Nonencapsulated Variant of *Cryptococcus neoformans*

I. Virulence Studies and Characterization of Soluble Polysaccharide

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A weakly virulent nonencapsulated variant of *Cryptococcus neoformans* is described. The chemical structure and antigenicity of the soluble polysaccharides produced by the variant strain and a typical virulent strain were compared. The soluble polysaccharides produced by both strains were composed of the same constituent monosaccharides; however, the virulent strain produced a polysaccharide having a greater uronic acid content and a larger molecular size than that of the variant strain. Soluble polysaccharides from the two strains are not closely related immunologically. Soluble polysaccharide obtained from the virulent strain did not affect persistence of the variant strain in mice.

Several small-capsule or nonencapsulated variants of *Cryptococcus neoformans* have been reported. Kase and Metzgar (16) isolated a small-capsule variant which was more virulent than the parent strain. A polymyxin B resistant strain isolated by Bodenhoff (6) had a reduced capsule and was avirulent for mice. Bulmer, Sans, and Gunn (7) isolated seven nonencapsulated mutants of *C. neoformans* which were avirulent for mice. Six of the seven isolates reverted to the encapsulated virulent state, and this reversion, in some cases, appeared to involve several stages of development. The authors speculated that prior to the reversion to the virulent phenotype, the capsules may have been in an immature state as far as being a functional virulence factor.

The purpose of this study was to compare the chemical structure and antigenicity of the soluble polysaccharides produced by a virulent encapsulated and a weakly virulent unencapsulated strain of *C. neoformans* and to examine the influence of the soluble polysaccharide of the virulent strain on the persistence of the weakly virulent strain in mice.

MATERIALS AND METHODS

Strains of *C. neoformans*. *Cryptococcus neoformans* strain 602 was isolated in 1957 at the University of Iowa Hospitals from spinal fluid of a patient with a clinical and laboratory diagnosis of cryptococcal meningitis. This strain of *C. neoformans*, although originally encapsulated, lost the ability to produce visible capsular material. India ink preparations made from in vitro cultures or from brain of mice inoculated with strain 602 reveal only unencapsulated cells.

Macroscopically, colonies of strain 602 appear dry and unlike the mucoid, glistening colonies commonly described for *C. neoformans*.

Cryptococcus neoformans strain 613 was isolated in 1961 from spleen and lung specimens obtained at autopsy from a patient with a clinical diagnosis of lobar pneumonia. The organism produced a capsule 1.2 μ m in width on Littman's medium. The median lethal dose (LD₅₀) for mice (60 days) by the intravenous route at the time of this study was about 4.9×10^3 viable units.

Stock cultures of *C. neoformans* strains 602 and 613 were maintained on Sabouraud's dextrose agar slants stored at 5 C. Regular transfers to fresh media were made at approximately 6-month intervals. Both strains satisfied the standard mycological classification of Kreger-van Rij (17). In addition, these strains assimilated creatinine, and they produced urease and deoxyribonuclease.

When the organisms were tested by the serologic grouping method of Wilson, Bennett, and Bailey (22), *C. neoformans* strain 613 reacted as a typical type D, whereas strain 602 reacted as an untypable strain. Cultures of *C. neoformans* serotypes A, B, C, and D were obtained from John E. Bennett, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Mice. Four-week-old male white Swiss mice from a Webster strain maintained by the Department of Microbiology at the University of Iowa were used throughout the study. Food and water were supplied to the animals ad libitum.

Cortisone treatment of mice. The injection schedule for cortisone treatment began on the day before challenge inoculation. Each mouse received a total of five 1-mg subcutaneous doses of cortisone at 4-day intervals. This strain of mice has consistently shown the ability to tolerate a total dosage of 5 mg of cortisone administered over a period of 18 days; 10 mg of

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cortisone administered over the same period resulted in death of 40% of the animals.

Antiserum production. *Cryptococcus neoformans* strains 602 and 613 were grown on a yeast extract dialysate medium (14) at room temperature for 48 hr. The cells were harvested by centrifugation, washed twice in saline (0.15 M sodium chloride) containing 1% formalin, and stored in formalinized saline overnight at 37 C. Finally, the cells were washed four times with saline and standardized by hemocytometer counts. Adult male New Zealand white rabbits obtained from a local commercial supplier were immunized with 10 daily intravenous injections of 2.5×10^7 cells. Animals were bled, and serum was collected 7 days after conclusion of the immunization schedule.

Purification of soluble polysaccharide. The method used was similar to that used by Evans and Theriault (10). Cultures were grown in 7-liter amounts for 4 days on yeast extract dialysate medium, and cells were subsequently separated from the medium by centrifugation at $1,000 \times g$ for 1 hr. The supernatant fluid was sterilized by filtration and concentrated to 200 ml in a Dia-flo pressure cell (Amicon Corp.) equipped with a XM-50 membrane. Sodium acetate crystals (10 g) and 1 ml of glacial acetic acid were added for each 100 ml of concentrated supernatant fluid, and the polysaccharide was precipitated by adding 2.5 volumes of 95% ethyl alcohol dropwise with constant mechanical stirring. The precipitate was redissolved in 350 ml of distilled water containing 1% glacial acetic acid and 10% sodium acetate. Deproteinization was accomplished by the method of Kabat and Mayer (15) for pneumococcal polysaccharides. The deproteinized polysaccharide was precipitated from solution by adding 2.5 volumes of ethanol dropwise with constant mechanical stirring. The precipitate was redissolved in 500 ml of distilled water, and the solution was cleared by dropwise addition of 1.5 volumes of ethanol followed by centrifugation at $16,000 \times g$ for 1 hr. The polysaccharide was precipitated from solution by addition of 50 g of sodium acetate and 5 ml of glacial acetic acid. After centrifugation, the polysaccharide was redissolved in 200 ml of distilled water, dialyzed against distilled water to remove sodium acetate, lyophilized, and dried to a constant weight in vacuo over anhydrous CaSO_4 .

Hydrolysis of soluble polysaccharide. A 1-mg sample of polysaccharide was dissolved in 1 ml of 1.0 N HCl and heated in a sealed glass vial at 100 C for 4 hr. The hydrolysate was lyophilized to remove HCl, and the residue was dissolved in 0.2 ml of distilled water.

Thin layer chromatography. Carbohydrates found in acid hydrolysates of cryptococcal polysaccharide were separated and identified by the use of thin layer chromatography. Thin layer plates were prepared in 0.250-mm thick layers from Adsorbosil 1 (Applied Science Lab.) in 0.02 M sodium acetate buffer. Before use, the plates were activated by heating at 120 C for 15 min. The solvent system used consisted of butanol-acetic acid-water (6:3:1, v/v). The indicator solution consisted of 1 g of diphenylamine and 1 ml of aniline dissolved in 100 ml of acetone. Immediately prior to spraying the plates, 1 ml of 85% phosphoric acid was mixed with each 10 ml of the acetone indicator solu-

tion. After the plates were sprayed, they were developed in a hot air oven at 120 C for 15 min.

Gel chromatography. Molecular-sieve chromatography was done on columns (2.5 by 50 cm) of Bio-Gel A-15m, 200 to 400 mesh (Calbiochem, Los Angeles, Calif.) with 0.15 M NaCl as eluant. The column was calibrated in terms of V_0 (Blue dextran 2,000) and V_i (sucrose). Samples of polysaccharide (2 ml) at a concentration of 1 mg/ml in 0.15 M NaCl were applied to the column. Fractions were collected, and the sugar content of each was determined by the phenol-sulfuric acid method (8).

Passive hemagglutination. Using a modification of the method of Baker, Stashak, and Prescott (2) for pneumococcal polysaccharides, sheep red cells stored in Alsever's solution were washed three times with saline and sensitized with polysaccharide. Saline (1 ml) containing 1.0 mg of purified polysaccharide was added to 0.6 ml of packed sheep erythrocytes. A fresh solution (1 ml) of saline containing 0.5 mg of chromium chloride ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$) was added dropwise with constant stirring. The cell suspension was incubated 7 min at room temperature, washed four times with saline, and adjusted to a final concentration of 0.5%.

All serum specimens were heat inactivated at 56 C for 30 min before use. Antisera was titrated by the microtiter method in "V" plates. Serial dilutions of serum were done in 25- μ l volumes in saline containing 1% inactivated normal rabbit serum (NRS). Results were recorded 2.5 hr after addition of 25 μ l of the 0.5% sensitized erythrocyte suspension to serum dilutions. Two controls were included to determine the specificity of any agglutination observed in titration of sera. Serum diluted in saline containing 1% NRS and a predetermined excess (100 μ g/ml) polysaccharide served as control for specificity of the observed agglutination. Hemagglutination due to a specific antigen-antibody reaction was inhibited by polysaccharide in the diluent. In addition, presence of natural sheep red blood cell agglutinins was determined by incubating serum dilutions with unsensitized sheep erythrocytes.

The ability of polysaccharides to inhibit the agglutination of sensitized erythrocytes by antiserum was determined in a manner similar to that used by Stavitsky and Arquilla (19). The antiserum was diluted in saline containing 1% NRS as in the PHA assay. To each dilution of antiserum was added 25 μ l of the inhibiting polysaccharide (100 μ g/ml in saline). A control consisted of serum incubated with 25 μ l of saline. After shaking, the mixtures were incubated at room temperature for 30 min, the sensitized erythrocytes were added, and the plates were incubated as in the PHA assay. The presence of a cross-reacting or identical polysaccharide was shown by a decrease in the PHA titer compared to the control.

Tissue population determination. Brains and spleens were aseptically removed from mice and placed in glass tissue homogenizers (Bellco Glass, Inc., Vineland, N.J.; model 1977). Weight of each tissue was determined, 1 ml of sterile 0.15 M NaCl was added, and the tissues were gently homogenized. Blood specimens were collected in heparinized tubes from

the ophthalmic venous plexus of mice prior to autopsy. Serial 10-fold dilutions were prepared of each specimen and pour plates of each dilution were prepared in potato dextrose agar. Calculations of the number of viable units present per 100 mg of tissue or 0.1 ml of blood were made on the basis of colony counts from plates incubated at room temperature for 4 days.

RESULTS

Virulence of *C. neoformans* strain 602. Only four deaths occurred in 60 mice given massive doses of strain 602 by the intraperitoneal, intravenous, or intracerebral routes. Two deaths, 34 days after challenge, occurred in the group receiving 10^7 cells intracerebrally, and two deaths also occurred 17 days after challenge in those mice treated with cortisone (Table 1). All other mice survived the 60-day period of observation, including those given 10^8 cells intraperitoneally in hog gastric mucin (20). This is in sharp contrast to the virulence of strain 613 whose LD₅₀ (60 days) for mice was approximately 4.9×10^8 viable units when administered intravenously.

Soluble polysaccharide of strain 602. Approxi-

mately 20 mg of purified soluble polysaccharide was isolated from 7 liters of culture fluid from strain 602. Cultures of strain 613 grown under identical conditions yielded 252 mg of purified soluble polysaccharide. A sample of each poly-

TABLE 1. Virulence of *Cryptococcus neoformans* strain 602^a

Route of injection and treatment	No. viable units injected	Deaths per total number of mice
Intraperitoneal	2×10^7	0/10
Intraperitoneal	10^8	0/10
Intracerebral	10^7	2/10
Intravenous	10^7	0/10
Intravenous, ^b cortisone	10^7	2/10
Intraperitoneal, ^c hog gastric mucin	10^8	0/10

^a Mice were observed for 60 days.

^b Mice received 5 mg of cortisone subcutaneously over 16 days.

^c Inoculum was emulsified in hog gastric mucin.

FIG. 1. Thin layer chromatographic separation of *Cryptococcus neoformans* capsular components. 1. Mixture of known compounds, 10 μ g each. 2. Hydrolysate of 50 μ g of 613 soluble polysaccharide. 3. Hydrolysate of 100 μ g of 602 soluble polysaccharide. 4. Mixture of known compounds, 10 μ g each.

saccharide was hydrolyzed, and the sugars were separated by thin layer chromatography to determine whether each of the polysaccharides contained the same constituent carbohydrates that Blandamer and Danishefsky (5) considered essential components of acid hydrolyzed cryptococcal polysaccharide. Figure 1 is an overlay tracing from a chromatogram of a mixture of known standards (10 μ g each of glucuronolactone, xylose, mannose, galactose, and glucuronic acid) and the hydrolysates of 50 μ g of 613 soluble polysaccharide and 100 μ g of 602 soluble polysaccharide. Glucuronolactone did not appear in hydrolysates of either strain 613 or 602. This is not surprising considering the reports of Evans and Theriault (10) and Blandamer and Danishefsky (5) that indicate only small amounts of the lactone will appear unless the hydrolysis is quite prolonged. The remaining four carbohydrates and a faint spot presumably due to oligosaccharides (5) appeared in hydrolysates of both polysaccharides. Based on intensity of the two spots, the hydrolysate from the soluble polysaccharide of strain 602 appeared to contain less glucuronic acid than that of strain 613. This observation was substantiated when the uronic acid content of each polysaccharide was determined by the Dische carbazole assay for uronic acid (15). The uronic acid content of the soluble polysaccharide was found to be 25% for strain 613 but only 8% for strain 602.

The soluble polysaccharide of strain 602 dissolved in water much more readily than 613

polysaccharide, suggesting that the molecular weight of 602 polysaccharide might be less than that of 613 polysaccharide. The relative molecular size of soluble polysaccharides produced by *C. neoformans* strains 613 and 602 was determined by gel chromatography on 4% agarose beads. The optical density (490 nm) of fractions after treatment with phenol and sulfuric acid is plotted against elution volume (Fig. 2). Soluble polysaccharide of strain 613 was excluded from the gel and migrated with the void volume; that of strain 602 penetrated the gel completely and migrated with the inner volume of the gel. Dialyzed culture filtrates of strain 613 and strain 602 showed elution profiles similar to that of purified polysaccharide preparations.

Soluble polysaccharides obtained from *C. neoformans* strains 602, 613 and known serotypes A, B, C, and D were tested by PHA for immunological activity with rabbit antiserum prepared against whole cells of strains 602 and 613 (Table 2). Antiserum to cells of strain 613 specifically agglutinated erythrocytes sensitized with all polysaccharide preparations but only at a low titer with 602 sensitized erythrocytes. Antiserum to cells of strain 602 agglutinated sheep erythrocytes coupled to 602, 613, and type D soluble polysaccharides, whereas specific hemagglutination was not observed with erythrocytes coupled to polysaccharides of serotypes A, B, and C. Polysaccharide-specific hemagglutination was neutralized by incorporating in the diluent an excess of the same polysaccharide used to sensitize erythro-

FIG. 2. Elution profile of soluble polysaccharides from *Cryptococcus neoformans* strains 613 and 602 on Bio-Gel A-15 m (200 to 400 mesh) column. Carbohydrate in each fraction was determined by the phenol-sulfuric acid assay and plotted in terms of absorbance at 490 nm.

TABLE 2. Agglutination of erythrocytes sensitized with *Cryptococcus neoformans* soluble polysaccharides by immune rabbit serum

Polysaccharide used for sensitization	Antiserum type	Reciprocal of antiserum dilution									Specificity control titer ^a	Sheep erythrocyte agglutinin titer ^b
		2	4	8	16	32	64	128	256	512 and 1024		
613	613	4 ^c	4	4	4	4	4	4	2	0	1/2	1/2
602	613	4	4	3	2	1	0	0	0	0	1/2	1/2
Type A	613	4	4	4	4	3	0	0	0	0	1/2	1/2
Type B	613	4	4	4	4	4	4	0	0	0	1/2	1/2
Type C	613	4	4	4	3	0	0	0	0	0	1/2	1/2
Type D	613	4	4	4	4	4	4	2	0	0	1/2	1/2
613	602	4	4	4	4	0	0	0	0	0	1/4	1/4
602	602	4	4	4	4	4	1	0	0	0	1/4	1/4
Type A	602	4	1	0	0	0	0	0	0	0	1/2	1/4
Type B	602	4	1	0	0	0	0	0	0	0	1/2	1/2
Type C	602	4	1	0	0	0	0	0	0	0	1/2	1/2
Type D	602	4	4	4	4	0	0	0	0	0	1/2	1/2

^a Titer of sera diluted in saline containing 100 μ liters/ml of the polysaccharide used for sensitization.^b Titer of sera for unsensitized sheep erythrocytes.^c 4+ = complete hemagglutination; 1+, 2+, 3+ = partial agglutination; 0 = no agglutination.

TABLE 3. Agglutination inhibition of 613 sensitized erythrocytes in homologous antiserum by 602 polysaccharide

Preincubations of antisera with ^a	Reciprocal of 613 antiserum dilution								Sheep erythrocyte agglutinin titer ^b
	2	4	8	16	32	64	128	256 to 1024	
Saline	4 ^c	4	4	4	4	4	1	0	1/2
602 polysaccharide (100 μ g/ml) in saline	4	4	4	2	1	0	0	0	1/2
613 polysaccharide (100 μ g/ml) saline	4	0	0	0	0	0	0	0	1/2

^a 0.25 μ liter of inhibitor was incubated 30 min at room temperature with each dilution of antiserum.^b Titer of sera for unsensitized sheep erythrocytes.^c 4+ = complete agglutination; 1+, 2+, 3+ = partial agglutination; 0 = no agglutination.

cytes. Low hemagglutination titers not neutralized by excess polysaccharide were shown to be due to natural sheep red blood cell agglutinins.

Further evidence for a weak immunologic relationship between polysaccharides of strains 602 and 613 was shown by inhibition of hemagglutination (Table 3). When dilutions of 613 antiserum were incubated with saline containing no inhibitor, the PHA titer was 1:64. Titration of the same antiserum in saline containing 100 μ g/ml of 602 polysaccharide reduced the titer to 1:8. When the antiserum was incubated with saline containing 100 μ g/ml of 613 polysaccharide, the titer was reduced to 1:2 which could be accounted for by the presence of natural sheep red blood cell agglutinins.

Effect of 613 polysaccharide on persistence of strain 602 in mice. A group of 40 mice was injected intravenously with 1 mg of soluble polysaccharide

of strain 613 per mouse. A second group of 40 mice received a placebo consisting of 1 mg of a mixture of glucuronic acid, mannose, xylose, and galactose in a ratio of 15:46:30:8 (w/w). The ratio of components in this mixture is similar to that reported for unhydrolyzed polysaccharide (5). After 24 hr all mice were challenged intravenously with 10^6 cells of *C. neoformans* strain 602. Each group of mice received injections of either the polysaccharide or placebo twice a week throughout the experiment. Five mice from each group were autopsied at varying intervals after the challenge inoculation. The number of viable cryptococci in the spleen, blood, and brain was determined as described. There was no significant difference between the group receiving the polysaccharide and the group receiving the placebo for any tissue studied (Fig. 3). No mice died during the 60-day experiment.

FIG. 3. Clearance of *Cryptococcus neoformans* 602 from tissue of mice. All animals were inoculated intravenously with 10^6 cells. Treated animals received, twice weekly, 1 mg of purified polysaccharide from *C. neoformans* 613. Control animals received, twice weekly, 1 mg of the constituent carbohydrates in cryptococcal polysaccharide. Cell counts from spleen and brain are in terms of viable units per 100 mg of tissue; counts from blood are in terms of number of viable units per 0.1 ml.

DISCUSSION

Cryptococcus neoformans strain 602 occasionally killed mice when injected intracerebrally in very high concentrations or when injected into mice treated with high doses of cortisone. Whereas the strain of mice used in this study is known to tolerate, without lethal effect, the concentration of cortisone administered, we cannot rule out the possibility that cortisone may have had some ill effect on the animals. It can not be concluded whether strain 602 is more or less virulent than the avirulent mutants described by Bulmer et al. (7) and Bodenhoff (6) since different experimental conditions existed in each case. Deaths did not occur, however, when strain 602 was administered in intraperitoneal (7) or intravenous (6) doses comparable to those used with the reported avirulent mutants, nor did deaths occur under experimental conditions used by other investigators (3, 20, 21) to determine virulence.

Thin layer chromatography studies on acid hydrolysates of the soluble polysaccharide produced by *C. neoformans* strain 602 showed that even though the yeast may have no visible capsule, it does produce a soluble polysaccharide containing all sugars found in the capsular polysaccharide of virulent strains. It would appear that

strain 602 possesses at least some of the genetic requirements for capsule production. Thin layer chromatographic results suggest that soluble polysaccharides of strains 602 and 613 are not identical. Hydrolysates of the polysaccharides differed in intensity of the glucuronic acid spots, and the uronic acid content of the soluble polysaccharide produced by strain 613 was found to be approximately three times that of the 602 polysaccharide.

Another marked difference between the two polysaccharides was in molecular size, with the polysaccharide of 602 being much smaller than that of 613. There are conflicting reports on the molecular weight of cryptococcal polysaccharide. Gadebusch, Ward, and Frenkel (12) found the molecular weight to be 1.2×10^6 to 1.5×10^6 on the basis of diffusion and sedimentation data. Fahri, Bulmer, and Tacker (11) using ultracentrifugation studies reported the molecular weight to be in the range of 10,000 to 15,000. Miyazaki (18) calculated the molecular weight at 6,600 on the basis of reducing end-group analysis. Using 4% agarose (Sephacrose 4B), Anderson, Dea, and Munro (1) reported on the chromatographic behavior of nine acidic polysaccharides in the fractionation range of approximately 3×10^5 to 30×10^5 . Since 613 polysaccharide was ex-

cluded from the 4% agarose gel, we must assume that it has a molecular weight in the upper fractionation range of the gel. This would make our data more compatible with that obtained by Gadebusch et al. (12). Purification techniques used by Miyazaki (18) were vigorous and may have resulted in degradation of the polysaccharide into smaller subunits.

The small size of the soluble polysaccharide of strain 602 might help explain the absence of a visible capsular gel around the organism. Evans (9) suggested that cryptococcal capsule was probably a highly concentrated polysaccharide gel or a complex between the soluble polysaccharide and one or more undefined substances. Goren and Middlebrook (13) felt that the capsule was a lightly crosslinked gel perhaps acting through the intervention of a basic protein.

If the capsule is comprised of long random chain molecules, which in highly concentrated solutions have become entangled, very long molecules would have a greater opportunity to become enmeshed and would have less tendency to separate than short molecules. If the gel is composed of a lightly cross-linked matrix (13), the stability of the gel would also depend upon the molecular size. As the length of each chain increases, the number of attachment points per chain would increase leading to a more highly crosslinked and, therefore, more stable gel.

PHA assays showed that strain 602 is most closely related to *C. neoformans* serotype D since antiserum to strain 602 will react only with type D sensitized erythrocytes. This pattern of serologic reactivity is unique among strains of *C. neoformans* since strains of serotype D cross-react with other serotypes (22). Passive hemagglutination inhibition results confirm that strain 602 is deficient in some antigenic determinants possessed by 613, a strain of serotype D.

Bulmer et al. (7) reported that all but one of their seven nonencapsulated avirulent mutants of *C. neoformans* would revert to the encapsulated virulent phenotype. This reversion appeared to involve several stages of development. Three of the mutants reverted to an encapsulated state but remained avirulent for mice. Another of the isolates progressed through at least two stages of development before an intact capsule could be seen. On the basis of these observations, one might speculate that there could be structural or antigenic differences between the capsules of the virulent and avirulent stages of the same strain. *Cryptococcus neoformans* strain 602 may be such a strain that has stabilized in one of these intermediate stages where soluble polysaccharide can be detected chemically and immunologically

despite the absence of a visible capsule. Our data appear to confirm that structural and antigenic differences may exist between the polysaccharides of virulent and weakly virulent strains of *C. neoformans*.

Unlike the results of Bennett and Hasenclever (4) who worked only with virulent strains of *C. neoformans*, injection of soluble cryptococcal polysaccharide into mice did not enhance the virulence of *C. neoformans* strain 602. This might imply that the polysaccharide is not a virulence factor. Rather, the genetic factors responsible for virulence may be closely linked to capsule formation, causing a genotypic loss of capsule production to accompany a loss of virulence. It is also possible that injected polysaccharide does not enhance virulence unless the cryptococcal cells are surrounded by an intact capsule.

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