Purification of the 115-Kilodalton Exoantigen of *Cryptococcus neoformans* and Its Recognition by Immune Sera

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Received 11 August 1992/Accepted 4 November 1992

A 115-kDa exoantigen produced by *Cryptococcus neoformans* recognized by the previously described murine monoclonal antibody 7C9 has been purified from culture filtrate by a combination of membrane ultrafiltration, isoelectric focusing, and preparative gel electrophoresis. It is produced in late-log-phase cultures and is present in greater amounts in cultures grown at 25°C than in those grown at 37°C. Recognition of the antigen by 7C9 on immunoblots is abolished by the proteolytic enzymes papain and trypsin. The antigen is a glycoprotein bearing N-linked oligosaccharides, of which mannose is an important constituent. It does not appear to have proteolytic activity and is acidic, with a pI of 3 to 3.2. Its relationship to previously described *C. neoformans* mannoprotein is unclear since 7C9 shows only very weak cross-reactivity with a purified sample of the latter. Sera from patients infected with *C. neoformans* exhibited strong recognition of the glycoprotein as shown by immunoenzyme development of Western immunoblots, indicating its possible significance as a marker of disease.

Cryptococcus neoformans is the causative agent of cryptococcosis, a disease typified by an initial pulmonary infection which can disseminate to produce an ultimately fatal form of meningitis (5). The disease has become an important infection in individuals suffering from AIDS (15) and is now recognized as the fourth most common life-threatening infection in this patient group (1). The diagnosis of the disease relies either on the isolation of the yeast or on the detection of capsular polysaccharide via the latex agglutination test with either cerebrospinal fluid or serum (12). Thus far, three molecular species have been defined as cryptococcal exoantigens in culture filtrate: high-molecular-weight glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoprotein (MP) (11). GXM makes up 88% of the capsule mass and has been purified, extensively characterized (3, 4, 10), and identified as a virulence factor (2). GalXM has also been extensively characterized (9, 16), as has the MP, although to a lesser extent (17). The role of GalXM and MP in pathogenicity has not been elucidated.

Recently we have produced two species-specific monoclonal antibodies (MAbs) against *C. neoformans* exoantigens: MAb 7C9 and MAb 3C2. MAb 7C9 recognizes a 110- to 120-kDa band by Western immunoblot (6), and MAb 3C2 recognizes a 34- to 38-kDa glycoprotein (7). In this communication we report on the purification of a 115-kDa glycoprotein by using MAb 7C9 as a probe, together with the pattern of recognition of this purified exoantigen by patient sera. The absence of substantial cross-reactivity between 7C9 and purified MP is also detailed, suggesting the novel nature of this glycoprotein.

MATERIALS AND METHODS

Organisms and growth conditions. Baffled flasks containing 1 liter of Sabouraud's broth were inoculated with 10^6 *C. neoformans* yeast cells (isolates B4131 [nonencapsulated]

and B3501 [encapsulated] from June Kwon-Chung, Mycology Department, National Institute of Allergy and Infectious Diseases, Bethesda, Md., and isolates NCPF 3168, 3169, and 3409, from the National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Colindale, England) and placed on an orbital incubator (Fisons, Loughborough, England) at 125 rpm at 37°C. At 12-h intervals (up to 168 h), 10 ml of fluid was aseptically removed from the cultures; 9 ml of this liquid was filtered through Whatman no. 1 paper prior to 25-fold concentration on a Centricon 30 centrifugal concentrator (Amicon, Stonehouse, England) and subsequent recognition by MAb 7C9 in an enzyme-linked immunosorbent assay (ELISA; see below). The remaining 1 ml was spun down ($13,000 \times g$ for 5 min), resuspended in 100 μ l of 0.01 M phosphate-buffered saline (PBS; pH 7.4), and subjected to cell count with a hemocytometer. Cultures were also grown at 25 and 30°C to equivalent cell densities (10^7 cells per ml) to ascertain the effect of temperature on the production of the 115-kDa antigen by measuring the relative reactivity of MAb 7C9 in an ELISA.

Purification of the 115-kDa antigen. *C. neoformans* nonencapsulated isolate B4131 was cultured as previously described until the cell density was at least 10^7 /ml; it was then filtered through Whatman no. 1 paper. Typically 6 liters of filtrate was then passed through a 100-kDa membrane in a Pellicon tangential-flow filter system (Millipore, Watford, England) as both a concentration and a preliminary purification step, and the retentate (150 ml) was then concentrated in 15-ml batches on Centricon 30 concentrators (spun at $1,500 \times g$). Samples were then loaded on a Rotofor isoelectric-focusing system (Bio-Rad, Hemel Hempstead, England) with ampholytes in the range pH 3 to 10, and the system was run at a constant power of 12 W until the voltage stabilized (4 to 6 h). The reactivity of the 20 Rotofor fractions to MAb 7C9 was determined by ELISA, and protein concentrations were determined by the Coomassie blue method (13). In addition, fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and West-

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TABLE 1. Human immune sera used in immunoblot studies of the 115-kDa antigen

Patient	Western blot lane ^a	Sex	Comments	Serum latex agglutination titer
1	3	M	HIV ⁻	1:5
	4			1:25
2	5	M	HIV ⁻	1:400
	6			1:50
3	7	F	HIV ⁻	1:2,000
	8			1:1,000
4	9	M	HIV ⁻	1:10,000
5	10	M	HIV ⁻	1:200
6	11	M	HIV ⁻	1:5
7	12	M	HIV ⁻	1:1,000
	13			Negative
8	14	F	HIV ⁻	1:2,000
9	15	F	HIV ⁻	1:800
10	16	M	HIV ⁻	1:8,000
11	17	M	HIV ⁺	1:200
12	18	M	HIV ⁺	1:800
13	19	M	HIV ⁺	1:25
14	20	M	HIV ⁺	1:1,000

^a Refers to lanes in Fig. 3.

ern blotting with a minigel system (Bio-Rad; see below) followed by immunoenzyme development of blots with patient sera. Fractions 1 to 3 from the Rotofor were pooled, reconcentrated on a Centricon 30 device, and then subjected to SDS-PAGE on a Bio-Rad dual vertical slab gel electrophoresis cell with a 7.5% acrylamide gel (200 V for 6 h) as the final purification step. By reference to molecular mass markers (high-molecular-mass markers; Sigma), a gel slice at 115 kDa was cut out and placed in sealed dialysis tubing together with buffer (25 mM Tris-glycine [pH 8.5], 0.1% [wt/vol] SDS). This was then immersed in the same buffer in a flat-bed tank, electrophoresed overnight at 80 V, subjected to polarity reversal for 30 s, and collected to yield the purified 115-kDa antigen. After another concentration in a Centricon 30 device, the antigen was subjected to SDS-PAGE and Western blotting on a minigel gel electrophoresis system. Blots were again subjected to immunoenzyme development with patient sera and MAb 7C9.

Serum samples and mannoprotein. Sera from patients with confirmed cryptococcosis and known latex agglutination titers were obtained from the Mycology Department, Public Health Laboratory, Colindale, England (Table 1). Controls consisted of 10 sera from healthy laboratory personnel. Purified mannoprotein was obtained from V. Vaishnav and R. Cherniak, Department of Chemistry, Georgia State University, Atlanta, and was subjected to SDS-PAGE, Western blotting, and immunoenzyme development with 7C9, together with a cytoplasmic extract of *C. neoformans* (6).

Electrophoresis. SDS-PAGE was performed with either 10 or 7.5% gels, and Western blotting and isoelectric focusing were performed with 10% polyacrylamide gels, as previously described (6, 7).

ELISA. ELISA was performed as previously described (6, 7) to determine fractions containing the 115-kDa antigen; MAb 7C9 was used as a probe (at a dilution of 1:1,000), and a goat anti-mouse immunoglobulin G peroxidase-linked conjugate (diluted to 1:4,000) (Jackson Immunochemicals, West Grove, Penn.) was used to recognize the MAb.

Immunoenzyme development. Immunoenzyme development was carried out as previously described (6, 7) with human sera at a dilution of 1:250 and goat anti-human

immunoglobulin G peroxidase-linked conjugate at a dilution of 1:2,000. Blots were also probed with MAb 7C9 (1:1,000) together with goat anti-mouse immunoglobulin G peroxidase-linked conjugate (1:2,000).

Substrate SDS-PAGE gels. A modification of the method of Heussen and Dowdle (8) was used. SDS-PAGE resolving gels (7.5% acrylamide) were copolymerized with either 0.25% bacterial gelatin (GIBCO, Paisley, Scotland) or 1% casein (Sigma, Poole, England). Stacking gels without substrate were then cast, and 2 µg of purified protein, unreduced and unboiled, made up in sample buffer (0.25 M Tris hydrochloride [pH 6.8], 20% [vol/vol] glycerol, 0.2% [wt/vol] SDS, 0.005% bromophenol blue) was loaded per track, together with molecular mass markers (Sigma). Gels were run at 25 mA per gel at 4°C and then agitated in 2.5% Triton X-100 for 30 min to remove SDS. Following a 60-min incubation in PBS, with two changes, the gels were placed in fresh buffer and left overnight at 37°C. Gels were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad) in water-methanol-acetic acid (5:5:1, vol/vol/vol) for 2 h and then destained in 45% (vol/vol) methanol-3% (vol/vol) acetic acid.

Deglycosylation of the 115-kDa antigen. Subsamples of fractions 1 to 3 from the Rotofor purification step, each containing a total of 1 µg of protein, were incubated overnight with each of the following deglycosylation enzymes used in volumes equivalent to 10 U of activity: peptide glycanase F in 22 µl of digestion buffer (300 µl of 100 mM 1,10-phenanthroline, 500 µl of 7.5% Nonidet P-40, 900 µl of 550 mM sodium phosphate [pH 8.6]) at 37°C, neuraminidase (BDH) in 0.1 M potassium acetate buffer (pH 4.5) supplemented with 2 mM CaCl₂ at 37°C, α- and β-mannosidase (Sigma) in 0.1 M sodium citrate buffer (pH 4) at 25°C, α- and β-galactosidase (Sigma) under the same conditions, and α-glucosidase (Sigma) in 0.1 M sodium acetate buffer supplemented with 0.05 M EDTA (pH 6) at 37°C. After treatment, samples were boiled for 3 min with 1% SDS and 1.6% 2-mercaptoethanol, run on an SDS-PAGE gel (7.5% polyacrylamide) at 200 V, and blotted onto Immobilon-P (Millipore) prior to immunoenzyme development with 7C9.

Proteolytic digestion of the 115-kDa antigen. Subsamples (2 µg) of the purified antigen were treated overnight at room temperature, either with trypsin (Sigma) in a solution of 0.67 M sodium phosphate buffer (pH 7.6) or with papain (Sigma), in a solution of 1 ml of 3 M NaCl in 10 ml of a mixture of 20 mM EDTA and 50 mM L-cysteine (pH 6.2). Samples were then treated for immunoenzyme development with 7C9 as above.

RESULTS

Purification of the 115-kDa exoantigen and immune recognition. In ELISA, the 115-kDa exoantigen became detectable by MAb 7C9 in culture medium after approximately 60 h under the conditions described, although typically cultures were harvested after 80 h, at which point the cell count was above 6×10^6 /ml (late log phase; data not shown). Purification of the antigen was straightforward, relying first on a concentration-purification step involving membrane filtration and then on isoelectric focusing with the Rotofor system. In the latter step the antigen was located at the anodic end in a range equivalent to a pI of 3 to 3.2 (fractions 1 to 3; Fig. 1). In addition to the 115-kDa band, there was a fainter band at around 200 kDa in these pooled fractions, together with a broad undefined smear of protein below 50 kDa (Fig. 2, lane A).

Western blot and immunoenzyme development with

FIG. 1. Purification of the 115-kDa antigen by using the Rotofor isoelectric-focusing system. The histogram represents ELISA reactivity with 7C9, the open circles represent pH gradient, and the solid squares represent protein concentration.

pooled sera from patients with cryptococcal infection and probed with an anti-immunoglobulin G conjugate revealed recognition of the 115-kDa band, together with reactivity to bands at approximately 200 and 100 kDa (Fig. 3, lane 1). Normal human serum was unreactive to these bands (Fig. 3, lane 2). Excision of a gel slice at 110 to 120 kDa from a preparative gel followed by electroelution and SDS-PAGE on a minigel revealed a single band (Fig. 2, lane B) of purified antigen which was reactive to 7C9 by immunoblot development (Fig. 2, lane C). MA b 7C9 failed to show significant cross-reactivity to a range of concentrations of purified mannoprotein by Western blot (Fig. 2, lane E). Table 2 illustrates the steps in purification together with protein yields and reactivity to 7C9 in ELISA.

The reactivities of 14 individual human cryptococcal serum samples (Table 1) to the purified 115-kDa antigen are shown in Fig. 3. Samples from all four of the human immunodeficiency virus-positive (HIV⁺) individuals demonstrated recognition of the 115-kDa antigen. Those from the 10 HIV⁻ patients showed various intensities of recognition, and only one, with a latex agglutination titer of 1:10,000, was negative. Sera from normal human controls demonstrated no recognition of the 115-kDa antigen (data not shown). Reactivity of 7C9 to culture filtrate produced at 25, 30, and 37°C was up to 50% higher at 25°C than at 37°C in ELISA (data not shown).

Enzymatic deglycosylation and proteolysis of the 115-kDa

FIG. 2. SDS-PAGE and immunoblots of the 115-kDa antigen demonstrating purification of the antigen. Lanes: A, Rotofor fractions 1 to 3 pooled and subjected to SDS-PAGE, Coomassie blue stained; B, SDS-PAGE of the band cut from the preparative gel and electroeluted, Coomassie blue stained; C, immunoblot of MA b 7C9 against the 115-kDa antigen purified by Rotofor fractionation and electroelution of the band from preparative gel; D, immunoblot of 7C9 against a crude cytoplasmic extract of *C. neoformans* (isolate B4131; 2 µg of total protein); (E) immunoblot of 7C9 against purified mannoprotein (2 µg total in the lane).

FIG. 3. Reactivity of Western blots of the purified 115-kDa antigen to human cryptococcal sera and normal human sera. Lanes: 1, pooled immune human sera against Rotofor fractions 1 to 3; 2, pooled normal human sera against fractions 1 to 3; 3 to 20, individual human sera against the purified 115-kDa antigen.

antigen. None of the enzymatic deglycosylation agents were able to completely abolish the recognition of the 115-kDa antigen by MA b 7C9. However, *N*-peptide glycanase F caused significant reductions, both in the apparent molecular mass of the antigen recognized by 7C9 and in the total antibody reactivity, indicating the presence of N-linked oligosaccharides on the molecule, along with the contribution of such groups to the epitope recognized by 7C9 (Fig. 4, lane B). α -Mannosidase caused reduction in the molecular mass of the antigen recognized by 7C9, suggesting that α -mannose is present on the glycoprotein (Fig. 4, lane D). The other agents caused apparently minor reductions in molecular mass (Fig. 4).

Treatment of the 115-kDa antigen with both trypsin and papain followed by development with 7C9 in immunoblots resulted in the disappearance of any discernible bands (data not shown). This is a result either of epitope disruption or of the generation of protein fragments too small to appear on such blots.

Substrate SDS-PAGE. There was no indication of any zones of proteolysis in substrate gels containing either gelatin or casein in the area of the 115-kDa antigen (data not shown).

DISCUSSION

The fall in molecular mass of the 115-kDa antigen identified by MA b 7C9 on Western blots after treatment with α -mannosidase and *N*-peptide glycanase F clearly indicates the presence of substantial amounts of N-linked oligosaccharides (as *N*-acetylglucosaminyl asparagine) on the antigen. In this context the α -mannosidase activity is probably directed against high-mannose-type N-linked oligosaccharides. In contrast to the epitope recognized by MA b 3C2 on

TABLE 2. Steps in the purification of the 115-kDa antigen

Step	Vol	Total protein (µg)	7C9 ELISA reactivity
Culture filtrate	6 liters	62,000	0.34
Ultrafiltration/Centricon 30	20 ml	14,000	0.61
Rotofor	4.5 ml	1,132	1.4
Preparative SDS-PAGE	250 µl	11	>2.0

FIG. 4. Effect of various deglycosylation agents on the reactivity of 7C9 as determined by Western blot. Lanes: A, control; B, peptide glycanase F; C, neuraminidase; D, α -mannosidase; E, β -mannosidase; F, α -galactosidase; G, β -galactosidase; H, α -glucosidase.

the 34- to 38-kDa glycoprotein of *C. neoformans* (7), which is totally destroyed by treatment with *N*-peptide glycanase F, recognition of the 115-kDa antigen by 7C9 is apparently only partially abolished by treatment with this deglycosylation agent. This suggests that the contribution of N-linked oligosaccharides to the epitope recognized by 7C9 is more limited than in the case of 3C2. The relationship, if any, between these two newly described exoantigens remains unclear. The effect of proteolytic agents in removing band recognition confirms the 115-kDa antigen as a glycoprotein and, as such, clearly distinguishes this molecule from the previously described *C. neoformans* carbohydrate exoantigens GalXM and GXM (11).

The presence of mannose in the 115-kDa antigen is initially suggestive of some affinity with the previously described MP (17), as is the fact that Reiss et al. have found antibodies to MP in the sera of patients with cryptococcosis (14) in the same way in which we have detected a significant immune response to the purified 115-kDa antigen. However, two lines of evidence suggest that the two antigens differ significantly. First, MAb 7C9 is substantially unreactive to purified MP by Western blot over a range of concentrations. Second, MP has been shown by immunoelectron microscopy to be a nonstructural element of the cell wall (17), with limited occurrence within the cytoplasm. We have previously reported that immunofluorescence reactivity of 7C9, which obviously equates with the distribution of the 115-kDa glycoprotein, is restricted to the yeast cytoplasm, with no apparent staining of the cell wall (6). It is also of note that there are no available data on the precise molecular mass of the MP recognized by immune sera, in contrast to the Western blot data we have assembled.

Only one of the patient serum samples assayed by Western blot was negative. It had the highest latex agglutination titer (1:10,000) of any of the sera used, raising the possibility that the high circulating antigen load had absorbed all

available serum antibody. Interestingly, one patient whose latex agglutination test became negative after therapeutic intervention was still antibody positive, an observation which is consistent with the persistence of serum antibody after infection has been cleared.

The function of the 115-kDa glycoprotein remains unclear, although according to substrate gel SDS-PAGE analysis, which demonstrated an absence of clearance zones at the relevant molecular mass, it is almost certainly not a proteolytic enzyme. It is hoped that N-terminal amino acid sequence data from the purified glycoprotein will enable our group to ascertain the identity of the 115-kDa antigen on the basis of sequence homology and, indeed, to construct oligonucleotides to probe a *C. neoformans* genomic DNA library. Future work will also be directed toward purifying the other high-molecular-mass antigens which colocalized in the Rotofor fractions with the 115-kDa glycoprotein and were recognized by human sera, with a view to ascertaining their function and their relationship to MP.

ACKNOWLEDGMENTS

We thank Colin Campbell, Medical Mycology Reference Laboratory, PHLS Laboratory, Colindale, England, and K. J. Kwon Chung, National Institute of Allergy and Infectious Diseases, Bethesda, Md., for supplying various fungal isolates and serum samples. Special thanks to Varsha Vaishnav and R. Cherniak, Department of Chemistry, Georgia State University, Atlanta, for the gift of purified mannoprotein.

This research was funded by a grant from the Wellcome Trust.

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