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D-Mannitol in Cerebrospinal Fluid of Patients with AIDS and Cryptococcal Meningitis

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Cryptococcal meningitis (CM) is associated with raised intracranial pressure which is linked with serious neurological sequelae. *Cryptococcus neoformans* produces D-mannitol in vitro and in experimental meningitis in rabbits. Mannitol present in the cerebrospinal fluid (CSF) of CM patients could exacerbate raised intracranial pressure and contribute to neurological damage. To link CSF mannitol to cryptococcal infection, levels of mannitol in the CSF of AIDS patients with CM were measured by gas-liquid chromatography. Mannitol was detected in 19 of 21 samples (range, 1.5 to 26.2 mg/liter), but there was no quantitative correlation between the mannitol concentration and the cryptococcal antigen titer.

Since the advent of the AIDS epidemic, the incidence of cryptococcal meningitis has risen dramatically. Prior to April 1988, 3,022 cases of extrapulmonary cryptococcosis in AIDS patients had been reported to the Centers for Disease Control in the United States (5). A proportion (6 to 10%) of patients with AIDS will develop cryptococcal meningitis, and in 40% of these patients it will be the first AIDS-defining infection (12). After human immunodeficiency virus dementia itself, toxoplasmosis and cryptococcal meningitis vie for the most common neurological presentation of AIDS (9).

In contrast to the diagnoses of other fungal diseases, such as disseminated candidosis or aspergillosis, the laboratory diagnosis of cryptococcal meningitis is relatively straightforward because of the availability of a sensitive and specific test for cryptococcal antigen in serum or cerebrospinal fluid (CSF) (4, 13). Despite the availability of this test and the ability to diagnose cryptococcal meningitis quickly, the disease is frequently associated with treatment failure or recrudescence, morbidity, and mortality. It has been suggested that factors other than antifungal treatment failure, such as intracranial hypertension, may be in part responsible for this (1).

Cryptococcal meningitis is associated with raised intracranial pressure (ICP). Some early cryptococcal meningitis deaths have been associated with high ICP; other serious effects of high ICP are reduced visual acuity, blindness, papilledema, and deafness (1). Survivors of cryptococcal meningitis are sometimes left with permanent neurological sequelae such as cranial nerve palsies, decreased mental capacity, and dependence on a ventriculoperitoneal shunt to relieve hydrocephalus (15). The pathogenesis of raised ICP in cryptococcal meningitis is obscure but tends to occur in those with severe or prolonged disease and high cryptococcal antigen titers. One postulated mechanism is increased CSF osmolality, which may be related to the high-molecular-weight cryptococcal polysaccharide reducing the outflow gradient of CSF. Alternatively, D-mannitol from *Cryptococcus* species may contribute to increased CSF osmolality.

Cryptococcus neoformans is known to produce D-mannitol in vitro and in rabbits with experimental meningitis. In the study of Wong et al. (17), all 12 isolates of *C. neoformans* studied produced extracellular D-mannitol. The concentration of D-mannitol in CSF correlated well with the severity of infection in the rabbit model. We therefore set out to measure, by gas-liquid chromatography, the mannitol concentrations in the CSF of patients suffering from cryptococcal meningitis and attempted a correlation of cryptococcal polysaccharide titers and severity of disease in humans.

We obtained samples of CSF from patients with AIDS and culture-proven cryptococcal meningitis. Samples were stored at −20°C until analyses for cryptococcal antigen and mannitol were performed.

Cryptococcal antigen detection. Detection of cryptococcal antigen was performed using the Meridian latex agglutination kit (Meridian Diagnostics Inc., Cincinnati, Ohio) as described previously (4). Samples of CSF were placed in a boiling water bath for 3 min. Twofold dilutions in glycine buffer (25 μl) were prepared, and the sample was then mixed on a dark slide with appropriate controls provided by the manufacturer. The slide was placed on a platform rotating at 120 rpm for 5 min prior to agglutination being read.

Mannitol detection: sample derivatization. Samples of CSF or aqueous standards were trimethylsilylated by the method of Kiehn et al. (6) as modified by Reeder (14) and Megson et al. (7). To 180 μl of undiluted CSF was added 20 μl of a 500-mg/liter aqueous solution of methyl α-D-mannopyranoside (Sigma) as an internal standard. This was equivalent to a final concentration of 50 mg/liter in a 200-μl volume. Spectroscopy-grade acetone (400 μl; BDH, Poole, United Kingdom) was added to the samples in order to precipitate any CSF protein. Samples were vortex mixed and centrifuged in a bench top centrifuge (12,000 × *g* for 5 min), and the supernatants were aspirated.

The supernatant was dried at 50°C under oxygen-free nitro-

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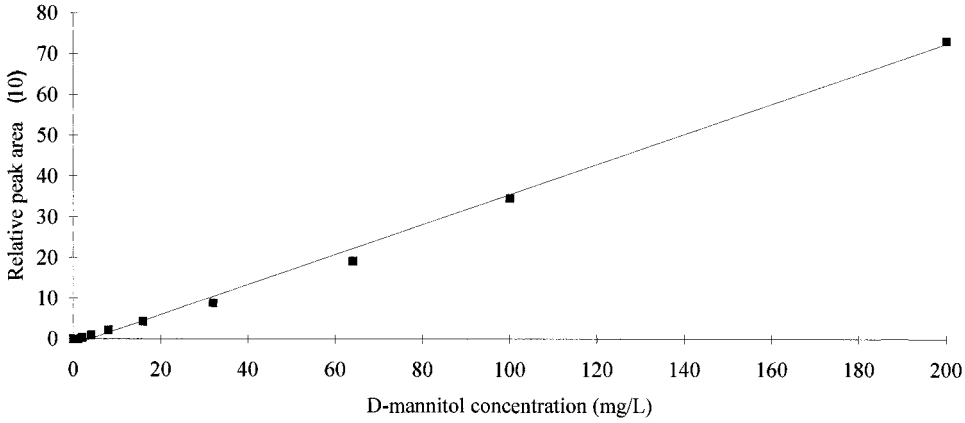

FIG. 1. Aqueous D-mannitol standard curve.

gen for 30 min prior to being trimethylsilylated with 100 μl of a cocktail composed of trimethylchlorosilane (Phase Separations Ltd., Deeside, United Kingdom), hexamethyldisilazane (Phase Separations), and pyridine (Phase Separations) in the volumetric proportions 2:4:6. Silylation proceded at 50°C for 30 min, after which the derivatization mixture was dried at room temperature under oxygen-free nitrogen for 30 min. The derivatized residue was resuspended in diethyl ether (BDH) prior to on-column injection on the chromatograph. All samples were analyzed in triplicate, and mean values were calculated.

Mannitol in CSF samples was identified by comparing the retention time of authentic D-mannitol with that of the suspected mannitol peak in CSF and by performing gas-liquid chromatography-linked mass spectroscopy (GC-MS) on chromatographed authentic D-mannitol (Sigma).

Chromatography. Chromatography was performed on an AI model 92 gas-liquid chromatograph (AI Instruments Ltd., Cambridge, United Kingdom) fitted with two glass columns (length, 1.8 m; internal diameter, 2 mm), packed with 3% SE-30 (a methyl-silicon gum; Phase Separations), and supported on Chromosorb 80-100 mesh (Phase Separations). The front and rear columns of the machine were designated reference and separation columns, respectively. The bleed of the reference column was adjusted to equal that of the separation column, inverted, and electronically subtracted from that of the separation column to give a flat baseline across the temperature range. This counteracted any tendency for the chromatogram baseline to rise because of stripping of the mobile phase from its support. The chromatograph was equipped with dual flame ionization detectors connected via an electronic amplifier to a Trilab 2000 multichannel data analysis system (Trivector Scientific, Beds, United Kingdom). The data analysis system was able to calculate retention time data and integrate the area under the chromatographic peaks of interest.

Routine separations were performed by holding the injection port at 200°C and the flame ionization detectors at 250°C. The chromatograph oven was programmed to rise from 140 to 170°C at a rate of 4°C/min and then from 170 to 200°C at 10°C/min. This temperature programming facilitated good peak separation within a reasonable time. Sample injections were performed on-column with a 5-μl glass microsyringe. Samples were analyzed in triplicate, and mean values were calculated.

Generation of standard curve. Standard curves for D-mannitol were generated by the derivatization and chromatography of the following aqueous D-mannitol standards: 0 (control), 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 100, and 200 mg/liter.

The size of each chromatographic peak was expressed relative to the size of the known concentration of internal standard according to the formula RPA = mannitol peak area/internal standard peak area, where RPA is relative peak area.

The values for relative peak areas were multiplied by a factor of 10 and plotted against the known standard concentrations to give the standard curve (Fig. 1). Linear regression and correlation analysis of the standard curve was done with the Minitab statistical software package. Values of 0.03, 0.3, and 0.998 were obtained for the abscissa intercept, slope, and correlation coefficient for D-mannitol, respectively. The regression data were substituted into an equation describing the standard curve: mannitol concentration = (sample RPA − 0.03)/0.3. This equation was used to calculate the unknown mannitol concentrations in patients' serum samples.

Confirmation of peak identity by gas-liquid chromatography-linked mass spectroscopy. To confirm the identity of the mannitol peak tentatively identified in the gas-liquid chromatography analyses, gas-liquid chromatography-linked mass spectroscopy analysis was performed. Prior to the production of any mass spectra, the resolution of D-mannitol and internal standard peaks was optimized on a Carlo Erba 4000 capillary column chromatograph that was attached to the mass spectrometer. This chromatograph was fitted with a glass capillary column (5 m by 0.22 mm) coated with OV-1 (methyl-silicon gum), 0.25 μm thick, and the hydrogen carrier gas flow rate was 1 ml/min. The chromatograph flame ionization detector and injector were both held at 300°C, and the column oven was programmed to rise from 50 to 290°C at a rate of 5°C/min. A 1-μl sample was introduced into the injector, which operated with a split ratio of 1:10.

Samples from the chromatograph were fed into a Kratos Concept mass spectrometer (Kratos Analytical) operated at 70 eV with a source temperature of 150°C. The instrument could be run in either electron impact or chemical ionization mode. The chemical ionization mode with ammonia reagent gas was found to be the most favorable technique for producing molecular ions and large molecular fragments.

When the constituent peaks in authentic D-mannitol standards had been separated, they were scanned in turn to provide a plot of relative peak intensities over a range of mass/charge (m/z) values. A mass spectrogram of the chromatographic peak thought to represent mannitol gave a high ion current at the m/z ratio corresponding to the D-mannitol molecular ion (Fig.

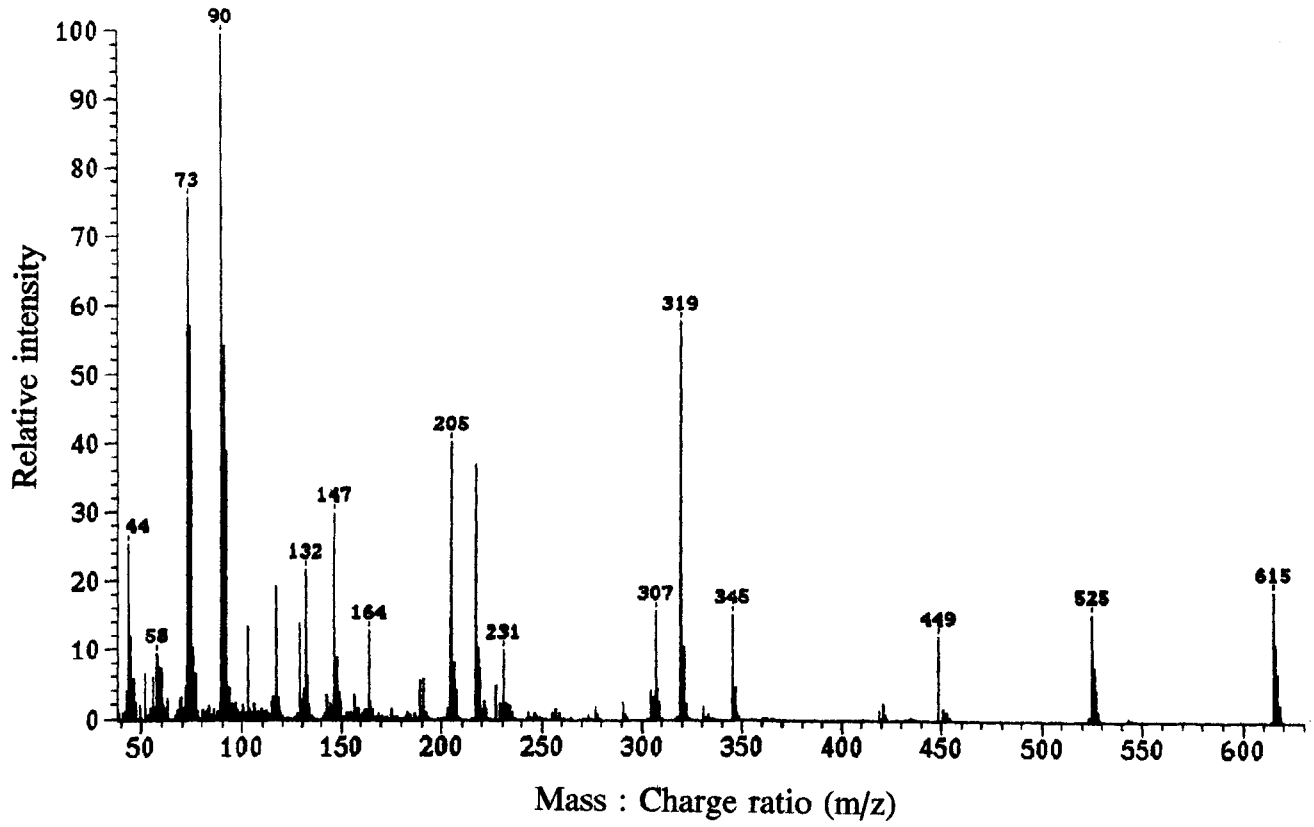

FIG. 2. Mass spectrogram of D-mannitol chromatographic peak.

2). This, in addition to the coincident retention time data, confirmed the identity of the chromatographic peak.

Mannitol was found in the CSF of infected individuals in concentrations ranging from 1.5 to 26.2 mg/liter (mean, 8.9 mg/liter). In two patients' samples, no mannitol was detected. The range of CSF cryptococcal antigen titer was 0 to 1:2,048. These results are shown in Fig. 3. No significant relationship between mannitol concentration and CSF cryptococcal antigen titer was found ($P = 0.15$ by analysis of variance).

Cerebrospinal fluid is produced by the brain of the normal subject at approximately 450 ml per day, and as the average CSF volume is 150 ml, the whole CSF volume is replaced three times per day. Much of this CSF is absorbed by the arachnoid villi and granulations on the cerebral surface, although a small amount may pass into the lymphatic system. This is in contrast to the absorption in the rabbit model, in which >90% of CSF absorption is into the lymph (3); consequently, the rabbit is a poor model for studies of intracranial hypertension and cryptococcal meningitis.

The effective therapies for preventing visual loss and other neurological sequelae of cryptococcal meningitis are those which focus on the reduction of ICP, such as ventriculoperitoneal shunt or frequent high-volume lumbar puncture (1). Frequent lumbar puncture or monitoring of CSF pressure by manometry techniques may lead to brain coning and death due to the rapid removal of large amounts of CSF; this may be

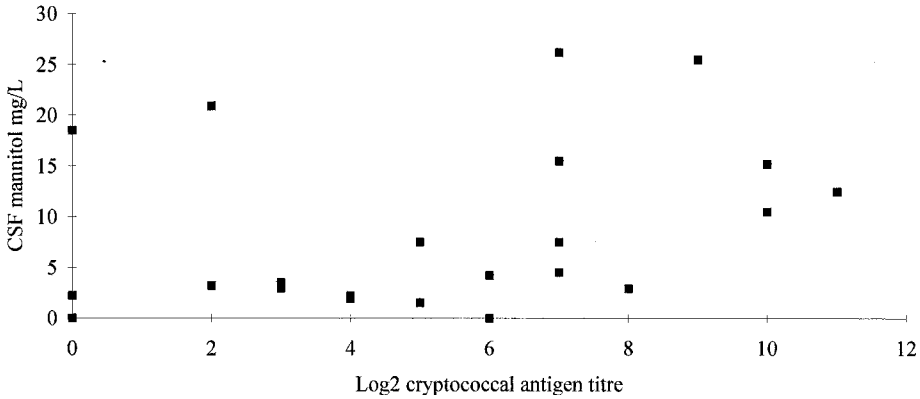

FIG. 3. Mannitol concentration versus $\log_2$ cryptococcal antigen titer in the CSF of patients with AIDS and cryptococcal meningitis.

avoided by using a strain gauge transducer for measuring opening pressure and alternative methods of pressure reduction (8). For the past 3 decades, intravenous D-mannitol has been used to reduce CSF pressure, primarily in neurosurgery, by setting up an osmotic gradient across the blood-brain barrier (osmotherapy). In neurosurgical practice, intravenous D-mannitol is given as a bolus whenever ICP rises above 25 to 30 mm Hg (normal resting adult ICP, 0 to 10 mm Hg) (11), although there is great interpatient variability in D-mannitol pharmacokinetics (16). If D-mannitol were present in the CSF of patients with cryptococcal meningitis, this would work in the opposite sense to D-mannitol osmotherapy, increasing the CSF pressure by osmosis and leading to cerebral edema. Additionally, D-mannitol is known to scavenge free radicals which may reduce the ability of CSF phagocytes to kill *C. neoformans* (2). Clearly, the presence of D-mannitol in the CSF of patients with cryptococcal meningitis is likely to have a detrimental effect on ICP and may also interfere with phagocytic cell function.

Previous work (17) had reported a good correlation between CSF D-mannitol concentration and severity of meningitis in a rabbit model. Hence, it was reasonable that a correlation between CSF mannitol concentrations and antigen titers might also be found. The lack of such a correlation may be due to the fact that cryptococcal antigen may persist in the CSF even after the yeasts are dead. As this antigen is a large and complex polysaccharide molecule and is thought to cause blockages in the arachnoid villi and lymphatic vessels (1), its clearance may be at a slower rate than that of D-mannitol, which is only produced by actively growing cells and is cleared rapidly (17). The fact that low concentrations of mannitol were found in CSF in the present study supports this hypothesis, as most samples were obtained, during maintenance therapy, from patients who may have had residual cryptococcal polysaccharides but few viable yeasts. A further difference between the results of the present study and those of Wong et al. (17) may be due to the different anatomical and physiological configurations of CSF outflow in rabbits and humans.

In this study we were not able to correlate mannitol concentration, cryptococcal antigen titer, and CSF pressure. It is possible that mannitol levels may correlate with CSF pressure more closely than do the antigen levels. It is noteworthy that one patient had a high CSF opening pressure (310 mm Hg) at the time that his CSF mannitol value was 25.5 mg/liter and his cryptococcal antigen titer was 1:512.

Mannitol is not a known product of mammalian metabolism and therefore is unlikely to be produced by normal metabolic processes or as part of an immunological response to meningitis (17). Hence, although it is regrettable that CSF mannitol data were not available for uninfected control patients, it is likely that the mannitol in the CSF is of fungal origin. However, two samples from patients undergoing maintenance therapy for cryptococcal meningitis contained no mannitol at all, making it extremely unlikely that the mannitol detected for the other patients was of any origin other than fungus. The role of fungal D-mannitol in the pathogenesis of cryptococcal meningitis remains to be established, but the fact that D-mannitol nonproducing strains may be less virulent than their D-mannitol-producing counterparts (10) adds weight to the premise that mannitol may contribute to the disease process.

It should be noted that D-mannitol may be produced by *Aspergillus* spp. in vivo (7), although this should not create diagnostic problems clinically. Detection of mannitol may be useful diagnostically for invasive aspergillosis and cryptococcal meningitis. However, the methodology applied here is not readily applicable in a routine clinical microbiology laboratory. Work is presently in progress commercially to generate a simple colorimetric assay for mannitol detection.

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