Polysaccharide-Coated Liposomal Amphotericin B for the Treatment of Murine Pulmonary Candidiasis

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*The Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852, *Department of Hospital Pharmacy, Nagasaki University Hospital, Nagasaki 852, and †Department of Polymer Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606*

MIYAZAKI, T., KOHNO, S., SASAYAMA, K., INOUE, Y., HARA, K., OGASAWARA, M., SATO, T. and SUNAMOTO, J. *Polysaccharide-Coated Liposomal Amphotericin B for the Treatment of Murine Pulmonary Candidiasis*. Tohoku J. Exp. Med., 1992, 168 (3), 483-490 — Amylopectin-coated liposomal amphotericin B was investigated in a murine model of pulmonary candidiasis. The LD₅₀ of amylopectin-coated liposomal amphotericin B in normal mice was more than 10.0 mg/kg, and that of conventional amphotericin B was 1.2 mg/kg. Amylopectin-coated liposomes showed twice the concentration in the lungs of conventional liposomes. *Candida albicans* was inoculated intratracheally into BALB/C mice. Twenty-four hours later, the number of *Candida* in the lungs of mice treated with amylopectin-coated liposomes was less than in those treated with conventional liposomes, and amylopectin-coated liposomes improved the survival rate of inoculated mice. Coating liposomes with amylopectin aids the targeting of amphotericin B to the lungs. — liposome; amphotericin B; amylopectin; pulmonary candidiasis; HPLC

Encapsulation of amphotericin B with liposomes decreases its toxicity (Graybill et al. 1982; Lopez-Berestein et al. 1984b). We also confirmed reduced toxicity and increased efficacy of liposomal amphotericin B in murine candidiasis (Miyazaki et al. 1990). Liposomes distribute mainly in the reticuloendothelial systems, but coating liposomes with polysaccharide reduced the distribution. Takada and co-workers reported that polysaccharide-coated liposomes distributed more preferentially in the lungs than conventional liposomes (Takada et al. 1984). In the present study, the higher concentrations of polysaccharide-coated liposomes in lungs suggested their therapeutic efficacy in respiratory infection. We used these polysaccharide-coated liposomes as an amphotericin B carrier for the therapy of murine pulmonary candidiasis.

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MATERIALS AND METHODS

Preparation of amylopectin-coated liposomes

Amylopectin-coated liposomes incorporated with amphotericin B were prepared according to the method described by Takada et al. (1984). Deoxycholate-free amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), egg phosphatidylcholine, and cholesterol were dissolved in methanol. The organic solvents were evaporated in a rotary evaporator and then dried in a vacuum desiccator. An aliquot of saline was added, and liposomes were allowed to form by shaking. The suspension was then centrifuged at 2,000 g, and the pellet was resuspended in saline. The coating of the liposomes with polysaccharide derivatives was carried out by the addition of an aqueous solution of aminoethylcarbamylnmethyl-amylopectin to multilamellar liposomes. The content of amphotericin B in the liposomes was measured at 405 nm by a spectrophotometer after solubilization in chloroform/methanol. The concentration of egg phosphatidylcholine in the final preparation was 30.0 mg/ml.

Tissue concentrations of amphotericin B

Tissue concentrations of amphotericin B in various organs of normal mice were examined by high-performance liquid chromatography (HPLC). HPLC was performed according to the method described by J.W. Mayhew et al. with slight modification (Mayhew et al. 1983). Twelve mice were randomly assigned into four groups. A tissue sample was collected from each three mice and homogenized in methanol at a concentration of 0.50 g/ml. The extraction mixture was then centrifuged at 15,000 g for 5 min and the supernatant was collected. The HPLC system consisted of Waters chromatography pump, auto sampler, a model 490 multiwave length detector and a model 740 data module. A Nova-Pak C18 reverse-phase column (Waters, Millipore, Bedford, MA, USA) was used. The mobile phase consisted of EDTA (0.005 M) and methanol (27 : 73, vol : vol), flowing at 1.0 ml/min. The injection size was 50 μ l. Absorption of the eluant was monitored at 405 nm.

Murine pulmonary candidiasis

Five-week-old male BALB/C mice, purchased from Shizuoka Laboratory Animal Center, were inoculated intratracheally with *Candida albicans* strain 7N. Mice were slightly anesthetized with pentobarbital sodium (50–75 mg/kg), and the trachea was surgically exposed under aseptic conditions. With a 27-gauge needle, 0.05 ml of saline containing *Candida albicans* strain 7N was injected. Inoculum size was confirmed by quantitative colony counts after incubation in Sabouraud dextrose agar at 37°C for 48 hr.

Treatment of murine pulmonary candidiasis by amylopectin-coated liposomal amphotericin B

Two hours after inoculation, animals were treated intravenously with either 0.1 ml of conventional liposomal amphotericin B (5 mg/kg), or amylopectin-coated liposomal amphotericin B (5 mg/kg). The survival rate was observed until day 10.

Number of Candida albicans in organs

The lungs, liver, spleen, heart, brain, and kidneys were removed and homogenized with 1.0 ml of sterile saline, and then serial dilutions were made to count the number of *Candida albicans* in organs (Miyazaki et al. 1990).

Statistical analysis

Median lethal doses (LD₅₀) were calculated by the method of Behrens-Karber. Survival curves were calculated by the method of Kaplan-Meier, and tests for differences in survival distributions were based on a generalized Wilcoxon test.

RESULTS

A model of pulmonary candidiasis

Median lethal doses (LD_{50}) in a model of pulmonary candidiasis, calculated by the method of Behrens-Karber, was 6.3×10^6 CFU (Fig. 1). Histologic examination of the lung showed filamentous fungi, surrounded by infiltration of monocytes and polymorphic nuclear cells. However, *Candida albicans* was not detected in any other organ by histologic and bacteriologic examination.

Toxicity of free amphotericin B (Fungizone®) and amylopectin-coated liposomal amphotericin B in normal mice

The LD_{50} of amylopectin-coated liposomal amphotericin B intravenously injected into normal BALB/C mice was greater than 10.0 mg/kg, and that of free amphotericin B (Fungizone), 1.2 mg/ml. The maximum dose without acute lethality of free amphotericin B was 0.8 mg/kg, whereas all mice injected with amylopectin-coated liposomal amphotericin B at a dose of 5.0 mg/kg survived more than 42 days (Fig. 2a and 2b).

Pharmacokinetics of amphotericin B

The concentrations of free and liposomal amphotericin B in various organs of normal mice are shown in Table 1. Encapsulation with liposomes enhanced drug concentration in various organs compared with non-encapsulated drugs. Coating liposomes with amylopectin enhanced the maximum concentration of amphotericin B in lungs from $25.4 \pm 7.78 \mu\text{g/g}$ to $55.33 \pm 0.71 \mu\text{g/g}$.

Fig. 1. Survival rate in murine pulmonary candidiasis.

Candida albicans strain 7N was inoculated to BALB/C mice intratracheally. $LD_{50} = 6.3 \times 10^6$ CFU/mouse. $n = 10$. Inoculum size: 1×10^6 CFU/mouse (●—●); 2×10^6 CFU/mouse (■—■); 7×10^6 CFU/mouse (□—□); 1×10^7 CFU/mouse (○—○).

Fig. 2. Toxicity to normal mice.

(A) Free amphotericin B. LD_{50} was 1.2 mg/kg. The maximum dose without acute lethality was 0.8 mg/kg. $n=10$. Dose of free amphotericin B: 0.8 mg/kg (○—○); 1.2 mg/kg (●—●); 2.0 mg/kg (□—□).

(B) Amylopectin-coated liposomal amphotericin B.

LD_{50} was greater than 10 mg/kg. All mice treated at a dose of 5.0 mg/kg were alive. $n=10$. Dose of amylopectin-coated liposomal amphotericin B: none (■—■); 2.5 mg/kg (□—□); 5.0 mg/kg (○—○); 10.0 mg/kg (●—●).

Number of Candida albicans in lungs

At 12 hr after inoculation of 9.3×10^6 CFU/mouse, there was no statistically significant difference in the number of *Candida albicans* in lungs among four groups of mice, which were treated respectively with liposomes alone, amphotericin B (0.8 mg/kg), liposomal amphotericin B (5.0 mg/kg) and amylopectin-coated liposomal amphotericin B (5.0 mg/kg) (Fig. 3). However, 24 hr later, the number of *Candida albicans* in lungs of mice treated with amylopectin-coated liposomes was less than that of those receiving liposomal amphotericin B ($p < 0.05$).

TABLE 1. Organ concentration of amphotericin B after injection of free, simple liposomal, and amylopectin-coated liposomal amphotericin B in normal mice

	Free-AMPH ^a		Simple-Lipo-AMPH ^b		Ap-Lipo-AMPH ^b	
	30 min ($\mu\text{g/g}$)	24 h ($\mu\text{g/g}$)	30 min ($\mu\text{g/g}$)	24 hr ($\mu\text{g/g}$)	30 min ($\mu\text{g/g}$)	24 h ($\mu\text{g/g}$)
Brain	ND ^c	ND	ND	ND	ND	ND
Heart	0.12 ± 0.02^d	ND	1.64 ± 0.41	0.85 ± 0.22	1.13 ± 0.46	0.35 ± 0.06
Lungs	0.34 ± 0.05	ND	25.42 ± 7.78	2.36 ± 0.5	55.33 ± 0.71	2.5 ± 0.38
Liver	0.94 ± 0.12	0.29 ± 0.07	14.2 ± 2.36	14.7 ± 1.28	10.46 ± 1.16	12.47 ± 1.07
Spleen	0.81 ± 0.17	0.23 ± 0.10	15.56 ± 2.93	13.61 ± 3.65	10.17 ± 0.76	10.39 ± 0.8
Kidneys	0.36 ± 0.07	0.09 ± 0.03	3.7 ± 1.05	1.88 ± 0.36	2.85 ± 0.18	0.43 ± 0.23

^aAmphotericin B 0.8 mg/kg weight.^bAmphotericin B 5.0 mg/kg weight.^cNot detectable (less than $0.09 \mu\text{g/g}$ tissue).^dThe mean $\pm$ S.D. Fig. 3. The number of *Candida albicans* in lungs at 12 and 24 hr after injection of amphotericin B, liposomal amphotericin B and amylopectin-coated liposomal amphotericin B in murine pulmonary candidiasis. Inoculum size was 9.3×10^6 CFU/mouse. $n=6$. Data represent the mean $\pm$ S.D.

The therapeutic efficacy of liposomal and amylopectin-coated liposomal amphotericin B

After intratracheal inoculation of 6.4×10^6 CFU/mouse, the survival rate of mice treated with liposomal amphotericin B at a dose of 5.0 mg/kg was 50%. In contrast, all mice treated with amphotericin B encapsulated in amylopectin-coated liposomes (5.0 mg/kg) survived (Fig. 4). In mice inoculated with 7.3×10^6 CFU/mouse, amylopectin-coated liposomes also showed better therapeutic efficacy

Fig. 4. Efficacy of liposomal amphotericin B and amylopectin-coated liposomal amphotericin B on survival rate in murine pulmonary candidiasis. Inoculum size: *Candida albicans* 7 N 6.4×10^6 CFU/mouse (A) and 7.3×10^6 CFU/mouse (B). Treatments at 2 hr after inoculation: amylopectin-coated liposomal amphotericin B 5 mg/kg (●—●); liposomal amphotericin B 5 mg/kg (□—□); empty liposome (○—○). $n=10$.

(50%) than liposomal amphotericin B (30%).

DISCUSSION

Amphotericin B is a good candidate for liposomal encapsulation because of its amphipathic properties. One hundred percent of the drug was incorporated in liposomes in our study (data not shown). We previously reported that encapsulation with liposomes enhanced the efficacy of amphotericin B in the treatment of murine candidiasis, due to decreased toxicity (Miyazaki et al. 1990). In this study, amphotericin B encapsulated in amylopectin-coated liposomes showed better efficacy than conventional liposomes for the treatment of murine pulmonary candidiasis.

The tissue concentration of amphotericin B after injection of a free agent was

examined by Tomita (1975). The concentrations of amphotericin B measured by microbiological assay in various organs were less than $1.85 \mu\text{g/g}$ in rats injected with non-liposomal amphotericin B (1.0 mg/kg). Liposomal amphotericin B showed much higher tissue distribution (Lopez-Berestein et al. 1984a). Polysaccharide-coated liposomal amphotericin B showed higher concentration in lungs than liposomal amphotericin B.

The multilamellar liposomes used in this study were smaller than erythrocytes, and no agglutination of liposomes was detected by electron microscopy. This suggested that the relatively high uptake of amylopectin-coated liposomes in the lung was not due to size effect (Takada et al. 1984; Sunamoto et al. 1985).

Sunamoto reported that alveolar macrophages, as well as blood monocytes, exhibited a marked enhancement in the internalization of liposomes when the liposomes are coated with amylopectin, compared with conventional liposomes (Sunamoto et al. 1985). He also reported phagocytosis of liposomes by alveolar macrophages in an electron microscopic study.

To determine whether liposomes can successfully traverse the wall of capillaries, Poste et al. examined the behavior of intravenously injected liposomes in the lung capillary bed (Poste et al. 1982). Their ultrastructural studies revealed that circulating phagocytic cells engulf the liposomes in the lung capillaries. They concluded that liposomes in the lung capillaries are engulfed by circulating blood phagocytes which subsequently migrate to the alveoli to become alveolar macrophages.

Coating liposomes with amylopectin effected twice the uptake of amphotericin B of conventional liposomes in lungs. This finding suggest that modification of the surface of liposomes represents a potential means of altering the tissue distribution of liposomes, and amphotericin B encapsulated in polysaccharide-coated liposomes could be a viable choice for the treatment of pulmonary fungal infections in humans (Lopez-Berestein et al. 1985).

References

- 1) Graybill, J.R., Craven, P.C., Taylor, R.L., Williams, D.M. & Magee, W.E. (1982) Treatment of murine cryptococcosis with liposome-associated amphotericin B. *J. Infect. Dis.*, **145**, 748-752.
- 2) Mayhew, J.W., Fiore, C., Murray, T. & Barza, M. (1983) An internally-standardized assay for amphotericin B in tissues and plasma. *J. Chromatogr.*, **274**, 271-279.
- 3) Miyazaki, T., Kohno, S., Kaku, M., Koga, H. & Yamaguchi, K. (1990) Liposome-encapsulated amphotericin B in the treatment of experimental murine candidiasis. *Tohoku J. Exp. Med.*, **161**, 273-281.
- 4) Lopez-Berestein, G., Rosenblum, M.G. & Mehta, R. (1984a) Altered tissue distribution of amphotericin B by liposomal encapsulation: Comparison of normal mice to mice infected with *Candida albicans*. *Cancer Drug Deliv.*, **1**, 199-205.
- 5) Lopez-Berestein, G., Hopfer, R.L., Mehta, R., Mehta, K., Hersh, E.M. & Juliano, R.L. (1984b) Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. *J. Infect. Dis.*, **150**, 278-283.
- 6) Lopez-Berestein, G., Fainstein, V., Hopfer, R., Mehta, K., Sullivan, M.P., Keating, M.,

- Rosenblum, M.G., Mehta, R., Luna, M., Hersh, E.M., Reuben, J., Juliano, L. & Bodey, G.P. (1985) Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: A preliminary study. *J. Infect. Dis.*, **151**, 704-710.
- 7) Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. & Fidler, I.J. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. *Cancer Res.*, **42**, 1412-1422.
- 8) Sunamoto, J., Goto, M., Iida, I., Hara, K., Saito, A. & Tomonaga, A. (1985) Unexpected tissue distribution of liposomes coated with amylopectin derivatives and successful use in the treatment of experimental Legionnaires' diseases. In: *Receptor-Mediated Targeting of Drugs*, edited by G. Gregoriadis, G. Poste, J. Senior & A. Trouet, Plenum Press, New York, pp. 359-371.
- 9) Takada, M., Yuzuriha, T., Katayama, K., Iwamoto, K. & Sunamoto, J. (1984) Increased lung uptake of liposomes coated with polysaccharides. *Biochim. Biophys. Acta*, **802**, 237-244.
- 10) Tomita, T. (1975) Amphotericin B concentration in rats following an intravenous administration. *Pharmacol. Ther.*, **13**, 1458-1462. (in Japanese)
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