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Cryptococcus neoformans Capsular Polysaccharide and Exopolysacchari Fractions Manifest Physical, Chemical, and Antigenic Differences▽

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ABSTRACT

The human pathogenic fungus *Cryptococcus neoformans* has a large polysaccharide (PS) capsule a copious amounts of PS into cultures and infected tissues. The capsular PS is a major virulence fact elicit protective antibody responses. PS recovered from culture supernatants has historically provid and convenient source of material for structural and immunological studies. Two major assumptio studies are that the structural features of the exopolysaccharide material faithfully mirror those of c that the isolation methods do not change PS properties. However, a comparison of exopolysacchar two isolation techniques with capsular PS stripped from cells with gamma radiation or dimethyl su revealed significant differences in glycosyl composition, mass, size, charge, viscosity, circular-dicl and reactivity with monoclonal antibodies. Our results strongly suggest that exopolysaccharides an are structurally different. A noteworthy finding was that PS made by cetyltrimethylammonium bro precipitation had a larger mass and a different conformation than PS isolated by concentration and suggesting that the method most commonly used to purify glucuronoxylomannan alters the PS. He method used to isolate PS can significantly influence the structural and antigenic properties of the findings have important implications for current views of the relationship between capsular PS and exopolysaccharides, for the generation of PS preparations suitable for immunological studies, and formulation of PS-based vaccines for the prevention of cryptococcosis.

Cryptococcus neoformans is an encapsulated fungus that is the causative agent of cryptococcosis, a threatening disease, particularly in situations of compromised immunity. The cryptococcal capsule structure that is considered the key virulence factor for this pathogen (8). The capsule is composed

polysaccharides (PSs), galactoxylomannan (GalXM) and glucuronoxylomannan (GXM). GalXM i galactan with branches of β(1,3)-galactose-α(1,4)-mannose-α(1,3)-mannose. Xylose units can be a branched mannose through β(1,3) or β(1,2) linkages (22). GalXM has an average mass of 100 kDa deleterious effects on immunological function (11, 12, 18). GXM is a high-molecular-mass PS wit structure. The weight-averaged mass (M_w) is 1,700 to 7,000 kDa (12), which makes up about 90% mass. Structurally, GXM consists of a linear α-(1,3)-mannan main chain with β(1,2)-glucuronic ac attached to every third mannose, on average. Mannosyl residues can also be 6-*O* acetylated and sul xylosyl units in β(1,2) or β(1,4) linkages (11). GXM and GalXM are released into culture medium cells as exopolysaccharides that can be recovered in sufficient quantities for physical and chemical

Although the biological and structural properties of GXM have been extensively studied, its physic remain relatively unexplored. Given that GXM is a macromolecule and that capsular assembly inv noncovalent attachment of PS fibrils to the cell wall (6, 19), it is likely that many properties of the directly related to physicochemical properties of the PS molecules. For example, there is evidence assembly is at least partly the result of inherent PS properties that promote self-assembly (12). GX to contribute to virulence by interfering with the host immune response by multiple mechanisms (1 almost certainly related to intrinsic PS structural properties. Some antibodies to GXM are protectiv can provide important components for a vaccine against cryptococcosis (5). Despite the extensive out with GXM, it is noteworthy that practically all of our information about *C. neoformans* capsul from studies of exopolysaccharide components released from cells and recovered from culture sup However, a correspondence of identity between the structures of capsular PS and exopolysacchari assumed without experimental verification.

In the present study, we report that different methods of purifying extracellular PS from strain 240 *neoformans* grown under the same conditions yield PS preparations with different physical, chemi serological properties. Comparison of soluble PS with PS directly released from the surface of *C. n* gamma radiation or dimethyl sulfoxide (DMSO) treatment revealed significant differences from exopolysaccharide material. The characterization of the physical chemical properties of cell-associ extracellular PS provides new insight into the relationship between exopolysaccharides and capsul

MATERIALS AND METHODS

***C. neoformans* cultures.** *C. neoformans* strain ATCC 24067 was grown in a minimal medium con glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μ Fungal cells were cultivated for 7 days at 30°C.

Isolation of PS from culture supernatants by cetyltrimethylammonium bromide precipitation (CT Extracellular PS (exopolysaccharide) was isolated as described by Cherniak et al. (4), with the min modifications proposed by Mc Fadden et al. (12). Briefly, supernatants were obtained by centrifug cultures and filtered through 0.45-μm-pore-size filters to remove remaining yeast cells and cell deb was then isolated from supernatants by addition of sodium acetate (10% [wt/vol], final concentrati solution pH was immediately adjusted to 7.0 with acetic acid to avoid destruction of acetyl groups. volumes of 100% ethanol was added to precipitate the PS. The PS precipitate was separated from t by centrifugation, and the pellet was then dissolved in water. The carbohydrate content was determ phenol sulfuric acid method (12). The PS solution was then adjusted with NaCl (0.2 M, final conce PS was selectively precipitated by adding 3 g of CTAB (Sigma, St. Louis, MO) per g of carbohydr

-PS complex, recovered by centrifugation, was then dissolved in 1 M NaCl and precipitated again
of 2.5 volumes of 95% ethanol. In this step, the addition of ethanol will precipitate the PS while m
CTAB remains in the NaCl solution. After centrifugation, the supernatant was discarded and the P
dissolved in 2 M NaCl, dialyzed extensively against 1 M NaCl to remove the CTAB, and then dial
distilled water for 2 days. The PS solution was then lyophilized, and the mass was determined in a
balance. Samples obtained by CTAB precipitation are referred to as CTAB-PS. The effects of CTA
also evaluated by addition of 0.1% CTAB solution to a 1-mg/ml solution of F-PS, GR-PS, and DM
samples will be referred to CTAB-F-PS, CTAB-GR-PS, and CTAB-DMSO-PS.

Isolation of PS from culture supernatants by filtration. Based on the ability of PS to self-aggregat
isolated by filtration and ultrafiltration as described recently (17). Briefly, *C. neoformans* cells wer
from culture supernatants by centrifugation and the resulting supernatant was concentrated approxi
with an Amicon (Millipore, Danvers, MA) ultrafiltration cell (cutoff of 100 kDa, total capacity of
stirring and Biomax polyethersulfone ultrafiltration discs (76 mm; Millipore, Danvers, MA). After
viscous film over the filtering disc, the fluid phase was discarded and the remaining jellified mater
recovered with a cell scraper. The final PS solution was lyophilized, and the dry PS mass was dete
Samples obtained by filtration were named F-PS.

Release of capsular components by gamma radiation. Capsular PS was isolated as described by
(10). Yeast cells were washed three times in Milli-Q water to remove shed capsular PS and suspen
water. The cells were then radiated with a Shepherd Mark I irradiator (JL Shepherd and Associates
Fernando, CA) at a dose rate of 1,388 rads/min for 60 min. Irradiated cells were removed by centri
the soluble phase was collected for lyophilization. PS fractions obtained from the cell surface of *C.*
by irradiation were named capsular GR-PS. The effects of gamma radiation on cell-free PS solutio
evaluated by irradiating PS fractions under the conditions described above. These samples were na
PS and R-F-PS.

Release of capsular components by DMSO. Capsular PS was isolated as described by Bryan et al.
cells were washed three times in Milli-Q water. The cells were suspended in 15 ml of DMSO and i
30 min twice. Cells were removed by centrifugation, and the supernatant was then dialyzed against
h, with water replacement with fresh water at 2-h intervals, and then extensively dialyzed against v
days. PS fractions obtained from the cell surface of *C. neoformans* by DMSO were named capsula
The effects of DMSO on PS were also evaluated by addition of DMSO to CTAB-PS, F-PS, and GI
samples are referred to as DMSO-CTAB-PS, DMSO-F-PS, and DMSO-GR-PS.

Effect of GalXM on PS. F- and R-F-PS solution was mixed with purified GalXM at a ratio 80:20 (v
parameters were measured as described below.

Glycosyl composition of PS-containing fractions. PS fractions were dissolved in methanol-1 M H
incubated at 80°C for 18 h. Methanolyzed samples were then per-*O*-trimethylsilylated by treatmen
(Pierce) for 30 min at 80°C. The per-*O*-trimethylsilylated derivatives were analyzed by gas chroma
coupled to mass spectrometry. The derivatized structures were first separated on an HP 5890 gas c
with a Supelco DB-1 fused silica capillary column (30 m by 0.25 mm [inside diameter]). Peaks det
were fragmented in a 5970 MSD mass spectrometer interfaced with the gas chromatograph. Carbo
standards included arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, manno
glucose, mannitol, dulcitol, and sorbitol. The average and standard deviation of three different extr
calculated.

Nuclear magnetic resonance (NMR) spectroscopy of capsular PS. The sample was partially depo probe sonication for 30 min at 0°C and de-*O*-acetylated by adjusting the solution to pH 11 with co ammonium hydroxide, followed by incubation at 25°C for 20 h. After lyophilization, the sample w exchanged by lyophilization from D_2O (99.9% D), dissolved in 0.28 ml D_2O (99.96% D), filtered transferred to an NMR tube with susceptibility plugs (Shigemi Inc., Allison Park, PA). A 1D proto spectrum was acquired on a Varian Inova 600-MHz spectrometer at 343 K (70°C). The spectral wi Hz, the acquisition time was 2.05 s, and 256 scans were collected. The spectrum was processed wi NMR software. Linear prediction to 16K and a Gaussian function (2-Hz line broadening) were app flame ionization detector to obtain a full spectrum. Linear prediction to 16K, a 0.65°-shifted sineb and a Gaussian function (2-Hz line broadening) were applied to obtain an enhanced-resolution part the region displaying the mannose anomeric protons. Chemical shifts were measured relative to int dimethyl-2-silapentane-5-sulfonic acid (δ = 0.00 ppm). Spectra were analyzed to identify the struct groups described by Cherniak et al. (4).

Molecular mass determination. Molecular masses were calculated by multiangle laser light scatte described by McFadden et al. (12). PS solutions were prepared with sterile-filtered, degassed, ultra samples were passed through an in-line 0.8-μm syringe filter to eliminate large aggregates and redu sources of refracted or scattered light. Differential refractometry with a 620-nm laser source (BI-D Brookhaven Instruments Corp., Holtsville, NY) was used to measure alterations in the refractive in the PS samples. Molecular masses were determined at 25°C by multiangle laser light scattering in weight analyzer (BI-MwA; Brookhaven Instruments Corp., Holtsville, NY) with a 675-nm laser so was used for system calibration, and 20-nm microspheres were used for normalization of Rayleigh (Duke Scientific Corp., Palo Alto, CA). The M_w was finally calculated by the Zimm equation, $Kc/$ $[P(\theta)] + 2\,A_2c$, where K is the optical constant, defined as the quotient of $4\pi^2 n_o^2 (dn/dc)^2)/N_A\lambda^4_o$, an excess Rayleigh factor, determined by comparing the sample and solvent values at angle θ and con (θ) represents the particle-scattering function, A_2 is the second virial coefficient, n_o is the refractive solvent, N_A equals Avogadro's number, and λ_o is the modal wavelength of the laser source. The $dn/$ adjusted according to the Cauchy equation ($dn/dc = A + B/\lambda^2$) by using a B coefficient for aqueous +0.0022 μm and wavelengths (λ) in micrometers to accommodate for the longer wavelength of the weight analyzer. Depolarization corrections were assumed to be negligible.

Zeta potential measurements. The zeta potential (ζ), particle mobility, and shift frequency of PS s calculated in a zeta potential analyzer (ZetaPlus; Brookhaven Instruments Corp., Holtsville, NY). ζ measurement of charge (in millivolts) defined as the potential gradient that develops across the inte a boundary liquid in contact with a solid and the mobile diffuse layer in the body of the liquid. It is the equation $\zeta = (4\pi\eta m)/D$, where D is the dielectric constant of the medium, η is the viscosity, and electrophoretic mobility of the particle.

Viscosity determinations. Brownian motion of polystyrene spheres (radius, 1.52 ± 0.05 μm) imme solutions was used to determine viscosity. A suspension of spheres (1 μl, 10% [vol/vol]; Sigma, St was diluted in 150 μl of dialyzed GXM (1 mg/ml) to a final concentration of 10^{-4}% (vol/vol), trans coverslip with an O ring 1 cm in diameter and 0.3 cm in width, and then sealed with a second cove were examined with an inverted Nikon Eclipse TE300 microscope connected by its epifluorescenc -YAG laser beam source (1,064-nm wavelength). When the laser beam passes through the objectiv optical trap near the objective focus, allowing the manipulation of small dielectric objects (~μm le samples were observed with a Plan Apo 100× NA1.4 objective. Digitized images were obtained by

coupled device camera connected to a Hamamatsu Argus and a Scion frame grabber. Images were
the ImageJ software (NIH, http://rsb.info.nih.gov/ij/). After positioning the bead at the desired heig
distance of the polystyrene sphere center to the glass coverslip), a small image area (50 by 50 pixel
for a frame capture rate of 27 to 28 frames/s. The optical tweezers were then alternately turned on
periods of circa 0.5 s by shutting the beam off.

The mean square displacement of the variation of the image center of mass position δρ in a time in
by the equation

$$<(\delta\rho)^2>=4Dt$$

where

$$D=\frac{k_BT}{\beta}$$

and β, the Stokes friction coefficient, is a function of the bead radius, *a*, and height, *h*. The height,
as the distance from the sphere center to the coverslip. β is given by the Faxen law as follows:

$$\beta=6\pi\eta a\left[1-\frac{9}{16}\left(\frac{a}{h}\right)+\frac{1}{8}\left(\frac{a}{h}\right)^3-\frac{45}{256}\left(\frac{a}{h}\right)^4-\frac{1}{16}\left(\frac{a}{h}\right)^5\right]^{-1}$$

For a fixed value of *h*, $<(\delta\rho)^2>$ was calculated by excluding the regions where δρ is <0.01 μm. Tho
correspond to the time intervals for which the bead was trapped. From the linear fit of the measure
values as a function of time, *t*, in equation 1, the diffusion coefficient, *D*, was obtained. With the v
Stokes friction coefficient, β, was determined by using equation 2. The sphere height was then cha
procedure was repeated. From the fit of the β values as a function of sphere height, *h*, by using equ
viscosity values were obtained. The method described had 0 to 20% uncertainty in viscosity detern

Circular-dichroism (CD) analysis of PS fractions. CD measurements of the different PS samples v
on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) with a 2-mm cell at 25°C. In all case
contained 3 mM PS dissolved in ultrapurified water. The CD spectra were recorded in the wavelen
185 to 250 nm and were expressed as molar ellipticity ([θ]), which is corrected for concentration u
cm^2/dmol.

Binding of monoclonal antibodies (MAbs) to PS in polystyrene plates. Binding of MAbs to the d
fractions was evaluated by enzyme-linked immunosorbent assay (ELISA) (3). Briefly, polystyrene
coated with 1 nM PS samples (with molecular masses determined in this study). The plates were b
phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Anti-PS MAbs 18B7 (imm
G1 [IgG1]), 2D10 (IgM), 12A1 (IgM), and 21D2 (IgM) were then added to the plates, followed by
antibody binding to PS with alkaline phosphatase-labeled secondary antibodies. The MAbs bind to
epitopes in PS. Colored reactions were developed after the addition of *p*-nitrophenyl phosphate (*p*-
(Sigma, St. Louis, MO), and quantitative determinations were performed by absorbance measurem
All incubations were carried out at 37°C for 1 h.

Binding of MAbs to PS by dot blot analysis. PS samples (1 nM) were placed on nitrocellulose me membranes were dried for 1 h at 37°C and then blocked with PBS containing 1% bovine serum alb Membranes were incubated with the antibodies described above at 10 μg/ml. After extensive wash membranes were incubated with alkaline phosphatase-conjugated antibodies and *p*-NPP solutions. were quantified by transfer of the soluble colored products to 96-well plates and reading at 405 nm

Binding of MAbs to PS by capture ELISA. Microtiter plates were coated with 50 μl of unlabeled a antibody at a concentration of 1 μg/ml in PBS and incubated for 1 h at 37°C. Afterward, the plates and the antibodies described above was added at 1 μg/ml. The plates were then incubated for 1 h a plates than were washed, and 1 nM PS was added. After 1 h of incubation at 37°C, MAb 18B7 wa wells at 1 μg/ml. After extensive washing, plates were incubated with alkaline phosphatase-conjug and *p*-NPP solutions. Reactions were quantified at 405 nm.

Statistical analysis. Statistical analyses were carried out with Bi-ZPMwA Zimm Plot Software (B Instruments Corp., Holtsville, NY) for M_w, *Rg*, and A_2. 90Plus/BI-MAS software was used for co effective-diameter, polydispersity, and diffusion coefficient values (Brookhaven Instruments Corp. NY). Zeta Plus software was used for comparisons of zeta potential, mobility, and frequency shift (Brookhaven Instruments Corp., Holtsville, NY).

RESULTS

Glycosyl composition analysis. PS samples were subjected to acidic methanolysis, and their mon constituents were analyzed by GC. This analysis allows the potential detection of four different fo sugar derivative, corresponding to the α and β forms of furanose and pyranose rings. In associatio spectrometry analysis, each peak can be identified precisely on the basis of the fragmentation profi Fragmentation of per-*O*-trimethylsilylated derivatives of hexoses usually generates diagnostic pea and 214. The (*m*/*z* 204)/(*m*/*z* 217) ratio of pyranose rings is >1, whereas that of furanose rings is <1 retention times corresponding to standard derivatives of the typical cryptococcal PS components m xylose (Xyl), and glucuronic acid (GlcA) were detected in the hydrolysates (Table 1). For CTAB a was the major monosaccharide constituent, followed by Xyl and GlcA. Different proportions of m constituents were observed in GR-PS and DMSO-PS, which presented, respectively, Man-Xyl-Glc molar ratios of 41.77:8.60:7.90 and 21.00:5.70:3.57, respectively. Galactose and glucose were the other than Man, GlcA, and Xyl detected in the samples. The average molar ratio of Gal was 0.80% 1.23% for F-PS, 0.37% for the GR-PS, and 0.53% for the DMSO-PS. The presence of galactose pr the presence of GalXM, another exocellular capsular PS of *C. neoformans*. A large amount of gluc measured in GR-PS and DMSO-PS, which may reflect release of glucans from the *C. neoformans* glucans make up >80% of the cell wall PSs (9). To investigate the origin of the glucose, the acapsu Cap67 cells were extracted with DMSO. The results showed an average molar ratio of 4.6:32.6:4.3 Xyl, Man, Gal, Glu, and *N*-acetylglucosamine, respectively, a finding suggesting a cell wall or cell for the glucose found in DMSO-PS. DMSO-PS also contained fatty acids that are presumably solu organic solvent.

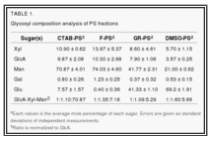

TABLE 1.

Glycosyl composition analysis of PS fractions

NMR spectroscopy of capsular PS. Based on previous reports on the structure of extracellular GX ATCC 24067 (1, 4), structural reporter groups and branches have been identified confirming that tl characterized molecule represents serotype D PS motif 1. Given the glycosyl heterogeneity of the l we studied the DMSO-PS (capsular PS) by NMR spectroscopy to relate our results to prior studies studies, the CTAB-PS fraction of strain 24067 was shown to be overwhelmingly composed of stru However, we recently described a new reporter group in the F-PS fraction of this strain (17). NMR DMSO-PS revealed the expected motif 1 GXM spectrum (data not shown).

Average molecular mass, radius of gyration, and second virial coefficients of PS. Multiangle las scattering was used to determine the average molecular mass (M_w) of PS from the different prepara masses of the different PS preparations were derived from Zimm plots of light-scattering data (Tab largest mass of PS was that of the DMSO-PS, followed by CTAB-PS, F-PS, and GR-PS. The radiu (*Rg*) and the second virial coefficient (A_2) were also calculated from the light-scattering data (Tabl the average distance from the center point of the PS to the outer edge of the molecule. The *Rg* mea the different PS samples manifested substantial differences. The *Rg* was used in conjunction with t calculate the mass density of each PS sample. This analysis revealed that the DMSO-PS was 412-f the PS obtained by filtration or released by gamma radiation. PS-CTAB was approximately 12-fol - and GR-PS. Overall, the results showed that F-PS and GR-PS were more similar to each other tha and CTAB-PS, with the caveat that each preparation manifested significant differences in one or m variables studied.

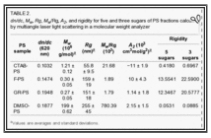

TABLE 2.

dn/dc, M_w, *Rg*, M_w/Rg, A_2, and rigidity for five and three sugars of PS fractions calculated by multiangle laser light scattering in a molecular weight analyzer

The rigidity of PS preparations was calculated as described by Sist et al. (21) from the M_w and *Rg* equation Rg^2/Nw, where *Nw* is the average mass of sugar residues in the PS, was applied to the dat sample. Rigidity was calculated on the basis of the five sugar residues (three Man, one GlcA, and motif 1 (M1) of the serotype D PS described by Cherniak et al. and confirmed in this study by NM corresponding to that of strain 24067 and based on only the three mannoses in the backbone (Table Considering only the mannose backbone, the pattern of rigidity followed that calculated for the co

Zeta potential, mobility, and frequency shift. Zeta potential determinations of PS preparations (Ta that the most negative values were obtained for DMSO-PS, followed by CTAB-PS, F-PS, and GR- negativity of mobility values and frequency shift of each sample followed the same pattern (Table

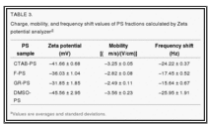

TABLE 3.

Charge, mobility, and frequency shift values of PS fractions calculated by Zeta potential analyzer[a]

Effect of CTAB on PS. To evaluate the effect of CTAB on the PS, F-PS, GR-PS, and DMSO-PS, tl preparations were incubated with a solution of 0.1% CTAB and the various physical parameters w After treatment with CTAB, the M_w of F-PS increased from 3×10^5 g/mol to 9×10^5 g/mol and the reduced by 1.6-fold, a phenomenon that may reflect aggregation or cross-linking of PS molecules (

GR-PS, addition of CTAB resulted in the M_w increasing from 2.7×10^5 to 1.1×10^6g/mol while th decreased approximately 61% (Fig. 1). For DMSO-PS, addition of CTAB resulted in an apparent i from 1.99×10^8 to 8×10^8g/mol, with the value of *Rg* decreasing approximately 47%. Values of ze mobility, and frequency shift for each preparation were more negative after addition of CTAB (Fig

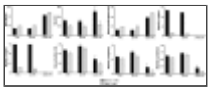

FIG. 1.
Effects of CTAB on physical and chemical parameters. Black bars represent PS fraction before CTAB addition. Gray bars represents PS fractions after CTAB addition.

Effect of gamma radiation on PS. Taking into consideration that some of the differences measured relative to the other PS fractions reflected radiation effects on PS, the CTAB- and F-PS preparation irradiated for 60 min and the physical parameters described above were determined. The M_w of CT increased from 1.2×10^6 g/mol to 3.2×10^8 g/mol after gamma irradiation, possibly by cross-linki Values for zeta potential, mobility, and frequency shift were less negative for both samples after ra 2).

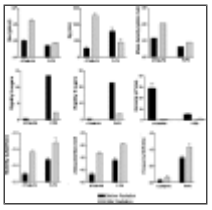

FIG. 2.
Effects of gamma radiation on physical and chemical parameters. Black bars represent PS fraction before radiation treatment. Gray bars represents PS fractions after radiation treatment.

Effect of DMSO on PS. To evaluate the effect of DMSO molecules on the PS, CTAB-PS, F-PS, an incubated with DMSO as described before and all of the physical parameters described above were and no significant differences were observed (Table 4).

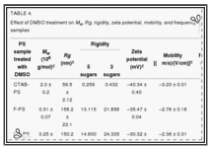

TABLE 4.
Effect of DMSO treatment on M_w, *Rg*, rigidity, zeta potential, mobility, and frequency shift of PS samples

Effect of GalXM on PS. To study the effect of GalXM on the PS, we added GalXM solution to F-P The results showed that addition of GalXM to F-PS and R-F-PS modified the physical properties o showing aggregation of PS fibers (Tables 5 and 6).

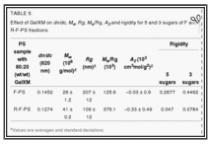

TABLE 5.
Effect of GalXM on *dn/dc*, M_w, *Rg*, M_w/Rg, A_2,and rigidity for 5 and 3 sugars of F and R-F-PS fractions

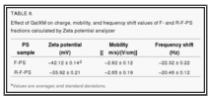

TABLE 6.
Effect of GalXM on charge, mobility, and frequency shift values of F- and R-F-PS fractions calculated by Zeta potential analyzer

CD of PS.

The secondary structure of PS was studied by CD spectroscopy (Fig. 3). The CD spectrum was dev bands, except for the far-UV region, consistent with the absence of chromophores in PS. Interestin demonstrated a large molar ellipticity band centered at 187 nm (Fig. 3A). Comparison of the CD sp irradiated and nonirradiated PS revealed radiation-induced changes consistent with secondary-struc (Fig. 3B). The effect in the CD spectra with CTAB and non-CTAB PS treatment revealed conform changes (Fig. 3C).

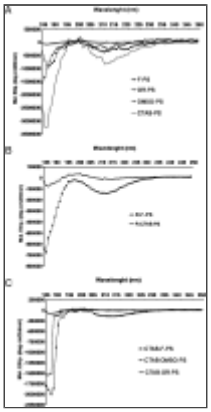

FIG. 3.

CD spectra of PS fraction before (A) and after (B) radiation and after CTAB treatment (C). The *x* axis represents the wavelength scanned. The *y* axis shows molar ellipticity (Mol. Ellip.) values expressed in deg cm^2/dmol.

Reactivity with MAbs. We evaluated the epitope contents of the various PS fractions by reactivity MAbs that bind different epitopes. Three different assays were used, i.e., ELISA with polystyrene- dot blot analysis with membrane-immobilized PS, and capture ELISA. The rationale for employing serological assays is that antibody reactivity for PS varies with the method used (7). Irrespective of used, we noted significant differences in the binding of MAb to PS fractions isolated by the variou 4).

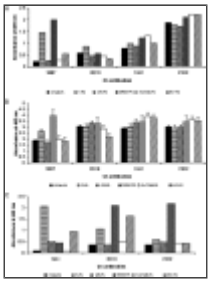

FIG. 4.

Binding of MAbs to PS fractions in polystyrene plates (A), dot blot analysis (B), and capture ELISA (C). The *x* axis represents different MAbs. The *y* axis represents absorbance at 405 nm. Cn, *Cryptococcus neoformans*.

DISCUSSION

Given the importance of the capsular PS in the pathogenesis of cryptococcal infections, it is not sur has been the subject of extensive studies. However, practically all structural and immunological stu utilized the soluble PS (exopolysaccharide) that accumulates in culture supernatants during in vitro this material is available in milligram quantities and is easily amenable to purification. However, th of many studies have been predicated on two assumptions that have not been validated experiment is generally thought that exopolysaccharide is derived from capsular PS and, consequently, that thi mirrors capsular PS with respect to structure and biological properties. However, our results showi differences in the physical and chemical properties of PS isolated from supernatant and capsule rai questions about the validity of this assumption. Secondly, it has been assumed that the methodolog purification delivers a PS product in its native state. Strong evidence against the second assumption by a recent study showing that exopolysaccharide isolated by concentrating and filtering culture su

significantly smaller than CTAB-purified PS from *C. neoformans* (17). Consequently, we have sys analyzed the physical, chemical, and antigenic properties of PS prepared by four different techniqu from the supernatants and the capsule.

Exopolysaccharide was isolated from culture supernatants by precipitation with ethanol and CTAE detergent, a standard protocol described in the literature (4), or by the recently described ultrafiltra (17). Sugar composition analysis showed Man, Xyl, and GlcA in all of the samples studied, consis proposed structures of cryptococcal PS. Gal was found in small amounts in all of the samples, and originated from the presence of GalXM since we analyzed the complete PS. Based on a previous r determining the molecular ratio of monosaccharides in this PS (22), we calculated the relative amo in each of the samples used in this study. The amount of GalXM in the capsular PS fractions was a (Table 1), since the galactose-containing PS is one of the components of the cryptococcal capsule. of small amounts of GalXM in exopolysaccharide fractions may indicate that these PSs can form n aggregates. To investigate this possibility, we added purified GalXM to PS at a PS-GalXM ratio of and noted a marked increase in molecular mass, suggesting that GalXM binds to PS or that it prom aggregation. Hence, the small amounts of GalXM in PS preparations could reflect coprecipitation heteropolymers, and the generation of purer PS preparations may require the use of GalXM-deficie strains (14). The relatively small amount of GalXM in the four samples indicates that the contribut to the average measurements presented in this study is small, with the caveat that it may contribute in apparent molecular mass if, indeed, it promotes PS aggregation. However, these results also indi assigning specific biological functions to PS preparations should be done cautiously, since GalXM immunomodulator (18) and even small amounts of GalXM in PS preparations may produce confou

Even considering the presence of GalXM in the PS fractions, the percent composition of each suga between samples. In all of the samples, the mole percentage of Man was higher than the mole perc This result is supported by a previous study (4) which showed only the presence of PS motif M1 (M Xyl) in the PS isolated from strain 24067. CTAB precipitation and filtration may yield qualitativel preparations than capsular PS from differences in sugar concentration and PS molecular mass (see PS isolated from the *C. neoformans* capsule by gamma radiation and DMSO extraction, the molar monosaccharides was similar but differed from that of the capsular PS. We measured large amount the two fractions of PS isolated from cells, namely, the DMSO-PS and GR-PS fractions. Glucose i component of GXM, and the capsule is not thought to contain glucans even though the attachment cell wall is thought to involve GXM-glucan interactions (19). The finding of glucose in capsular PS implies either the presence of glucan moieties in the capsule or the release of glucans from the cell DMSO and gamma radiation. Evidence for non-PS components in the capsule is provided by the o it contains chitin-like components (20). Nevertheless, our finding of a significant amount of glucos PS in DMSO-extracted material from acapsular cap67 cells strongly suggests that the glucose in D derived from PS of cell wall or cell body origin. Glycosyl analysis of DMSO-PS material fractiona exclusion chromatography revealed that the larger molecules with higher molecular weights that c mannose and galactose, but no glucose, are almost certainly PS from the capsule and not glucans fi wall. In contrast, glucose was found in the lower-molecular-weight fractions, where galactose was the basis of these results, we identify the large fibrils found in the DMSO-PS as GXM combined w

The CTAB method widely used for GXM purification is plagued by the difficulty in removing this the PS preparation. The usual method of removing CTAB from GXM is prolonged dialysis against water, but there is no good way to establish whether all of the cationic detergent is removed by dia

finding that CTAB-purified PS had a mass that was about 10-fold greater than that of F-PS implies changes were induced by detergent binding, possibly in the form of PS aggregation. In this regard, to promote the aggregation of PS molecules and these may remain in an aggregated state even afte presumably removed. CD spectral analysis revealed larger molar ellipticity signals in the far-UV re CTAB-PS than F-PS, indicating that CTAB purification induced a conformational change in PS.

Analysis of PS fractions by several physical techniques revealed additional differences in the fracti Measurements of molecular weight, radius of gyration, mass density, second virial coefficient, rigi zeta potential, mobility, and frequency shift each indicated that significant differences for PS were the different methods described here. In fact, our results demonstrate that radiation also has an imp properties of PS, since clear differences in its physical chemical features were induced after treatm gamma radiation. Gamma radiation generates highly reactive OH and H radicals through the radiol which may promote the removal of parts of the PS capsule by a chemical reaction that breaks the p smaller fragments and/or disrupts noncovalent interactions holding the capsule together (2). Smalle generated via free-radical attack may also exist as free radicals for a period of time sufficient to rea PS molecules, resulting in a cross-linking effect (2). Taken together, these results indicate that diffe effects of PS could be expected with different PS preparations. In addition, different physical chem could result, among other effects, in different profiles of receptor and antibody binding. To address we compared the reactivities of different MAbs to PS against the PS preparations analyzed in this s different serological methods. Irrespective of the method used, significant differences were noted i of PS, indicating that the structural differences inferred from the various physical and chemical ana translated into antigenic differences.

The finding of antigenic differences between CTAB- and F-PS has important implications for vacc this regard, it is noteworthy that two PS vaccines have been studied, each eliciting very different ty antibody responses. An earlier vaccine made from exopolysaccharide isolated from culture supern alcohol precipitation) was not protective despite eliciting high antibody titers. In contrast, a conjug made with CTAB-purified PS was protective in mice (6). Given our findings that F- and CTAB-PS significant physical, chemical, and antigenic differences, it is intriguing to consider that CTAB pur alter the PS to enhance its potential usefulness as a vaccine antigen. The different profiles of antibo each of the samples confirmed that the particular structural features may have an impact on PS bio properties. Although the MAbs used in this study were generated from mice immunized with CTA they bound more intensively to F-PS. This observation may be explained by differential epitope ac the filtered fraction. Radiation of PS increased antibody binding, supporting the hypothesis that epi in PS may vary according to parameters such as the rigidity, electronegativity, and diameter of the These results could be related to the well-known differences observed in the pattern of antibody bi *neoformans* cells (15, 16).

In summary, our results reveal significant differences between capsular PS and exopolysaccharide. chemical, and antigenic properties of PS varied with the purification method used, and this observa major questions about the relationship between the structures of native and purified PS and the rele and immunomodulatory activities in vivo.

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FOOTNOTES

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REFERENCES

1. Bacon, B. E., R. Cherniak, K. J. Kwon-Chung, and E. S. Jacobson. 1996. Structure of the O-dea
glucuronoxylomannan from Cryptococcus neoformans Cap70 as determined by 2D NMR spectros
Carbohydr. Res. 28395-110. [PubMed]

2. Bryan, R. A., O. Zaragoza, T. Zhang, G. Ortiz, A. Casadevall, and E. Dadachova. 2005. Radiolo
reveal radial differences in the architecture of the polysaccharide capsule of *Cryptococcus neoform*
Cell 4465-475. [PMC free article] [PubMed]

3. Casadevall, A., J. Mukherjee, and M. D. Scharff. 1992. Monoclonal antibody based ELISAs for
polysaccharide. J. Immunol. Methods 15427-35. [PubMed]

4. Cherniak, R., H. Valafar, L. C. Morris, and F. Valafar. 1998. Cryptococcus neoformans chemoty
quantitative analysis of ^{1}H nuclear magnetic resonance spectra of glucuronoxylomannans with a cc
simulated artificial neural network. Clin. Diagn. Lab. Immunol. 5146-159. [PMC free article] [Pub

5. Datta, K., and L. A. Pirofski. 2006. Towards a vaccine for Cryptococcus neoformans: principles
FEMS Yeast Res. 6525-536. [PubMed]

6. Devi, S. J. 1996. Preclinical efficacy of a glucuronoxylomannan-tetanus toxoid conjugate vaccir
Cryptococcus neoformans in a murine model. Vaccine 14841-844. [PubMed]

7. Duro, R. M., D. Netski, P. Thorkildson, and T. R. Kozel. 2003. Contribution of epitope specifici
binding of monoclonal antibodies to the capsule of Cryptococcus neoformans and the soluble form
polysaccharide, glucuronoxylomannan. Clin. Diagn. Lab. Immunol. 10252-258. [PMC free article]

8. Gates, M. A., P. Thorkildson, and T. R. Kozel. 2004. Molecular architecture of the Cryptococcu
capsule. Mol. Microbiol. 5213-24. [PubMed]

9. James, P. G., R. Cherniak, R. G. Jones, C. A. Stortz, and E. Reiss. 1990. Cell-wall glucans of Cr
neoformans Cap 67. Carbohydr. Res. 19823-38. [PubMed]

10. Maxson, M. E., E. Dadachova, A. Casadevall, and O. Zaragoza. 2007. Radial mass density, cha
epitope distribution in the *Cryptococcus neoformans* capsule. Eukaryot. Cell 695-109. [PMC free a
[PubMed]

11. McFadden, D., O. Zaragoza, and A. Casadevall. 2006. The capsular dynamics of Cryptococcus
Trends Microbiol. 14497-505. [PubMed]

12. McFadden, D. C., M. De Jesus, and A. Casadevall. 2006. The physical properties of the capsul polysaccharides from Cryptococcus neoformans suggest features for capsule construction. J. Biol. 2811868-1875. [PubMed]

13. Monari, C., F. Bistoni, and A. Vecchiarelli. 2006. Glucuronoxylomannan exhibits potent immu properties. FEMS Yeast Res. 6537-542. [PubMed]

14. Moyrand, F., T. Fontaine, and G. Janbon. 2007. Systematic capsule gene disruption reveals the galactose metabolism on Cryptococcus neoformans virulence. Mol. Microbiol. 64771-781. [PubM

15. Nakouzi, A., and A. Casadevall. 2003. The function of conserved amino acids in or near the co determining regions for related antibodies to Cryptococcus neoformans glucuronoxylomannan. Mo 40351-361. [PubMed]

16. Nakouzi, A., P. Valadon, J. Nosanchuk, N. Green, and A. Casadevall. 2001. Molecular basis fo immunoglobulin M specificity to epitopes in *Cryptococcus neoformans* polysaccharide that elicit p nonprotective antibodies. Infect. Immun. 693398-3409. [PMC free article] [PubMed]

17. Nimrichter, L., S. Frases, L. P. Cinelli, N. B. Viana, A. Nakouzi, L. R. Travassos, A. Casadeva Rodrigues. 2007. Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is divalent cations. Eukaryot. Cell 61400-1410. [PMC free article] [PubMed]

18. Pericolini, E., E. Cenci, C. Monari, M. De Jesus, F. Bistoni, A. Casadevall, and A. Vecchiarelli Cryptococcus neoformans capsular polysaccharide component galactoxylomannan induces apopto cells through activation of caspase-8. Cell. Microbiol. 8267-275. [PubMed]

19. Reese, A. J., and T. L. Doering. 2003. Cell wall α-1,3-glucan is required to anchor the Cryptoc neoformans capsule. Mol. Microbiol. 501401-1409. [PubMed]

20. Rodrigues, M. L., M. Alvarez, F. L. Fonseca, and A. Casadevall. 26 November 2007, posting d the wheat germ lectin to *Cryptococcus neoformans* suggests an association of chitin-like structures budding and capsular glucuronoxylomannan. Eukaryot. Cell doi:.10.1128/EC.00307-07 [PMC free [PubMed] [Cross Ref]

21. Sist, P., P. Cescutti, S. Skerlavaj, R. Urbani, J. H. Leitao, I. Sa-Correia, and R. Rizzo. 2003. Ma and solution properties of cepacian: the exopolysaccharide produced by a strain of *Burkholderia ce* from a cystic fibrosis patient. Carbohydr. Res. 3381861-1867. [PubMed]

22. Vaishnav, V. V., B. E. Bacon, M. O'Neill, and R. Cherniak. 1998. Structural characterization o galactoxylomannan of Cryptococcus neoformans Cap67. Carbohydr. Res. 306315-330. [PubMed]