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Aerosol and Parenteral Pneumocandins Are Effective in a Rat Model of Pulmonary Aspergillosis

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The pneumocandins are semisynthetic analogs of echinocandin-like compounds that have shown efficacy in animal models of systemic candidiasis, disseminated aspergillosis, and pneumocystis pneumonia. However, the most common form of *Aspergillus* infection in susceptible patients is pulmonary aspergillosis, which was not directly tested in the mouse models used in the past. We have evaluated three pneumocandins, L-693,989, L-731,373, and L-733,560, in a rat model of pulmonary aspergillosis. Male Sprague-Dawley rats were treated for 2 weeks with cortisone and tetracycline and fed a low-protein diet before being inoculated via the trachea with 10^6 conidia of *Aspergillus fumigatus* H11-20. In the absence of drug treatment, the animals developed a progressive, rapidly fatal bronchopneumonia. All three pneumocandins at doses of 5 mg/kg (intraperitoneally [i.p.] every 12 h [q12h]) were effective in delaying mortality in this model. Survival at day 7 postinfection was 20% among controls ($n = 10$ for all groups), while it was 60, 80, and 90% in groups that were treated with L-693,989, L-731,373, and L-733,560, respectively. In another trial, survival at day 7 postinfection was 25% among controls ($n = 8$ for all groups); it was 87.5% in a group treated with amphotericin B (0.5 mg/kg i.p. q12h) and was 100% in a group treated with L-733,560 (0.625 mg/kg i.p. q12h). In a separate trial, aerosol L-693,989 administered 2 h before infection (5 mg/kg) delayed mortality. Eight of the 10 animals treated with aerosol L-693,989 survived for 7 days, whereas only 2 of 10 control animals survived. We conclude that the pneumocandins we tested were highly effective in an animal model of pulmonary aspergillosis.

The semisynthetic pneumocandins analogs L-731,373 and L-733,560 are members of a new class of antifungal agents which, unlike previously studied echinocandins and pneumocandins, have a broad spectrum of in vivo activity against *Candida* and *Aspergillus* spp. and *Pneumocystis carinii* (2, 7, 22, 30, 32). These new pneumocandin analogs are efficacious in mouse models of disseminated candidiasis (1, 2) and aspergillosis (2), are extremely potent in a rat model of pneumocystis pneumonia (32, 33), and prolong survival in a rat model of pulmonary aspergillosis (10). The in vivo anticandida activity is not surprising in view of the superior in vitro activity of these compounds in antifungal susceptibility testing (7). The amino-containing analogs also have potent activity against *Aspergillus* spp. in an agar diffusion assay (8) or a modified MIC assay with reduced growth and profound morphological alterations as the end point (28, 29).

For *Candida* and *Aspergillus* spp., there is now ample evidence that the pneumocandins inhibit the synthesis of 1,3-β-D-glucan, a cell wall polymer vital to the structural integrity of these fungi (4, 19, 20, 34, 38–41). Confirmation of the mechanism of antifungal activity derives from in vitro enzyme inhibition, whole-cell labeling (4, 13), ultrastructure studies (12–14, 18, 21, 28), and genetic analysis (17). Inhibition of cell wall synthesis is a particularly attractive attribute for an antifungal drug candidate, because the likelihood of mechanism-based toxicity and cross-resistance to currently used agents with different modes of action should theoretically be reduced.

In view of the potential of the pneumocandin analogs as antiaspergillus agents, we have studied the effectiveness of L-693,989, L-731,373, and L-733,560 in a rat model of pulmonary aspergillosis which may mimic the route and course of human infection more closely than intravenous infection. Rats pretreated with cortisone and inoculated via the trachea with *Aspergillus fumigatus* develop rapidly progressive bronchopulmonary aspergillosis. Infection remains confined to the lungs. Prevention or delay of mortality in this model is a sensitive and reliable indicator of significant activity (36).

(A portion of this work has been presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, 1993 [10].)

MATERIALS AND METHODS

Antifungal compounds. Figure 1 shows the chemical structures of the natural product pneumocandin B_0 (37) and the semisynthetic derivatives L-731,373 (11), L-733,560 (11), and L-693,989 (5) (provided by scientists at Merck Research Laboratories, Rahway, N.J.). All compounds were shown by high-performance liquid chromatography to be >95% pure. Amphotericin B (AmB) (Fungizone) was from Bristol-Myers Squibb.

In vitro antifungal activity. The in vitro susceptibility to the pneumocandins was determined for *A. fumigatus* H11-20, the strain used for the in vivo test, by both agar diffusion and a morphological assay (28, 29). The diffusion assay used 10 ml of potato dextrose medium seeded with 10^6 conidia of *A. fumigatus* in petri dishes. Pneumocandins (128 to 0.06 μg/ml) were applied to 5-mm-diameter wells in the agar surface, and zones of inhibition were measured after 24 h of incubation at 30°C. The critical concentration was determined for each compound as described previously (3). Yeast Nitrogen Base (Difco) with 2% glucose was used for determinations of MICs and minimum effective concentrations (MECs) by the broth microdilution assay described previously (27). Briefly, 10^4 conidia were inoculated into 0.15 ml of medium containing twofold serial dilutions of test compound in a 96-well microtiter plate. Growth was monitored visually after incubation for 48 h at 30°C for the MIC assay. The morphological assay used the same medium, inocula, and growth conditions as the standard MIC determination. The MEC was considered to be the lowest concentration of drug to produce a change in morphology observable by eye (28).

Animal model. All animal procedures related to efficacy studies were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Memorial Sloan-

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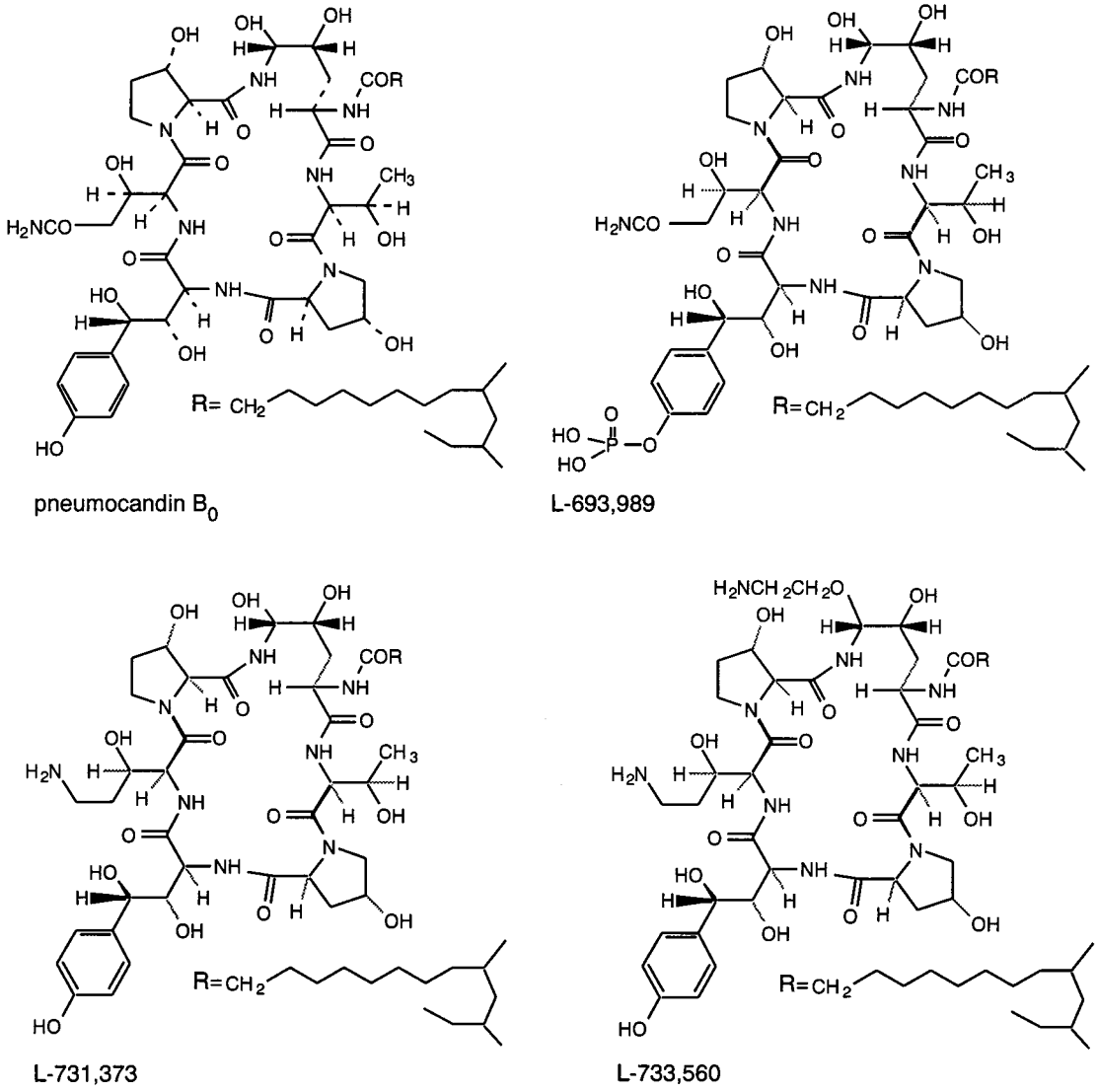

FIG. 1. Chemical structures of pneumocandin analogs.

Kettering and Merck Institutional Animal Care and Use Committees. The rat model of pulmonary aspergillosis has been described previously (35). In brief, male Sprague-Dawley rats weighing 140 to 150 g received cortisone acetate (100 mg/kg, subcutaneously, thrice weekly), tetracycline via drinking water, and a low-protein diet. After 2 weeks on this regimen, animals were infected via the trachea with a suspension of 10^6 conidia of *A. fumigatus* H11-20 in 0.1 ml of sterile saline. In every trial, each treatment or control group consisted of 8 or 10 animals. Most trials included a total of six groups. The outcome was judged by survival analysis and histopathologic examination of the lungs. Tissues from animals at necropsy were homogenized in saline (9 ml/g), and slides were stained with toluidine blue-O. All animals were anesthetized with enflurane during all surgical procedures and dosing. Moribund animals were sacrificed with carbon dioxide gas. Survival analysis was done by Kaplan-Meier plots and the log rank test. A P value of less than 0.05 was considered significant.

Treatment regimens. For parenteral treatment, animals continued to receive the immunosuppressive regimen for 1 week postinfection and were treated with antifungal agents or a placebo intraperitoneally every 12 h for 7 days. The pneumocandins were dissolved in saline and sterilized by filtration; AmB solutions were prepared by diluting AmB in sterile 5% glucose in water.

For single-dose aerosol treatment, groups of four or five rats were placed in a glass chamber filled with a stream of nebulized L-693,989 created by air flowing at 8 liters/min through a nebulizer (Cadema Medical Products, Middletown, N.Y.) as described previously (35). Two hours later, the rats were infected with a lethal inoculum of *A. fumigatus* H11-20. Under these conditions, the drug solution was aerosolized at a rate of 0.3 ml/min, and particles with an estimated mean mass aerodynamic diameter of 1.0 μm were generated. The dose of aerosolized L-693,989 or AmB was calculated from the product of the concentration of the drug in the chamber, the minute volume of the rats (lung volume × respiratory rate), and the time of exposure. In these experiments, the exposure time was 15 min; 4.5 ml of water containing 150 mg of L-693,989 in solution was nebulized, and the minute volume was assumed to be 70 ml. The mean weight of the rats was 130 g. The final dose for AmB was 0.8 mg/kg. The calculated dose provides an estimate of the amount of drug inhaled; it does not predict the amount that will be retained by the lung. Previous experiments with this procedure and AmB showed that the dose retained in the lung was 4% of the administered dose (35), which is equivalent to 15 μg/g of lung tissue.

RESULTS

In vitro antifungal susceptibility assays. To determine the susceptibility of the *Aspergillus* isolate used in the animal model (*A. fumigatus* H11-20) to the pneumocandin analogs being evaluated, two assays were used. As has been previously noted (19, 20, 26, 28, 29, 31), the pneumocandin class of compounds do not produce a clear MIC against *Aspergillus* spp. The data in Table 1 show that all of the pneumocandin analogs tested had MICs of >128 μg/ml, while the MICs of AmB were between 0.2 and 0.8 μg/ml. However, the compounds were quite active against strain H11-20 when tested in an agar dif-

TABLE 1. Susceptibility of *A. fumigatus* H11-20 to pneumocandin B_0, L-731,373, and L-733,560

Compound	Agar diffusion (μg/ml)	MIC (μg/ml)	MEC (μg/ml)
AmB	0.4	0.2–0.8	ND[a]
Pneumocandin B_0	<0.06	>128	0.5
L-731,373	<0.06	>128	0.25
L-733,560	0.50	>128	0.06

[a] ND, not done.

fusion assay or in a morphological assay. Pneumocandin B_0 was used rather than the phosphate prodrug derivative L-693,989 since previous tests had shown that L-693,989 was not active in in vitro tests, presumably because of the lack of sufficient phosphatase activity to liberate the active compound under the assay conditions used (data not shown).

In vivo parenteral studies. A total of seven trials with different doses of the three pneumocandins were completed. In all trials, the placebo-treated controls developed rapidly progressive pulmonary aspergillosis. The overall mortality rate among controls in all trials was 83% ($n = 58$).

Mortality curves with animals treated with the phosphate prodrug L-693,989 showed that the drug-treated animals survived longer than the control animals (90 versus 10% survival at 7 days). This compared quite favorably with the survival of animals given the conventional dose of AmB (0.5 mg/kg), which was 60% at 7 days postinfection in this trial. In rats treated with a combination of the two drugs, the effects appeared to be additive, with 100% of the animals still alive at the end of the observation period. There was no evidence of antagonism with AmB in this test or in other trials (data not shown).

In view of the superior in vitro and in vivo activities of the amino-modified analogs, L-731,373 and L-733,560, against *Candida albicans* (6–8) and *Aspergillus* spp. in disseminated-infection models (1, 2, 27–29), we compared the efficacies of these compounds with L-693,989 in the pulmonary aspergillosis model. All three compounds were effective in prolonging the survival of infected animals (Fig. 2). The relative potencies of the compounds at 7 days postinfection were L-733,560 > L-731,373 > L-693,989, with 90, 80, and 60% survival, respectively. Although the differences did not reach statistical significance, both amino-modified analogs were better than L-693,989 in preventing mortality. These results are consistent with the ranking of the three compounds in the target organ kidney model of disseminated candidiasis in mice (7) and in a survival model for disseminated aspergillosis in immunocompromised mice (1, 2).

The excellent survival of animals treated with 5 mg of L-733,560 per kg prompted a comparison between therapy with low doses of L-733,560 and therapy with standard doses of AmB. Nine of 10 rats treated with 0.625 mg of L-733,560 per kg and all rats (10 of 10) treated with 0.5 mg of AmB per kg survived for 7 days postinfection, while survival was 20% (2 of 10) among controls, indicating that the two therapies were equally potent in protecting rats from death (data not shown).

Histopathologic examinations of the lungs from necropsied animals showed that the lungs of animals treated with the pneumocandins were normal in size and appearance. Only rare mycelia were seen on microscopic examination, and these were notable for their stunted, aberrant morphology (Fig. 3C and D).

In vivo aerosol studies. Ten animals were treated with a single dose of aerosolized L-693,989 (10 mg/kg), another 10 animals were treated with aerosolized AmB (0.8 mg/kg), and 10 more were administered aerosolized saline as controls. Two hours after the dosing, the rats were inoculated intratracheally with *Aspergillus* conidia, and survival was monitored (Fig. 4). The first death among the L-693,989-treated animals occurred on day 5, by which time five of the control animals had died. By day 7, 2 of 10 saline-treated animals survived, while 8 of 10 of the L-693,989-treated animals were still alive. All of the AmB-treated animals were alive at the end of the 7-day observation period.

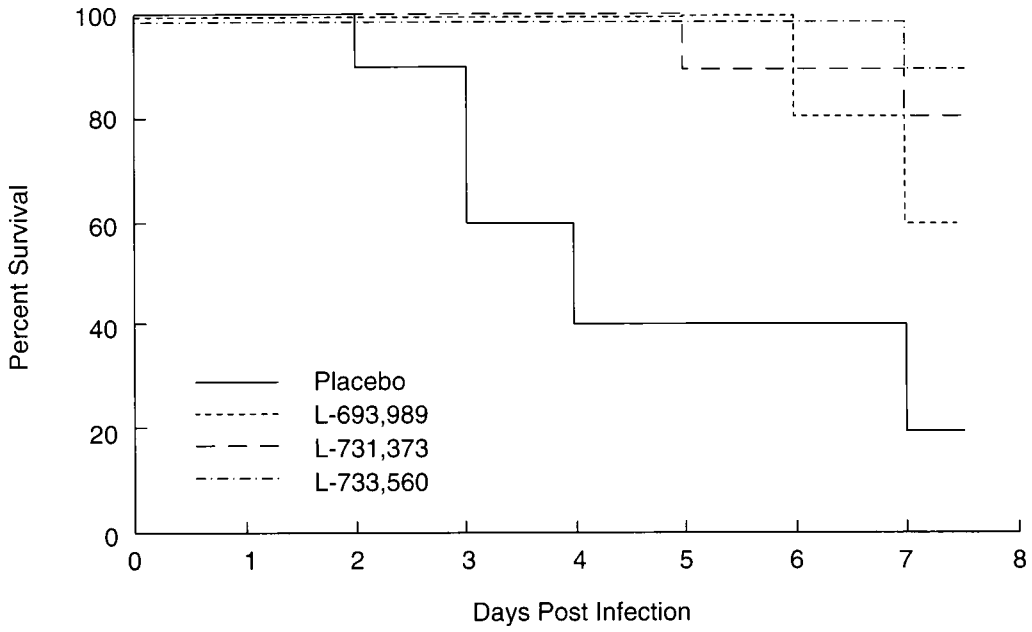

FIG. 2. Cumulative mortality of rats treated with three pneumocandin analogs (5 mg/kg). Each group consisted of 10 animals. Treatment was initiated at the time of infection and continued for 7 days.

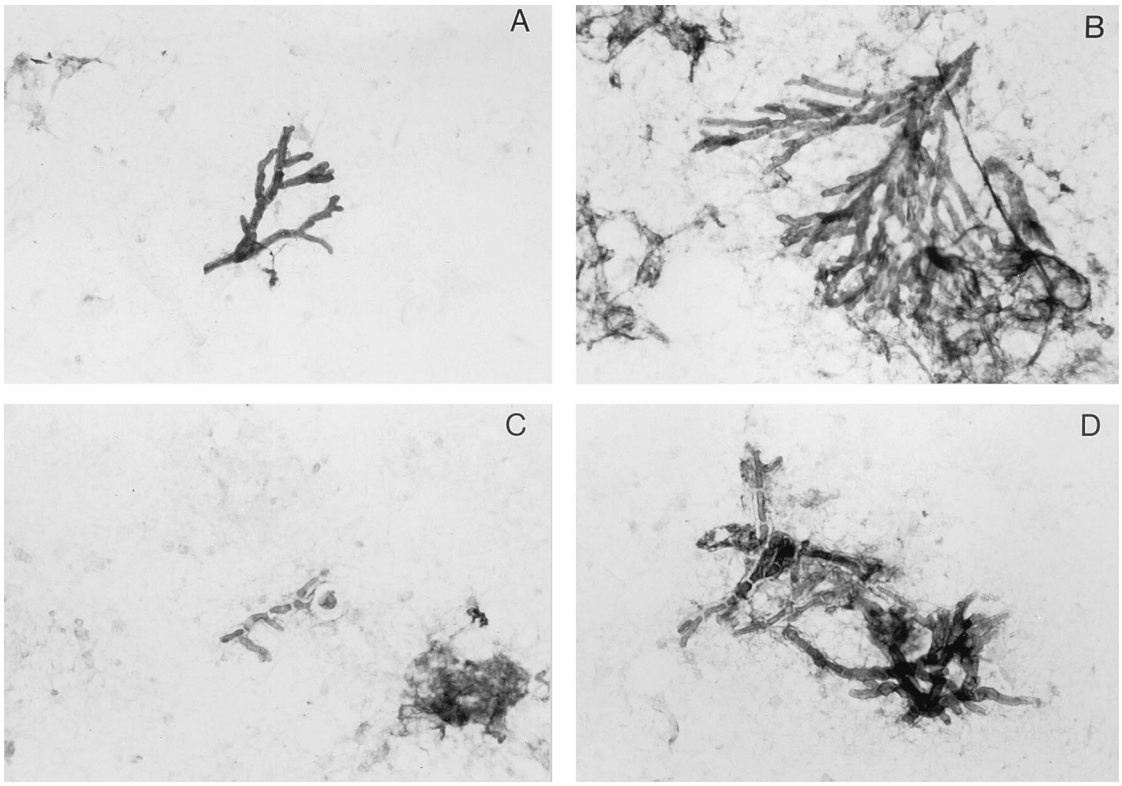

FIG. 3. Light micrographs of *A. fumigatus*-infected rat lung tissue stained with toluidine blue-O. (A and B) Sham-treated control; (C and D) animal treated with 0.625 mg of L-733,560 per kg.

DISCUSSION

This report describes the in vivo activity of pneumocandin antifungal compounds in an experimental rat model of pulmonary aspergillosis caused by *A. fumigatus*. The relative ranking of these compounds compares well with the in vivo efficacies in a model of disseminated aspergillosis in the mouse (1, 2).

As has been demonstrated previously, the pneumocandins showed no clear MIC against *A. fumigatus* in a microtiter assay with lack of visible growth as an end point (2). However, the isolate used in the animal model was sensitive to the analogs in both an agar diffusion assay and a morphological assay described previously (28, 29) (Table 1). The MEC results with *A. fumigatus* correlate well with previous reports of relative potencies of these compounds against *C. albicans* (8, 30) and also correlate with the in vivo efficacies in a model of disseminated aspergillosis in the mouse (1, 2).

Evidence is accumulating that the pneumocandins and echinocandins severely inhibit the growth of *Aspergillus* species, probably by inhibiting cell wall glucan synthesis (9, 16, 28). Although early in vivo experiments showed that >62.5 mg of cilofungin per kg was needed to prolong survival when therapy was delayed for 24 h in a mouse model of disseminated disease (15), lower doses were required in a 10-day treatment regime initiated shortly after infection (50% effective dose of 20.6 mg of cilofungin per kg) (9). Better activity has been demonstrated for more-potent analogs of echinocandin B (42) and amino-containing pneumocandins (1, 2, 42) in similar mouse models of aspergillosis. Among the compounds we tested, we found that the more-potent inhibitors, as judged by the morphology assay, required lower doses to be effective in vivo. The results show that L-733,560 can prolong survival at 0.625 mg/kg, which approaches the dose of AmB (0.5 mg/kg) required for similar efficacy. The ranking of potency in this pulmonary aspergillosis model correlates well with relative potencies in a model of disseminated infection in immunocompromised mice that used an intravenous injection of spores (1). In the disseminated-infection model, L-733,560 was the most potent compound, with a 50% effective dose estimated at 0.028 mg/kg. Earlier work with itraconazole in this model showed that between 40 and 80 mg/kg was required to achieve a similar level of survival (36). Given that only AmB and itraconazole have been used successfully to treat human pulmonary aspergillosis and that this rat model correlates with the outcome in humans (25, 36), compounds such as L-733,560 should be evaluated for treatment of this life-threatening disease.

Histopathologic examination of the lung tissue of pneumocandin-treated animals indicated that, unlike in control infected animals, fungal cells were rare. The few mycelia seen in the pneumocandin-treated tissues were stunted and morphologically abnormal (Fig. 3). The accumulating biochemical and genetic evidence (9, 12, 13, 17, 26–28, 38, 39, 42) indicates that the lipopeptide class of compounds produce cell wall abnormalities in *Candida* and *Aspergillus* spp. by inhibition of 1,3-β-glucan synthesis. Under the high osmotic pressure of the cell contents, the weakened cell wall of a drug-treated cell balloons into abnormal structures. In the case of *Candida* spp., cell lysis can be observed (12, 13). The aberrant *A. fumigatus* forms seen in vivo in this study resemble the altered germlings and mycelia seen in vitro (28) and suggest that the mode of action of the pneumocandins in vivo is the same as it is in vitro. This suggests that the pneumocandins may facilitate clearance of the aspergillus infection rather than just prevent hyphal proliferation.

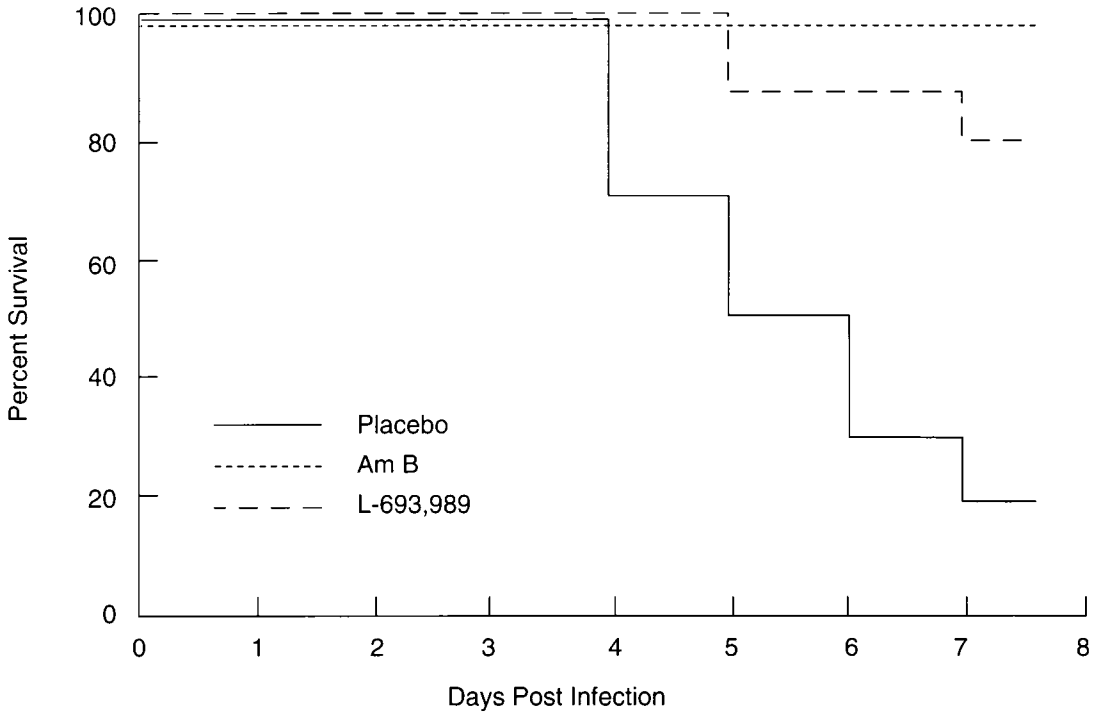

FIG. 4. Single-dose aerosol treatment of pulmonary aspergillosis with L-693,989. Groups of 10 animals received aerosol doses of AmB (0.8 mg/kg) or L-693,989 (10 mg/kg). Single doses were administered at 2 h prior to infection.

Combination therapy with L-733,560 and AmB resulted in better protection from mortality than was obtained with either agent alone. The results suggest additivity of the two drugs; no evidence of antagonism was seen in any trials. Combination studies have been performed with AmB and cilofungin, a semisynthetic echinocandin B derivative with the same mode of action as the pneumocandins (15, 23). In a mouse model of disseminated rather than pulmonary aspergillosis, the combination of AmB (3.3 mg/kg) and cilofungin (62.5 mg/kg) resulted in greater mortality than was observed with either agent alone (15, 35). It is possible that the dose of AmB contributed to the mortality, since the same combination but with a lower level of AmB (0.625 mg/kg) was synergistic in a mouse model of candidiasis (23). Neither the mechanism of antagonism nor that of synergy for these compounds has been established (15, 23). Some in vitro synergy against *C. albicans* was demonstrated (23). The additivity in the rat model of pulmonary aspergillosis indicates that concurrent L-733,560–AmB therapy may be of value in the clinic.

The dramatic improvement in survival with aerosolized L-693,989 was encouraging in view of the fact that a single dose was administered prior to infection. It will be interesting to determine if the more potent analogs such as L-733,560 are more effective than L-693,989 in this model. Noteworthy in this regard is the reduced incidence of human fungal disease when aerosolized AmB is used prophylatically (24).

We conclude that the pneumocandins are highly active against *Aspergillus* species in a model which correlates well with human experience in the clinic. The addition of AmB or another therapy with activity against aspergillosis may also be of value in treating life-threatening disease. On the basis of our data, clinical trials with a potent pneumocandin such as L-733,560 appear warranted.

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