Production of the Hexitol D-Mannitol by Cryptococcus neoformans In Vitro and in Rabbits with Experimental Meningitis

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We studied the ability of Cryptococcus neoformans to produce the hexitol D-mannitol in vitro and in rabbits with experimental meningitis. Twelve of twelve human isolates of C. neoformans produced D-mannitol in yeast nitrogen base plus 1% glucose and released D-mannitol into the medium. In a pilot study, pooled cerebrospinal fluid (CSF) from cortisone-treated rabbits given 3 × 10⁷ C. neoformans H99 intracisternally contained more D-mannitol (identified by gas chromatography and enzymatically) than CSF from normal controls or cortisone-untreated rabbits with self-limited meningitis. In a second experiment, cortisone-treated rabbits given C. neoformans intracisternally had significantly higher CSF D-mannitol concentrations than controls given cortisone alone at 4, 6, and 8 days after infection. Moreover, log₁₀ CSF D-mannitol correlated well with log₁₀ CSF CFU (r = 0.81) and log₁₀ CSF cryptococcal antigen titer (r = 0.78). Lastly, the initial volume of distribution and elimination half-life of D-mannitol given intracisternally to normal rabbits suggested that D-mannitol was distributed in total CSF and was removed by CSF bulk flow. Thus, C. neoformans produces D-mannitol in vitro and in vivo, and D-mannitol is a quantitative marker for experimental cryptococcal meningitis. D-Mannitol produced by C. neoformans may also contribute to brain edema and interfere with phagocyte killing by scavenging hydroxyl radicals.

The prevalence of cryptococcal meningoencephalitis has increased markedly in recent years, primarily because the human immunodeficiency virus epidemic has greatly expanded the population of patients with profoundly deficient cellular immunity. A serious problem among patients with the acquired immune deficiency syndrome (AIDS) and cryptococcosis is that most of these patients relapse after receiving antifungal therapy that cures most patients with other underlying diseases (11, 28). This problem is compounded by the fact that AIDS patients who will eventually relapse cannot be differentiated reliably from those who will not by clinical or available laboratory criteria. It has therefore been proposed that all AIDS patients with cryptococcal meningitis should receive prolonged or even lifelong suppressive therapy with the toxic antifungal agent amphotericin B (11, 28, 29). Much of the drug toxicity, inconvenience, and expense that this approach necessarily entails might be avoided if better quantitative methods for assessing the effects of treatment on fungal load were available.

It has long been known that many fungi produce large amounts of acyclic polyols (23), and recent studies have shown that Candida and Aspergillus species produce sufficient amounts of their polyol metabolites in infected mammalian hosts to cause increased body fluid concentrations. For example, the Candida species responsible for almost all cases of human infection produce large amounts of the pentitol D-arabinitol in vitro (2, 3, 10), and animals (24) and humans (3, 9, 10, 20, 25, 27) with invasive candidiasis have more D- or Dl-arabinitol in the serum than uninfected controls. It is also known that Candida albicans produces D-arabinitol in infected animals directly in proportion to fungal load (24). Similarly, the Aspergillus species responsible for most cases of human aspergillosis produce large amounts of the hexitol D-mannitol in vitro (S. J. Flaherty, E. M. Bernard, E. McKinney, B. Wong, and D. Armstrong, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, F7, p. 293). Moreover, rats with experimental aspergillosis have higher D-mannitol levels in serum and tissue than uninfected controls, and these levels correlate well with severity of infection as assessed by histology (26).

In 1968, Onishi and Suzuki (14) reported that a strain of Candida neoformans produced D-mannitol in culture, and the study by Flaherty et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1984) confirmed this observation in a few additional strains. However, it is not known whether all or almost all strains of C. neoformans produce D-mannitol in vitro or whether any C. neoformans strain can produce appreciable amounts of D-mannitol in vivo. Therefore, we measured D-mannitol production by cultures of 12 human isolates of C. neoformans. We also analyzed cerebrospinal fluid (CSF) samples from rabbits with experimental cryptococcal meningitis to assess the ability of C. neoformans to produce D-mannitol in vivo.

(Materials and Methods)

In vitro studies. We studied the abilities of 12 human isolates of C. neoformans to produce D-mannitol in vitro and to release it into the extracellular environment as follows. These strains included C. neoformans H99 (serotype A) and 11 strains from the Mycology Laboratory at the University of Cincinnati Hospital (4 type A, 1 type B, 2 type C, 1 type D, and 3 of unknown serotype). Each isolate was inoculated at 10⁵ yeast cells per ml in yeast nitrogen base plus 1% glucose. The cultures were incubated in air with slow shaking at 37°C, and samples were removed after 12, 24, 36, 48, 72, and 96 h. The cells were enumerated in a hemacytometer. Colony counts were determined by culturing serial

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10-fold dilutions on Sabouraud glucose agar at 37°C for 24 to 48 h. Extracellular D-mannitol in the cell-free supernatants was measured by gas chromatography (GC). The samples obtained at 24, 48, and 96 h were also heated to 100°C for 10 min to release intracellular polyols (2), and total D-mannitol (intracellular and extracellular) was measured.

Experimental cryptococcal meningitis. The ability of C. neoformans H99 to produce d-mannitol in vivo was studied by analyzing CSF from rabbits with cryptococcal meningitis (16). New Zealand White rabbits (2 to 3 kg) were housed in individual cages and received Purina rabbit chow and water ad lib. Beginning 1 day prior to infection and daily thereafter, each rabbit received either 2.5 mg of cortisone acetate per kg intramuscularly or no cortisone. On the day of infection, the rabbits were anesthetized with 100 to 150 mg of ketamine and 15 to 25 mg of xylazine intramuscularly, and 0.3 ml of a suspension of 10^8 C. neoformans H99 yeast cells per ml of 0.015 M phosphate-buffered saline was injected into the cisternum magna. The rabbits were reanesthetized, and CSF was obtained by cisternal puncture at intervals thereafter.

Fungal colony counts were determined by culturing serial 10-fold dilutions of 0.1 ml of CSF in phosphate-buffered saline on Sabouraud agar (containing 100 μg of chloramphenicol per ml) at 37°C for 24 to 48 h. The capsular polysaccharide antigen of C. neoformans was measured by testing serial twofold dilutions of CSF in sterile water (starting at 1:20) by latex agglutination (Wampole Laboratories, Cranbury, N.J.). D-Mannitol was measured by GC in the cell-free supernatants of the CSF samples.

In a pilot study, D-mannitol was measured in pooled CSF samples (approximately five to eight rabbits per pool) that had been stored at −70°C for as long as 3 years. These samples included (i) CSF specimens from four groups of uninfected normal rabbits, (ii) serial CSF specimens from cortisone-treated rabbits with experimental cryptococcal meningitis, and (iii) serial CSF specimens from two groups of non-cortisone-treated rabbits with experimental cryptococcal meningitis. CSF colony counts had previously been determined in the samples from the infected rabbits.

D-Mannitol was next measured in CSF from individual cortisone-treated rabbits with experimental cryptococcal meningitis and from uninfected controls. Nine rabbits received cortisone daily and 3 × 10^7 C. neoformans H99 yeast cells intracisternally as described above, and 11 controls received cortisone alone. Approximately 0.5 ml of CSF was obtained from as many infected and control rabbits as was technically feasible on the day before and 2, 4, 6, and 8 days after infection. Fungal colony counts and cryptococcal antigen titers were determined in the specimens from the infected rabbits.

Distribution and elimination of intracisternal D-mannitol. Six rabbits were anesthetized and given 0.275 μmol (50 μg) of D-mannitol in 0.3 ml of phosphate-buffered saline by intracisternal injection. D-Mannitol was measured in CSF obtained after 1, 2, 4, 8, 24, and 48 h and in serum obtained after 2, 4, 8, 24, and 48 h. The apparent volumes of distribution and elimination half-lives were determined from plots of logarithmic concentrations versus time (14).

D-Mannitol measurements. D-Mannitol in serum was measured by GC as described previously (26), and it was measured in culture supernatants and CSF as follows. α-Methylmannoside and α-methylglucoside (1.03 μmol [200 μg] per ml of culture supernatant or 0.103 μmol [20 μg] per ml of CSF) were added as internal standards to 0.2 ml of culture supernatant or to 0.025 to 0.2 ml of CSF (depending on the amount available). The samples were deproteinized by adding 1.0 ml of acetone and by centrifugation at 1,000 × g for 5 min. The supernatants were evaporated to dryness in an N2 stream at 55°C, and the trimethylsilyl ether derivatives were formed by adding 0.1 ml of trimethylsilylimidazole and 0.1 ml of N,N-dimethylformamide to the sample residues and by heating to 50°C for 15 min.

The derivatives were extracted into 0.20 to 0.25 ml of dry hexane. The extracts of derivatized culture supernatants (2 μl) were analyzed by GC with a fused silica CP-Sil 5CB column (25 m by 0.32 mm; 1.2-μm film thickness; Chrompack, Inc., Raritan, N.J.), helium carrier at 35 cm/s, splitless injector at 250°C (septum purge and inlet splitter valves closed for the first 30 s after injection), and a flame ionization detector at 275°C. The column oven temperature was 120°C for 1 min after injection, after which it was increased by 20°C/min to 180°C and by 3°C/min to 240°C. The hexane extracts of derivatized CSF (2 μl) were analyzed with a fused silica SPB-5 column (60 m by 0.32 mm; 1.0-μm film thickness; Supelco, Inc., Bellefonte, Pa.), helium carrier at 40 cm/s, splitless injector at 250°C (septum purge and inlet splitter valves closed for the first 30 s after injection), and a flame ionization detector at 275°C. The oven temperature was 120°C for 1 min after injection, after which it was increased by 25°C/min to 195°C and by 3°C/min to 240°C.

D-Mannitol was quantified in culture supernatants by comparing its peak area with that of α-methylmannoside and in CSF by comparing its peak area with that of the better-resolved internal standard. Relative response factors were determined daily from standard curves derived from five samples of culture medium or three samples of pooled normal rabbit CSF to which known amounts of D-mannitol were added. The standard curves were linear throughout the relevant ranges.

Selected CSF samples were also analyzed after treatment for 30 min at 21°C with the Klebsiella pneumoniae NAD: oxidoreductase D-arabinitol dehydrogenase, rabbit muscle lactate dehydrogenase, sodium pyruvate, and NAD as described previously (25). D-Arabinitol dehydrogenase converts D-mannitol to D-fructose by oxidizing the C2 hydroxyl group (13), and it depletes serum of at least 98% of D-mannitol when NAD is regenerated by a coupled reaction (25, 26). Since the only known substrates of this enzyme are D-arabinitol and D-mannitol (13), disappearance of a peak that coelutes with D-mannitol from D-arabinitol dehydrogenase-treated specimens confirms that the compound of interest is D-mannitol (26).

Statistical methods. The Mann-Whitney test was used to calculate the significance of differences between groups, and the least-squares method was used for linear regression analyses.

RESULTS

In vitro studies. All of the C. neoformans strains studied produced D-mannitol in culture. The range of extracellular D-mannitol concentrations at 96 h was 140 to 2,700 μM (25 to 500 μg/ml). Differences in growth rates were responsible for much of the variation in D-mannitol production; strains that grew faster generally produced and released more D-mannitol than those that grew more slowly. D-Mannitol production by most strains continued after the cultures stopped growing; indeed, several strains produced and released more D-mannitol during the stationary phase than during logarithmic growth. Lastly, higher proportions of total D-mannitol were released into the culture medium as the cultures aged;
extracellular d-mannitol represented 0.54 ± 0.24 of total d-mannitol at 24 h, 0.83 ± 0.12 at 48 h, and 0.95 ± 0.19 at 96 h (means ± standard deviation [SD]) (Fig. 1).

Identification of d-mannitol in CSF. When the pooled CSF specimens were analyzed by GC, a compound with the same retention time as authentic d-mannitol was found in much larger amounts in the samples from the cortisone-treated, infected rabbits than in those from normal uninfected or non-cortisone-treated, infected rabbits. Treatment of the CSF samples with d-arabinitol dehydrogenase, lactate dehydrogenase, NAD, and sodium pyruvate resulted in the almost complete disappearance of the compound of interest, thus confirming its identification as d-mannitol (Fig. 2).

d-Mannitol production by C. neoformans in vivo. Among the rabbits studied in retrospect, the CSF d-mannitol concentrations corresponded well with severity of infection. The CSF colony counts declined over time in the non-cortisone-treated rabbits given C. neoformans intracisternally, and CSF d-mannitol concentrations in these rabbits were no higher than the values in uninfected controls. In contrast, the CSF colony counts and CSF d-mannitol concentrations increased over time in the cortisone-treated rabbits given C. neoformans intracisternally (Fig. 3). These results suggested that C. neoformans produced d-mannitol in proportion to fungal load, but specimens from cortisone-treated controls were unavailable.

Therefore, we next studied individual cortisone-treated rabbits given C. neoformans H99 intracisternally and cortisone-treated, uninfected controls. The fungal colony counts, cryptococcal antigen titers, and d-mannitol concentrations in the infected and control rabbits are summarized in Fig. 4. The CSF d-mannitol concentrations rose substantially over time in the infected rabbits but not in the controls. The mean CSF d-mannitol concentrations (±SD) in the infected and control rabbits, respectively, were as follows: 1.91 ± 0.52 and 1.84 ± 0.34 μM on day −1 (n = 9 and 11, respectively; P = 0.675); 3.01 ± 0.56 and 2.60 ± 0.43 μM on day 2 (n = 7 and 9; P = 0.136); 7.03 ± 6.21 and 2.20 ± 0.40 μM on day 4 (n = 6 and 8; P = 0.008); 36.9 ± 21.6 and 2.14 ± 0.38 μM on day 6 (n = 5 and 8; P = 0.002); and 53.7 ± 37.4 and 2.43 ± 0.66 μM on day 8 after infection (n = 4 and 10; P = 0.001).

Among the infected rabbits, the CSF d-mannitol concentrations correlated well with severity of infection as assessed by CSF fungal colony counts and CSF cryptococcal antigen titers (r = 0.81 for log10 d-mannitol versus log10 CFU, n = 21...
FIG. 3. CSF colony counts and D-mannitol concentrations in rabbits studied in retrospect. Groups of approximately five to eight rabbits received (i) either 2.5 mg of cortisone acetate per kg per day intramuscularly beginning on day −1 (○) or no cortisone (△, □) and (ii) 3 × 10⁷ C. neoformans H99 cells intracisternally on day 0. The serial colony counts and D-mannitol concentrations in the pooled CSF samples are shown in panels A and B, respectively. The mean ± 2 SD CSF D-mannitol concentrations in four pools of normal rabbit CSF are also shown (●).

paired observations; \( r = 0.78 \) for log₁₀ D-mannitol versus log₁₀ antigen titer, \( n = 20 \) (Fig. 5).

**Distribution and elimination of D-mannitol in CSF.** Since the rate at which a compound appears in a body compartment cannot be estimated from its concentration unless its distribution and rate of clearance are also known, we studied the distribution and elimination of D-mannitol in CSF. Figure 6 shows the CSF D-mannitol concentrations in four rabbits given 0.275 μmol of D-mannitol intracisternally; the results in two rabbits were excluded because sufficient samples were unobtainable or because of incomplete intracisternal injection of D-mannitol. Clearance of exogenous D-mannitol from the CSF was biphasic; approximately 90% disappeared within 4 h (apparent volume of distribution = 2.9 ml); elimination half-life = 1.35 h), and the remainder disappeared more slowly. The mean D-mannitol concentration (±SD) in serum was 1.1 ± 0.51 μM at 2 h and did not change significantly thereafter.

**DISCUSSION**

The objective of this study was to determine whether C. neoformans produces D-mannitol in culture and in infected animals. We found that cultures of 12 of 12 human isolates of C. neoformans produced D-mannitol. D-Mannitol production by C. neoformans differed from D-arabinitol production by Candida spp. in that most of the C. neoformans strains produced more D-mannitol during the stationary than during the logarithmic phase of the cultures, whereas C. albicans is a net producer of D-arabinitol during the logarithmic phase and a net user of D-arabinitol thereafter (2). We also found that C. neoformans released most of the D-mannitol it produced into the medium. C. albicans also releases most of the D-arabinitol it produces into the medium (2), but most of the D-mannitol found in 48-h cultures of several Aspergillus species was intracellular (Flaherty et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984). These differences in the relative proportions of intracellular and extracellular polyols may explain in part why rabbits with cryptococcal meningitis and rats with invasive candidiasis (24) had much higher body fluid polyol levels than rats with disseminated aspergillosis (26).

The ability of C. neoformans H99 to produce D-mannitol in vivo was studied in rabbits with experimental meningitis. In a pilot study of stored CSF specimens, the CSF D-
mannitol levels corresponded well with severity of infection. There was no more D-mannitol in the CSF of non-cortisone-treated rabbits with self-limited infection than in normal controls. In contrast, the CSF D-mannitol levels rose substantially in cortisone-treated rabbits with progressive infection. In a subsequent experiment in which individual rabbits given cortisone and C. neoformans H99 were compared with controls given cortisone alone, the CSF D-mannitol concentrations were much higher in the infected rabbits. Moreover, the CSF D-mannitol levels in infected rabbits rose directly in proportion to fungal load as assessed by CSF colony counts or CSF cryptococcal antigen titers.

We also examined the distribution of D-mannitol within the CSF and its rate of elimination to facilitate interpretation of CSF D-mannitol concentrations. We found that (i) approximately 90% of D-mannitol given intracisternally was cleared during the first 4 h, (ii) the initial apparent volume of distribution approximated total CSF, and (iii) the initial elimination half-life was consistent with removal by bulk CSF flow. D-Mannitol was cleared more slowly after 4 h; possible explanations include uptake and slow release by tissues adjacent to the CSF or slow elimination from another body compartment that is in equilibrium with the CSF.

These results were similar to those of Prockop et al. (19), who showed that (i) 8.1% of [14C]mannitol given intravenicularly or intracisternally to rabbits remained in the CSF after 6 h and (ii) mannitol was cleared slightly faster than compounds that are cleared by unidirectional CSF bulk flow. The authors concluded that mannitol exits the CSF primarily by bulk flow but that some is eliminated by other means such as "slow penetration into brain tissue." Although we did not measure D-mannitol clearance from the CSF of infected rabbits, Prockop and Fishman (18) have shown that [14C]mannitol entered the CSF more rapidly after intravenous administration and disappeared from the CSF more rapidly after intracisternal administration in dogs with experimental pneumococcal meningitis than in uninfected controls. Taken together, our results and those of Prockop et al. imply that continuous production of large amounts of D-mannitol within the central nervous system is necessary for maintenance of high CSF D-mannitol concentrations.

These findings strongly suggest that C. neoformans H99 can produce large amounts of D-mannitol in vivo as well as in vitro. An alternative explanation for the results is that the excess D-mannitol in the CSF of infected rabbits may have been produced by the host as part of the inflammatory or immune response. We consider this unlikely, because (i) D-mannitol is not a known product of mammalian metabolism, (ii) the CSF D-mannitol levels were highly correlated with fungal load, and (iii) intracisternal inoculation of C. neoformans H99 results in lower CSF leukocyte counts and less intense meningeal inflammation in rabbits given cortisone than in cortisone-untreated controls (8, 16). On the basis of these considerations, we conclude that the excess D-mannitol observed in the CSF of the infected rabbits was produced in vivo by C. neoformans.

One implication of these results is that D-mannitol is a quantitative marker for experimental cryptococcal meningitis in rabbits. It remains to be determined whether these results also apply to humans. It is not yet known, for example, whether the variable abilities of different C. neoformans strains to produce and release D-mannitol in vitro imply that different strains also produce variable amounts of D-mannitol in vivo. We also do not yet know whether D-mannitol production or release by C. neoformans or D-mannitol clearance by an infected host is influenced in humans by factors such as host inflammatory or immune responses, renal failure (which is associated with increased.

FIG. 5. Evidence that log₁₀ CSF D-mannitol concentrations were highly correlated with the log₁₀ CSF colony counts (A) and the log₁₀ CSF cryptococcal antigen titers (B) in rabbits with experimental cryptococcal meningitis (see legend to Fig. 4 for methods).
serum and CSF D-mannitol concentrations [17, 22]), or immunosuppressive or antifungal drugs.

Nevertheless, it is likely that CSF D-mannitol measurements should be more useful for some purposes than for others. Cryptococcosis differs from most other opportunistic mycoses in that reliable initial diagnostic methods are already available. C. neoformans can be isolated from the CSF of almost all infected patients, and the latex agglutination test for cryptococcal capsular polysaccharide antigen is a reliable and widely used initial diagnostic test. Moreover, increased CSF D-mannitol concentrations were not observed in the non-cortisone-treated infected rabbits or in the cortisone-treated infected rabbits until 4 days after infection. It therefore appears unlikely that CSF D-mannitol measurements will improve our ability to diagnose cryptococcal meningitis initially.

On the other hand, it is very difficult to assess the severity of cryptococcal meningitis by current diagnostic methods, especially during treatment (1, 5, 7). For example, sterilization of the CSF within 2 weeks, pretreatment CSF cryptococcal antigen titers, and changes in CSF cryptococcal antigen titers over time correlated poorly with final outcome in a recent large treatment trial in patients with cryptococcal meningitis and a variety of underlying diseases (7). Persistence of viable fungi outside the subarachnoid space and prolonged antigen shedding by dead or dying fungi were probably responsible for these findings.

In contrast, only actively metabolizing cryptococci produce D-mannitol, and dead fungi are unlikely to release substantial amounts of D-mannitol because little is conserved intracellularly. Also, since D-mannitol is cleared rapidly from the CSF, changes in fungal viability or metabolic activity should result in prompt changes in CSF D-mannitol concentrations. Thus, serial CSF D-mannitol measurements may provide a more precise means for quantifying the load of metabolically active fungi than is currently available, at least when the CSF D-mannitol concentrations are elevated initially. This approach should be most useful in patients in whom treatment is least likely to be successful (such as those with AIDS). In such patients, serial CSF D-mannitol measurements might eventually be used to individualize the dosages or durations of initial or suppressive antifungal therapy. This approach may also be helpful for assessing the likelihood of relapse or for comparing the effects of different forms of treatment on fungal viability or metabolic activity or both.

The results of the present study may also have pathogenic implications. We have shown that D-mannitol accumulates in the CSF of infected rabbits despite an efficient clearance mechanism. Since D-mannitol is not metabolized by mammalian cells and since it crosses the blood-brain barrier by passive diffusion alone (18), it is probably cleared less efficiently from brain tissue than from CSF. This suggests that there may be much more D-mannitol in tissues immediately surrounding actively metabolizing cryptococci than in CSF. One consequence of D-mannitol accumulation in brain tissue may be increased turgor and edema. Cerebral edema is common in cerebral cryptococcosis (4), but its pathogenesis is poorly understood. Our results suggest that fungal production of a low-molecular-weight solute that is not easily cleared may be a contributing factor. It is also known that oxygen-dependent products of phagocytic cells (e.g., H$_2$O$_2$) kill C. neoformans in vitro (6). Moreover, D-mannitol is an effective scavenger of hydroxyl radicals, and D-mannitol inhibits the abilities of hydroxyl radicals generated by xanthine oxidase and acetaldelyde to kill Staphylococcus aureus (10) and of monocytes and macrophages to kill Toxoplasma gondii (12). Thus, accumulation of D-mannitol in infected tissues may also contribute to pathogenesis by interfering with optimal killing of C. neoformans by phagocytes.

In summary, we have shown that C. neoformans can produce large amounts of D-mannitol in vitro and in vivo. These results suggest that serial CSF D-mannitol measurements may be useful for quantifying fungal load in cryptococcal meningitis and that fungal D-mannitol production may contribute to the pathogenesis of cryptococcosis.

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LITERATURE CITED


