Aerosolized Amphotericin B-Liposomes for Treatment of Systemic *Candida* Infections in Mice

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Mice lethally infected with *Candida albicans* were exposed to small-particle aerosols containing amphotericin B-liposomes. The drug, when administered twice daily for 2 h (0.58 mg/kg of body weight per day) on days 1, 2, and 3 postinoculation, significantly reduced the numbers of *Candida* organisms in the kidneys. Aerosol treatment increased the survival time of mice given 2 2-h treatments once a week for 4 weeks. A twice-weekly, 2-h small-particle aerosol administration of amphotericin B-liposomes for 1, 2, or 3 weeks significantly increased both the mean time of survival and percent survival.

Fungal infections of immunocompromised patients are a major source of morbidity and mortality. Of the fungi causing these infections, *Candida albicans* has been demonstrated to be the most common causative agent of oral candidiasis in patients infected with human immunodeficiency virus (12). *Candida albicans* may account for more than 20% of the fatal infections in patients with leukemia and for 13% of those in patients with lymphomas (15). Thus, in these immunocompromised individuals, both mucocutaneous and/or systemic disease can be caused by *Candida* infection.

Amphotericin B (AmpB) (Fungizone) has been used in the treatment of *Candida* infections. However, the toxicity associated with this drug has limited its usefulness and has led to the development of preparations of AmpB-liposomes (AmpB-Lip) which retain antifungal activity but which have reduced toxicity (1, 7, 13, 15). We have shown recently (6) that AmpB-Lip can be successfully aerosolized, generating particles with mass median aerodynamic diameters of 1.8 μ m which are capable of depositing the drug throughout the respiratory tract (9, 11). AmpB-Lip aerosol was effective in the treatment of mice infected intranasally with *Cryptococcus neoformans* (6). Aerosol administration reduced the number of cryptococci in the lungs and brain and increased the mean duration of survival. These studies indicated that AmpB-Lip could be effective in treating both local, pulmonary *Cryptococcus* disease and systemic disease.

In the present studies, a clinical isolate of *Candida albicans* (14, 15) was used to infect 6- to 8-week-old (25- to 28-g), random-bred CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The animals were housed in cages covered with barrier filters and were fed mouse chow and water ad libitum. Mice were inoculated intravenously in the tail vein with 7×10^5 CFU of *Candida albicans* in 0.2 ml (day 0) (15). Five to six mice per group were used in the quantitation of organisms colonizing the kidneys, and 15 mice per group were used in the mortality experiments. Experiments were terminated 14 days after the last animal to die had died (note that time points have been truncated in Fig. 2 and 3 for clarity).

Mice were exposed to small-particle aerosols (SPA) contain-

ing AmpB-Lip (4, 19). A Collison aerosol generator was used with a reservoir modified to allow for collection of Lip at the bottom (6, 10, 19). The estimated amount of drug retained by the mice was calculated from the amount of drug in the aerosol, the minute volume, and duration of treatment (4, 6, 19). Lip were composed of AmpB (1.6-mg/ml final pure concentration; 80% pure; Sigma Chemical Co., St. Louis, Mo.) and egg yolk phosphatidylcholine (30-mg/ml final concentration; Avanti Polar Lipids, Inc., Pelham, Ala.) (3, 6). Control Lip were prepared similarly but without AmpB. Lip preparations were kept at 4°C for up to 3 days.

The concentration of AmpB generated in the aerosol and the particle size distribution were determined with an all-glass impinger and an Andersen sampler, respectively (5, 6). The mass median aerodynamic diameter of the aerosol particles was $1.8 \pm 0.2 \mu$ m, with a geometric standard deviation of $3.4 \pm 0.6 \mu$ m. The aerosol contained $10.3 \pm 2.4 \mu$ g of AmpB per liter (6). On the basis of this value, the estimated retained amount of AmpB for a twice-daily, 2-h treatment period was calculated to be 0.58 mg/kg of body weight per day (4, 6, 19).

To quantitate *Candida* organisms in organs, both kidneys and spleens were removed, rinsed of any adhering blood, and homogenized in 5 ml of phosphate-buffered saline, pH 7. Serial 10-fold dilutions of the homogenates were plated (at 0.1 ml) on petri dishes containing Sabouraud's agar. Following incubation at 35°C for 2 to 3 days, colonies were quantitated. Results are presented as means $\pm$ standard deviations. Variation in the quantitations of *Candida* organisms in kidneys ranged from standard deviations of $\pm 17\%$ to $\pm 57\%$ when at least 10 organisms were counted per plate, except as noted. Statistical

TABLE 1. Effect of aerosolized AmpB-Lip on colonization of organs by *Candida albicans* administered intravenously

Expt no.	CFU/organ (mean $\pm$ SD) ^a in:			
	Control mice		Treated mice	
	Kidneys	Spleen	Kidneys	Spleen
1	5,300 $\pm$ 3,020 ^b	430 $\pm$ 254	1,150 $\pm$ 1,059 ^b	333 $\pm$ 367
2	6,480 $\pm$ 1,944 ^b	ND ^c	1,340 $\pm$ 1,286 ^b	ND

^a Results are for 5 to 6 mice.

^b For control versus treated mice, $P < 0.003$ (two-tailed student's t test).

^c ND, not done.

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FIG. 1. Effect of dosing schedule on *Candida* organisms in mouse kidneys following treatment with aerosolized AmpB-Lip. The drug was administered (to five mice per group) once for 0.5 h (1×0.5 HR) at 0.08 mg/kg/day, once for 1 h at 0.15 mg/kg/day, once for 2 h at 0.3 mg/kg/day, and twice for 2 h at a total of 0.6 mg/kg/day.

analysis of the data was performed with the True Epistat statistical package from Epistat Services, Richardson, Tex. *P* values are based on analysis of these data by Student's *t* test with analysis of variance, by Fisher's exact test, or by life table analysis.

The effect of AmpB-Lip aerosol on the colonization of the kidneys was evaluated for mice ($n = 5$ to 6 per group) treated with AmpB-Lip aerosol for 2 h twice daily (total of 0.58 mg/kg/day) on days 1, 2, and 3. On day 4, both kidneys (a major site for infection [14]) and the spleen were removed and the organisms were quantitated. A statistically significant reduction in the number of organisms in the kidneys was associated with the aerosol treatment ($P < 0.003$; Table 1). A low number of yeasts were recovered from the spleen, with no observed differences between control and treated mice. Additional experiments for dosing schedules indicated that a minimum treatment of 2 h (0.3 mg/kg/day) initiated on day 1 reduced colonization of the kidneys compared with that in the controls ($P = 0.064$; Fig. 1). Shortening the duration of treatment on day 1 to 0.5 or 1 h did not reduce the number of organisms in

the kidneys ($P = 0.094$ and 0.221 , respectively). Delaying treatment until day 3 increased the number of *Candida* organisms colonizing the kidneys ($P = 0.007$); however, the higher-than-usual variation in the number of organisms in the kidneys of the control mice may account for this apparent increase. Multiple, 2-h, once- or twice-daily treatments were most effective compared with treatments given controls ($P = 0.039$ and 0.018 , respectively). There was no statistical difference in the multiple treatments given once or twice daily beginning on day 1 ($P = 0.709$).

Aerosol administration of AmpB-Lip also was effective in protecting mice from a highly lethal, intravenous challenge with *Candida albicans*. The degree of protection depended on the treatment regimen. A single 2-h treatment, once a week starting on day 1 and continuing for 4 weeks, was as effective as a single 2-h treatment given only on days 1 to 3. Compared with those for the controls, the mean durations of survival increased by 9 and 13 days, respectively ($P < 0.001$) (Fig. 2); however, treatment did not increase the percent survival. Treatment extended to twice-a-week, 2-h SPA administration

FIG. 2. Effect of dosing schedule on mortality of mice infected systemically with *Candida* organisms and treated with aerosolized AmpB-Lip. Mice ($n = 15$) were treated with SPA (2 mg of AmpB/ml in the reservoir) once for 2 h on days 1, 2, and 3 (■) or on days 1, 8, 15, and 22 (▲) or were left untreated (●). The drug was given once for 2 h at 0.3 mg/kg/day.

FIG. 3. Effect of dosing schedule on mortality of mice infected systemically with *Candida* organisms and treated with aerosolized AmpB-Lip. Mice ($n = 15$) were treated with SPA (2 mg of AmpB/ml in the reservoir) for 2 h twice a week (Tuesday and Friday) on week 1 (■), weeks 1 and 2 (▲), or weeks 1, 2, and 3 (○) or were left untreated (●). The drug was given once for 2 h at 0.3 mg/kg/day.

of AmpB-Lip for 1 week, for 1 and 2 weeks, or for 1, 2, and 3 weeks significantly increased the mean duration of survival by 12, 11, and 19 days, respectively ($P < 0.002$). The 3-week treatment also was effective in increasing the percent survival ($P < 0.001$) (Fig. 3).

The murine model of systemic candidiasis used by us has been well characterized (14). Intravenous inoculation with *Candida* organisms causes a very lethal infection with involvement of most organ systems, including severe histopathological changes in the kidneys which affect renal function. AmpB has been shown to be effective in reducing, but not eliminating, mortality (1, 8, 15). Because of the toxicity associated with AmpB, various liposomal preparations of AmpB have been tested and, when administered intravenously, shown to effectively protect mice from lethal *Candida* infections and to have reduced toxicity (1, 7, 8, 15).

Previously, we have shown that SPA administration of AmpB-encapsulated Lip was effective in treating both local, pulmonary *Cryptococcus* disease and systemic disease (6). It seemed reasonable that similar AmpB-Lip SPA treatment of *Candida* disease, especially in immunocompromised AIDS patients, in which oral as well as systemic infection frequently occurs (2, 12), should also be effective. This is particularly likely since SPA delivers AmpB directly to the oral cavity and respiratory tract, where it appears to diffuse from the lungs to the kidneys and other organs. Aerosolized AmpB in deoxycholate has been tested in the rat model of pulmonary aspergillosis (16, 17). Prophylaxis and treatment of pulmonary aspergillosis with aerosolized AmpB significantly prolonged the mean duration of this difficult-to-treat disease but not the percent survival. Pharmacokinetics of AmpB in the lungs of these rats suggested that the drug may accumulate following multiple treatments.

In our previous studies, we have shown that SPA administration of liposomal AmpB achieved estimated concentrations in the lung of 40 $\mu\text{g}/\text{ml}$ of respiratory secretion or about 5 $\mu\text{g}/\text{g}$ of lung tissue (6). This value is approximately 50 times the levels in lungs achieved by intravenous injection (1 mg/kg/day) of AmpB-lipid complex (1) or unilamellar Lip (7) and is well above the inhibitory concentration for *Candida* organisms (14, 18), even in human immunodeficiency virus-infected patients, from whom clinical isolates may demonstrate significantly less in vitro susceptibility to AmpB (12).

The fact that aerosol treatment in this and the *Cryptococcus*

models (6) had an effect on systemic infection indicates that AmpB found in high concentrations in the lungs was capable of getting from the lungs to distant organs and at therapeutic levels. Thus, AmpB delivered to the lungs may be useful for treating other diseases with systemic components.

The data obtained in the present study indicate that SPA administration of AmpB-Lip is an effective way of treating systemic *Candida* infection in mice and that limited treatments spread over many weeks may be the most effective regimen. Because of the higher-than-usual variability in the quantitation of organisms observed for the controls in Fig. 1 (standard deviation: $\pm 17\%$ to $\pm 49\%$ versus $\pm 93\%$ in this experiment), there appeared to be significant enhancement of colonization of the kidneys when treatment was delayed to day 3. We do not believe that this occurs since multiple treatments spread over the week protected mice from this lethal disease and was never observed to enhance it (Fig. 2 and 3).

We believe that systemic *Candida* infections may be effectively treated with aerosolized AmpB-Lip. The advantages of using aerosols for drug delivery are the selective deposition of the drug throughout the respiratory tract without the use of invasive procedures and the subsequent release of the drug into the systemic circulation. On the basis of this and the previous *Cryptococcus* study, we believe that aerosolized AmpB-Lip should be further evaluated.

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REFERENCES

- Clark, J. M., R. R. Whitney, S. J. Olsen, R. J. George, M. R. Swerdel, L. Kunselman, and D. P. Bonner. 1991. Amphotericin B lipid complex therapy of experimental fungal infections in mice. *Antimicrob. Agents Chemother.* 35:615-621.
- Diamond, R. D. 1991. The growing problem of mycoses in patients infected with the human immunodeficiency virus. *Rev. Infect. Dis.* 13:480-486.
- Garcon, N. M., H. R. Six, J. Frazer, C. Hazlewood, B. E. Gilbert, and V. Knight. 1989. Liposomes of enviroxime and phosphatidylcholine: definition of the drug-phospholipid interactions. *Antiviral Res.* 11:89-98.

4. Gilbert, B. E., and V. Knight. 1986. Biochemistry and clinical applications of ribavirin. *Antimicrob. Agents Chemother.* **30**:201–205.
5. Gilbert, B. E., H. R. Six, S. Z. Wilson, P. R. Wyde, and V. Knight. 1988. Small particle aerosols of enviroxime-containing liposomes. *Antiviral Res.* **9**:355–365.
6. Gilbert, B. E., P. R. Wyde, and S. Z. Wilson. 1992. Aerosolized liposomal amphotericin B for treatment of pulmonary and systemic *Cryptococcus neoformans* infections in mice. *Antimicrob. Agents Chemother.* **36**:1466–1471.
7. Gondal, J. A., R. P. Swartz, and A. Rahman. 1989. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. *Antimicrob. Agents Chemother.* **33**:1544–1548.
8. Hospenthal, D. R., A. L. Rogers, and E. S. Beneke. 1989. Effect of attachment of anticandidal antibody to the surfaces of liposomes encapsulating amphotericin B in the treatment of murine candidiasis. *Antimicrob. Agents Chemother.* **33**:16–18.
9. Knight, V., and B. Gilbert. 1988. Antiviral therapy with small particle aerosols. *Eur. J. Clin. Microbiol. Infect. Dis.* **7**:721–731.
10. Knight, V., B. E. Gilbert, and S. Z. Wilson. 1986. Ribavirin small particle aerosol treatment of influenza and respiratory syncytial virus infections, p. 37–56. *In* T. Stapleton (ed.), *Studies with a broad spectrum antiviral agent*. Royal Society of Medicine Services, Ltd., New York.
11. Knight, V., C. P. Yu, B. E. Gilbert, and G. W. Divine. 1988. Estimating the dosage of ribavirin aerosol according to age and other variables. *J. Infect. Dis.* **158**:443–448.
12. Korting, H. C., M. Ollert, A. Georgii, and M. Fröschl. 1988. In vitro susceptibilities and biotypes of *Candida albicans* isolates from the oral cavities of patients infected with human immunodeficiency virus. *J. Clin. Microbiol.* **26**:2626–2631.
13. Lopez-Berestein, G., V. Fainstein, R. Hopfer, K. Mehta, M. P. Sullivan, M. Keating, M. G. Rosenblum, R. Mehta, M. Luna, E. M. Hersh, J. Reuben, R. L. Juliano, and G. P. Bodey. 1985. Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: a preliminary study. *J. Infect. Dis.* **151**:704–710.
14. Lopez-Berestein, G., and R. L. Juliano. 1987. Application of liposomes to the delivery of antifungal agents, p. 253–276. *In* M. J. Ostro (ed.), *Liposomes: from biophysics to therapeutics*. Marcel Dekker, Inc., New York.
15. Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh, and R. Juliano. 1983. Treatment and prophylaxis of disseminated infection due to *Candida albicans* in mice with liposome-encapsulated amphotericin B. *J. Infect. Dis.* **147**:939–945.
16. Niki, Y., E. M. Bernard, H.-J. Schmitt, W. P. Tong, F. F. Edwards, and D. Armstrong. 1990. Pharmacokinetics of aerosol amphotericin B in rats. *Antimicrob. Agents Chemother.* **34**:29–32.
17. Schmitt, H. J., E. M. Bernard, M. Häuser, and D. Armstrong. 1988. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of pulmonary aspergillosis. *Antimicrob. Agents Chemother.* **32**:1676–1679.
18. van Etten, E. W. M., N. E. van de Rhee, K. M. van Kampen, and I. A. J. M. Bakker-Woudenberg. 1991. Effects of amphotericin B and fluconazole on the extracellular and intracellular growth of *Candida albicans*. *Antimicrob. Agents Chemother.* **35**:2275–2281.
19. Wyde, P. R., S. Z. Wilson, B. E. Gilbert, and R. H. A. Smith. 1986. Protection of mice from lethal influenza virus infection with high dose-short duration ribavirin aerosol. *Antimicrob. Agents Chemother.* **30**:942–944.