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Cerebrospinal Fluid and Plasma (1→3)-β-D-Glucan as Surrogate Markers for Detection and Monitoring of Therapeutic Response in Experimental Hematogenous *Candida* Meningoencephalitis[▽]

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The treatment, diagnosis and therapeutic monitoring of hematogenous *Candida* meningoencephalitis (HCME) are not well understood. We therefore studied the expression of (1→3)-β-D-glucan (β-glucan) in cerebrospinal fluid (CSF) and plasma in a nonneutropenic rabbit model of experimental HCME treated with micafungin and amphotericin B. Groups studied consisted of micafungin (0.5 to 32 mg/kg) and amphotericin B (1 mg/kg) treatment groups and the untreated controls (UC). Despite well-established infection in the cerebrum, cerebellum, choroid, vitreous humor (10^2 to 10^3 CFU/ml), spinal cord, and meninges (10 to 10^2 CFU/g), only 8.1% of UC CSF cultures were positive. By comparison, all 25 UC CSF samples tested for β-glucan were positive (755 to 7,750 pg/ml) ($P < 0.001$). The therapeutic response in CNS tissue was site dependent, with significant decreases of the fungal burden in the cerebrum and cerebellum starting at 8 mg/kg, in the meninges at 2 mg/kg, and in the vitreous humor at 4 mg/kg. A dosage of 24 mg/kg was required to achieve a significant effect in the spinal cord and choroid. Clearance of *Candida albicans* from blood cultures was not predictive of eradication of organisms from the CNS; conversely, β-glucan levels in CSF were predictive of the therapeutic response. A significant decrease of β-glucan concentrations in CSF, in comparison to that for UC, started at 0.5 mg/kg ($P < 0.001$). Levels of plasma β-glucan were lower than levels in simultaneously obtained CSF ($P < 0.05$). CSF β-glucan levels correlated in a dose-dependent pattern with therapeutic responses and with *Candida* infection in cerebral tissue ($r = 0.842$). Micafungin demonstrated dose-dependent and site-dependent activity against HCME. CSF β-glucan may be a useful biomarker for detection and monitoring of therapeutic response in HCME.

Hematogenous *Candida* meningoencephalitis (HCME) is a common complication of candidemia in pediatric patients, particularly premature neonates (3, 4, 5, 9). Neonatal HCME results in significant morbidity, including mental retardation, seizures, and adverse neurological outcomes (5, 10). Recent studies show that central nervous system (CNS) infection caused by *Candida* spp. occurs more often than is detected by positive blood and/or cerebrospinal fluid (CSF) cultures (6, 11). Currently, there are relatively few options for the treatment of HCME. The use of amphotericin B deoxycholate, despite its broad-spectrum antifungal activity and demonstrated efficacy, is often limited by drug-related nephrotoxicity as well as poor penetration of the blood-brain barrier (12, 13, 18). There is an urgent requirement for better diagnostic tools as well as more-efficacious and less-toxic antifungal therapies.

Echinocandins (caspofungin, anidulafungin, and micafungin) are a class of semisynthetic lipopeptide antifungal noncompetitive inhibitors of (1→3)-β-D-glucan (β-glucan) synthase, an enzyme involved in the synthesis of glucan, which is the major component of the structural integrity of the fungal cell wall and is required for normal cell growth and division (7, 8, 19, 22, 35). The inhibition of β-glucan synthesis results in cell wall damage, osmotic instability and, ultimately, cell death. The utility of echinocandins in CNS infection remains undefined, and their penetration into the intact blood-brain barrier is low. Despite concerns about the CNS penetration of the echinocandins, there are both experimental and clinical data which suggest that they may have a role in the treatment of HCME (14, 27).

The detection and therapeutic monitoring of CNS candidiasis are challenging. Quantitative CSF cultures are not a sensitive marker for the diagnosis of HCME (2, 6), nor is clearance of CSF a reliable indicator for the eradication of *Candida* spp. from CNS tissue. We hypothesized that the biomarker of cell wall β-glucan may aid in the diagnosis and monitoring of patients with HCME. Detection of β-glucan is possible using a colorimetric assay read at 405 nm, based upon *para*-nitroanilide absorption at that wavelength (26, 28, 32).

We therefore studied the utility of β-glucan levels in CSF and plasma for the diagnosis and monitoring of therapeutic responses with a nonneutropenic rabbit model of experimental HCME treated with micafungin. To our knowledge, this is the first such study to demonstrate the utility of β-glucan in HCME.

MATERIALS AND METHODS

Animals. Female New Zealand White rabbits (Covance Research Products, Inc., Denver, PA) weighing 2.4 to 3.7 kg at the time of intravenous candidal challenge were used in all experiments of the nonneutropenic rabbit model of

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experimental HCME, which has been previously described (13). All rabbits (n = 119) were individually housed and maintained with water and standard rabbit feed ad libitum. They were monitored under human care and use standards in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, according to the guidelines of the National Research Council for the care and use of laboratory animals and under the approval of the Animal Care and Use Committee of the National Cancer Institute (24). Vascular access was established under general anesthesia by the surgical placement of a Silastic tunneled central venous catheter as previously described (33). The Silastic catheter permitted nontraumatic venous access for repeated blood sampling for studies of biochemical and hematological parameters, plasma pharmacokinetics, and the administration of inoculum and parenteral agents. Rabbits were euthanized according to Animal Care and Use Committee-approved, prespecified humane end points by intravenous (i.v.) administration of pentobarbital (65 mg of pentobarbital sodium/kg of body weight; Beuthanasia-D Special [euthanasia solution]; Schering-Plough Animal Health Corp., Union, NJ) at the end of each experiment, 0.5 h after administration of the last dose of study drug.

Organism and inoculation. A well-characterized clinical isolate, *Candida albicans* NIH 8621 (ATCC MYA-1237), which was obtained from a neutropenic patient with disseminated candidiasis, as later proven by autopsy, was used for all experiments.

The MICs for *C. albicans* were determined according to current CLSI standards (23). The MIC of micafungin (Astellas Pharma US, Inc., Deerfield, IL) was 0.125 μg/ml (30), and that of amphotericin B (Amphocin, manufactured for Pharmacia and Upjohn Co., a subsidiary of Pharmacia Corporation, Kalamazoo, MI, by Cardinal Health, Albuquerque, NM) was 0.125 μg/ml.

For preparation of the inoculum, the *C. albicans* isolate was subcultured from a frozen stock culture stored at −80°C on potato dextrose agar slants (K-D Medical, Inc., Columbia, MD) on Sabouraud dextrose agar (SGA) plates (K-D Medical, Inc., Columbia, MD) and incubated at 37°C for 24 h. Three to five well-isolated colonies were sampled from freshly grown culture plates and suspended into 50 ml of Emmon's modified Sabouraud glucose broth (K-D Medical, Inc., Columbia, MD) (pH 7.0) in a 250-ml Erlenmeyer flask. The suspension was incubated in a gyratory water bath at 80 oscillations per min at 37°C for 18 h. The *Candida* suspension was then centrifuged at 1,600 × *g* for 10 min and washed three times with sterile 0.9% normal saline (K-D Medical, Inc., Columbia, MD). The concentration was adjusted by use of a hemacytometer and was confirmed by quantitative cultures of a 10-fold serial dilution. The final inoculum of 1×10^6 blastoconidia (suspended in a 5-ml volume of sterile normal saline slowly administered to each rabbit via an indwelling Silastic central venous catheter) was designed to establish a nonlethal nonneutropenic rabbit model of HCME over the course of 7 days (13). The inoculum size was confirmed by plating serial dilutions onto SGA plates.

Antifungal compounds and treatment groups. The treatment groups in the model of experimental HCME consisted of untreated (control) rabbits (n = 72) and rabbits treated with micafungin (n = 41) or amphotericin B (n = 6). Therapy was initiated 48 h postinoculation and continued throughout the course of the experiments, for 7 days.

Micafungin powder was reconstituted and diluted further in sterile 0.9% normal saline to achieve the desired concentration of 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 5 mg/ml, or 10 mg/ml. Treatment groups consisted of rabbits receiving micafungin at dosages of 0.5 (n = 6), 2 (n = 6), 4 (n = 6), 8 (n = 6), 16 (n = 8), 24 (n = 4), and 32 (n = 5) mg/kg i.v. once daily.

Amphotericin B deoxycholate was resuspended in sterile water, maintained at 4°C, and diluted at a 1:4 ratio with sterile 5% dextrose solution (Abbott Labs, North Chicago, IL) to achieve a final concentration of 1 mg/ml, immediately prior to use, according to the manufacturer's instructions. Amphotericin B was administered i.v. at 1 mg/kg slowly (0.1 ml every 15 s) once daily (n = 6).

Quantitation of *C. albicans* in the blood, CSF, brain tissues, aqueous humor, and vitreous humor. Levels of antifungal activity in the model of HCME were determined by the quantitative clearance of *C. albicans* from the blood, CSF, brain tissues, aqueous humor, and vitreous humor. Blood cultures were drawn on a daily basis. Depending upon the anticipated concentration of organisms in the bloodstream, aliquots of 1,000 μl, 500 μl, or 100 μl of whole blood and serial dilutions of 10^{-1} to 10^{-2} in sterile 0.9% normal saline were plated on Emmon's modified SGA plates containing chloramphenicol and gentamicin.

The aliquots of 1,000 to 1,500 μl of CSF were collected after euthanasia from each rabbit by inserting a 23-gauge needle into the cisterna magna. The puncture was in the midline halfway between the cranial edges of the wings of the atlas and below the external occipital protuberance at the atlantooccipital site.

For dissection of meninges, the skin on the top of the skull was dissected in the middle and pulled away to the sides to open the cranial portion of the skull that contains the brain. The dissection of the skull was carefully performed to avoid damage to the three protective meningeal layers (dura mater, arachnoidal mater, and pia mater) in the area of the transverse sinus. The incision in the skull was performed with a bone cutter through the superior nuchal line separating the parietal, interparietal, and supraoccipital bones. The parietal, frontal bones of the skull were then removed. A small incision was made in the meninges, and the dissection was performed in the midline only, through the meninges, so as not to damage the brain tissue. Both sides of the meninges were then removed from the cerebrum.

Representative sections of tissues (meninges, cerebrum, cerebellum, and spinal cord) were weighed, and each tissue sample was then homogenized (Stomacher 80; Tekmar Corp., Cincinnati, OH) in sterile reinforced polyethylene bags (Nasco Whirl-Pak, Atlanta, GA) with 1 (for meninges), 2, or 5 ml of sterile 0.9% normal saline for 30 s (34).

Antifungal activity in treatment of *Candida* infection of the eyes was also assessed postmortem. First, the aqueous humor was aspirated from the anterior chamber through the cornea of each eye into a 3-ml sterile syringe (Becton Dickinson and Co., Franklin Lakes, NJ). The globes of the eyes were then carefully dissected using aseptic techniques and transferred to a sterile petri dish (Falcon; Becton Dickinson Labware, Becton Dickinson and Co., Franklin Lakes, NJ). The cornea and iris were dissected with sharp scissors from the globe, the lens was removed, and 500 to 1,000 μl of vitreous humor was slowly aspirated into a sterile tuberculin syringe (Sherwood Medical, St. Louis, MO). The specimens of vitreous humor from both globes were pooled and processed together. The sclera from each globe was divided into two parts, and the choroid was scraped from each part for quantitation of *C. albicans* and pharmacokinetic studies. The choroid sample used for the quantitation of *C. albicans* was placed into 2-ml Sarstedt tubes and weighed, an aliquot of 1000 μl of sterile normal saline was added, and the mixture was vortexed.

The CSF, aqueous humor, or vitreous humor specimen or each tissue homogenate was serially diluted to 10^{-1},10^{-2}, or 10^{-4} in sterile 0.9% normal saline. Aliquots (100 μl) of CSF, aqueous humor, vitreous humor, or undiluted tissue homogenates and serial dilutions of 10^{-1},10^{-2}, or 10^{-4} in sterile 0.9% normal saline were separately plated onto Emmons modified SGA plates. Culture plates were incubated at 37°C for 24 h, after which CFU were counted and the numbers of CFU/ml (for CSF and vitreous humor) or CFU/g (for brain tissues) were calculated. Potential carryover of drug was minimized by performing serial dilutions (17). The limit of detection was ≥10 CFU/ml or ≥10 CFU/g. The culture-negative plates were counted as 0 CFU/ml or 0 CFU/g. Data were graphed as the means of $\log_{10}$ (CFU/ml or CFU/g) ± standard errors of the means.

β-Glucan assay. Blood from each rabbit infected with *C. albicans* was collected every day for determination of plasma β-glucan concentrations. CSF was collected postmortem from infected and noninfected animals as described above. The assay (Fungitell; Associates of Cape Cod, Inc., Falmouth, Mass.) for detection of β-glucan is licensed for the diagnosis of invasive fungal infections. Lipopolysaccharide and β-glucan initiate the coagulation cascade in the horseshoe crab (*L. polyphemus* or *T. tridentatus*) by activating different serine protease zymogens, factors C and G. Lipopolysaccharide specifically activates factor C, while β-glucan activates factor G. The specificity of β-glucan is ensured by using factor C-depleted *L. polyphemus* amebocyte lysate. The assay was performed according to the manufacturer's instructions. Briefly, aliquots of 5 μl of plasma or CSF were added to duplicate wells of a 96-well microtiter plate and pretreated for 10 min at 37°C with an alkaline reagent (20 μl; 0.125 M KOH/0.6 M KCl). An aliquot of 25 μl of the standards (100 to 6.25 pg/ml pure pachyman, a linear β-glucan) was then added to each well. An aliquot of 100 μl of Fungitell reagent (lyophilized β-glucan-specific *Limulus* amebocyte lysates) was reconstituted with 2.8 ml of glucan-free reagent-grade water, followed by 2.8 ml of Pyrosol reconstitution buffer (2 M Tris-HCl, pH 7.4), and 100 μl of this mixture was added to each sample. The plate was monitored at 405 nm (with 490-nm background subtraction) for 40 min at 37°C by using a Bio-Tek ELx808 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) equipped with KC4 software (Bio-Tek Instruments, Inc., Winooski, VT). The mean rate of optical density change was determined for each well, and the glucan concentration was determined by comparison to a standard curve. When absorbance was outside the range of the standard curve, the plasma or CSF samples were serially diluted in reagent-grade water and tested again. Interpretation of β-glucan values, according to the manufacturer's instructions for use, was as follows: <60 pg/ml, negative; 60 to 79 pg/ml, indeterminate; ≥80 pg/ml, positive. The correlation coefficient (r) of the standard curve was ≥0.9992 (range, 0.9980 to 0.9998). The mean β-glucan level in CSF from 20 noninfected rabbits was 1.36 ± 0.55 pg/ml (range, 0 to 9.0 pg/ml), and that in the serum from 60 noninfected rabbits was 13 ± 1.21 pg/ml (range, 0 to 35.2 pg/ml).

Pharmacokinetic studies. The plasma pharmacokinetics of micafungin were investigated with four to six infected rabbits per dosage group using minimal

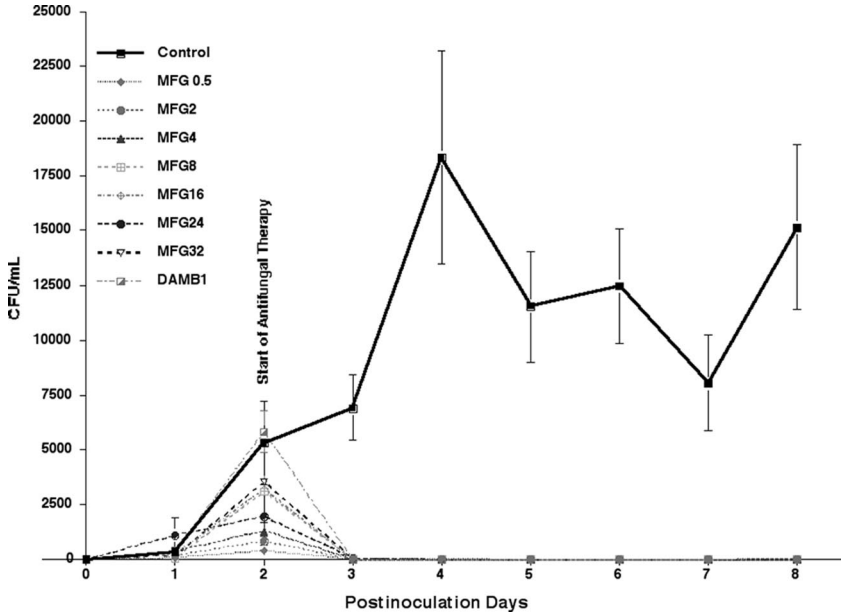

FIG. 1. *C. albicans* fungemia in nonneutropenic untreated control and treated rabbits. Animals were treated with micafungin (MFG) and amphotericin B (DAMB); dosages (in milligrams/kilogram) are indicated after each drug abbreviation. Treatment was started 48 h (day 2) after inoculation.

plasma sampling and determined on day 6 (120 to 144.5 h) after initiation of antifungal therapy, part of which has been previously described (15). The pharmacokinetics of 0.5, 2, 4, 8, 16, 24, and 32 mg/kg doses were studied. Micafungin was administered once daily as a steady i.v. bolus over 4 min. Blood samples were collected into heparinized syringes before the administration of drug at 0 h (baseline) and then at 0.07, 0.25, 0.5, 2, 4, 6, 8, and 24 h after the drug administration. Plasma also was obtained 0.5 h after the administration of the last dose of micafungin, before animals were sacrificed. Plasma samples were stored at −80°C prior to analysis.

Micafungin was extracted from plasma using solid-phase extraction with C_8 Bond Elut cartridges (100 mg, 1 ml) (Varian, Palo Alto, CA) and a Vac-Elut vacuum manifold (Analytichem International, Harbor City, CA). Cartridges were conditioned by completely draining 500 μl of high-performance liquid chromatography (HPLC)-grade acetonitrile and then half-draining 1 ml of acetonitrile-ammonium acetate (phosphate buffer, pH 4.0) (10:90 [vol/vol]). An aliquot of 300 μl of plasma was applied to the half-filled cartridge, together with 25 μl of the internal standard (50 mg/liter anidulafungin; Vicuron Pharmaceuticals, Inc., King of Prussia, PA). Acetonitrile-ammonium acetate (10:90 [vol/vol]) (pH 4.0) was then added to completely fill the cartridge (300 μl), and the sample was drained slowly under vacuum.

After drainage, C_8 cartridges were washed with 1 ml of acetonitrile-ammonium acetate (10:90 [vol/vol]) and dried under vacuum. The cartridges remained at high vacuum for 30 s to dry. Subsequently, 1 ml of 100% chloroform (Mallinckrodt, Phillipsburg, NJ) was added, and the cartridges were drained and thoroughly dried under vacuum for 1 min. Micafungin was eluted with 1 ml of acetonitrile-ammonium acetate (70:30 [vol/vol]) (50 mM, pH 4.0), followed by brief full vacuum pressure to get the draining started, and then vacuum pressure was reduced to allow material to drain slowly into 12- by 75-mm disposable culture test tubes. After all the eluent in the cartridge was pulled into culture test tubes, vacuum was applied briefly (approximately 5 s) to deplete any eluent remaining in the guide needles attached to the vacuum. The eluent in the culture tubes was then evaporated under nitrogen using a Zymark Turbo Vap LV evaporator (American Laboratory Trading LLC, Niantic, CT) at 40°C for 90 min or until the tubes were dry.

Dried eluent was reconstituted in 150 μl of methanol-ammonium acetate (50:50 [vol/vol]; 50 mM, pH 4.0; methanol was from J.T. Baker, Phillipsburg, NJ) in a culture test tube, which was vortexed for 1 min. The reconstituted eluent was transferred to Eppendorf tubes and centrifuged for 5 min at 2,600 × *g* before being transferred into a microvial insert for HPLC injection and placed in the autosampler at 9°C.

Concentrations of micafungin were determined using a reversed-phase HPLC (Waters 2695 separation module). Acetonitrile-ammonium acetate (50:50 [vol/vol]; 50 mM, pH 4.0) was used as the mobile phase, with an isocratic flow rate of 0.5 ml/min. A C_8 analytical column (150 by 4.6 mm, 5 μm) (Alltech Inertsil; Alltech Associates, Deerfield, IL) maintained at 50°C was preceded by a C_8 column guard (7.5 by 4.6 mm, 5 μm) (Alltech Inertsil; Alltech Associates, Deerfield, IL). The injection volume was 75 μl. Micafungin and the internal standard, anidulafungin, were detected using UV light (wavelength of 271 nm) and eluted between 7.6 and 8.1 and between 12.7 and 13.1 min, respectively. Quantitation was based on the ratio of peak area of micafungin relative to that of the internal standard.

Standard curves that encompassed the expected experimental range of micafungin concentrations were constructed in their respective matrix. The lower limit of quantification was ≤0.075 mg/liter. The coefficient of determination (r^2) was ≥0.996. The intra- and interday coefficients of variation were both <14%.

The pharmacokinetic data from individual rabbits were modeled using a population methodology by means of the NPAG (nonparametric adaptive grid) program with adaptive γ (20). An open two-compartment model with zero-order time-delimited input and first-order elimination from the central compartment was used. The data were weighted by the inverse of the estimated variance of the drug assay (16). Models were discriminated on the basis of the log-likelihood value, the mean weighted error, the bias-adjusted weighted mean squared error, and a visual inspection of the regression of observed versus predicted values obtained after the Bayesian step. The Bayesian estimates of the pharmacokinetic parameters were estimated using the "population of one" utility in NPAG. For each dosage group, these Bayesian estimates were collected and the average was obtained. The area under the plasma concentration-time curve from 0 to 24 h ($AUC_{0\text{-}24}$) at steady state was determined by integration.

Histopathology analysis. Representative sections of the cerebrum, cerebellum, and spinal cord were prepared for histology studies. Tissue specimens were excised and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and then stained with either periodic acid-Schiff stain (PAS) or Grocott-Gomori methenamine-silver stain (GMS). Tissues were microscopically examined for structural changes in *C. albicans*, underlying histopathology, and correlation with microbiological clearance.

Toxicity studies. Chemical determinations of creatinine, urea nitrogen, alanine aminotransaminase, aspartyl aminotransaminase, and potassium concentrations in plasma were performed by the Department of Laboratory Medicine in the NIH Warren Grant Magnuson Clinical Center on the penultimate sample drawn from each rabbit.

Statistical analysis. Comparisons between groups were performed by analysis of variance (ANOVA) with Dunn's correction for multiple comparisons, Bonferroni's correction for multiple comparisons, the Mann-Whitney U test, or the Spearman rank correlation, as appropriate. All *P* values were two-sided, and a *P* value of <0.05 was considered to be significant. Values are expressed as means and standard errors of the means.

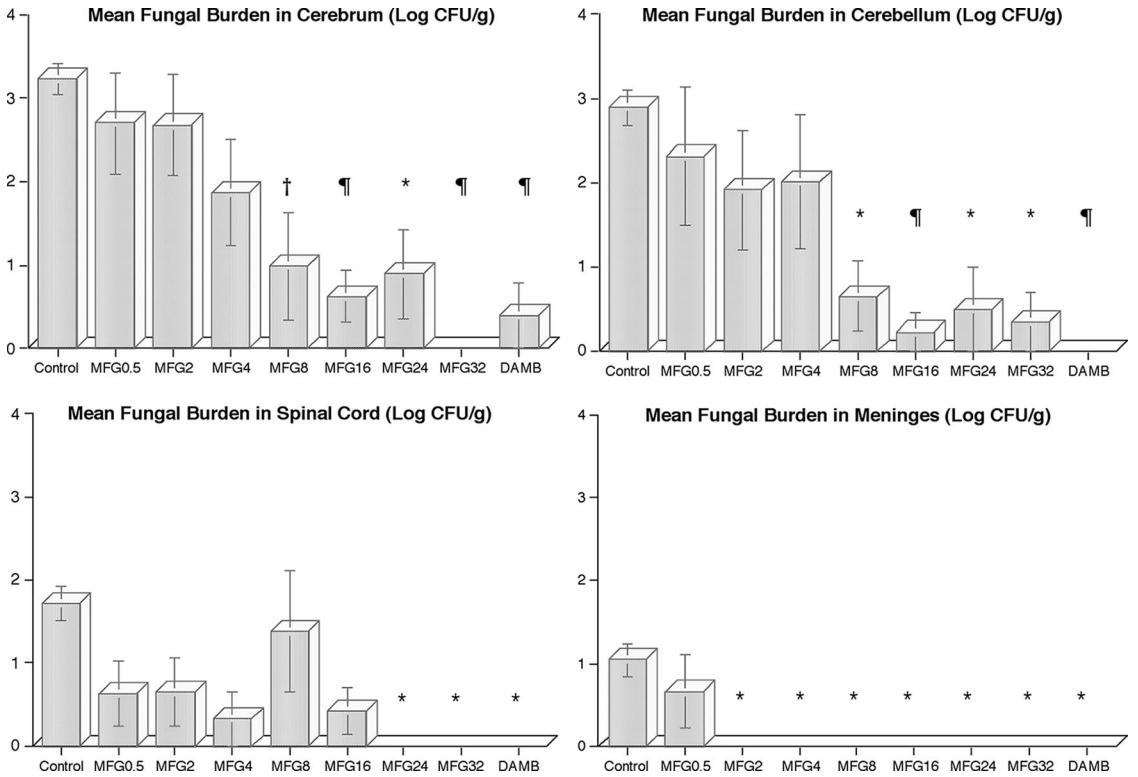

FIG. 2. Response of HCME in nonneutropenic rabbits to antifungal therapy measured by mean log (CFU/g) concentration of organism in cerebrum, cerebellum, spinal cord, and meninges in untreated controls ($n = 58$), micafungin-treated rabbits at 0.5 mg/kg (MFG0.5; $n = 6$), 2 mg/kg (MFG2; $n = 6$), 4 mg/kg (MFG4; $n = 6$), 8 mg/kg (MFG8; $n = 6$), 16 mg/kg (MFG16; $n = 8$), 24 mg/kg (MFG24; $n = 4$), 32 mg/kg (MFG32; $n = 5$), and amphotericin B-treated at 1 mg/kg (DAMB; $n = 6$). Values are given as means ± standard errors of the means. (P values were <0.05 [*], <0.01 [†], or <0.001 [¶] in comparison to untreated controls using ANOVA with Bonferroni's multiple comparisons test.)

RESULTS

Quantitative blood cultures. All infected nonneutropenic rabbits had positive blood cultures for *C. albicans* at 24 h (day 1) and 48 h (day 2) after inoculation (Fig. 1). Fungemia persisted in all untreated control animals throughout the experiments. Positive blood cultures converted to negative in rabbits treated with micafungin and amphotericin B 1 day after treatment initiation. At the end of therapy with micafungin or amphotericin B, all blood cultures in treated animals were negative.

Despite negative blood cultures in rabbits treated with micafungin or amphotericin B, CNS tissues remained positive for *C. albicans*. Among rabbits receiving the different dosages of micafungin, positive fungal cultures in one or more CNS tissue sites were found for 5 of 6 rabbits (83.3%) at 0.5 mg/kg, 5 of 6 (83.3%) at 2 mg/kg, 5 of 6 (83.3%) at 4 mg/kg, 3 of 6 (50%) at 8 mg/kg, 4 of 8 (50%) at 16 mg/kg, 3 of 4 (75%) at 24 mg/kg, and 2 of 5 (40%) at 32 mg/kg. Among amphotericin B-treated rabbits, 1 of 6 (16.7%) also had positive fungal cultures from the cerebrum samples.

Quantitative CSF cultures. Despite well-established fungal infection in the cerebrum (10^2 to 10^3 CFU/g), cerebellum (10^2 to 10^3 CFU/g), spinal cord (10 to 10^2 CFU/g), and meninges (10 to 10^2 CFU/g) in all control animals, CSF cultures were positive in 5 of 62 (8.1%) CSF samples from untreated control rabbits at autopsy and positive in 1 of 6 (16.7%) rabbits treated with micafungin at 0.5 mg/kg. CSF cultures were negative in all other micafungin-treated rabbit dosage groups.

In light of these culture data, we studied levels of β-glucan in CSF in comparison to culture. Among 25 CSF samples from untreated rabbits analyzed for concentrations of β-glucan, only 2 samples were culture positive, whereas β-glucan was present within a range of 755 to 7,750 pg/ml in all 25 samples (8.1% versus 100% testing positive, respectively; $P < 0.001$).

Antifungal therapy. Micafungin demonstrated significant dose-dependent antifungal efficacy in the treatment of HCME (Fig. 2). There was a significant decrease in the fungal burden in the cerebrum and cerebellum starting at a dosage of 8 mg/kg ($P < 0.01$ and $P < 0.05$, respectively) and in meninges at 2 mg/kg ($P < 0.05$). A higher dosage of 24 mg/kg of micafungin was required to achieve a significant effect in the spinal cord ($P < 0.05$).

All aqueous humor cultures were negative in untreated rabbits and in rabbits treated with micafungin or amphotericin B (data are not shown). A statistically significant decrease in the residual fungal burden in the vitreous humor was achieved in micafungin-treated rabbits at 4 mg/kg ($P \leq 0.05$) and in the choroid at 24 mg/kg ($P < 0.05$) (Fig. 3).

β-glucan levels. Levels of β-glucan in plasma collected from micafungin-treated and untreated rabbits were lower than levels of β-glucan in simultaneously obtained CSF samples ($P < 0.05$) (Fig. 4). The mean concentration of β-glucan in the CSF

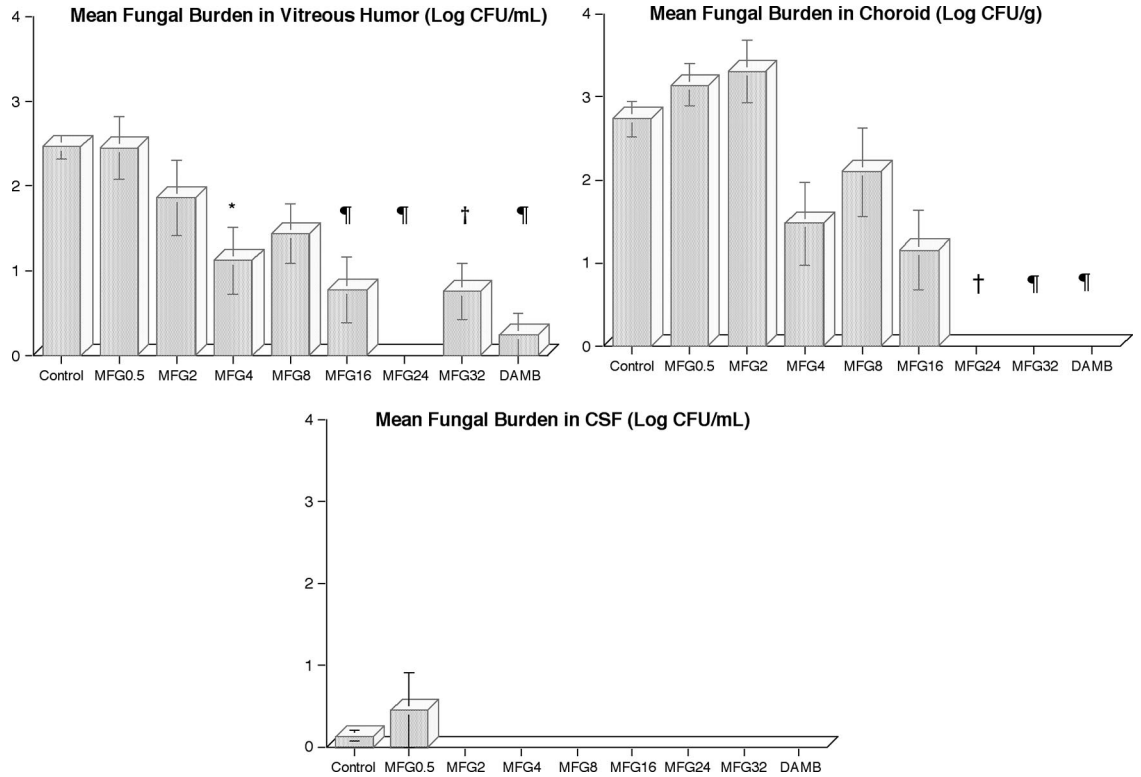

FIG. 3. Response of disseminated candidiasis and HCME in nonneutropenic rabbits to antifungal therapy measured by mean log (CFU/g) concentration of organism in the vitreous humor, choroid, and CSF in untreated controls ($n = 58$; $n = 48$ in CSF), micafungin-treated rabbits at 0.5 mg/kg (MFG0.5; $n = 6$), 2 mg/kg (MFG2; $n = 6$), 4 mg/kg (MFG4; $n = 6$), 8 mg/kg (MFG8; $n = 6$), 16 mg/kg (MFG16; $n = 8$), 24 mg/kg (MFG24; $n = 4$), and 32 mg/kg (MFG32; $n = 5$), and amphotericin B-treated rabbits at 1 mg/kg (DAMB; $n = 6$). Values are given as means ± standard errors of the means. (P values were <0.05 [*], <0.01 [†], or <0.001 [¶] in comparison to untreated controls using ANOVA with Bonferroni's multiple comparisons test.)

of untreated rabbits was 3,801 ± 465 pg/ml (range, 754 to 7,064). By comparison, the mean concentration of β-glucan in untreated rabbit plasma obtained simultaneously with CSF was 852 ± 305 pg/ml (range, 107 to 1,819). A significant decrease of β-glucan concentrations in CSF was observed for rabbits treated with micafungin at 0.5 mg/kg in comparison to that of untreated animals ($P < 0.001$). As the dosage of micafungin increased to ≥2 mg/kg, the plasma β-glucan levels declined significantly, whereas the CSF levels of β-glucan demonstrated a significant but more gradual decrease across the dosage range of 0.5 to 32 mg/kg. Although there was a significant decrease in β-glucan concentrations in CSF in micafungin- or amphotericin B-treated rabbits, those levels stayed above the positive limit, while plasma concentrations remained in the negative range.

CSF concentrations of β-glucan correlated in a dose-dependent pattern with therapeutic response and with residual *Candida* infection in cerebral tissue ($r = 0.842$; $P < 0.001$) (Fig. 5). As the log concentration of residual cerebral *Candida* increased, the CSF β-glucan levels also increased.

The kinetic profiles of plasma β-glucan of rabbits treated with micafungin or amphotericin B rabbits were analyzed for plasma β-glucan levels (Fig. 6). The concentrations of β-glucan in plasma obtained from all rabbits before inoculation ranged from 5 to 30 pg/ml. After inoculation of *C. albicans*, the plasma β-glucan concentration of untreated rabbits reached the highest level, 1,593 ± 570 pg/ml (range, 246 to 3,602 pg/ml), on day 5 of the experiment and then started to decline to the level of 453 ± 126 pg/ml at the end of experiment (range, 113 to 1,564 pg/ml). The levels of β-glucan in the plasma of rabbits treated with micafungin at 0.5 mg/kg peaked on day 4, when the level of 399 ± 330 pg/ml (range, 70 to 728 pg/ml) started to decline to the level of 240 ± 67 pg/ml (range, 27 to 510 pg/ml). The plasma β-glucan concentrations of rabbits treated with micafungin at 2, 4, 8, 16, and 24 mg/kg were below the positive limit (80 pg/ml) by days 7 and 8 of the experiment. Concentrations of β-glucan in the plasma of rabbits treated with micafungin at 32 mg/kg became negative on day 5 of the experiment, while those from amphotericin B-treated rabbits became negative for β-glucan on day 7.

Pharmacokinetics of micafungin. Micafungin exhibited linear plasma pharmacokinetics over the dosage range used in the study. The fit of the pharmacokinetic model to the data was excellent, with highly acceptable measures of bias (mean weighted error, −0.53) and precision (bias-adjusted mean weighted squared error, 7.43) and a coefficient of determination of 0.95 after the Bayesian step. The observed-predicted relationships for plasma drug concentrations of micafungin after the Bayesian step are shown in Fig. 7. The fit of the pharmacokinetic model to the data was excellent, with an r^2 of 0.974, along with acceptable measures of precision and bias. The estimates for the means and standard

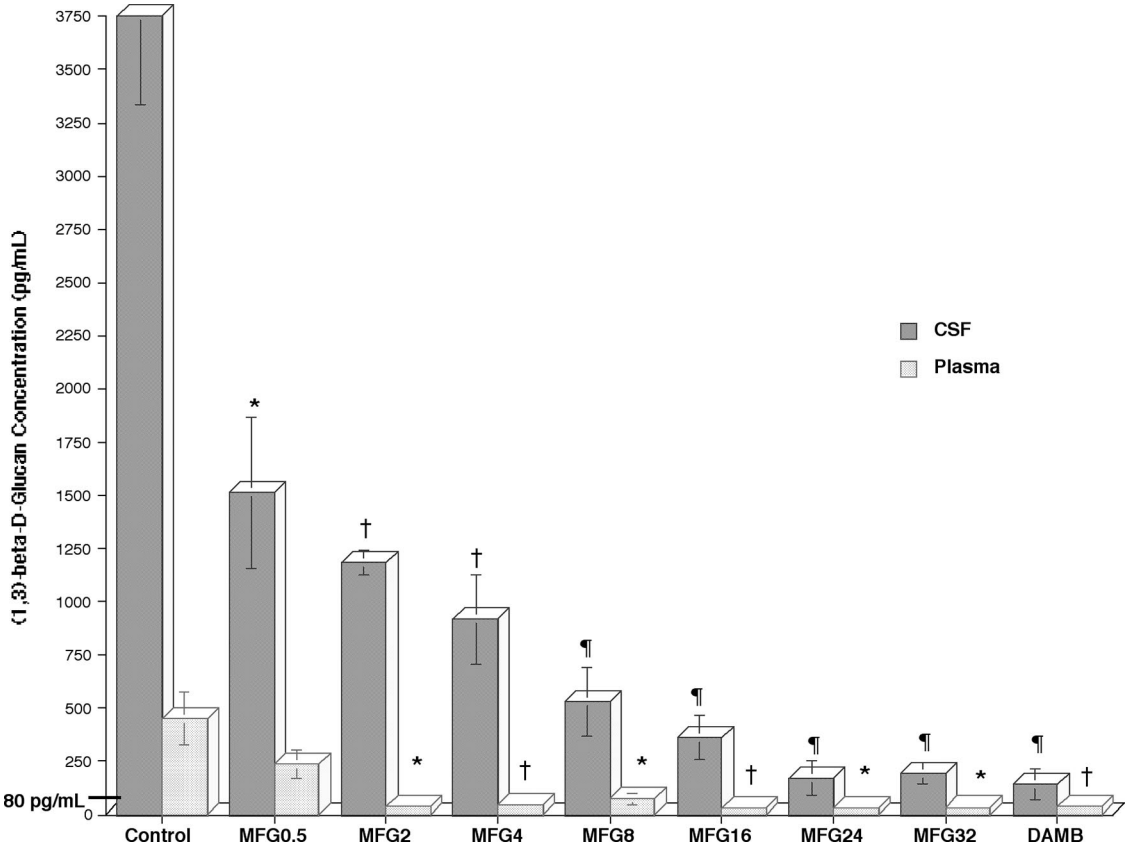

FIG. 4. Comparison of β-glucan concentrations in CSF and the last plasma sample obtained from the rabbit before euthanasia. Drug and dosage abbreviations are as described in the legend to Fig. 3. (*P* values were <0.05 [*], <0.01 [†], or <0.001 [¶] in comparison to untreated controls using ANOVA with Bonferroni's multiple comparisons test.)

deviations of the pharmacokinetic parameters from the population analysis, with an estimated elimination half-life of 7.65 h, are summarized in Table 1.

Histopathology. There was a dosage-dependent effect of fewer lesions per low-power field (×40) being detected in tissues. The number of lesions was markedly reduced in tissues from untreated controls and treated rabbits; lesions were barely detectable in tissues from animals treated with micafungin at 4 or 16 mg/kg and nonexistent in tissues from rabbits treated with micafungin at 32 mg/kg. At a high magnification (×400), inspections of the lesions demonstrated a marked effect on the structures of hyphae, pseudohyphae, and blastoconidia. Hyphae and pseudohyphae were disrupted, truncated, and distorted (Fig. 8A to C). In sections of cerebral lesions with PAS, microabscesses consisting of polymorphonuclear leukocytes and monocytes surround and damage hyphae, pseudohyphae, and blastoconidia of *C. albicans* (Fig. 8D to F).

Toxicity studies. Rabbits treated with micafungin or amphotericin B and untreated control rabbits had no elevated concentrations of creatinine, urea nitrogen, hepatic transaminases, or potassium in plasma.

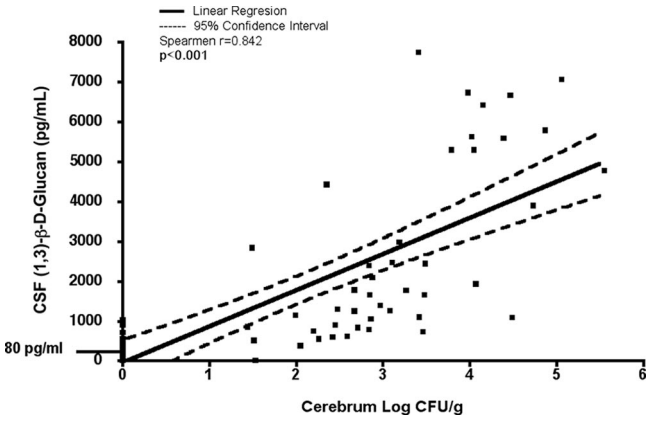

FIG. 5. Linear regression of β-glucan concentrations in CSF and fungal burden (log CFU/g) in cerebrum tissue.

DISCUSSION

HCME is an important complication in children with candidemia that may result in abscesses, intraventricular hemorrhage, mental retardation, seizures, and neurological deficits. Amphotericin B deoxycholate has long been the preferred agent for candidemia and HCME in infants and children; however, its use is often limited by drug-induced azotemia and hypokalemia. Thus, new, safe, and effective therapeutic agents and diagnostic modalities are needed for this infection.

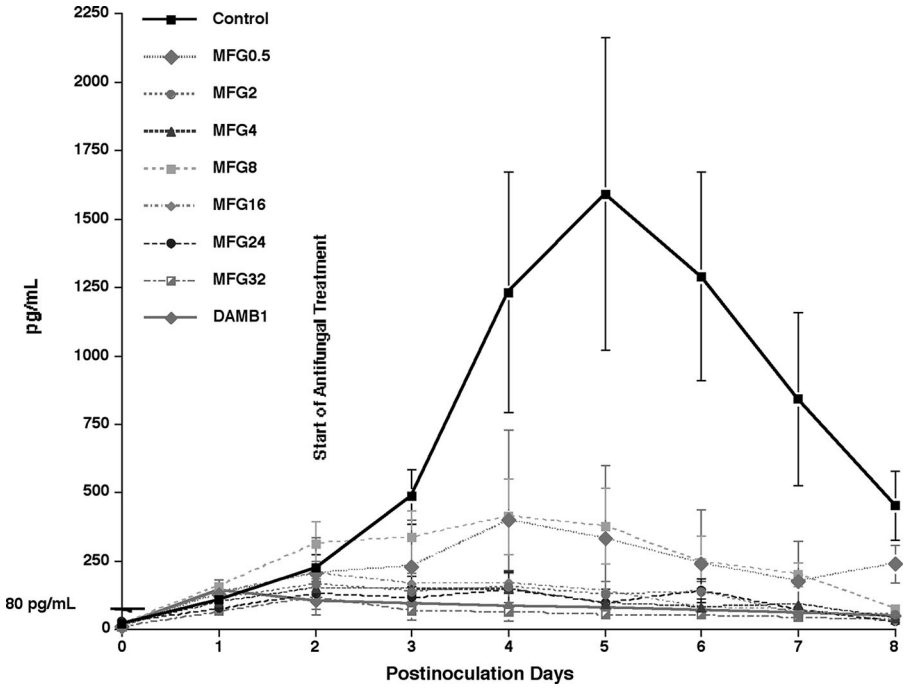

FIG. 6. Expression of β-glucan concentrations in HCME nonneutropenic rabbit plasma in the treatments with micafungin (MFG) and amphotericin B (DAMB); dosages (in milligrams/kilogram) are indicated after each drug abbreviation.

Micafungin demonstrated dosage-dependent antifungal activity with a significant decrease in the residual fungal burden in the cerebrum, cerebellum, meninges, and vitreous humor. Higher dosages were required to achieve a significant effect in the spinal cord and choroid. We further characterized the expression of β-glucan in CSF and plasma as a potential biomarker for HCME. CSF cultures were seldom found to be positive (8.1%) despite extensive CNS tissue infection, which is consistent with clinical observations. The finding that all CSF samples (25/25) were positive for β-glucan suggests that this biomarker may be a useful diagnostic tool for HCME.

The utility of echinocandins in human CNS infection is undefined. One patient was found to have developed candidal endophthalmitis and another HCME during echinocandin therapy (1, 29). However, an infant with HCME refractory to deoxycholate amphotericin B was successfully treated with caspofungin (27).

The experimental data in this report are consistent with those of our previous study describing the activity of micafungin in the treatment of HCME (15). Both studies underscore the dose dependency and site dependency of echinocandins within the CNS. This report demonstrates that the vitreous humor, choroid, and spinal cord require relatively high dosages for clearance of *C. albicans*. The highest dosage required was that for the vitreous humor. This may relate to differential diffusion of antifungal agents from the choroidal vasculature into the vitreous humor, whereas diffusion through the vitreous matrix may be slow. Thus, any consideration of the dosage of echinocandin should consider penetration into the vitreous humor. These data are compatible with previous clinical observations of development of candidal endophthalmitis in patients being treated for candidemia (1). Importantly, however, randomized clinical trials of echinocandins versus amphotericin or fluconazole have not shown an excess of endophthalmitis in adult patients, which may be due to some activity at relatively low drug exposures (21, 31).

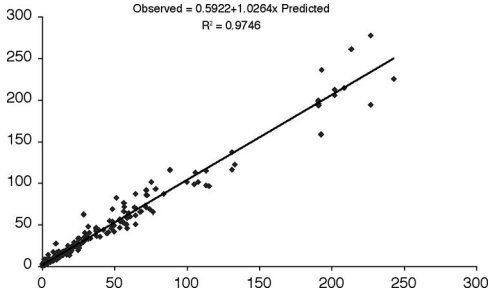

FIG. 7. Observed-predicted relationships for plasma drug concentrations of micafungin after the Bayesian step.

The measurement of β-glucan concentrations in serum have been developed and validated in clinical trials (28). However, little is known about the expression of β-glucan in the CSF in patients with HCME and the utility of this biomarker for the diagnosis and therapy of premature neonates.

The β-glucan assay in CSF was significantly more sensitive than quantitative cultures of CSF in the rabbit model of HCME; β-glucan was highly positive in all 25 animals with HCME. These data suggest that the release of cell wall carbohydrate fragments occurs more readily than that of whole organisms from microabscesses.

Clearance of *C. albicans* from blood cultures was not predictive of the eradication of organisms from the CNS. The

TABLE 1. Pharmacokinetic parameters of micafungin in nonneutropenic rabbits with HCME from the population analysis after multiple daily doses over 6 days[a]

Dosage (mg/kg)	Vc (liters)	Kcp (h^{-1})	Kpc (h^{-1})	SCL (liters/h)	$AUC_{0-\infty}$ ($\mu g/ml \cdot h$)
0.5	0.535 (0.064)	0.542 (0.135)	0.281 (0.046)	0.129 (0.012)	10.75 (0.589)
2	0.450 (0.088)	0.664 (0.430)	0.350 (0.320)	0.113 (0.016)	49.55 (7.88)
4	0.633 (0.136)	0.484 (0.182)	0.514 (0.124)	0.144 (0.029)	77.83 (20.24)
8	0.649 (0.153)	0.743 (0.933)	0.587 (0.364)	0.175 (0.049)	137.22 (30.05)
16	0.659 (0.122)	0.666 (0.743)	0.622 (0.286)	0.158 (0.010)	305.71 (24.96)
24	0.611 (0.377)	0.852 (1.013)	0.617 (0.299)	0.146 (0.084)	767.33 (466.23)
32	0.577 (0.165)	0.537 (0.304)	0.509 (0.349)	0.141(0.032)	788.5 (230.83)
Overall model	0.588 (0.185)	0.612 (0.739)	0.454 (0.361)	0.143 (0.041)	

[a] All values are expressed as means, and standard deviations are shown in parentheses. Vc, volume of the central compartment; SCL, clearance; Kcp and Kpc, first-order intercompartmental rate constants; $AUC_{0-\infty}$, area under the plasma concentration-time curve from time zero to infinity.

clearance of blood cultures and persistence of CNS tissue infection is observed for children with HCME (2, 5, 6, 25). By comparison, concentrations of β-glucan in CSF were predictive of the therapeutic response. There was a dose-dependent reduction in β-glucan following micafungin administration. There was a direct correlation between the levels of β-glucan in CSF and the tissue fungal burden as measured for the cerebrum, which contributes the bulk of *Candida* to the overall infection in the brain. That levels of β-glucan in plasma were lower than levels in simultaneously obtained CSF suggests a compartmentalization of this large polymeric carbohydrate, with higher concentrations within the CNS not readily crossing the blood-brain barrier.

To our knowledge, this study is the first to illustrate the potential diagnostic utility of the β-glucan concentration in CSF as well as its possible role in the therapeutic monitoring of HCME. That CSF β-glucan concentrations correlated directly with levels of *C. albicans* in tissue in response to antifungal therapy suggests that this biomarker may be a useful tool for monitoring the therapeutic response.

The safety of high doses of up to 32 mg/kg of micafungin was similar to that of dosages as low as 2 mg/kg, with no alterations in levels of bilirubin, aspartyl aminotransaminase, alanine aminotransaminase, or in renal function. These findings are consistent with recent observations of higher dosages of micafungin as being relatively safe for pediatric patients (P. B. Smith, T. J. Walsh, W. W. Hope, A. Takada, L. Kovanda, G. L. Kearns, D. Kaufman, T. Sawamoto, D. N. Buell, and D. K. Benjamin, Jr., abstr. 3761.6, presented at 2008 Pediatric Academic Societies and Asian Society for Pediatric Research Joint Meeting, Honolulu, HI, 2 to 6 May 2008). Thus, the higher dosages of micafungin that are required to successfully treat CNS infection may be safely achieved in patients without causing apparent hepatic or renal dysfunction.

In summary, micafungin demonstrated dose-dependent and site-dependent activity against HCME throughout the CNS. These antifungal effects of micafungin were comparable to those of deoxycholate amphotericin B. There was no evidence of hepatic or renal toxicity due to micafungin therapy at the dosages studied. Moreover, β-glucan was a sensitive marker

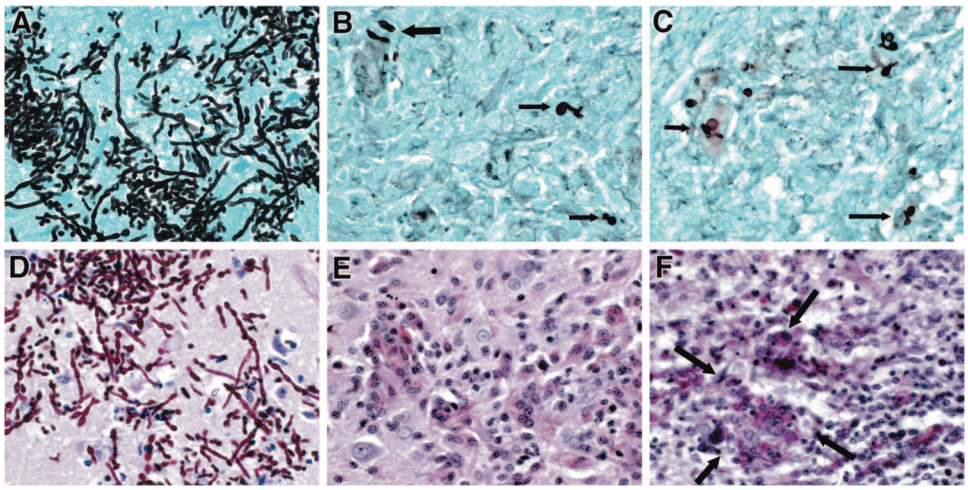

FIG. 8. Antifungal effect of micafungin on microscopic morphology of the cell structure of *C. albicans* in cerebral tissue. (A) untreated control (original magnification, ×400) (GMS), extensive infiltration of tissue by hyphae, pseudohyphae, and blastoconidia; (B) micafungin at 4 mg/kg (×400) (GMS), distorted blastoconidia (thin arrows) and hyphal fragments (thick arrow); (C) micafungin at 16 mg/kg (×400) (GMS), distorted blastoconidia (thin arrows); (D) untreated control (×400) (PAS); (E) micafungin at 4 mg/kg (×400) (PAS), microabscess consisting mononuclear cells and scatted polymorphonuclear leukocytes; (F) micafungin at 16 mg/kg (×400) (PAS), microabscesses consisting of mononuclear cells, macrophages, polymorphonuclear leukocytes, and necrotic foci (thick arrows).

for the detection and therapeutic monitoring of CNS candidiasis.

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