Long-Circulating Immunoliposomal Amphotericin B against Invasive Pulmonary Aspergillosis in Mice

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We investigated the efficacy of long-circulating immunoliposomal amphotericin B (AmB) against invasive pulmonary aspergillosis in mice using three types of liposomal AmB: conventional liposomal AmB (AmBisome), a long-circulating liposomal AmB and prepared by coating the liposome surface with polyethylene glycol (PEG; PEG-L-AmB), long-circulating immunoliposomal AmB (34A-PEG-L-AmB). The survival rates for mice with invasive pulmonary aspergillosis treated with an intravenous dose of 2 mg of AmBisome, PEG-L-AmB, or 34A-PEG-L-AmB per kg of body weight were 16.7, 83.3, and 100%, respectively. Treatment with 34A-PEG-L-AmB produced a marked reduction in the number of Aspergillus fumigatus organisms in the lungs. Pharmacokinetic studies showed the presence of high AmB concentrations in the plasma of mice treated with PEG-L-AmB (40.8 μg/ml) and in the lungs of mice treated with 34A-PEG-L-AmB (42.3 μg/g). We conclude that 34A-PEG-L-AmB, a long-circulating immunoliposomal AmB, is a promising form of AmB against invasive pulmonary aspergillosis.

There has been a dramatic rise in the number of invasive fungal infections in immunocompromised patients in recent years. Accordingly, there is an urgent need to improve the treatments for invasive fungal infections because the overall prognosis for patients with these infections remains poor. Amphotericin B (AmB) is a broad-spectrum and potent antifungal agent, but its clinical use is sometimes limited due to adverse reactions, such as renal toxicity, hypokalemia, and anemia. Furthermore, it is usually necessary to initiate therapy with a small dose of AmB, and it usually takes a few days to achieve effective therapeutic concentrations in serum. A promising approach to the treatment of invasive fungal infections is the use of liposome-encapsulated AmB (4, 12). The development of a drug carrier that encapsulates AmB allows for the administration of a high dose of AmB due to the reduced toxicity of AmB (7, 11). Liposomal AmB (AmBisome) is commercially available (5, 16); however, certain problems with the liposomal formation of AmB still make the drug an easy target for the reticuloendothelial system (RES). Thus, an increased dosage is needed to establish adequate therapeutic effects (19, 21).

Immunoliposomal AmB is a newly developed form of AmB modified by monoclonal antibodies for active targeting to specific sites. A few investigators have already examined the efficacies of immunoliposomes in animals and humans (6, 18). While immunoliposomes show a high degree of efficiency in vitro, their targeting efficiency in vivo is relatively low (15). This is probably due to the appearance of antibodies on the surfaces of liposomes, leading to enhanced uptake of immunoliposomes by the RES. In our previous reports (10, 14), the use of a long-circulating liposome prepared by coating the liposome surface with polyethylene glycol (PEG) (PEG-L) allows the liposome to evade the RES. Van Etten et al. (20) reported that the therapeutic efficacy of liposomal AmB coated with PEG (PEG-L-AmB) was better than that of AmBisome against invasive candidiasis in neutropenic mice. We reported previously that monoclonal antibodies could be attached to the distal ends of chains of long-circulating liposomes prepared by coating the liposome surface with PEG (15). Monoclonal antibody 34A recognizes surface glycoproteins (8) that are expressed on the luminal surface of the pulmonary capillary vessel wall in the mouse lung (9, 17). Binding of this immunoliposome to the lung is very rapid, and it is not captured by the RES. In this study, we compared the in vivo efficacy of long-circulating immunoliposome (34A-PEG-L-AmB) with those of AmBisome and long-circulating liposome (PEG-L-AmB) in mice with invasive aspergillosis.

MATERIALS AND METHODS

Compounds. AmB was kindly donated by Bristol-Myers Squibb Pharmaceutical Research (Tokyo, Japan). AmBisome was obtained from Vestar Inc. (San Dimas, Calif.) as a sterile lyophilized product. Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylethanol amine (DSPE), monomethoxy polyethylene glycol succinimidyl succinate (PEG-OSS), and polyethylene glycol bisuccinimidyl succinate (PEG-2OSS) were kindly provided by Nippon Oil and Fats (Tokyo, Japan). Cholesterol was purchased from Wako Pure Chemicals (Osaka, Japan). The average molecular weights of PEG-OSS and PEG-2OSS measured by gel permeation chromatography were 2,219 and 2,230, respectively. The other chemicals used were of high grade.

DSPE-PEG and DSPE-PEG-COOH were synthesized by the methods described previously (10, 15). In brief, 1 ml of 5% DSPE in chloroform-methanol (3:1, vol/vol) was added to 9.5 ml of 10% PEG-OSS or PEG-2OSS in chloroform, followed by the addition of 20.5 μl of triethylamine. The reaction mixture was stirred vigorously overnight at room temperature. Full conversion of the primary amino group in DSPE was confirmed by negative ninhydrin reactivity after separation of the products by thin-layer chromatography. The phospholipid phosphorus assay showed the appearance of new phosphate-positive spots at a higher Rf value than the value for phosphate-positive spots from DSPE. A small amount of water was added to the evaporated reaction residues to form micelles. The micelles were dialyzed for 5 days against water by using a dialysis bag with large pores (Spectra-por CE 300000 MWCO; Spectrum Medical, Houston, Tex.) and were then lyophilized.

Rat immunoglobulin G2a (IgG2a) antibody 34A was a generous gift from S. Kennel (Oak Ridge National Laboratory, Oak Ridge, Tenn.). The antibody recognizes surface glycoproteins (gp112) which are expressed exclusively and...
Preparation of liposomes. To prepare PEG-L-AmB, a chloroform-lipid solution (DPPC, 7.92 mg; cholesterol, 2.08 mg) was mixed with a methanol solution (3.0 mg of AmB (1.50 mg) and DSPE-PEG (2.88 mg) and evaporated in a round-bottom flask at 65°C. The lipid film was hydrated by vortex mixing in 1.2 ml of 9% saccharose. The procedure of freezing and thawing was repeated four times. Liposomes were extruded through a Nucleopore polycarbonate membrane (Costar Science Co. Cambridge, Mass.), with a resultant average particle size of 125 nm (range, 115 to 140 nm), as measured by dynamic light scattering (model NISD; Coulter, Hialeah, Fla.). This was followed by centrifugation (2 × 10^9 × g for 15 min) to separate nonentrapped AmB.

We also used the method described above to prepare 34A-PEG-L-AmB, except that DSPE-PEG was replaced by DSPE-PEG-COOH. The centrifuged liposomes were resuspended in 1.0 ml of 5 mM morpholinethesulfonic acid (MES) buffer (pH 5.5), and this combination was mixed with 240 μl of 0.25 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce, Rockford, Ill.) and 240 μl of N-hydroxysulfosuccinimide (Pierce). After allowing the mixture to stand at room temperature for 10 min, the reaction mixture was adjusted to pH 7.5 with 1 N NaOH and was then mixed with antibody (0.25 μg) at a weight ratio of 1/4 in antibody-lipid, and the whole mixture was incubated for 2 h at room temperature. Immunoliposomes were separated from the unbound protein and were concentrated by centrifugation (2 × 10^9 × g for 15 min). The centrifuged liposomes were suspended in 9% saccharose to prepare predetermined concentrations of AmB for injection. AmBisome was suspended in 5% glucose for injection.

Animals and fungus. Male BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan) and were used in the present experiments at the age of 6 weeks. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine. All mice were housed in a pathogen-free environment and received sterilized food and water at the Laboratory Animal Center for Biomedical Research of Nagasaki University School of Medicine. Aspergillus fumigatus MF-13 was isolated from the sputum of a patient with a pulmonary aspergilloma at the Nagasaki University Hospital and was maintained at −80°C. The strain was subcultured on Sabouraud dextrose agar (Becton Dickinson, Cockeysville, Md.) at 30°C for 7 days; single black conidia were harvested with sterile saline containing 0.02% Tween 80 (Wako Pure Chemical Industries), counted in a hemocytometer, and diluted with sterile saline for inoculation.

Effect of liposomal AmB against murine invasive pulmonary aspergillosis. The experimental design of the study was basically similar to that for a previously described model of pulmonary aspergillosis in mice (2), with several modifications. The initial step of the experiment included the implementation of immunosuppression in the mice by injecting 2.5 mg of cortisone acetate (Wako Pure Chemical Industries) subcutaneously every day for 5 days, beginning 4 days before infection. With the mice under general anesthesia induced by intraperitoneal injection of pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.), 0.08 ml containing 2 × 10^5 conidia of A. fumigatus was instilled intratracheally. Animals were housed in groups of three mice per cage and were given drinking water containing tetracycline (250 mg/800 ml; Achromycin; Ledane, Duluth, Ga.) and water at the Laboratory Animal Center for Biomedical Research of Nagasaki University School of Medicine (Hamamatsu, Japan) and were used in the present experiments at the age of 6 weeks.

Preparation of liposomes. Liposomes were resuspended in 9% saccharose to prepare predetermined concentrations of AmB for injection. AmBisome was suspended in 5% glucose for injection.

RESULTS

Efficacy of each form of liposomal AmB against invasive pulmonary aspergillosis. In the first step, we investigated the therapeutic efficacy of various doses of AmBisome against experimental invasive pulmonary aspergillosis (Fig. 1). All mice in the control group treated with 5% glucose died within 6 days of infection. The effect of AmBisome on the survival rate was dependent on the dose; all mice treated with 10 or 20 mg/kg/day survived. In the next series of experiments, we compared the efficacies of AmBisome, PEG-L-AmB, and 34A-PEG-L-AmB and their effects on the survival rate (Fig. 2). The survival rates following treatment with 2 mg of AmBisome, PEG-L-AmB, and 34A-PEG-L-AmB per kg/day were 16.7, 83.3, and 100%, respectively. These results indicated that 34A-PEG-L-AmB was the most effective type of liposomal AmB among the three types of liposomal AmB used in the present study.

Mycological, histopathological, and pharmacokinetic studies. The number of A. fumigatus organisms in the lungs 3 days after the administration of AmBisome was lower than that in the lungs of control mice treated with 5% glucose only. The number of fungi in the lungs of PEG-L-AmB-treated mice was lower than that in the lungs of AmBisome-treated mice, but the difference was not significant (P = 0.2478). On the other hand, the number of fungi in the lungs of 34A-PEG-L-AmB-treated mice was significantly lower than that in the lungs of AmBisome-treated mice (P < 0.01) and PEG-L-AmB-treated mice (P < 0.01). The number of fungi in the lungs of A. fumigatus organisms in the lungs of mice treated with control (5% glucose) and AmBisome-, PEG-L-AmB-, and 34A-PEG-L-AmB-treated mice were 6.24 ± 0.08 log10 CFU/g, 5.79 ± 0.13 log10 CFU/g (P < 0.01 compared with the control group), 5.70 ± 0.05 log10 CFU/g (P < 0.01 compared with the control group), 5.70 ± 0.05 log10 CFU/g (P < 0.01 compared with the control group) taking 2 mg of 1-amino-4-nitronaphthalene (Aldrich, Milwaukee, Wis.) per ml. AmB was extracted from supernatants of the homogenates with BOND-ELUT (Varian, Harbor City, Calif.) and was measured by high-pressure liquid chromatography (PU-980; Nihon Bunko, Tokyo, Japan). A Wako 5C18 column (4.0 mm by 30 cm; Wako Pure Chemical Industries) was used for all analyses. A mobile phase of 40% acetonitrile in 10 mM acetic acid buffer (pH 4.0) and a flow rate of 1.0 ml/min were used. The retention times of AmB and internal standard were 8.0 and 11.0 min, respectively. The absorbance of the column effluent was monitored at 408 nm.

The experimental design of the study was basically similar to that for a previously described model of pulmonary aspergillosis in mice (2), with several modifications. The initial step of the experiment included the implementation of immunosuppression in the mice by injecting 2.5 mg of cortisone acetate (Wako Pure Chemical Industries) subcutaneously every day for 5 days, beginning 4 days before infection. With the mice under general anesthesia induced by intraperitoneal injection of pentobarbital (Nembutal; Abbott Laboratories), the iliac arteries were dissected and cut. This was followed by thoracotomy and infusion of 2 ml of sterile saline containing 0.02% Tween 80 (Wako Pure Chemical Industries), counted in a hemocytometer, and diluted with sterile saline for inoculation.
control group), and $5.39 \pm 0.11 \log_{10} \text{CFU/g} (P < 0.01$ compared with the control group, $P < 0.01$ compared with the AmBisome-treated group, and $P < 0.01$ compared with the PEG-L-AmB-treated group) (Student’s $t$ test), respectively.

Pathological examination of the lungs of control mice showed hyphal invasion in the lung parenchyma through the bronchial wall (Fig. 3A), and only a few hyphae were seen in mice treated with 34A-PEG-L-AmB (Fig. 3D). Hyphal proliferation was observed inside the bronchi of mice treated with AmBisome and PEG-L-AmB (Fig. 3B and C).

The results of the pharmacokinetic study with AmBisome, PEG-L-AmB, and 34A-PEG-L-AmB are summarized in Table 1. Injection of PEG-L-AmB resulted in plasma AmB concentrations at 1 and 6 h higher than those after injection of AmBisome and 34A-PEG-L-AmB. Studies examining the distribution of AmB in tissue showed that injection of 34A-PEG-L-AmB resulted in the highest concentration of AmB in the lung at 1 and 6 h compared with those after the injection of AmBisome and PEG-L-AmB. The concentration of AmB in the liver was very low at both time intervals in mice treated with 34A-PEG-L-AmB.

**DISCUSSION**

In the present study, we investigated the efficacy of long-circulating immunoliposomal AmB (34A-PEG-L-AmB) against experimental invasive aspergillosis and compared its effects with those of AmBisome and long-circulating liposomal AmB (PEG-L-AmB). The development of AmBisome was intended to circumvent the toxicity and enhance the efficacy of AmB by allowing for the administration of higher doses of the drug.
TABLE 1. Concentrations of AmB in plasma and tissues of mice with invasive pulmonary aspergillosis a

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc (µg/ml) in plasma</th>
<th>1 h</th>
<th>6 h</th>
<th>Conc (µg/g) in lung</th>
<th>1 h</th>
<th>6 h</th>
<th>Conc (µg/g) in liver</th>
<th>1 h</th>
<th>6 h</th>
</tr>
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<tbody>
<tr>
<td>AmBisome</td>
<td>15.2</td>
<td>10.0</td>
<td>2.8</td>
<td>2.5</td>
<td>11.1</td>
<td>18.8</td>
<td></td>
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</tr>
<tr>
<td>PEG-L-AmB</td>
<td>40.8</td>
<td>20.4</td>
<td>10.1</td>
<td>10.4</td>
<td>8.7</td>
<td>13.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34A-PEG-L-AmB</td>
<td>4.8</td>
<td>2.6</td>
<td>42.3</td>
<td>23.0</td>
<td>4.9</td>
<td>3.3</td>
<td></td>
<td></td>
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</tbody>
</table>

a All liposomes were injected at a dose of 2 mg/kg intravenously once on the third day after infection. The concentrations of AmB are given as the mean at 1 h and 6 h after injection (n = 4).

Previous studies have shown that 5 mg of AmBisome per kg/day was effective against invasive candidiasis but was only marginally effective against invasive pulmonary aspergillosis (3, 16). Our results suggested that a high dose of AmBisome is necessary for the treatment of invasive pulmonary aspergillosis.

Unfortunately, AmBisome is an easy target for the RES (19), thus disallowing its use to achieve the high concentrations of AmB necessary to kill Aspergillus spp. in the lungs. In the present studies, we used two types of liposomal compounds against pulmonary aspergillosis. The first preparation was a long-circulating liposomal compound prepared by coating the liposome surface with amphipathic PEG (10). This coating of the liposome allows the liposome to evade uptake by the RES, allowing AmB to remain in the systemic circulation for a long period of time. We reported previously that the half-life for the clearance of liposomes from the blood of healthy mice is estimated to be 1.5 h for conventional liposomes and 5.6 h for PEG-coated liposomes (14). The present study extended these early results by demonstrating that the concentration of AmB in the sera of mice with invasive pulmonary aspergillosis treated with PEG-L-AmB was approximately 250% of that in the sera of mice treated with AmBisome. The pharmacokinetic difference may explain the high degree of efficacy of PEG-L-AmB against invasive pulmonary aspergillosis compared with that of AmBisome. It should be noted, however, that histological examination of the lung tissue showed no significant differences in the number of A. fumigatus organisms in the lungs of mice treated with AmBisome or PEG-L-AmB. It is possible that the difference in the size of the fungal population in the lung tissue could be due to the timing of examinations.

The other liposomal preparation was the long-circulating immunoliposomal AmB prepared by coating the PEG-coated liposomal surface with a monoclonal antibody. Our results indicated that the peak concentration of immunoliposome in blood lasted for a short period of time compared with that for the long-circulating liposome, because the immunoliposome is easily captured by the RES due to the opsonization induced by the surface antibody (13, 15). In the present study, we evaluated a new type of long-circulating immunoliposome using monoclonal antibody 34A. This monoclonal antibody recognizes surface glycoproteins that are expressed on the pulmonary vessel. The immunoliposome with monoclonal antibody 34A rapidly binds to the lung tissue and evades the RES (15). This explains why 34A-PEG-L-AmB was more effective than AmBisome and PEG-L-AmB against invasive pulmonary aspergillosis. High concentrations of 34A-PEG-L-AmB accumulated in the lungs, causing a larger decrease in the numbers of A. fumigatus organisms in murine lungs compared with the decreases associated with AmBisome and PEG-L-AmB treatments.

It should be noted, however, that immunoliposomes, which have already been used as anticancer agents (14), have certain disadvantages, e.g., inactivation by anti-idiotype antibodies, a process that develops rapidly after injection. Several methods are being developed to combat this phenomenon, e.g., modification of the liposome and antibody. The Fab priming method that deletes the Fc portion from the IgG antibody (18) and the chimera antibody method, which combines the Fc portion of human IgG and the Fab portion of IgG of other species (1), have been proposed as means of overcoming the problem of inactivation by anti-idiotype antibodies.

In conclusion, our encouraging results obtained with the long-circulating immunoliposomal AmB against murine pulmonary aspergillosis suggest that the agent may be effective against invasive pulmonary aspergillosis in humans.

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