

## Radiological Studies Reveal Radial Differences in the Architecture of the Polysaccharide Capsule of *Cryptococcus neoformans*

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**The polysaccharide capsule of the pathogenic fungus *Cryptococcus neoformans* is an important virulence factor, but relatively little is known about its architecture. We applied a combination of radiological, chemical, and serological methods to investigate the structure of this polysaccharide capsule. Exposure of *C. neoformans* cells to gamma radiation, dimethyl sulfoxide, or radiolabeled monoclonal antibody removed a significant part of the capsule. Short intervals of gamma irradiation removed the outer portion of the cryptococcal capsule without killing cells, which could subsequently repair their capsules. Survival analysis of irradiated wild-type, acapsular mutant, and complemented mutant strains demonstrated that the capsule contributed to radioprotection and had a linear attenuation coefficient higher than that of lead. The capsule portions remaining after dimethyl sulfoxide or gamma radiation treatment were comparable in size, 65 to 66  $\mu\text{m}^3$ , and retained immunoreactivity for a monoclonal antibody to glucuronoxylomannan. Simultaneous or sequential treatment of the cells with dimethyl sulfoxide and radiation removed the remaining capsule so that it was not visible by light microscopy. The capsule could be protected against radiation by either of the free radical scavengers ascorbic acid and sorbitol. Sugar composition analysis of polysaccharide removed from the outer and inner parts of the capsule revealed significant differences in glucuronic acid and xylose molar ratios, implying differences in the chemical structure of the constituent polysaccharides. Our results provide compelling evidence for the existence of two zones in the *C. neoformans* capsule that differ in susceptibility to dimethyl sulfoxide and radiation and, possibly, in packing and composition.**

The capsule of the human-pathogenic fungus *Cryptococcus neoformans* is a complex polysaccharide structure that surrounds the cells of this organism in vitro and in vivo. The capsule is composed of several polysaccharides, of which glucuronoxylomannan (GXM) is the major constituent (2, 10). Minor components of the capsule are galactoxylomannan and mannoprotein (9). The structure of the capsule has attracted considerable attention because it is a distinctive feature of this organism and an important virulence factor (2, 3, 6, 17). Despite intense interest in capsule-related studies, we know remarkably little about the architecture of the assembled capsule because there are relatively few techniques for studying this fragile structure in *C. neoformans* and other encapsulated microbes. One of the most striking characteristics of the capsule is that it changes size depending on the environmental conditions experienced by the fungus. Several factors, such as CO<sub>2</sub>, limited iron concentration, and infection in mice, are known to increase capsule size (1, 12, 26). In our laboratories, we have been studying the role of the capsule in complement- and antibody-mediated phagocytosis of *C. neoformans* (38) and, more recently, described experimental conditions that reliably promote rapid capsular growth in vitro (37, 36) and facilitate studies of capsule growth and architecture.

Suspension of encapsulated *C. neoformans* cells in dimethyl sulfoxide (DMSO) is known to remove a significant proportion of the capsule (21). Recently, Gates et al. used the combina-

tion of DMSO capsule removal and diffusion of macromolecules into the capsule to establish that the capsule differs in porosity as a function of radial distance from the cell wall, with the inner layers being more tightly packed (20). That finding and the observations that soluble polysaccharide could bind to acapsular strains to form a small capsule and that the capsular polysaccharide was shed into solution during growth suggested that the capsule is loosely attached to the fungal cell (24). Consistent with this view, Reese and Doering recently demonstrated that the capsular polysaccharide is attached to  $\alpha$ -1,3-glucan in the cell wall through a noncovalent interaction (29). However, the fact that addition of exogenous polysaccharide to acapsular mutants does not reconstitute the full-size capsular structure implies that the naturally assembled structure has significantly greater complexity and is possibly qualitatively different.

Three decades ago Dembintzer et al. reported the phenomenon of *C. neoformans* capsule disappearance after irradiation with large doses of gamma rays (16). We hypothesized that by using much lower doses of radiation, we could use this nonchemical method for removing the capsule gradually and thus study its structure. On the other hand, while carrying out experiments to develop radioimmunotherapy for *C. neoformans* infection, we noted that this organism was extremely resistant to external gamma radiation and, conversely, demonstrated a comparatively 1,000-fold greater susceptibility to particulate radiation when incubated with radiolabeled capsule binding monoclonal antibody (14). Since monoclonal antibody can alter capsular structure (11, 28) and the capsule protects *C. neoformans* cells against many insults (6), we also entertained the notion that the capsule may protect the fungus against

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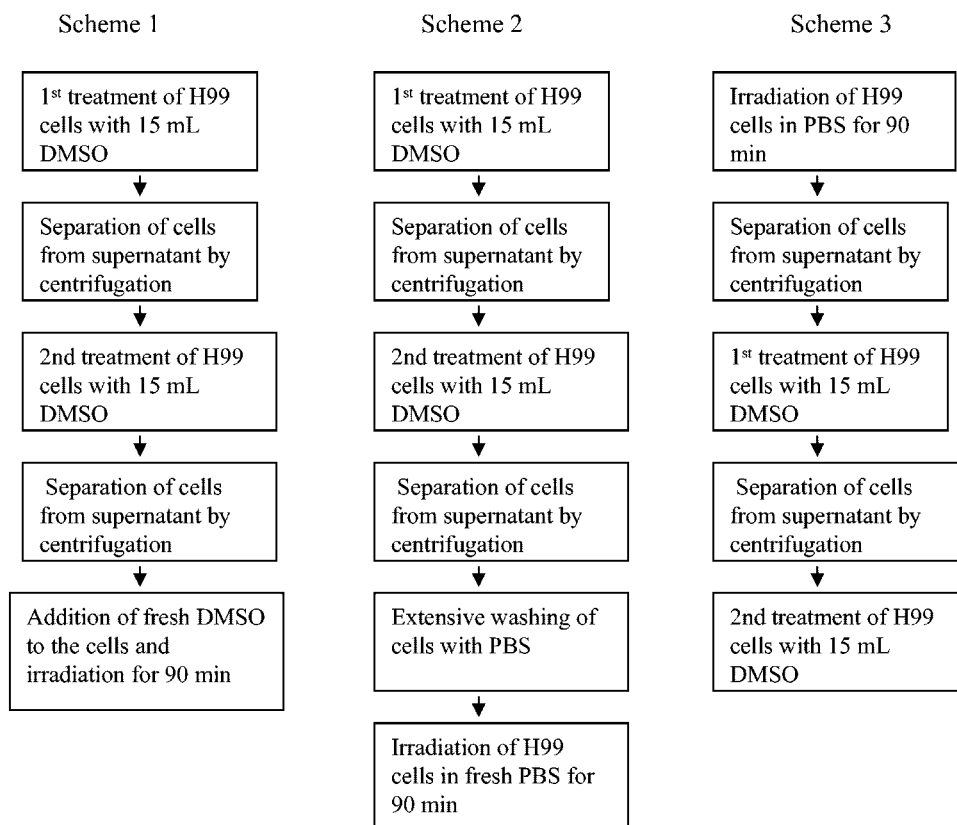


FIG. 1. Schematics of the protocols for H99 decapsulation by combined or sequential treatments with DMSO and radiation used in this study.

ionizing radiation and that radiolabeled capsule-binding monoclonal antibodies might have profound effects on the capsule.

In this study we have investigated the interaction of the capsule of serotype A *C. neoformans* with capsule-binding monoclonal antibody 18B7 labeled with rhenium-188 ( $^{188}\text{Re}$ ), external gamma radiation, and the known decapsulating agent DMSO (21) by microscopic, serological, and biochemical techniques. The use of radiation to probe capsule structure provides a new approach for this complex problem. The results offer new insights into the architecture of the capsule and indicate that the polysaccharide capsule provides protection against radiation.

#### MATERIALS AND METHODS

***C. neoformans* strains and growth conditions.** Capsule induction was performed with the experimental conditions described before (36). Briefly, the serotype A *C. neoformans* strain H99 was grown overnight in Sabouraud broth at 30°C with shaking (150 rpm). To induce capsule growth, the cells were subcultured into 10% Sabouraud broth in 50 mM HEPES, pH 7.3, and grown overnight at 37°C with or without shaking. Wild-type serotype D *C. neoformans* B-3501, acapsular mutant cap59 C536, and complemented cap59 mutant C538 (8), referred to in the text as the cap59 strain set, were grown in YNB (yeast nitrogen base without amino acids)-galactose in a rotary shaker (150 rpm) until late log to early stationary phase at 30°C (19). Galactose was added to the YNB medium because cap59 mutant C538 is complemented with a *gal7* promoter and needs galactose for induction of capsule growth.

**Treatment of *C. neoformans* cells with gamma radiation, DMSO, and radiolabeled monoclonal antibody 18B7.** H99 and cap59 strain set cells were washed three times with phosphate-buffered saline (PBS). Cells were then suspended in

PBS at a density of  $10^8$  cells/ml and exposed to  $^{137}\text{Cs}$ , which emanated gamma radiation at a constant dose rate of 14 Gy/min for 13 or 45 s or 10, 20, 40, 80, and 90 min. The kinetics of capsule removal from H99 by gamma radiation was analyzed with Prism software (GraphPad, San Diego, Calif.) by nonlinear regression. H99 cells were also irradiated in the presence of two compounds with free radical-scavenging properties, ascorbic acid (1 mg/ml) and sorbitol (50 mg/ml).

Alternatively, H99 cells at the same density were treated with DMSO by adding 15 ml of DMSO to 0.5 ml of  $10^8$  *C. neoformans* cell suspension, collecting cells by centrifugation, and then suspending the cells again in 15 ml of DMSO with each incubation lasting 30 min ( $2 \times 15$  ml DMSO treatment). In some experiments the DMSO and gamma radiation treatments were combined or administered sequentially (Fig. 1, schemes 1 to 3). The H99 cells were treated with  $2 \times 15$  ml DMSO, collected by centrifugation, suspended in fresh DMSO and immediately irradiated at the above dose rates for 90 min (Fig. 1, scheme 1). Alternatively, the DMSO-treated H99 cells were washed with PBS, then suspended in fresh PBS, and exposed to gamma radiation for 90 min (Fig. 1, scheme 2) or irradiated in PBS and subsequently treated with  $2 \times 15$  ml DMSO (Fig. 1, scheme 3). Following irradiation, treatment with DMSO or combined treatment with irradiation and DMSO,  $10^5$  H99 or cap59 strain set cells were plated on Sabouraud agar to assess cell viability as determined by CFU.

To investigate whether cells decapsulated by gamma radiation were capable of repairing their capsules, H99 cells were irradiated for 40 min in PBS and then placed in capsule-inducing medium (10% Sabouraud broth in 50 mM HEPES, pH 7.3) and grown overnight at 37°C with shaking. Cells were then collected, washed with PBS, and examined by microscopy for capsule size.

To assess if particulate radiation delivered to the capsule by capsule-binding monoclonal antibody affected capsule size,  $10^7$  H99 cells in 3 ml of PBS were treated with the increasing activities of  $^{188}\text{Re}$ -labeled monoclonal antibody 18B7 (specific activity, 1.15 mCi/mg) for 1 h at 37°C, followed by centrifugation and resuspension of the cells in fresh PBS and further incubation at 4°C for 48 h. The latter is necessary to provide sufficient time (almost three half-lives) for  $^{188}\text{Re}$  to deliver most of its radiation to the capsule and the cells. Monoclonal antibody

18B7 was labeled with  $^{188}\text{Re}$  as described (14). For a control, H99 cells were treated under identical conditions with the same concentrations of unlabeled monoclonal antibody 18B7.

**Determination of the linear attenuation coefficient of *C. neoformans* polysaccharide.** To compare the radioprotective properties of polysaccharide with those of well-known radioprotectors such as lead, we calculated its linear attenuation coefficient according to the equation  $I = I_0 e^{-\mu x}$ , where  $I_0$  and  $I$  are the radiation intensity before and after shielding, respectively,  $\mu$  is the linear attenuation coefficient (per centimeter), and  $x$  is the thickness of the capsule (in centimeters). The reduction in radiation intensity was calculated from the linear parts of cap59 strain set survival curves assuming that a 10% increase in survival is equivalent to a 10% decrease in radiation intensity. The thickness of the capsule was determined as described below.

**Measurement of total polysaccharide amount and its concentration per H99 cell.** H99 cells grown in capsule-inducing medium were collected, washed with PBS, and irradiated for different times up to 90 min. Control and irradiated cells were separated from supernatants by centrifugation, and the supernatants were then filtered through a 0.22- $\mu\text{m}$  filter, and the total polysaccharide concentration in the supernatants was initially determined semiquantitatively by latex agglutination (Latex-Crypto antigen detection system, Immuno-Mycologics, Norman, Okla.).

To determine the amount of total polysaccharide released by radiation alone,  $2 \times 10^9$  H99 cells were irradiated in PBS for 90 min. The capsule material released by radiation alone was concentrated into  $\text{H}_2\text{O}$  with Centricon-50 (molecular weight cutoff,  $5 \times 10^4$  Da) microconcentrators. For determination of total polysaccharide released by combined treatment with DMSO and radiation,  $4.6 \times 10^8$  H99 cells were treated with  $2 \times 15$  ml DMSO, collected by centrifugation, and irradiated in 15 ml of PBS for 90 min (Fig. 1, scheme 2). DMSO and PBS supernatants were collected. The capsule material released by DMSO was transferred from DMSO into PBS with Centricon-50 and then the post-DMSO and postirradiation PBS supernatants were concentrated in  $\text{H}_2\text{O}$ . The concentration of total polysaccharide in the above supernatants was determined by the phenol-sulfuric acid colorimetric technique (18).

The amount of polysaccharide per cell was calculated by dividing the concentration of released polysaccharide per milliliter by the cell density per milliliter; the concentration of polysaccharide in the capsule of a H99 cell was obtained by dividing the amount of polysaccharide per cell by the capsule volume calculated as below; the number of polysaccharide molecules per H99 cell was computed assuming a molecular mass of  $1.5 \times 10^6$  Da (D. McFadden and A. Casadevall, Abstr. 103rd Annu. Meet. Am. Soc. Microbiol., abstr. F003, 2003).

**Glycosyl composition analysis of H99 polysaccharide.** For performing the glycosyl composition analysis of polysaccharides removed from H99 cells by radiation alone or by DMSO and radiation, the cells were irradiated for 90 min or treated with the sequence of  $2 \times 15$  ml fractions of DMSO and then radiation (Fig. 1, scheme 2), supernatants were collected, concentrated in  $\text{H}_2\text{O}$  with Centricon-50 microconcentrators, and analyzed at the Complex Carbohydrate Research Center, University of Georgia (Atlanta) by combined gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methylglycosides produced from the sample by acidic methanolysis. Methyl glycosides were first prepared from the polysaccharide samples by methanolysis in 1 M HCl in methanol at  $80^\circ\text{C}$  (18 to 22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at  $80^\circ\text{C}$  (0.5 h). These procedures were carried out as described (35).

Gas chromatography/mass spectrometry analysis of the trimethylsilyl methylglycosides was performed on an HP 5890 GC interfaced to a 5970 MSD with a Supelco DB-1 fused silica capillary column (30 m by 0.25 mm inner diameter). This analysis yielded the amounts of mannose, xylose, and galactose in the samples but measured unexpectedly low levels of glucuronic acid, which was presumably destroyed in the analytical process. Consequently, the glucuronic acid content of the polysaccharide samples was determined spectrophotometrically via hydrolysis with sulfuric acid, reduction with tetraborate, followed by a coloring step with *m*-hydroxydiphenyl (34). Total polysaccharide was determined by the phenol-sulfuric acid colorimetric technique (18). Subsequently, the molar percentages of mannose, xylose, glucuronic acid, and galactose in the samples were calculated. For comparison, the glycosyl composition of H99 exopolysaccharide was determined. Exopolysaccharide was obtained from 10-day-old H99 culture supernatants by addition of sodium acetate and precipitation of exopolysaccharide by ethanol.

**HPLC analysis of H99 polysaccharide.** To get insights into the heterogeneity and composition of H99 fractions released by the different decapsulation treatments, all samples used for glycosyl composition analysis as well as GXM purified by precipitation with hexadecyltrimethylammonium bromide as described (9)

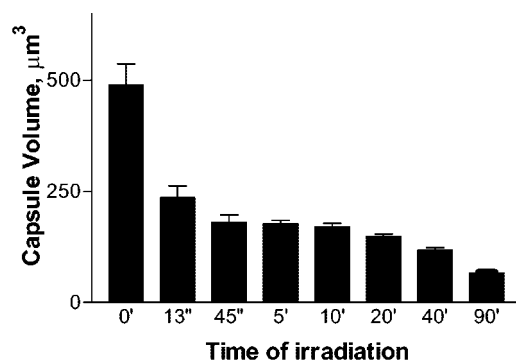


FIG. 2. H99 cells capsule size as a function of time in response to treatment with gamma radiation at 14 Gy/min. Bars represent averages of 50 measurements and error bars represent the standard error.

were also analyzed by high-pressure liquid chromatography (HPLC) on TSK-3000 size exclusion column (Toso-Haas) eluted with PBS at 1 ml/min. The absorbance of the eluate was measured at 254 nm. The dependence of elution time upon the molecular weight of the polysaccharides for TSK-3000 column was established with dextrans of different molecular weights (Molecular Probes).

**Visualization of *C. neoformans* capsule.** The *C. neoformans* polysaccharide capsule was visualized by light and fluorescence microscopy. For light microscopy the cells were fixed in ice-cold 70% ethanol for several hours following irradiation or DMSO treatment and washed and suspended in NS buffer (0.25 M sucrose, 10 mM Tris, pH 7.2, 1 mM EDTA, 1 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{ZnCl}_2$ , 7 mM  $\beta$ -mercaptoethanol) (25). For light microscopy, 10  $\mu\text{l}$  of the cell suspension was mixed with a drop of India ink and observed in the Olympus AX70 microscope. At least five different fields were randomly chosen and photographed, and 40 to 60 cells were measured.

For immunofluorescence, the cells were blocked for nonspecific protein binding with 1% bovine serum albumin and 0.5% fetal bovine serum and then incubated with 10  $\mu\text{g}$  of monoclonal antibody 2H1, an immunoglobulin G1 which binds GXM (7), per ml. Tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse immunoglobulin G (5  $\mu\text{g}/\text{ml}$ , Southern Biotechnology Associates, Inc, Birmingham, Ala.) was used as the secondary antibody to produce fluorescent staining on any cells that retained GXM.

**Measurement of capsule volume.** The diameters of the whole cell and the cell body were measured from light microscopy images with Adobe Photoshop 7.0, and capsule volume was defined as the difference between the volume of the whole cell (yeast cell and capsule) and the volumes of the cell body (as limited by the cell wall). The volume was calculated with the equation  $(4\pi/3)(D/2)^3$ , where  $D$  is the diameter of the cell body or of the whole cell. Forty to sixty cells were measured for each determination.

**Statistics.** For statistical analyses, the Student two-tailed unpaired  $t$  test and nonlinear regression analysis were performed with Prism software (GraphPad, San Diego, Calif.).

## RESULTS

**Irradiation removed a major part of the capsule from H99 cells.** Exposing H99 cells grown in capsule-inducing conditions to gamma radiation at 14 Gy/min removed a major part of the capsule. Figure 2 shows the time course of capsule removal by radiation. A 13-s exposure (3 Gy) removed half of the capsule ( $P < 0.0001$ ). Additional exposure for up to 40 min (560 Gy) removed another half of the remaining capsule ( $P = 0.0002$ ). Prolonged irradiation for 90 min further reduced the capsule volume to  $66 \mu\text{m}^3$  ( $P < 0.001$ ). Hence, a significant proportion of the capsule is susceptible to removal by gamma radiation, so that the initial volume was reduced from 490 to  $1,200 \mu\text{m}^3$  to  $65 \mu\text{m}^3$ . Depending on the initial size of the capsule, up to 95% of the capsule was removed by radiation. The kinetics of capsule removal by radiation can be described by a two-phase exponential decay equation ( $R^2 = 0.98$ ) with initial fast disap-

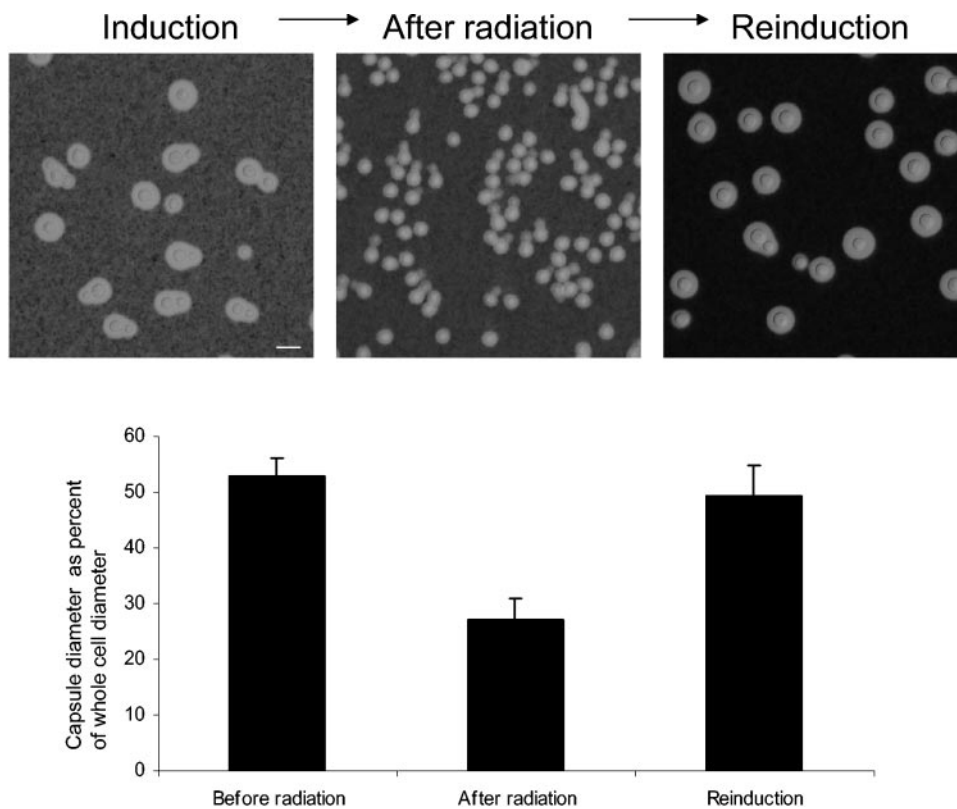


FIG. 3. *C. neoformans* cells decapsulated with radiation are able to repair their capsules when placed in capsule-inducing medium. The top panels show light microscopy images before and after irradiation and placement in capsule-inducing conditions (bar represents 10  $\mu\text{m}$ ). The bottom panel shows a histogram of capsule width as a percentage of the whole cell diameter after each condition. The calculation was performed according to the formula  $(W - C)/W \times 100$ , where  $W$  is the diameter of the whole cell and  $C$  is the diameter of the cell body. Bars represent averages of 50 measurements, and error bars represent the standard deviation.

pearance of capsule with a half-life of 0.1 min, followed by much slower removal with a half-life of 18.5 min. The result indicates that the outer and inner layers of the capsule differ in their susceptibility to gamma radiation as measured by dosimetry or the kinetics of capsular removal.

To establish if gamma radiation removed the capsular polysaccharide from H99 cells or just caused the capsule to collapse, we measured the concentration of polysaccharide in the cell suspension supernatant before and after gamma irradiation. Supernatants from unirradiated cells contained less than 3 ng of polysaccharide per ml, while those from cells irradiated for 10, 20, or 40 min had a polysaccharide concentration of  $\approx 5 \mu\text{g/ml}$  as measured by the Latex-Crypto antigen detection system. The 1,000-fold increase in polysaccharide concentration in the supernatant of irradiated cells indicated that the reduction in capsule size following gamma radiation was a consequence of radiation-mediated stripping of the polysaccharide capsule from the cell into the solvent.

**H99 cells remained viable postirradiation and capable of repairing their capsules.** There was no significant decrease in cell viability in the dose range administered (up to 560 Gy for 40 min of irradiation) as measured by CFU. This result confirms the remarkable radioresistance of *C. neoformans* measured in our earlier study (14). Irradiation-decapsulated cells could repair their capsules when placed in capsule-inducing medium, consistent with the observation that these cells were

viable and not significantly damaged by radiation (Fig. 3). In contrast, decapsulation of cells with DMSO for 30 min or irradiation of cells in DMSO killed all cells (results not shown). The fungicidal effects of DMSO presumably reflect disruption of cell membranes and other cellular structures by the organic solvent.

**Combined or sequential treatment with radiation and DMSO removed the capsule.** Incubation in DMSO removed a significant proportion of the *C. neoformans* capsule (21, 20). Here we compared the capsules of DMSO-treated and gamma radiation-treated cells. The effects of different treatments on capsule size are summarized in Table 1, and the light microscopy images are presented in Fig. 4. Incubation of H99 cells in DMSO for 30 min produced an effect on capsule size similar to that observed with 90 min of irradiation, with the capsule volume shrinking from 1,276 to 65 to 66  $\mu\text{m}^3$ . The efficient removal of capsule required two sequential applications of DMSO, which may be due to saturation of DMSO with polysaccharide. There was no statistically significant difference between the capsule volumes of gamma-irradiated and DMSO-treated cells ( $P = 0.8$ ). However, combining DMSO and gamma radiation treatments (Fig. 1, scheme 1) significantly reduced the remaining capsule structure. Exposing *C. neoformans* cells to gamma radiation in the presence of DMSO removed additional capsule material and reduced the remaining capsule volume to 32  $\mu\text{m}^3$ . Sequential treatments with

TABLE 1. Effects of different treatment conditions on capsule volume of H99 *C. neoformans* cells<sup>a</sup>

Sample	Capsule volume ( $\mu\text{m}^3$ )
Untreated control	1,276 $\pm$ 95
DMSO (2 $\times$ 15 ml, 30 min each)	65 $\pm$ 8
Treated with 90 min of radiation in PBS at 14 Gy/min	66 $\pm$ 3
Suspended in DMSO and irradiated for 90 min at 14 Gy/min (Fig. 1, scheme 1)	32 $\pm$ 7
Treated with DMSO, extensively washed with PBS, followed by 90 min of radiation in PBS at 14 Gy/min (Fig. 1, scheme 2)	33 $\pm$ 9
Treated with 90 min of radiation in PBS at 14 Gy/min followed by DMSO treatment (Fig. 1, scheme 3)	32 $\pm$ 6

<sup>a</sup> Cell volume in all treatment groups was  $62 \pm 5 \mu\text{m}^3$ . Capsule volume and cell volume are expressed as means with standard errors of the means. Capsule volume was defined as a difference between the volume of the whole cell (yeast cell plus capsule) and the volume of the cell body (as limited by the cell wall).

DMSO and radiation and, conversely, with radiation and DMSO (Fig. 1, schemes 2 and 3) resulted in the same reduction of capsule volume to 32 to 33  $\mu\text{m}^3$  ( $P = 0.6$  to 0.7).

**Capsule protected cells against ionizing radiation.** The cap59 strain set was irradiated to determine whether the capsule protected the fungus against ionizing radiation. The survival of acapsular cap59 mutant C536 was significantly lower than that of the wild-type strain (Fig. 5a). The complemented strain was less susceptible to radiation than the acapsular mutant but was not as resistant as the wild-type strain. We further analyzed these differences by considering cellular volume and capsule size. The cell volume of the acapsular and complemented strains is larger than that of the wild-type strain (19), which should make such cells larger targets for radiation-generated toxic free radicals and, consequently, should increase radiation-mediated damage. Free radicals are produced both outside and inside the cell wherever gamma photons pass through water or cytoplasm. Thus, a larger cell diameter will

result in more radicals being produced inside the cell and should be accounted for.

To account for increased volume-related susceptibility, we normalized the survival data by dividing percent survival by cell volume, which resulted in every point on a survival curve's becoming statistically different ( $P < 0.01$ ) from the corresponding points on the other survival curves (Fig. 5b). Furthermore, we measured the size of the capsule in the wild-type and complemented strains and found that the wild-type structure was almost twice the size ( $144 \pm 32 \mu\text{m}^3$ ) of that of the complemented strain ( $82 \pm 20 \mu\text{m}^3$ ). From these data, we calculated the linear attenuation coefficient of capsular polysaccharide according to the equation given in Materials and Methods as  $1.7 \times 10^3 \text{ cm}^{-1}$ .

**Ascorbic acid and sorbitol protected the capsule against radiation.** Since gamma radiation generates highly reactive free radicals in aqueous solutions through radiolysis of water (22), which could be involved in decapsulation, we hypothesized that addition of antioxidants to the cell suspension may protect the capsule. The presence of the free radical scavengers ascorbic acid and sorbitol protected the capsule against gamma radiation (Fig. 6c). Ascorbic acid was more effective than sorbitol in protecting the capsule, which remained practically intact in the presence of this antioxidant.

**Interaction of capsule-binding <sup>188</sup>Re-labeled monoclonal antibody 18B7 with H99 capsule.** Given that radiation removed a significant portion of the *C. neoformans* capsule, we evaluated the effect of radiolabeled monoclonal antibody on capsule size. Incubation of *C. neoformans* cells with 75  $\mu\text{Ci}$  of radiolabeled monoclonal antibody reduced the diameter of the capsule by more than threefold, while the same concentration (22  $\mu\text{g/ml}$ ) of unlabeled monoclonal antibody had no effect on capsule volume (Fig. 7). Increasing the activity of <sup>188</sup>Re-labeled 18B7 did not result in further reduction of the capsule size. However, incubation of *C. neoformans* cells with unlabeled monoclonal antibody at concentrations of 44 and 88  $\mu\text{g/ml}$  also produced a reduction in capsule size, though the effect was not statistically significant in comparison with untreated samples.

**Immunoreactivity of H99 after decapsulation treatments.** Immunofluorescence with monoclonal antibodies to capsular polysaccharide was done to determine if the residual capsule after radiation, DMSO, or combined or sequential treatment retained immunoreactivity. Monoclonal antibody 2H1 bound to the capsule remaining after either radiation or DMSO treatment. However, minimal monoclonal antibody binding was observed for cells exposed to combined or sequential treatments with DMSO and gamma radiation. The fluorescence observed in cells irradiated in DMSO was very heterogenous, with most of the cells showing an irregular punctuate pattern. In addition, we noted some cells with no signal at all, and some cells had only a weak fluorescence signal, consistent with total decapsulation (Fig. 8d).

**Measurement of total polysaccharide amount per cell.** Using Centricon-50 microconcentrators, we separated the polysaccharides generated by sequential treatment of H99 cells with DMSO and radiation (Fig. 1, scheme 2) by molecular weight. Two sequential treatments with DMSO followed by 90 min of irradiation of  $4.6 \times 10^8$  cells (Fig. 1, scheme 2) produced  $1.74 \pm 0.25$ ,  $0.35 \pm 0.12$ , and  $0.064 \pm 0.010$  mg, respec-

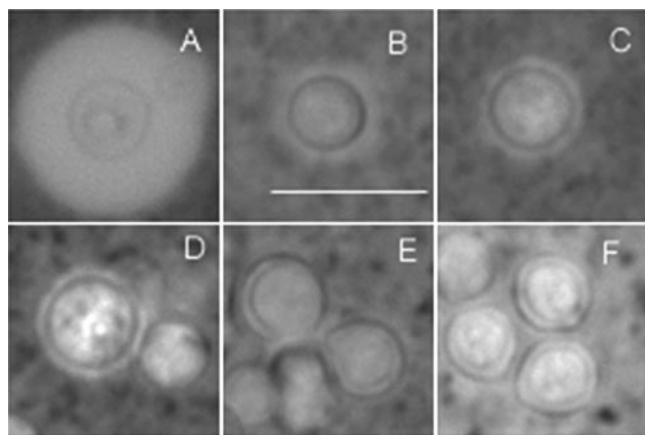


FIG. 4. Light microscopy images of H99 cells subjected to different treatments and stained with India ink: A untreated; B DMSO; C 90 min of radiation in PBS; D DMSO, wash, 10 min of radiation in PBS; E DMSO, wash, 90 min of radiation in PBS; F 90 min of radiation in PBS and then DMSO. The bar represents 10  $\mu\text{m}$ .

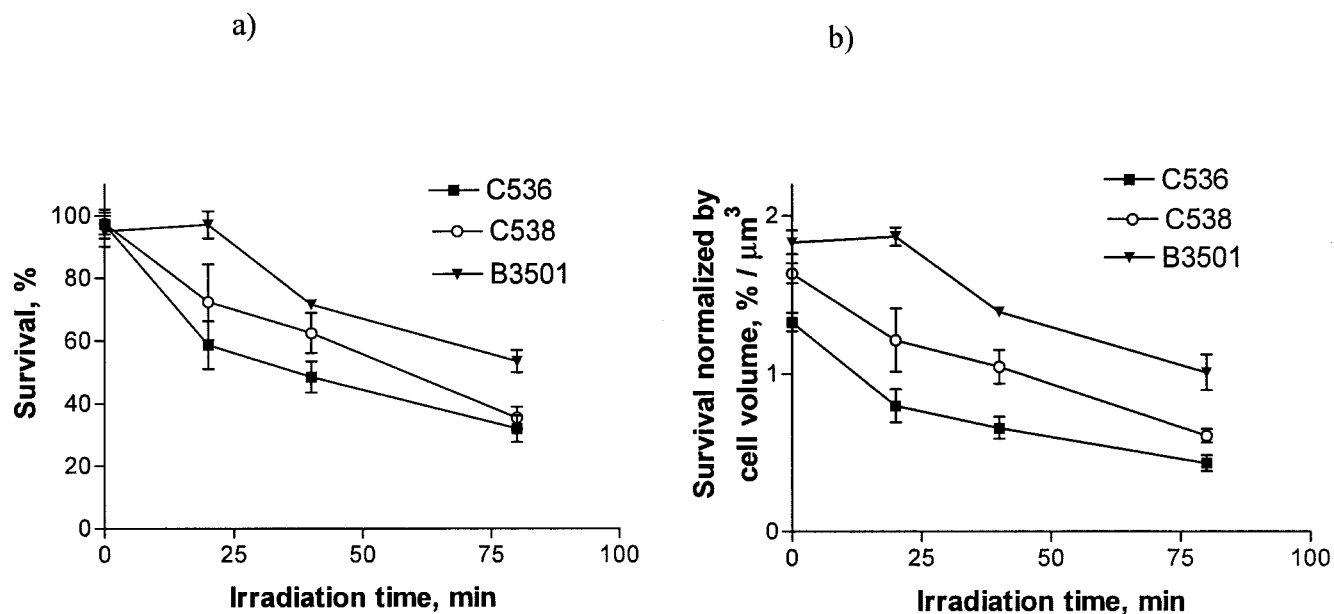


FIG. 5. Survival of the cap59 strain set after gamma irradiation. a) Dependence of survival on irradiation time. Every sample was plated in triplicate. Error bars represent the standard deviation. There was a statistically significant difference in survival ( $P < 0.01$ ) between wild-type B-3501 and acapsular mutant cap59 C536 for every irradiation time and between wild-type B-3501 and complemented mutant cap59 C538 ( $P < 0.005$ ) at 20 and 80 min. b) Dependence of survival normalized to cellular volume on irradiation time. There was a statistically significant difference ( $P < 0.01$ ) for all irradiations times when wild-type B-3501, acapsular mutant cap59 C536, and complemented mutant cap59 C538 were compared.

tively, of polysaccharide species with molecular mass exceeding  $5 \times 10^4$  Da and  $1.54 \pm 0.19$ ,  $0.16 \pm 0.05$ , and 0 mg, respectively, of polysaccharide species with molecular mass below  $5 \times 10^4$  Da. Thus, high-molecular-mass species constituted 4.7 pg of polysaccharide per H99 cell and low-molecular-mass species constituted 3.7 pg of polysaccharide per cell, with the overall amount being 8.4 pg/cell.

The concentration of polysaccharide in the H99 capsule was calculated to be 6.6 mg/ml. Assuming the molecular mass of GXM to be  $1.5 \times 10^6$  Da, the number of GXM molecules per H99 cell was calculated to be  $1.8 \times 10^6$ . Assuming a molecular mass of  $5 \times 10^4$  we estimate that the number of molecules of low molecular mass to be at least  $4.5 \times 10^8$  per cell. Given that the cell surface area of a nonencapsulated *C. neoformans* strain is approximately  $79 \mu\text{m}^2$ , the surface density of GXM is at least

$2.3 \times 10^4$  molecules/ $\mu\text{m}^2$  for high-molecular-mass GXM and  $5.7 \times 10^6$  molecules/ $\mu\text{m}^2$  for all forms of GXM.

HPLC analysis of capsule fractions revealed two major species of  $1.5 \times 10^6$  to  $2.0 \times 10^6$  (elution time, 4.5 min) and  $8.0 \times 10^5$  (elution time, 12 min) in each of the chromatograms of ethanol-precipitated material, purified GXM, and the different fractions removed by either DMSO or radiation or combined DMSO and radiation treatment (Fig. 9). We measured the absorbance of the eluate at 254 nm when the absorbance of protein standards in comparison with dextran standards was minimal. There were also species with molecular weights in excess of  $2.0 \times 10^6$  which is beyond the resolution of the TSK-3000 size exclusion column, as well as fractions that eluted at 9 and 15 min with molecular weights of  $3.5 \times 10^5$  and  $10^4$ , respectively, attesting to the compositional complexity of

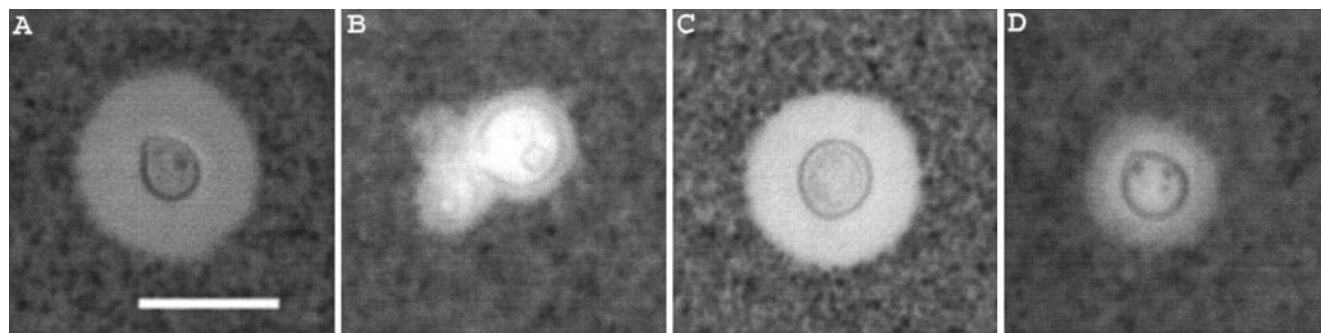


FIG. 6. Protective effects of ascorbic acid and sorbitol on H99 capsule during radiation: A) untreated; B) 40 min of radiation in PBS; C) 40 min radiation in PBS with 1 mg of ascorbic acid per ml; D) 40 min of radiation in PBS with 50 mg of sorbitol per ml.

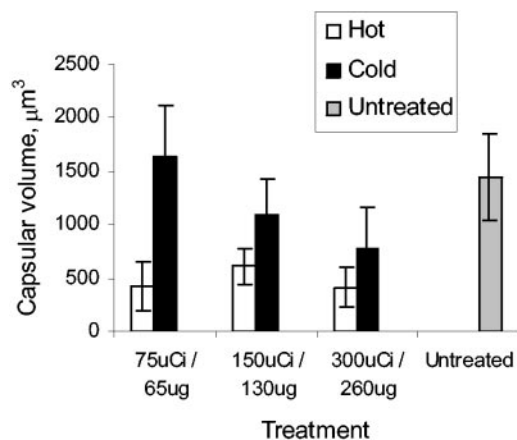


FIG. 7. Effect of incubating *C. neoformans* cells with radiolabeled monoclonal antibody 18B7 (hot) or an equivalent amount of unlabeled monoclonal antibody 18B7 (cold). H99 cells in 3 ml of PBS were treated with increasing activities of  $^{188}\text{Re}$ -labeled monoclonal antibody 18B7 for 1 h at 37°C followed by centrifugation and resuspension of the cells in fresh PBS and further incubation at 4°C for 48 h. Bars represent averages of 60 measurements and error bars represent the standard deviation. There was a significant difference ( $P < 0.02$ ) between all groups treated with labeled 18B7 and untreated cells as well as between groups treated with 75 and 150  $\mu\text{Ci}$  of labeled 18B7 and matching concentrations of unlabeled 18B7. There was no statistically significant difference between all groups treated with unlabeled 18B7 and untreated cells or between 300  $\mu\text{Ci}$  of labeled 18B7 and a matching concentration of unlabeled 18B7.

these fractions. It is impossible, however, to assign these peaks to either galactoxylomannan or GXM on the basis of elution profile data.

**Glycosyl composition analysis of H99 polysaccharide.** The fractions from H99 cells treated with gamma radiation alone; combined radiation and DMSO treatment according to the protocol in Fig. 1, scheme 2, and naturally shed exopolysaccharide were analyzed for glycosyl composition. All samples treated with DMSO had significant concentrations of glucose, while samples treated with radiation alone or naturally shed by

H99 cells had only trace amounts of glucose. Glucose is not a component of GXM or galactoxylomannan and is likely released from cellular stores by DMSO-mediated membrane disruption; accordingly, we limited our analysis to comparing the molar amounts of glucuronic acid, xylose, mannose, and galactose, since these are the recognized sugar components of GXM and galactoxylomannan.

The ratios of xylose to mannose in fractions obtained by DMSO or radiation alone or combined treatment were comparable and close to 1 and somewhat lower in naturally shed exopolysaccharide, 0.7, though there was no statistically significant difference in the percentage of mannose between any pair of samples. The percentage of glucuronic acid in all samples was approximately 20 mol%, with the exception of the “post-radiation” sample, the deepest layer of polysaccharide which can be removed only by combined or sequential DMSO and radiation treatment, where the glucuronic acid content was only 10%. The following differences were statistically significant: xylose content in the deepest layer in comparison with naturally shed exopolysaccharide ( $P = 0.03$ ), and glucuronic acid content in the deepest layer in comparison with both the outer layer ( $P = 0.02$ ) and the naturally shed exopolysaccharide ( $P = 0.01$ ). The ratio of galactose to mannose was close to 0.1 in most fractions, although galactose was absent in the fraction recovered from cells subjected to radiation alone.

## DISCUSSION

The application of new investigational methods in the form of radiation combined with other techniques could provide new information on the architecture of the capsule. Dembintzer et al. reported the disappearance of the polysaccharide capsule after irradiation of *C. neoformans* with gamma rays (16). However, that study employed lethal radiation doses (6,000 to 10,000 Gy) for *C. neoformans* (14) and reported no measurements of the capsule size. In this study we used significantly lower radiation doses (3 to 1,260 Gy delivered at 14 Gy/min), which allowed gradual removal of the capsule without killing the cells. Remarkably, half of the capsule disap-

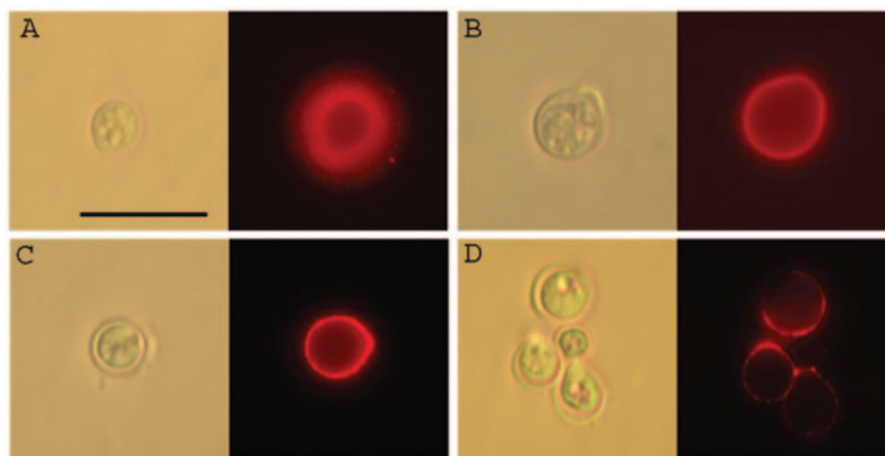


FIG. 8. Immunofluorescence of H99 cells subjected to different treatments and stained with GXM-specific monoclonal antibody 2H1. Left panel, light microscopy image; right panel, fluorescent image: A) untreated; B) irradiated in PBS for 40 min; C) treated with DMSO alone; D) irradiated in DMSO for 40 min. The bar represents 10  $\mu\text{m}$ .

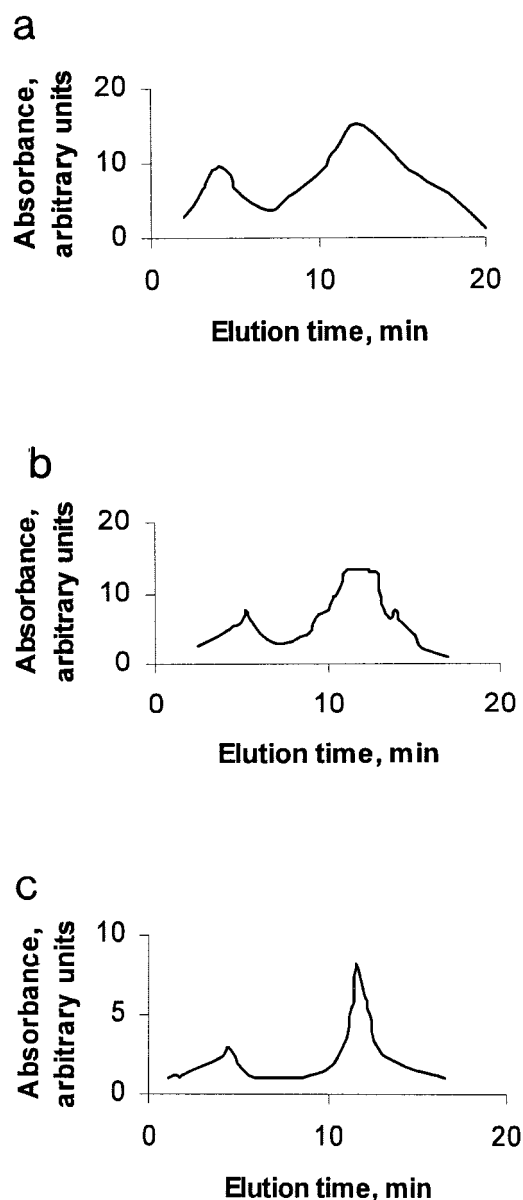


FIG. 9. HPLC elution profiles of H99 capsule fractions a) released by gamma radiation in PBS or b) released by DMSO and c) purified GXM. The TSK-3000 size exclusion column was eluted with PBS at 1 ml/min. The absorbance of the eluate was measured at 254 nm. The column was calibrated with dextrans of different molecular weights.

peared after just 3 Gy, and up to 95% of the capsule was gone after 140 Gy. The kinetics of this process followed two-phase exponential decay, with capsule removal being almost 150 times faster during the first phase (half-life, 0.1 min) than during the second phase (half-life, 18.5 min). Since the radiation dose rate was constant, this difference in capsule removal kinetics provides strong evidence for differences in composition and/or architecture for the inner and outer capsular layers.

The capsule of *C. neoformans* cells exhibits a considerable degree of plasticity in its ability to undergo reversible changes in volume depending on the ionic strength of the solvent (20). To exclude the possibility that capsule volume changes after

radiation were a consequence of a collapse of the capsule onto the cell wall, we measured the concentration of polysaccharide in the cell supernatant before and after irradiation and found that soluble polysaccharide increased more than 1,000-fold. This result indicates that radiation caused the release of the capsule into the cell solvent. Hence, exposing *C. neoformans* cells to gamma radiation provides a nonchemical method for removing a significant portion of the polysaccharide capsule, which leaves the cell viable for subsequent studies.

Given that the capsule disappeared gradually with prolonged irradiation, we investigated whether the capsule protected *C. neoformans* cells against radiation. For that experiment we used the cap59 strain set, which includes wild-type, acapsular, and complemented strains, exposed them to various doses of radiation, and determined their survival. The complemented strain was less susceptible to radiation than the acapsular mutant but was not as resistant as the wild-type strain. The higher susceptibility of the acapsular strain relative to the wild-type and complemented strains implies that the capsule has radioprotective properties. Consistent with this conclusion was the observation that the complemented strain was more susceptible to radiation than the wild type and had a capsule that was almost half the size of that of the wild-type strain.

Since gamma radiation induces the radiolysis of water, which produces reactive cytotoxic free radicals, a likely mechanism for the protective capsular effect is free radical quenching by carbohydrate moieties. In this regard, carbohydrates are efficient sinks for hydroxyl free radicals (27, 30, 31), and the arabinogalactan from *Tinospora cordifolia* confers significant protection against gamma radiation-induced damage (33). Hence, we investigated whether the capsule itself could be protected from radiation by compounds known to quench free radicals. The concentration of polysaccharide in the capsule of an H99 cell was estimated to be 6.6 mg/ml, and this concentration range was used for ascorbic acid and sorbitol. Both ascorbic acid and sorbitol protected the *C. neoformans* capsule, a finding that strongly supported a radiation-mediated decapsulation mechanism based on free radical damage (see below).

We conclude that the capsule is an efficient radioprotective material and has an effective linear attenuation coefficient of  $1.7 \times 10^3 \text{ cm}^{-1}$ . Since the attenuation coefficient of lead is  $27 \text{ cm}^{-1}$  (31), we construe that capsular polysaccharide is a much more efficient radioprotector than lead, when hydrated in aqueous medium, where the radiation-induced microbicidal molecules are likely to be highly reactive oxygen radical species. In this regard we note that the capsule is known to confer protection against amoeboid predators (32), which can produce an oxidative burst during phagocytosis (4). Hence, the radioprotective properties of the capsule may be a consequence of selection pressures in the environment other than background radiation.

Comparison of the effect of gamma radiation on the capsule with that of particulate radiation delivered locally to the capsule by capsule-binding monoclonal antibody radiolabeled with  $^{188}\text{Re}$  revealed that both reduced the capsule size. Incubation with  $^{188}\text{Re}$ -labeled monoclonal antibody 18B7 reduced H99 cell capsule size by threefold. At monoclonal antibody concentrations of  $>40 \mu\text{g/ml}$ , unlabeled monoclonal antibody showed a trend to affect capsule size. However, the mechanisms of this reduction by  $^{188}\text{Re}$ -labeled and unlabeled monoclonal anti-



TABLE 2. Glycosyl composition analysis of H99 capsule

Monosaccharide	Polysaccharide prepn <sup>a</sup> (mol %)				H99 total polysaccharide
	DMSO1	DMSO2	Postradiation	Radiation alone	
Xylose	38 ± 4	37 ± 6	43 ± 4	37 ± 5	29 ± 4
Glucuronic acid	19 ± 6	16 ± 4	10 ± 3	20 ± 7	23 ± 7
Mannose	39 ± 5	43 ± 7	45 ± 9	43 ± 7	41 ± 6
Galactose	3 ± 0.5	4 ± 1	3 ± 1	0	6 ± 2

<sup>a</sup> The values were calculated by using only the values for xylose, glucuronic acid, mannose, and galactose since these are the known sugar components for GXM and GalXM. The results are the means and standard deviations of two different experiments, with each sample analyzed in duplicate, to yield four values that were averaged. H99 cells were decapsulated by two sequential treatments with  $2 \times 15$  ml DMSO (DMSO1 and DMSO2). Supernatants were collected and concentrated in H<sub>2</sub>O with Centricon-50 microconcentrators. Postradiation material originated from DMSO-treated cells followed by irradiation in PBS for 90 min. Radiation alone, material originated from H99 cells exposed to gamma radiation for 90 min. Total polysaccharide was obtained from H99 cell culture supernatant by precipitation with ethanol.

body 18B7 are most likely very different. In the case of <sup>188</sup>Re-labeled monoclonal antibody 18B7, the capsule is bombarded with high-energy ( $E_{\max} = 2.1$  MeV) beta-particles, which can damage the polysaccharide molecules and shave a significant amount of capsule, while in the case of unlabeled 18B7, the reduction in capsule volume probably results from monoclonal antibody-mediated capsule contraction caused by cross-linking of polysaccharide fibers unaccompanied by physical loss of polysaccharide (20). This significant reduction in capsule size by radiolabeled monoclonal antibody might contribute to the mechanism of efficacy of radioimmunotherapy against *C. neoformans* (15) and may have even wider implications for the emerging field of radioimmunotherapy of infection in general (5), as many pathogens are encapsulated and potentially useful monoclonal antibodies which bind to capsule antigens are available (13).

Incubation of encapsulated *C. neoformans* with DMSO yielded cells with the same capsule volume as cells exposed to gamma radiation (Table 1). Both DMSO and radiation reduced the capsule to approximately the same volume of 65 to 66  $\mu\text{m}^3$  regardless of the initial cell volume, which ranged from 450 to 1,280  $\mu\text{m}^3$  in various experiments, strongly suggesting that both treatments removed the same type of capsular material. This is supported by the results of sugar composition analysis, which showed similar compositions for the fractions removed by DMSO and radiation alone (Table 2) in regard to the xylose/mannose ratio. The higher capsule density in the region closest to the cell wall (20) could make this portion of the capsule more resistant to removal by either physical or chemical methods.

The precision with which either radiation or DMSO reduced the capsule volume to 65 to 66  $\mu\text{m}^3$  was striking, since the mechanisms of capsule removal by these agents are almost certainly quite different. DMSO is a dipolar aprotic solvent with a dielectric constant  $\epsilon$  of 45, and DMSO-mediated solubilization of the capsule is most likely the result of disruption of noncovalent interactions between polysaccharide molecules. In contrast, gamma radiation generates highly reactive OH radicals through the radiolysis of water, which may promote the removal of parts of the polysaccharide capsule by a chemical reaction that either breaks the polymer into smaller fragments or disrupts noncovalent interactions holding the capsule together. The observation that both of the free-radical scavengers ascorbic acid and sorbitol protect the capsule against radiation is consistent with and supportive of this mechanism.

The chemical composition of the naturally shed exopolysaccharide was similar to that of the outer layer of the capsule and close to the one predicted from Cherniak's classic analysis (10). In addition we noted the absence of galactose in the fraction removed by radiation alone. Galactose presumably originates from galactoxylomannan, a minor component of capsular polysaccharide which is also made by acapsular cells and whose role in capsule architecture, if any, is unknown. The compositions of the outer layer of the capsule removed by either DMSO or radiation alone were similar to each other (Table 2), consistent with the view that DMSO and radiation remove the same part of the capsule. Neither radiation nor DMSO alone removed the portion of the capsule in the region closest to the cell wall.

One potential explanation for this phenomenon is that the inner radiation- and DMSO-resistant capsule and the outer radiation- and DMSO-susceptible regions have different chemical composition, branching or different density of packed molecules. In this regard, the glycosyl analysis established differences in the proportion of different sugar components, with the inner capsule closest to the cell wall having more xylose and less glucuronic acid relative to the outer capsule (Table 2). We note that arabinogalactan protects against radiation (33) and it is possible that galactoxylomannan in the inner capsule contributed to the radioresistance of that layer. While total glycosyl analysis can measure differences in the chemical composition of polysaccharides, it is unlikely to detect differences in branching. Although branching has not been described for *C. neoformans* GXM, other microbes have more complicated backbone substitutions. In this regard the GXM of *Cryptococcus flavescens* may have side chains consisting of more than two xylose residues (23).

The fact that HPLC analysis of inner and outer capsule fractions revealed similar molecular weight elution profiles is consistent with the thesis that different branching, packing, and/or substitution and not molecular weight distinguishes the two capsular layers. Tighter molecular packing could result in a more stable structure near the cell wall whereby such molecules are tightly held together, presumably, by multiple intermolecular hydrogen bonds. In fact, the fibers of the polysaccharide cellulose are known for their tensile strength, which is attributed to molecules connected to each other by multiple hydrogen bonds. We suggest that the mechanism for the combined or sequential action of DMSO and radiation involves damage to the polysaccharide by OH radicals, which allow

deep penetration of DMSO into the structure, where it completes the decapsulation process by interfering with hydrogen bonding.

Serological analysis of the residual capsule after different treatments demonstrated the ability of residual capsule postradiation and postDMSO treatment to bind monoclonal antibody 2H1, indicating that the epitope recognized by this antibody remained intact after exposure of *C. neoformans* to radiation or organic solvent. In contrast, the combined and sequential action of gamma radiation and DMSO removed the residual capsule from H99 cells, such that it was not visible by either light or immunofluorescence microscopy.

From the determination of total polysaccharide by the phenol-sulfuric acid method per H99 cell we conclude that almost half (44%) of the total polysaccharide in the capsule is present as a low molecular mass species ( $>5 \times 10^4$  Da), which is easily removed by either radiation or DMSO. The relationship between the high and low polysaccharide species is unknown but we suspect that the low-molecular-mass species may be precursors of the large molecules, given recent evidence that GXM components are synthesized intracellularly and traffic to external spaces where the capsule is presumably assembled (19). After the outer layer is removed, the inner layer consisting mostly of the high molecular mass polysaccharide can be removed with additional amounts of DMSO or irradiation time. Although our decapsulation studies indicate the presence of two layers in the capsule, it is also possible that the density and composition of the capsule are continuous, such that changes occur as a function of radial position. Interestingly, the amount of total polysaccharide per cell (8.4 pg) obtained in this work is very close to the number calculated by us from the data reported by Goren and Middlebrook several decades ago, 8 pg per cell (21).

In conclusion, we employed a combination of radiological, chemical and serological techniques to investigate the structure and composition of *C. neoformans* capsule. In aggregate, our results provide strong evidence that the capsule of *C. neoformans* cells has two layers that differ in their physical and chemical properties. Furthermore, our findings have significant implications for the use of radiation as an anti-infective modality, since they suggest that capsules can protect against external gamma radiation while providing a target for the delivery of microbicidal particulate radiation by radiolabeled capsule-binding antibodies.

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#### REFERENCES

- Bergman, F. 1965. Studies on capsule synthesis of *Cryptococcus neoformans*. *Sabouraudia* **4**:23–31.
- Bhattacharjee, A. K., J. E. Bennett, and C. P. J. Glaudemans. 1984. Capsular polysaccharides of *Cryptococcus neoformans*. *Rev. Infect. Dis.* **6**:619–624.
- Bose, I., A. J. Reese, J. J. Ory, G. Janbon, and T. L. Doering. 2003. A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot. Cell* **4**:655–663.
- Brooks, S. E., and D. L. Schneider. 1985. Oxidative metabolism associated with phagocytosis in *Acanthamoeba castellanii*. *J. Protozool.* **32**:330–333.
- Casadevall, A., E. Dadachova, and L. Pirofski. 2004. Passive antibody therapy for infectious diseases. *Nat. Rev. Microbiol.* **2**:695–703.
- Casadevall, A., and J. R. Perfect. 1998. *Cryptococcus neoformans*, p. 147–151. ASM Press, Washington, D.C.
- Casadevall, A., M. DeShaw, M. Fan, F. Dromer, T. R. Kozel, and L. A. Pirofski. 1994. Molecular and idiotypic analysis of antibodies to *Cryptococcus neoformans* glucuronoxylomannan. *Infect. Immun.* **62**:3864–3872.
- Chang, Y. C., and K. J. Kwong-Chung. 1994. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol. Cell. Biol.* **14**:4912–4919.
- Cherniak, R., Morris, L. C., Anderson, B. C., and S. A. Meyer. 1991. Facilitated isolation, purification, and analysis of glucuronoxylomannan of *Cryptococcus neoformans*. *Infect. Immun.* **59**:59–64.
- Cherniak, R., and J. B. Sundstrom. 1994. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. *Infect. Immun.* **62**:1507–1512.
- Cleare, W., and A. Casadevall. 1999. Scanning electron microscopy of encapsulated and non-encapsulated *Cryptococcus neoformans* and the effect of glucose on capsular polysaccharide release. *Med. Mycol.* **37**:235–243.
- Cruickshank, J. G., R. Cavill, and M. Jelbert. 1973. *Cryptococcus neoformans* of unusual morphology. *Appl. Microbiol.* **25**:309–312.
- Dadachova, E., T. Burns, R. A. Bryan, C. Apostolidis, M. W. Brechbiel, J. D. Nosanchuk, A. Casadevall, and L. Pirofski. 2004. Feasibility of radioimmunotherapy of experimental pneumococcal infection. *Antimicrob. Agents Chemother.* **48**:1624–1629.
- Dadachova, E., R. W. Howell, R. A. Bryan, A. Frenkel, J. D. Nosanchuk, and A. Casadevall. 2004. Susceptibility of human pathogens *Cryptococcus neoformans* and *Histoplasma capsulatum* to gamma radiation versus radioimmunotherapy with alpha- and beta-emitting radioisotopes. *J. Nucl. Med.* **45**:313–320.
- Dadachova, E., A. Nakouzi, R. Bryan, and A. Casadevall. 2003. Ionizing radiation delivered by specific antibody is therapeutic against a fungal infection. *Proc. Natl. Acad. Sci. USA* **100**:10942–10947.
- Dembitzer, H. M., I. Buza, and F. Reiss. 1973. Biological and electron microscopic changes in gamma-radiated *Cryptococcus neoformans*. *Mycopathol. Mycol. Appl.* **47**:307–315.
- Doering, T. L. 2000. How does *Cryptococcus* get its coat? *Trends Microbiol.* **8**:547–553.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350–356.
- Garcia-Rivera, J., Chang, Y. C., Kwon-Chung, K. J., and A. Casadevall. 2004. *Cryptococcus neoformans* CAP59 (or Cap59p) is involved in the extracellular trafficking of capsular glucuro-noxylomannan. *Eukaryot. Cell* **3**:385–392.
- Gates, M. A., P. Thorkildson, and T. R. Kozel. 2004. Molecular architecture of the *Cryptococcus neoformans* capsule. *Mol. Microbiol.* **52**:13–24.
- Goren, M. B., and G. M. Middlebrook. 1967. Protein conjugates of polysaccharide from *Cryptococcus neoformans*. *J. Immunol.* **98**:901–913.
- Hall, E. 2000. Radiobiology for a radiologist. Lippincott, Philadelphia, Pa.
- Ikeda, R., and T. Maeda. 2004. Structural studies of the capsular polysaccharide of a non-*neoformans* *Cryptococcus* species identified as *C. laurentii*, which was reclassified as *Cryptococcus flavescens*, from a patient with AIDS. *Carbohydr. Res.* **339**:503–509.
- Kozel, T. R., and C. A. Hermerath. 1984. Binding of cryptococcal polysaccharide to *Cryptococcus neoformans*. *Infect. Immun.* **43**:879–886.
- Lengeler, K. B., G. M. Cox, and J. Heitman. 2001. Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Infect. Immun.* **69**:115–122.
- Love, G. L., G. D. Boyd, and D. L. Greer. 1985. Large *Cryptococcus neoformans* isolated from brain abscess. *J. Clin. Microbiol.* **22**:1068–1070.
- Morelli, R., S. Russo-Volpe, N. Bruno, and R. Lo Scalzo. 2003. Fenton-dependent damage to carbohydrates: free radical scavenging activity of some simple sugars. *J. Agric. Food Chem.* **51**:7418–7425.
- Mukherjee, J., W. Cleare, and A. Casadevall. 1995. Monoclonal antibody mediated capsular reactions (quellung) in *Cryptococcus neoformans*. *J. Immunol. Methods* **184**:139–143.
- Reese, A. J., and T. L. Doering. 2003. Cell wall alpha-1,3-glucan is required to anchor the *Cryptococcus neoformans* capsule. *Mol. Microbiol.* **50**:1401–1409.
- Sharpaty, V. A. 1999. Radiochemistry of polysaccharides. *Radiat. Biol. Radioecol.* **39**:156–161.
- Sorenson, J. A., and M. E. Phelps. 1987. Physics in nuclear medicine. W. B. Saunders Company, Philadelphia, Pa.
- Steenbergen, J. N., H. A. Shuman, and A. Casadevall. 2001. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci. USA* **98**:15245–15250.
- Subramanian, M., G. J. Chintalwar, and S. Chattopadhyay. 2002. Antioxidant properties of a *Tinospora cordifolia* polysaccharide against iron-mediated lipid damage and gamma-ray induced protein damage. *Redox Rep.* **7**:137–143.
- van den Hoogen, B. M., P. R. van Weeren, M. Lopes-Cardozo, L.M. van

- Golde, A. Barneveld, and C. H. van de Lest.** 1998. A microtiter plate assay for the determination of uronic acids. *Anal. Biochem.* **257**:107–111.
35. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. *Methods Enzymol.* **118**:3–40.
36. **Zaragoza, O., and A. Casadevall.** 2004. Experimental modulation of capsule size in *Cryptococcus neoformans*. *Biol. Proc.* **6**:10–15.
37. **Zaragoza, O., B. C. Fries, and A. Casadevall.** 2003. Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO<sub>2</sub>. *Infect. Immun.* **71**:6155–6164.
38. **Zaragoza, O., C. Taborda, and A. Casadevall.** 2003. The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. *Eur. J. Immunol.* **33**:1957–1967.