°C for 2 h and centrifuged. The supernatant was dialyzed extensively for 48 h against distilled water and lyophilized.

Preparation of monospecific rabbit immunoglobulin G (IgG) to the *C. albicans* CR2. Pools of partially purified material from DEAE-Trisacryl or concanavalin A (ConA) columns (1) containing components that inhibited the rosetting of sheep erythrocytes coated with C3d by hyphae of *C. albi-*

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cans were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) preparative gels. The horizontal band in the region of 60 kDa, earlier proven to have *C. albicans* CR2 activity, was excised and electroeluted (model 422 electroeluter; Bio-Rad, Richmond, Calif.). After dialysis and lyophilization, the purity and the size (60 kDa) of the eluted protein were determined. This protein was designated CR2 and used as an immunogen.

New Zealand White rabbits were immunized subcutaneously with 150 μg of the CR2 immunogen emulsified with complete Freund's adjuvant. Three booster injections, each of 70 μg of protein emulsified in incomplete Freund's adjuvant, were given at 3-week intervals. The rabbits were bled 1 week after each immunization. The monospecificity of the antisera was assessed by double immunodiffusion and crossed immunoelectrophoresis against the immunogen. Tests with the DTT hyphal extract showed only one precipitate in double immunodiffusion, but in crossed immunoelectrophoresis a faint line with a faster mobility was seen in addition to the major precipitate. Sera from each of the rabbits were pooled. IgG was isolated from the pooled rabbit sera by means of protein A affinity chromatography. Immuno Pure IgG buffers and affinity Pak columns of immobilized protein A covalently coupled to cross-linked agarose were used in accordance with the manufacturer's protocol (Pierce, Rockford, Ill.). The IgG samples were lyophilized and used preparatively to isolate the *C. albicans* CR2 from the DTT hyphal extract.

Isolation of the *C. albicans* CR2 by immunoaffinity chromatography. The DTT hyphal extract was subjected to purification on AFFI-Gel HZ agarose beads (Bio-Rad) conjugated with monospecific rabbit IgG to the *C. albicans* CR2 in accordance with the instructions in the product guide. In brief, 10 mg of the purified IgG was dissolved in 3 ml of coupling buffer, and the mixture was oxidized and coupled to 2 ml of AFFI-Gel HZ beads. This suspension was poured into a chromatography column (1 by 10 cm), and the column was thoroughly washed and equilibrated with five bed volumes of antigen application buffer (20 mM Tris, 150 mM NaCl [pH 7.4]). The DTT hyphal extract was dissolved in 1 ml of application buffer at a concentration of 7 to 10 mg of protein per ml, and the mixture was applied to the column and incubated for 1 h at room temperature.

Unbound material was removed from the column with two bed volumes of application buffer at 0.5 M NaCl and washed with application buffer. The bound CR2 was eluted with 5 ml of 3 M $MgCl_2$ in 20 mM Tris buffer (pH 7.4), dialyzed against 0.01 M phosphate-buffered saline (PBS) for 24 h, and lyophilized.

SDS-PAGE and immunoblotting. SDS-PAGE under reducing conditions and Western blotting (immunoblotting) were performed by established procedures (1, 17, 27, 31). Rabbit IgG to the *C. albicans* CR2 and MAb CA-A (17) were used as primary antibodies, and protein A-alkaline phosphatase (Organon Teknika, West Chester, Pa.) was used as the secondary reagent. The blots were also incubated with two anti-human CR2 MAbs, B2 (an IgM MAb from Coulter Immunology, Hialeah, Fla.) and HB-5 (an IgG2a MAb from Becton Dickinson, Mountain View, Calif.). These commercial preparations (1 mg/ml) were used at 1:40 and 1:20 dilutions, respectively. A biotinylated goat $F(ab')_2$–anti-mouse IgG-IgM–streptavidin HPR conjugate (TAGO Inc., Burlingame, Calif.) was used with both MAbs B2 and HB-5 to detect the reactive proteins. In addition, a rabbit antiserum to the human C3d ligand (Calbiochem, La Jolla, Calif.) and an anti-C3 (C3d) MAb (Quidel, San Diego, Calif.) were used as described by Tronchin et al. (29), with the exception that a protein G streptavidin HPR conjugate (Bio-Rad) was used for detection (1:2,500). Staining of SDS-PAGE proteins transferred to nitrocellulose was performed with Aurodye (Janssen Life Sciences, Piscataway, N.J.), and the identification of ConA-binding proteins was carried out by previously described methods (12, 17).

Protein determinations. Total protein was determined by the method of Lowry et al. (18).

Infection of mice and preparation of lymphocytes. Mice were infected with blastoconidia grown in Sabouraud dextrose broth medium for 24 h 37°C (Difco, Detroit, Mich.). Cells were collected by centrifugation, washed, resuspended in PBS, and adjusted to a cell density of 2×10^6 cells per ml. Male mice (BALB/c ByJ; Jackson Laboratory, Bar Harbor, Maine) were injected subcutaneously (at the right anterior chest wall) with 5×10^5 viable cells per mouse and reinjected 14 days after the primary injection with the same number of cells. Another group of mice received two subcutaneous doses of the purified CR2 (10 μg of protein per 0.25 ml of PBS per injection) at the aforementioned times. Control mice (designated normal) received an equal volume of PBS. Five days following the second inoculation, lymph nodes and spleens were removed, teased apart in RPMI 1640 (GIBCO, Grand Island, N.Y.), and passed through a 100-mesh screen to obtain a single-cell suspension of each.

Lymphoblastogenesis assay. Cells from lymph nodes draining the site of infection or spleens were pooled from at least three mice, washed three times with RPMI 1640, and adjusted to 10^7 cells per ml in complete medium (RPMI 1640 with 10% fetal calf serum; GIBCO), and 2×10^5 cells were dispensed into wells of 96-well flat-bottomed microtiter plates (Costar, Cambridge, Mass.). The *C. albicans* CR2 (0.027 to 0.27 μg of protein per 50 μl of RPMI 1640) and the DTT hyphal extract (0.004 to 0.125 μg of protein per 50 μl of RPMI 1640) were added to the appropriate wells. The mitogens used included phytohemagglutinin (PHA) (GIBCO) at a 1:100 dilution, ConA (Sigma) at 1.0 μg per well, lipopolysaccharide (LPS; Difco) at 1.0 μg per well, and pokeweed mitogen (PWM; GIBCO) at a 1:100 dilution. All lymphocyte cultures were incubated at 37°C in an atmosphere of 5% CO_2. At designated times, 0.5 μCi of [^{3}H]thymidine (ICN Biochemicals, Irvine, Calif.) was added to each well. Cultures were incubated for an additional 18 h and harvested on glass filter paper discs (Whatman Inc., Clifton, N.J.). The filters were extensively washed with distilled water. Thymidine uptake was measured with a liquid scintillation counter. Data are expressed as stimulation indices (SI) calculated according to the following equation: cpm of lymphocytes stimulated with antigen or mitogen/cpm of unstimulated lymphocytes. All experiments were repeated at least two times.

Statistical analyses. All assays were performed in triplicate. The results are expressed as the mean of two or more independent observations ± the standard deviation (SD). Statistical significance was estimated with the Student *t* test.

RESULTS

Characterization of the *C. albicans* CR2. As an alternative to previous methods (1, 17), the *C. albicans* CR2 antigen was purified from a DTT hyphal extract with an immunoaffinity column prepared with monospecific rabbit IgG to CR2 conjugated to AFFI-Gel HZ beads. The purified CR2 preparations were pooled and lyophilized. Comparison of the CR2 antigen and the source extract by means of SDS-PAGE

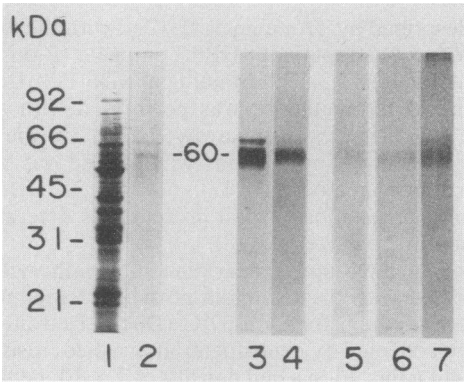

FIG. 1. SDS-PAGE and Western blot analyses of the *C. albicans* CR2. Electrophoresis was performed with 10% gels, and proteins were transferred to nitrocellulose membranes. Aurodye staining revealed the protein moieties in the candidal hyphal extract and the CR2 antigen in lanes 1 and 2, respectively. Lanes 3 to 7 illustrate, as immunoblots, the interactions between the CR2 antigen and five different, specific antibodies: rabbit IgG to CR2 (lane 3), anti-*C. albicans* CR2 MAb CA-A (lane 4), anti-mammalian CR2 MAb B2 (lane 5), rabbit antiserum to the human C3d ligand (lane 6), and anti-C3 (C3d) MAb (lane 7). The dominant band (60 kDa) in all blots is indicated, and the positions of separated molecular mass markers are designated on the left.

and transfer to and Aurodye staining of the nitrocellulose membranes revealed a doublet at 60 and 68 kDa, as described earlier for purified CR2 (17); this result was in contrast to the multiple protein bands representing the source extract (Fig. 1, lanes 2 and 1, respectively).

Immunoblots of the purified CR2 are shown in Fig. 1. Rabbit IgG to CR2 (lane 3) and MAb CA-A against the *C. albicans* CR2 (lane 4) revealed doublets similar to those revealed by the CR2 protein separation. The anti-mammalian CR2 MAb B2 showed low-affinity binding only to the 60-kDa protein (lane 5), while this band was detected even less discernibly by the anti-mammalian CR2 MAb HB-5 (data not shown). In blotting assays with antibodies to the C3d ligand, the rabbit antiserum to the human C3d ligand reacted only with the 60-kDa protein, whereas the anti-C3 (C3d) MAb detected both components in the doublet (lanes 6 and 7). The glycosylated nature of the CR2 was indicated by its reactivity with ConA, confirming previous observations (17) (data not shown).

Effect of the CR2 on lymphoblastogenesis. Using the CR2 as an antigen, we compared the reactivities of regional lymph node lymphocytes from mice immunized by infection with *C. albicans* blastoconidia and from uninfected mice (Table 1). As a positive control, proliferation was also determined with a complex hyphal extract (DTT). Both T-cell mitogens (ConA and PHA) and B-cell mitogens (LPS and PWM) were included in the assays. The results in Table 1 indicate that the SI were significantly higher in the infected mice for both the CR2 and the DTT hyphal extract ($P < 0.01$, infected versus uninfected). The mean incorporation ± SD of [^{3}H]thymidine in CR2-stimulated cultures was 813 ± 107 cpm, corrected for incorporation in cultures without the antigen (data not shown). The responses of both groups of mice to all mitogens were similar, although PHA-induced lymphoproliferation appeared lower in infected mice (Table 1, $P < 0.02$, infected versus uninfected). We also measured the proliferative responses of splenic lymphocytes from the same groups of mice. The kinetics of the CR2-induced

TABLE 1. Proliferative responses of lymphocytes from lymph nodes of normal and infected mice[a]

Antigen (μg of protein/ml)	SI ± SD for the following mice:	
	Normal	Infected
CR2 (0.54)	1.0 ± 0.3	3.1 ± 1.5[b]
CR2 (2.50)	0.8 ± 0.5	2.8 ± 1.2[b]
CR2 (5.40)	0.7 ± 0.4	1.4 ± 1.0[b]
DTT (0.08)	1.1 ± 0.3	1.8 ± 0.9
DTT (0.25)	0.9 ± 0.3	2.3 ± 0.8[b]
DTT (2.50)	1.0 ± 0.3	5.0 ± 1.6[b]
ConA	728 ± 148	613 ± 218
PHA	680 ± 117	487 ± 143[c]
PWM	42 ± 5	27 ± 6
LPS	34 ± 7	35 ± 5

[a] Lymph node cells from normal (uninfected) mice or mice infected subcutaneously with *C. albicans* cells were incubated with antigen or mitogen for 48 h and pulsed with [^{3}H]thymidine.
[b] $P < 0.01$.
[c] $P < 0.02$.

proliferative responses of splenic cells from both normal and immunized mice are shown in Table 2. The optimum concentration of the CR2 for proliferation in immunized (infected) mice was 0.54 μg of protein per ml after 72 h of incubation of splenic cells with the antigen. Proliferation was highest for all concentrations of the CR2 at this time and generally decreased thereafter. At all times, the responses of cells from immunized (infected) mice were higher than those of cells from unimmunized mice, although significant responses were observed in the latter. The mean incorporation ± SD of [^{3}H]thymidine for splenic cells incubated with CR2 was 3,484 ± 832 cpm, corrected for incorporation in cultures without the antigen (data not shown).

PHA-induced blastogenesis was also measured with splenocytes from uninfected mice or from mice infected with whole cells or immunized with the purified CR2. A significantly higher response (SI) was observed with the splenocytes from uninfected mice than with those from *C. albicans*-infected mice (SI = 56.3 ± 6.1 in uninfected versus 33.3 ± 3.2 in infected; $P < 0.001$) or those from mice immunized with the CR2 (SI = 42.4 ± 7.1; $P < 0.01$) (data not shown). Suppression of the splenocyte response to PHA was also greater in *C. albicans*-immunized mice than in mice immunized with the CR2 ($P < 0.01$).

TABLE 2. Kinetics of the CR2-induced proliferative responses of splenocytes from nonimmunized and immunized mice[a]

Mice	CR2 (μg/ml)	SI ± SD at the following time (h):		
		48	72	96
Nonimmunized	0.54	1.4 ± 0.7	4.9 ± 2.0	4.5 ± 0.4
	2.5	2.2 ± 1.2	5.3 ± 1.3	1.9 ± 0.4
	5.4	2.2 ± 1.2	2.9 ± 1.2	1.2 ± 0.5
Immunized	0.54	4.5 ± 0.8[b]	8.0 ± 1.2	5.4 ± 0.8
	2.5	4.8 ± 1.6[b]	7.0 ± 0.3	3.2 ± 1.0
	5.4	3.8 ± 1.2[c]	4.2 ± 1.2	1.8 ± 1.1

[a] The mean ± SD for three experiments is shown. Splenocytes from nonimmunized (normal) and immunized (*C. albicans*-infected) mice were incubated with the CR2 at the indicated concentrations. At designated times cultures were pulsed wtih [^{3}H]thymidine, and 18 h later they were harvested and counted.
[b] $P < 0.01$.
[c] $P > 0.05$.

DISCUSSION

The CR2 used in this study was purified with a monospecific rabbit antiserum to the 60-kDa mannoprotein. In a Western blot assay, the CR2 purified with the monospecific antiserum reacted with a MAb to the *C. albicans* CR2 which we have previously described (1) (Fig. 1, lane 4). Additionally, reactivity of the CR2 of *C. albicans* was observed with a MAb which recognizes the mammalian CR2 (MAb B2; Fig. 1, lane 5) and with an antiserum to the human C3d ligand (Fig. 1, lane 6). Thus, reactivity of both anti-*C. albicans* CR2 and anti-mammalian CR2 antibodies as well as direct C3d binding have established the identity of the protein used in this study.

Cell surface complement receptors of *C. albicans* which recognize the C3 conversion products C3bi (CR3) and C3d (CR2) have been described by several investigators (1, 7, 8, 10, 11, 13, 17). The role(s) of these receptors, however, is not completely understood. Recent investigations indicate that the *C. albicans* CR3 behaves as an adhesin in the recognition of endothelial cells (11). The *C. albicans* CR2 may be associated with adherence to plastic (30) and also recognizes host cell matrix proteins such as laminin (29). Each of these studies was based on in vitro observations, however, so that the expression of the CR2 or the CR3 by *C. albicans* during an infection was not reported. Recently, however, Kanbe et al. (14) used a monospecific rabbit antiserum to the *C. albicans* CR2 and immunoelectron microscopy to identify the CR2 on the cell surface of hyphal forms of the organism from animals infected intraperitoneally (intraperitoneal lavage fluid) or intravenously (kidney tissue). Thus, it would appear that the CR2 is expressed in vivo.

As an additional approach to addressing the question of expression in vivo, we immunized mice by establishing a subcutaneous infection with blastoconidia. Subsequently, lymph node and splenic lymphocytes were isolated, and each was found to be reactive with the purified CR2. The reactivity was significantly higher in infected animals than in uninfected animals. This observation indicates that an epitope(s) specific for the CR2 of *C. albicans* is recognized by the host. As an additional observation, lymphocytes from uninfected mice responded to PHA to a significantly higher extent than did those from infected or CR2-immunized animals, indicating that immunosuppression had occurred. However, since the reactivity of lymph node lymphocytes to another T-cell mitogen (ConA) was similar regardless of the treatment group, we are uncertain of the significance of the decreased reactivity to PHA in infected mice compared with normal, uninfected mice.

Candidiasis has been frequently found in patients with a depressed immune system, such as AIDS patients (20). However, a number of investigators have reported that *C. albicans* contributes to immunosuppression. For example, in some cases of candidiasis, clinical improvement seems to parallel a restoration of normal cell-mediated immune system activity (2, 15, 21). Also, transiently reduced in vitro responses to PHA and ConA occurred following the intravenous administration of Formalin-killed *C. albicans* (23) or live blastoconidia in mice (25) or rats (24). Cuff et al. (3) observed that splenocytes obtained from normal mice and cultured with Formalin-killed cells of *C. albicans* acquired significant suppressor activity in 3 days. Both a PHA and an anti-sheep erythrocyte antibody response were suppressed by the cultured splenocytes. More recently, Cuff et al. (4) demonstrated a similar suppressive effect on anti-sheep erythrocyte antibody responses by using a DTT extract of blastoconidial cell walls. The suppressor cells were thought to be of the $L3T4^+$ $Ly\text{-}1^+$ $Lyt\text{-}2^-$ lineage.

The nature of the immunosuppressive component from *C. albicans* is not completely characterized, but this component appears to be a mannoprotein (5, 6). In one study (5), intravenously administered antigen suppressed delayed hypersensitivity to the same antigen given prior to sensitization. In vitro studies of immunosuppression of human peripheral blood lymphocytes by a polysaccharide from *C. albicans* has been reported (22). Normal peripheral blood lymphocytes, when cultured with this polysaccharide, suppressed a mixed lymphocyte reaction but did not affect the reactivity of fresh peripheral blood lymphocytes with ConA, PHA, or PWM. The suppressor cells were thought to be of the T-cell lineage.

In conclusion, we demonstrated that the purified CR2 stimulated the proliferation of both lymph node and splenic lymphocytes from *C. albicans*-infected mice. While the CR2 suppressed lymphoblastogenesis in response to PHA, the significance of this immunosuppressive activity has yet to be determined.

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