JOURNAL OF BACTERIOLOGY, Dec. 1989, p. 6850–6852
0021-9193/89/126850-03$02.00/0

Vol. 171, No. 12

Localization of Mannoprotein in *Cryptococcus neoformans*

S. E. VARTIVARIAN,[1,2]†* G. H. REYES,[3] E. S. JACOBSON,[1,2] P. G. JAMES,[3] R. CHERNIAK,[3] V. R. MUMAW,[4] AND M. J. TINGLER[1,2]

Departments of Medicine[1] and Pathology,[4] Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298; Research Service (151), McGuire Veterans Administration Medical Center, Richmond, Virginia 23249[2]; and Department of Chemistry, Georgia State University, Atlanta, Georgia 30303[3]

Received 8 June 1989/Accepted 18 August 1989

Cell wall mannoprotein of nonpathogenic yeasts is surface exposed, since the cells are agglutinated by concanavalin A and antimannoprotein antibodies. However, nonencapsulated cells of *Cryptococcus neoformans* were agglutinated neither by concanavalin A nor by antimannoprotein antibodies. Immunogold electron microscopy located most mannoprotein in the inner cell wall. Chemical analysis of purified cell walls showed the lack of mannose, xylose, and galactose residues. These data indicate that cryptococcal mannoprotein recovered from the cultural supernatant is a nonstructural element of the cell wall.

Mannoprotein (MNP) is a major structural and antigenic component of the cell walls of *Saccharomyces* and *Kluyveromyces* spp. (1, 2). The surface location of cell wall MNP of these nonpathogenic yeasts is supported by immunochemical and agglutination data (1).

MNP can be recovered in small amounts from cultural supernatants of the pathogenic yeast *Cryptococcus neoformans* (6, 14). MNP is known to be immunogenic in cryptococcal infections, since Reiss et al. have found antibodies to MNP in the sera of patients with cryptococcosis (10). Murphy et al. have found that cryptococcal MNP elicits a significantly stronger delayed-type-hypersensitivity response in mice than capsular polysaccharide does (9). Although the function of MNP in the cryptococcal cell and its cellular location are not known, analogy with the above examples suggests that MNP may come from the outer surface of the cell wall. We report here a study of the distribution of MNP in *C. neoformans* as determined by lectin and antibody agglutination, immunogold electron microscopy, and chemical analysis of the cell wall. The present results do not support the *Saccharomyces* model.

C. neoformans Cap67 (an acapsular mutant derived from NIH B-3501) was grown in 5 ml of brain heart glucose broth overnight at 37°C with shaking. Cells were harvested, washed twice with saline, and suspended for 1 h in 0.1 ml of fluorescein-labeled concanavalin A (fl-ConA; catalog no. C-7642; Sigma Chemical Co., St. Louis, Mo.)(0.25 mg/ml of saline) with occasional shaking. The cells were then washed twice and suspended in saline. Labeling was determined visually with a Zeiss Standard 18 microscope with epifluorescence. Fl-ConA labeled the cell surface. However, when we tested for agglutination with unlabeled ConA (catalog no. C-7875; Sigma; 0.01 to 3,000 μg/ml with 1 mM $MnCl_2$ or $CaCl_2$) at various cell densities, no agglutination was detected. ConA-agarose beads (catalog no. C-6904; Sigma) suspended in sodium acetate buffer (0.01 M, pH 6.2) did not cause agglutination of cryptococcal cells. Agglutination of 2% fresh human type A erythrocytes and *Kluyveromyces lactis* cells was obtained in positive controls. Rabbit anti-MNP serum (9) also failed to agglutinate nonencapsulated yeast cells. Equal volumes of exponential-growth-phase cells and antiserum (1/1,000 dilution) were used. The lack of agglutination of cryptococcal cells suggested that MNP was not exposed on the cell surface.

The isolated cell wall and the soluble fraction were obtained as part of a concomitant study (R. Cherniak, P. G. James, R. G. Jones, and E. Reiss, submitted for publication). Glucose was the only neutral hexose detected in isolated, hydrolyzed cell walls, and it constituted 86% of the dry weight of the cell walls (Cherniak et al., submitted). The soluble fraction was obtained by centrifugation after the initial fracture of the *C. neoformans* cells. The supernatant was decanted, concentrated in vacuo below 40°C, dialyzed versus distilled water, and lyophilized. Thirty-eight grams (wet weight) of cells yielded 950 mg (dry weight) of sample. The constituent monosaccharides of the soluble fraction were identified and quantitated by gas-liquid chromatography following acid hydrolysis (5, 13). The four monosaccharides present were glucose and galactose (51%), mannose (10%), and xylose (3%). A separate quantitation of glucose and galactose was not possible because of the large amount of glucose present in the sample. The soluble fraction was applied to an affinity column of ConA–Sepharose CL-6B (6, 14). Sixty-two percent of the sample was not bound by the ConA column (galactoxylomannan). Ten percent was recovered by elution with methyl-α-D-mannopyranoside (14). The fraction eluted from the ConA column and an authentic sample of MNP formed an immunoprecipitation line of identity with rabbit anti-MNP serum by double diffusion in gel (9). Therefore, MNP and galactoxylomannan are probably not covalently bound to the cell wall.

Rabbit anti-MNP serum was prepared as previously described (9). The titer and specificity of anti-MNP serum were determined by an enzyme-linked immunosorbent assay. Microdilution plates were prepared by adding 0.1 ml of antigen (10 μg/ml of 0.06 M $NaCO_3$ [pH 9.6]) to each well. The plates were incubated for 3 h at 37°C and stored overnight at 4°C. The *C. neoformans* MNP and the purified mannan antigens from *Candida albicans* 20A, *C. albicans* 526B, and *Candida tropicalis* 83-4806 were used. (The purified mannans were a gift from E. Reiss, Centers for Disease Control, Atlanta, Ga.) The remainder of the enzyme-linked immunosorbent assay procedure was done as described previously (4). The titer of the anti-MNP serum in the assay was $60,000^{-1}$ versus MNP, while the titers versus the other

* Corresponding author.

† Present address: Section of Infectious Diseases, University of Texas M. D. Anderson Hospital and Tumor Institute, 1515 Holcombe Blvd., Houston, TX 77030.

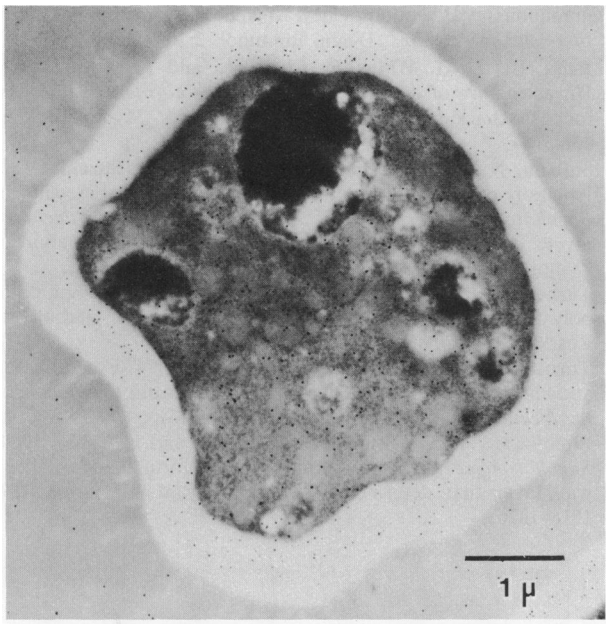

FIG. 1. Distribution of MNP in *C. neoformans* cell wall. Rabbit antibody to MNP was localized with gold-protein A.

mannans were all $1,000^{-1}$ or less. The titers of a control serum, rabbit anti-*C. albicans* A, were $8,000^{-1}$ versus the homologous mannan and 0 versus *C. neoformans* MNP.

The anti-MNP serum was used to determine the location of MNP by immune electron microscopy on sections of wild-type cryptococcal cells fixed in 2% glutaraldehyde in 0.1 M phosphate-buffered saline for 1 h. Cells were postfixed for 30 min in OsO_4, rinsed three times with phosphate-buffered saline, and dehydrated with ethanol. Propylene oxide was used as the intermediate fluid. Cells were embedded in 100% Polybed (Polysciences Inc., Warrington, Pa.) as the epoxy resin. 2,4,6-Tri(dimethylaminomethylphenol) was added 24 h later as the accelerator, with shaking for 3 to 5 h. Embedding of cells and polymerization of resin were allowed to proceed for another 48 h at 60°C. Thin sections were cut and placed on Formvar-coated nickel grids placed on sodium metaperiodate for 1 h. The grids were rinsed, blotted, and placed on a drop of 5% bovine serum albumin for 1 h. They were then incubated with rabbit anti-MNP serum (9) for 1 h, rinsed, and placed on a drop of protein-A-coated 10-nm-diameter colloidal gold (Janssen Pharmaceutica, Piscataway, N.J.) for 15 min. Sections were then rinsed gently with running tap water for 2 min, stained with uranyl acetate and lead citrate (LKB Instruments, Inc., Gaithersburg, Md.), and examined with a Philips EM 400 electron microscope. Negative controls included the use of normal rabbit serum or phosphate-buffered saline instead of antiserum. Omission of sodium metaperiodate or OsO_4 resulted in poorer labeling. Gold particles were seen in the cytoplasm and the cell wall (Fig. 1). The density of gold particles was four times greater in the inner two-thirds of the cell wall than in the outer third (Table 1), and little or no gold was apparent on the surface of the cell.

In *Candida* spp., the MNPs contribute to the architecture of the yeast cell and the mycelial cell walls (7). In *Saccharomyces* and *Candida* spp., there is evidence for covalent linkage or close aggregation of MNP with glucan of the cell wall surface (8, 12). In *Saccharomyces* spp., the wall MNPs

TABLE 1. MNP density in *C. neoformans* cell wall

Micrograph no.	No. of gold particles in cell wall		Ratio of particle densities in cell wall (inner 2/3:outer 1/3)[a]
	Inner 2/3	Outer 1/3	
11	225	38	2.96
862	158	40	1.98
863	551	58	4.75
4,293 (1)	96	14	3.43
4,293 (2)	90	6	7.5
4,293 (3)	91	9	5.06
4,294	271	37	3.66
4,296 (1)	152	54	1.41
4,296 (2)	148	17	4.35
4,297	195	15	6.5

[a] Average = 4.16.

form an external layer that serves as a permeation barrier that discriminates among dissolved polymers primarily on the basis of size (15). Our results suggest that the MNP of *C. neoformans* migrates slowly through the denser inner layer of the cell wall but that it migrates more rapidly and is released into the environment when it confronts the less dense outer layer of the wall. This view is consistent with the asymmetric distribution of MNP in the cell walls observed by immunogold electron microscopy (3, 11) (Fig. 1). The physical fracturing and fragmentation of *C. neoformans* cell walls enhanced the rate at which MNP was released into the soluble phase.

The fluorescent labeling of cryptococcal cells by fl-ConA remains puzzling. Glucans were probably the residues labeled; however, agglutination was not observed. The reason for this apparent discrepancy is not clear at this time. Hypothetically, if the nonreducing glucosyl residues are embedded deep in the matrix of the cell wall, then it is possible to obtain monovalent binding of the residue by ConA (hence labeling by fl-ConA) without obtaining the divalent binding necessary for agglutination caused by steric hindrance.

In *Saccharomyces* spp., MNPs are a heterogeneous group which includes enzymes such as invertase (2, 12). Again by analogy, MNP recovered from cryptococcal culture supernatants may not represent all antigenic types of MNPs, so that antibodies to extracellular MNP may not react with all cellular MNPs. However, the absence of mannose, xylose, and galactose in purified cell walls indicated that no major cell wall MNP escaped detection. It therefore seems likely that the extracellular MNP of *C. neoformans* represents soluble, nonstructural proteoglycan.

This work was supported by Public Health Service grant AI-21767 and the Veterans Administration.

LITERATURE CITED

1. **Ballou, C.** 1976. Structure and biosynthesis of the mannan component of the yeast envelope. Adv. Microb. Physiol. **14:** 93–158.
2. **Cabib, E., R. Roberts, and B. Bowers.** 1982. Synthesis of the yeast cell wall and its regulation. Annu. Rev. Biochem. **51:** 763–793.
3. **Cassone, A., N. Simonetti, and V. Strippoli.** 1974. Wall structure and bud formation in *C. neoformans*. Arch. Microbiol. **95:** 205–213.
4. **Cherniak, R., M. M. Cheeseman, G. H. Reyes, E. Reiss, and F. Todaro.** 1988. Enhanced binding of capsular polysaccharides of *Cryptococcus neoformans* to polystyrene microtitration plates

for enzyme-linked immunosorbent assay. Diagn. Immunol. **5:** 346–348.

5. **Cherniak, R., R. G. Jones, and E. Reiss.** 1988. Structure determination of *Cryptococcus neoformans* serotype A-variant glucuronoxylomannan by ^{13}C-n.m.r. spectroscopy. Carbohydr. Res. **172:**113–138.
6. **Cherniak, R., E. Reiss, and S. H. Turner.** 1982. A galactoxylomannan antigen of *Cryptococcus neoformans*. Carbohydr. Res. **103:**239–250.
7. **Elorza, M. V., A. Murgui, and R. Sentandreu.** 1985. Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell walls. J. Gen. Microbiol. **131:**2209–2216.
8. **Murgui, A., M. V. Elorza, and R. Sentandreu.** 1985. Effect of papulacandin B and calcofluor white on the incorporation of mannoproteins in the wall of *Candida albicans* blastospores. Biochim. Biophys. Acta **841:**215–222.
9. **Murphy, J. W., R. L. Mosley, R. Cherniak, G. H. Reyes, T. R. Kozel, and E. Reiss.** 1988. Serological, electrophoretic, and biological properties of *Cryptococcus neoformans* antigens. Infect. Immun. **56:**424–431.
10. **Reiss, E., R. Cherniak, R. Eby, and L. Kaufman.** 1984. Enzyme immunoassay detection of IgM to galactoxylomannan of *Cryptococcus neoformans*. Diagn. Immunol. **2:**109–115.
11. **Reiss, E., E. H. White, R. Cherniak, and J. E. Dix.** 1986. Ultrastructure of acapsular mutant *C. neoformans* Cap67 and monosaccharide composition of cell extracts. Mycopathologia **93:**45–54.
12. **Shibata, N., K. Mizugami, K. Takano, and S. Suzuki.** 1983. Isolation of mannan-protein complexes from viable cells of *Saccharomyces cerevisiae* X2180-1A wild type and *Saccharomyces cerevisiae* X2180-1A-5 mutant strains by the action of Zymolyase-60,000. J. Bacteriol. **156:**552–558.
13. **Turner, S. H., and R. Cherniak.** 1981. Total characterization of polysaccharides by gas-liquid chromatography. Carbohydr. Res. **95:**137–144.
14. **Turner, S. H., R. Cherniak, and E. Reiss.** 1984. Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. Carbohydr. Res. **135:**343–349.
15. **Zlotnik, H., M. P. Fernandez, B. Bowers, and E. Cabib.** 1984. *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. J. Bacteriol. **159:** 1018–1026.