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Effects of Pentoxifylline or Dexamethasone in Combination with Amphotericin B in Experimental Murine Cerebral Cryptococcosis: Evidence of Neuroexcitatory Pathogenic Mechanisms

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In a murine model of intracerebral infection by *Cryptococcus neoformans* the therapeutic effects of pentoxifylline or dexamethasone were studied alone and in combination with amphotericin B. Assessed parameters were mean survival time, brain histopathology index, amounts of glutamate and γ-aminobutyric acid in the brain, and yeast CFU per brain. Survival increased significantly in mice treated with dexamethasone, amphotericin B, amphotericin B plus dexamethasone, and amphotericin B plus pentoxifylline; the latter had significantly longer survival than other treated groups. Indices of histopathological damage were similar in all treated groups. In infected untreated mice, the amounts of glutamate in the brain were decreased, presumably by depletion. In mice treated with amphotericin B plus dexamethasone, glutamate levels returned to the range of control mice. No differences in the amounts of γ-aminobutyric acid were found between control and treatment groups. Brain fungal counts were significantly lower in mice treated with amphotericin B, amphotericin B plus dexamethasone, and amphotericin B plus pentoxifylline than in untreated animals. In this model, pentoxifylline in combination with amphotericin B improved survival, decreasing the fungal burden, and has potential as adjuvant therapy in cerebral cryptococcosis.

Meningeal cryptococcosis (MC) is one of the most devastating opportunistic infections. It is estimated that 10% of patients with AIDS in the United States will develop MC (23), with mortality rates of up to 40% (19). The pathophysiology of MC is incompletely understood (17). Clinical experience has shown that most deaths in advanced cases occur in the first 2 weeks after diagnosis despite antifungal treatment (8, 19). Baseline factors potentially predictive of a poor outcome appear to be correlated with a high fungal burden in the cerebrospinal fluid (CSF) at the time of diagnosis (8, 10, 17, 19, 28). Patients usually have elevated opening pressure of the CSF (8, 17), and therapy of increased intracranial pressure has been restricted to CSF shunts and neurosurgical decompression (6, 25, 26).

Neuroexcitatory damage due to release of agonist excitatory amino acids is considered to be an important pathogenic mechanism in central nervous system ischemia (18), and it seems to participate in the pathogenesis of bacterial meningitis (11). This mechanism of brain damage has not been studied for MC. In infants and children 2 months of age or older with *Haemophilus influenzae*-caused meningitis a short course of dexamethasone at the onset of antibiotic therapy reduces the likelihood of deafness without delayed sterilization of CSF cultures (1). Pentoxifylline, a dimethylxanthine known by its fibrinolytic activity and ability to inhibit platelet aggregation, decreases leukocyte recruitment into the CSF in experimental bacterial meningitis (20).

The purpose of this study was to adapt an experimental murine model of intracerebral cryptococcosis (2, 3) to an advanced stage of the infection at the onset of therapy, similar to that in patients with AIDS or with factors predictive of a poor outcome, in order to evaluate adjunctive therapy with pentoxifylline and dexamethasone, which were chosen because in vitro observations have demonstrated that both drugs block the release of tumor necrosis factor, interleukin 1, and interleukin 6 in murine microglial cell cultures when added concomitantly with lipopolysaccharide (7) and could protect the central nervous system against cytokine-mediated injury. Dexamethasone and the standard antifungal agent amphotericin B in doses similar to those with proved therapeutic effects in human infection were administered. The dose of pentoxifylline was taken from a previous animal study (5). Several parameters were selected to evaluate the drug effects along with the possible participation of neuroexcitatory amino acids in brain damage.

MATERIALS AND METHODS

Swiss albino NIH female mice 6 to 8 weeks old, weighing between 20 and 25 g, were used. Animals were housed under standard laboratory conditions.

Pharmacological compounds. Parenteral amphotericin B desoxycholate (Bristol-Myers-Squibb, Princeton, N.J.), dexamethasone phosphate (Merck, Mexico City, Mexico), and pentoxifylline (Trental; Hoechst-Roussel, Mexico City, Mexico) were used.

Drug treatments. Animals were treated with daily nonpyrogenic saline, dexamethasone at 0.6 mg/kg of body weight per day, pentoxifylline at 17 mg/kg/day, or amphotericin B at 2 mg/kg every 48 h, according to their assigned groups. All drugs were diluted in nonpyrogenic saline, except for amphotericin B, which was diluted in 5% dextrose; all drugs were administered by intraperitoneal injection

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TABLE 1. Survival of mice infected intracerebrally with 4×10^4 CFU of *C. neoformans*[a]

Drug[b]	Survival (days)	Mean	SD	SE	P[c]
None	10, 13, 14, 14, 14, 14, 18, 22, 26, 41	18.6	9.2	2.9	
Amphotericin B	15, 15, 26, 28, 29, 30, 32, 37, 42, 60	31.4	13.1	4.1	<0.05
Dexamethasone	14, 14, 24, 24, 25, 26, 29, 31, 34, 42	26.3	8.5	2.7	<0.05
Pentoxifylline	12, 12, 14, 15, 19, 19, 22, 24, 27, 30	19.4	6.3	2.0	
Amphotericin B + dexamethasone	13, 19, 20, 25, 31, 31, 32, 35, 36, 49	29.1	10.3	3.3	<0.05
Amphotericin B + pentoxifylline	26, 35, 41, 43, 43, 44, 47, 52, 60, 60	45.1	10.5	3.3	<0.01

[a] All mice receiving nonpyrogenic saline or autoclaved cryptococci survived for at least 60 days.
[b] 21 days of therapy, or less if the animal died.
[c] Kruskal-Wallis analysis of variance followed by multiple comparisons of all treatment groups with the infected control group by the Mann-Whitney U test.

in a 0.2-ml volume. Treatments began 7 days after intracerebral inoculation of *Cryptococcus neoformans* and had a duration of 7 days for sacrificed animals and 21 days for the survival subgroups.

Cryptococcal strain. A capsulated strain of *C. neoformans* var. *gattii* serotype B (INNN-20) obtained from the CSF of a non-AIDS patient was used. The strain has a large capsule and demonstrated consistent virulence in preliminary mouse challenge studies. Stock cultures were maintained by twice-a-week passages on Sabouraud dextrose agar; slants were kept at room temperature.

Intracerebral inoculation. Yeast cells of the cryptococcal strain were harvested from slants and were suspended in nonpyrogenic saline. Suspension density was adjusted with a spectrophotometer and counted on a hemocytometer to contain 4×10^4 CFU in 40 μl, verified by serial dilution and plate counts. Each animal was anesthetized with ether and via a disposable insulin-type syringe, a precision plunger, and 27-gauge needle received an injection of 40 μl in the right lateral ventricle according to a previously described method (2). Prestudy tests with 10 mice were performed with toluidine blue-O as a marker for accurate ventricular injection with macroscopic and histopathological examination. After inoculation, viability and size of inoculum were confirmed by serial dilution and plate counts.

Pilot group. A group of 10 mice was used to assess the virulence of the strain with the standard inoculum. The first death occurred on day 10, and nine animals were dead by day 26. Histopathological examination of the brains of these mice revealed massive quantities of encapsulated yeasts, subependymal inflammation, cryptococcomas, and brain edema.

Experimental protocol. Eight groups of 25 mice each were used. Ten mice in each group (survival subgroups) were observed daily for 60 days, with the day of their death recorded; the day of intracerebral inoculation was counted as day 0. Mean survival time was calculated for each group. On day 14 after inoculation all 15 remaining mice from each group were sacrificed. Five mice were used for histopathological analysis, five were used for brain fungal counts, and five were used for neurochemical evaluation. The first three groups (controls) received intracerebral inoculation of nonpyrogenic saline, autoclaved cryptococci, and live cryptococci, respectively, and were treated with intraperitoneal nonpyrogenic saline. The other five groups received intracerebral inoculation with live cryptococci and were treated with amphotericin B, dexamethasone, pentoxifylline, amphotericin B plus dexamethasone, and amphotericin B plus pentoxifylline (treatment groups).

Histopathological evaluation. Mice were sacrificed by cervical dislocation and perfused with a solution of 10% formalin and 4.5% glutaraldehyde. The brain was dissected and fixed in the same solution for 2 weeks and then sectioned, dehydrated, embedded in paraffin, and stained with the hematoxylin-and-eosin, periodic acid-Schiff, and Grocott stains. All slides were evaluated blindly by a neuropathologist, who graded each animal in the following categories: subependymal inflammation (0 to 3), cryptococcomas in brain parenchyma (0 or 1), brain edema (0 to 3), and quantity of observed yeasts (0 to 3). Grades were summed to obtain an index of histopathological severity from 0 to 10 for each animal. A score was calculated for each group.

Neurochemical evaluation. Tissue concentrations of glutamate and γ-aminobutyric acid (GABA) were measured for five animals from each group by high-performance liquid chromatography (HPLC) with fluorescence detection. The animals received one intraperitoneal injection of 1.2 mol of 3-mercaptopropionic acid per kg 90 s before sacrifice to inhibit postmortem GABA increase (27). After sacrifice by cervical dislocation the brain was dissected on ice, weighted, and homogenized in 15 volumes of methanol-water (85%, vol/vol). Samples were centrifuged at $3,000 \times g$ for 15 min, and small volumes of the supernatant were stored at −70°C until analyzed. Precolumn derivatization was performed as previously described (22). One hundred microliters of the OPA reagent (containing 5 mg of *o*-phthaladehyde, 625 μl of methanol, 5.6 ml of borate buffer 0.4 M [pH 9.5], and 25 μl of 2-mercaptoethanol) was added to 100 μl of the supernatant. The mixture was stirred and allowed to stand for 1 min; then, it was injected with a 25-μl Hamilton syringe into a Perkin-Elmer series 3B liquid chromatograph. An adsorbosphere All-tech reversed phase column was used with a particle size of 3 μM and a length of 100 mm. Buffer A consisted of 50 mM sodium acetate aqueous solution, pH 5.9, with 1.5% (vol/vol) tetrahydrofuran. Solvent B was HPLC-grade methanol. Gradient programming was made in two stages at a linear increase of solvent B: from 10 to 65% in 15 min and then from 65 to 99% in 5 min. Standard curves were constructed for glutamate and GABA by using known concentrations. Results were expressed in micrograms of the amino acid per g (wet weight) of brain. Means were then obtained for glutamate and GABA in all groups.

Brain fungal counts. After sacrifice the brain was aseptically dissected and homogenized with 2 ml of sterile nonpyrogenic saline. Serial dilutions were made and plated in duplicate in dicloxacillin-supplemented Sabouraud agar plates. CFU were counted after 48 h of incubation at room temperature. The total number of CFU per brain was the mean of the two samples from each mouse. Means were obtained for each group.

Statistical analysis. Results are expressed as means ± standard deviations and ± standard errors. The means of each variable were compared among all groups by the analysis of variance, Mann-Whitney U, and Tukey tests with SPSS software; $P < 0.05$ was considered significant.

RESULTS

Survival. Mean survival for the group of animals treated with pentoxifylline was similar to that for infected controls. Animals treated with dexamethasone or amphotericin B alone or with amphotericin B plus dexamethasone or amphotericin B plus pentoxifylline had significantly increased survival times compared with those for infected controls. The group treated with amphotericin B plus pentoxifylline had the highest survival time, significantly higher than those for other treatment groups (Table 1).

Histopathology. Infected animals without treatment had similar findings to those in the pilot group. Among the groups of treated animals only the group treated with amphotericin B revealed a modest reduction in the index of histopathological damage. No histological alterations were found for the group that received intracerebral nonpyrogenic saline. Hemosiderin-loaded macrophages were observed for the group inoculated with autoclaved cryptococci.

Neurochemistry. Table 2 shows the amounts of glutamate and GABA in the brain. Intracerebral inoculation of autoclaved cryptococci and live cryptococci resulted in a significant depletion of glutamate content in the brain in comparison with animals inoculated with nonpyrogenic saline. The only treatment that antagonized this depletive effect was the combination amphotericin B plus dexamethasone. In this group the levels of glutamate were similar to those of controls (nonpyrogenic saline). No differences in GABA contents in the brain were found between controls and treatment groups, thus suggesting that GABA levels are not affected by cryptococcal intracerebral infection.

Brain fungal counts. Significantly lower levels of CFU were found for all groups treated with amphotericin B, either alone or combined with dexamethasone or pentoxifylline, compared with infected controls (Table 3).

TABLE 2. Concentrations of glutamate and GABA in the brain in mice 14 days after inoculation with 4×10^4 CFU of *C. neoformans*

Treatment	Mean concn (μg/g[a]) + SD[b]	
	Glutamate	GABA
Nonpyrogenic saline	2,230 ± 460†‡	199 ± 23
Autoclaved cryptococci	1,346 ± 574*	190 ± 39
Live cryptococci		
No drug	1,086 ± 315*	136 ± 14
Amphotericin B	1,320 ± 375*	154 ± 22
Dexamethasone	1,203 ± 317*	132 ± 42
Pentoxifylline	1,085 ± 181*	190 ± 58
Amphotericin B + dexamethasone	2,658 ± 1,042†‡	181 ± 45
Amphotericin B + pentoxifylline	768 ± 148*	130 ± 69

[a] Wet weight.
[b] Statistical significance ($P < 0.05$; analysis of variance followed by the Tukey test) is indicated as follows: *, †, and ‡, different from the corresponding value for the group receiving nonpyrogenic saline, autoclaved cryptococci, or live cryptococci with no drug, respectively.

DISCUSSION

This study provides evidence that in a lethal model of intracerebral cryptococcosis treatment with pentoxifylline in combination with amphotericin B significantly increases survival, in comparison with animals treated with amphotericin B alone. In contrast, mice that received pentoxifylline as a single agent had survival rates and brain fungal counts similar to those of untreated animals. Therapy was started 7 days after inoculation, once the disease was in an advanced stage, in an attempt to simulate the events that occur in patients with a high fungal burden in the brain at the onset of therapy. Our observations may have practical importance for the use of adjunct therapy along with antifungal agents in treatment of MC. It was an unexpected finding that mice treated with dexamethasone as a single drug had better survival than untreated animals, with similar fungal counts per brain in both groups. It has been shown that, in mice with experimental cryptococcus infection induced by the intravenous route, cortisone acetate did not change survival rates (14). The groups of mice treated with the combination of amphotericin B with either dexamethasone or pentoxifylline had parallel reductions of fungal counts similar to those of the group that received amphotericin B alone. The histopathological indices did not correlate with survival or fungal counts and were similar among treatment groups, indicating that the histopathological index has a lower sensitivity in comparison with the other assessed variables.

TABLE 3. Quantitative cultures of mouse brain tissue 14 days after intracerebral inoculation with 4×10^4 CFU of *C. neoformans*[a]

Drug[b]	CFU/brain (10^6)	Mean[c]	SD	SE
None	2.0, 1.8, 1.9, 1.4, 2.2	1.8	0.3	0.1
Amphotericin B	0.5, 0.04, 0.4, 0.2, 1.4	0.5[d]	0.5	0.2
Dexamethasone	1.8, 1.4, 2.0, 1.6, 2.2	1.8	0.3	0.1
Pentoxifylline	2.0, 3.7, 0.9, 2.3, 3.2	2.4	1.1	0.5
Amphotericin B + dexamethasone	0.2, 0.3, 0.5, 0.3, 0.07	0.3[d]	0.2	0.06
Amphotericin B + pentoxifylline	0.02, 0.4, 0.4, 0.1, 0.4	0.3[d]	0.2	0.08

[a] All mice receiving nonpyrogenic saline or autoclaved cryptococci had 0 CFU of *C. neoformans.*
[b] Given for 7 days.
[c] Of duplicate cultures of each brain.
[d] Significantly different from the corresponding value for the no-drug control group ($P < 0.05$; one-way analysis of variance followed by the Tukey test for multiple comparisons).

Involvement of neuroexcitatory mechanisms in cryptococcal infection is suggested by the reduced glutamate contents in the brain in the group that did not receive antifungal or adjunctive therapy. It seems plausible that cryptococcal brain infection induced a depletion of glutamate levels caused by components of the host immune response in the brain, as the autoclaved cryptococci and the live cryptococci induced similar responses. Therefore, it is not surprising that amphotericin B failed to prevent such action, since the toxicity of certain antifungal agents is related to their ability to induce the production of cytokines by mononuclear phagocytes. Recently Louie et al. (13) reported that amphotericin B elicited tumor necrosis factor alpha production by murine macrophages, this response was dependent on the concentration of amphotericin B, and this effect was attenuated by pretreating macrophages with pentoxifylline or dexamethasone. In our study, only the combination of amphotericin B plus dexamethasone reversed the depletive effect on glutamate content in the brain. The depletion of glutamate in cryptococcal intracerebral infection is probably due to excessive glutamate release, as has been found with other brain infectious or toxic disorders (12, 18).

In human cryptococcosis, patients immunosuppressed by pharmacological doses of corticosteroids are prone to develop MC, and the continuation of corticosteroid therapy during antifungal treatment has been identified as an adverse factor for survival (10). In patients with AIDS, corticosteroids used as adjunctive treatment produce reactivation of viral and fungal infections (4, 15). Pentoxifylline has been suggested as a therapeutic agent for some specific conditions of patients with human immunodeficiency virus infection (9). A recent in vitro observation indicated that pentoxifylline reduces tumor necrosis factor alpha secretion and increases mycobacterial load in macrophages from patients with AIDS with disseminated *Mycobacterium avium-M. intracellulare* complex infection (21). Both dexamethasone and pentoxifylline have potential risks for patients with AIDS in whom severe and life-threatening concurrent infections are common. Although the *Cryptococcus* strain chosen for this study rarely infects patients with AIDS, it was selected because it is consistently virulent for mice and represents about 10% of clinical isolates in countries like Mexico, Australia, and Brazil (16).

Additional experimental studies with either dexamethasone or pentoxifylline as an adjunct to antifungal therapy are warranted for MC in an attempt to reduce the acute mortality associated with a high fungal burden in the brain. The mechanisms of action of pentoxifylline may be multiple either at the microglial level (7) or by modifying the activation of macrophages or neutrophils induced by amphotericin B (13, 24). The role of endogenous cytokines in the pathogenic mechanisms of cerebral cryptococcosis remains to be studied.

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