Treatment of a murine model of systemic candidiasis with liposomal amphotericin B bearing antibody to Candida albicans

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Summary. Survival of mice infected with an intravenous injection of Candida albicans was observed in a short-term (21-day) survival study. Concentration of C. albicans in the kidneys, liver, and spleen was determined at various times. The effects of treatment with the commercial formulation of amphotericin B (fAMB), liposomal amphotericin B (LAMB), and liposomal amphotericin B bearing external antibody specific for C. albicans (LAMB-Ab) were compared. In single intravenous treatment dosages of 0.6 mg of amphotericin B/kg, the liposomal forms of the drug (LAMB and LAMB-Ab) enhanced the percentage survival and mean survival time of mice in comparison with those treated with the unencapsulated antifungal compound, fAMB (p < 0.03 and p < 0.001, respectively). LAMB-Ab, at this dosage, produced an increase in the survival (p < 0.007) of mice over that produced by LAMB. LAMB-Ab treatment caused a greater than 3-fold increase over fAMB. The percentage of LAMB-Ab-treated mice which survived for 21 days was almost double that of the LAMB-treated mice. The increase in survival following this treatment did not, however, lead to the eradication of C. albicans in all mice which survived to the end of the experiment.

Introduction

In the treatment of opportunistic infections caused by Candida albicans, amphotericin B (AMB) is administered intravenously in its emulsified commercial form, its clinical use being limited by its various toxic effects (Graybill and Craven, 1983). Despite the undesirable effects, AMB is currently the drug of first choice for most life-threatening mycoses including candidiasis. Recent research has investigated formulations to deliver AMB in a less toxic, yet equally active form. Attempts to produce derivatives of AMB (Bannatyne and Cheung, 1977) or to discover less toxic carriers of the compound (Kirsh et al., 1988; Lopez-Berestein et al., 1983) have been described. Of this research, the encapsulation of AMB in vesicles consisting of phospholipid bilayers assembled into closed membrane systems, termed liposomes by Bangham (1980), has aroused greater interest. The encapsulation of AMB in liposomes (LAMB) has been shown to reduce toxicity without decreasing activity. Tests of LAMB in vitro and in vivo in mice yielded data that suggest that encapsulation increases the efficacy of AMB by allowing larger doses to be used safely (Juliano et al., 1985). Initial clinical trials by Lopez-Berestein et al. (1987, 1985) have produced encouraging results in patients with haematological malignancies, most of whom had shown no previous response to the unencapsulated drug (fAMB).

Liposomes targeted by antibody incorporated onto their surfaces have been shown to aid in the specific targeting of compounds to antigen-specific sites (Heath et al., 1983; Leserman et al., 1983). Recently, liposomes containing AMB and bearing C. albicans antibody have been produced in our laboratory (Hospenthal et al., 1988). These liposomes, designated LAMB-Ab, have been shown in vitro to be as effective against C. albicans, but less toxic to human cells than fAMB. In initial murine studies with an intraperitoneal candidiasis model (Hospenthal et al., 1989), increased survival rates produced by LAMB-Ab were shown to be caused by the attachment of specific antibody to the surface of LAMB. This effect was not produced by the attachment of nonspecific antibody to LAMB, nor
by the inclusion of unattached specific antibody in the LAMB treatments. The current investigations were performed to establish a new intravenous model of candidiasis for this and future studies, and to conduct further preliminary tests of LAMB-Ab in vivo.

Materials and methods

Animals and material

White Swiss mice, each weighing 18–22 g, were from Charles River Laboratories, Portage, MI. Liposomes were prepared with dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) (Sigma Chemical Co., St Louis, MO). Amphotericin B, as the commercial preparation (Fungizone) (FAMB) and in the nonemulsified form (AMB) were supplied by Squibb Pharmaceutical, New Brunswick, NJ. The antibody preparation used was produced from rabbit antisera to Candida albicans (Difco Laboratories, Detroit, MI).

Yeast cells and infection

C. albicans AK785, a clinical isolate, was maintained on frozen slants of Sabouraud glucose agar. Cells from these were incubated overnight at 37°C on fresh Sabouraud glucose agar, and inocula from these were transferred to Tryptic Soy Broth containing glucose 4% and incubated (37°C) on a rotary shaker for 14 h to produce the stationary phase. Yeast cells were then recovered by centrifugation (2000g, 10 min) and washed three times in phosphate-buffered saline before injection. Systemic candidiasis was produced by the injection of $3 \times 10^5$ cfu into a lateral caudal vein of each mouse as a single bolus contained in 0.2 ml of phosphate-buffered saline.

Yeast viability

Counts of C. albicans in the kidneys, liver, and spleen of individual mice were performed by dilution plating of these organs after homogenisation. The mice were either killed by cervical dislocation or had died of the candidal infection. The liver, spleen, and kidneys (each pair prepared as one unit) were removed aseptically and homogenised in sterile distilled water. The homogenates were serially diluted in four ten-fold dilutions in sterile distilled water. Spleen dilutions were from 1 in 5 to 1 in 25,000, liver from 1 in 20 to 1 in 200,000, and kidneys 1 in 100 to 1 in 100,000. Dilutions were then plated on Mycosel Agar (BBL Microbiology Systems, Cockeysville, MD), incubated at 37°C of 48 h, and the colonies were counted. Organ concentrations of C. albicans were recorded in colony forming units (cfu). Only plates on which between 10 and 500 colonies had grown were used in the calculation of cfu. On this criterion, yeast concentrations <50 cfu ($\log_{10} 1.7$) in the spleen, 200 cfu ($\log_{10} 2.3$) in the liver, 1000 cfu ($\log_{10} 3.0$) in the kidneys were not detected or included in this report.

Liposome preparation

Liposomes were produced with a 7:3 molar ratio of DMPC and DMPG by a reverse-phase evaporation procedure previously described (Hospenthal et al., 1988). Briefly, the liposomes were formed in phosphate-buffered saline as the organic solvents suspending the phospholipids and the unemulsified AMB were removed by evaporation under N2. The product of this evaporation was sonicated to reduce the size of the liposomes, dialysed overnight, and recovered by centrifugation. Liposomes bearing antibody (LAMB-Ab) were produced with rabbit antisera which was modified by the covalent attachment of palmitic acid residues. The antiserum, containing modified antibody, was included in the liposome preparation before dialysis with an emulsifier to allow the insertion of the fatty-acid residues into the liposome surfaces. This antiserum was present in LAMB-Ab in a concentration of 1:2 w:w protein:AMB. Previous studies revealed that the C. albicans-specific immunoglobulin content in the modified antiserum was approximately 10% (Hospenthal et al., 1989). Quantitation of liposomes was by AMB content, determined by spectroscopy at 405 nm. Free drug and liposomal preparations (LAMB and LAMB-Ab) were diluted to injection concentrations in phosphate-buffered saline and stored refrigerated (4°C) until the day of treatment. No liposomal preparations were stored for more than one day in this study.

Therapy

Groups of 8–10 mice were treated with a single injection 2 days after the initiation of the infection with C. albicans and observed for 21 days for survival. Liposome and free drug preparations were incubated for 20 min at 37°C before injection into the caudal veins of the mice to further reduce the toxicity of the liposomal compounds (Szoka et al., 1987). Treatment dosages of 0.6 and 1.2 mg of AMB/mouse kg of fAMB, LAMB, and LAMB-Ab were compared. These dosages were selected because the maximum tolerated dose of fAMB has been reported by Lopez-Berestein et al. (1983) to be 0.8 mg AMB/kg in the size of mouse used in this study.

Statistical analysis

The 21-days survival patterns of mice treated with the AMB preparations and untreated controls were compared at each dose by a generalised Wilcoxon test (Gehan, 1965).

Results

Candidiasis model

The number of viable C. albicans in the kidneys, liver, and spleen of untreated mice was examined
Fig. 1. *C. albicans* present in the kidneys (●) and livers (▲) of untreated mice killed during the first 7 days of infection. Animals were infected with $3 \times 10^5$ cfu of the yeast. Yeast concentrations < log$_{10}$ 2 cfu were below the sensitivity of the study design.

Fig. 2. The effect produced by anticandidal antibody bearing liposomal AMB on the survival of mice infected with *C. albicans*. Two days after initiation of the infection the animals were treated with a single dose of 0·6 mg of AMB/kg as LAMB-Ab (■), LAMB (▲) or fAMB (●), or were not treated (▼).

Therapy of candidiasis

The combined 21-day survival pattern in two studies of mice treated with 0·6 mg of AMB/kg is shown in fig. 2. All amphotericin-B formulations improved the percentage survival and mean survival time of mice over the control group at this dose (p < 0·001). LAMB-Ab and LAMB increased the survival over mice that received the free drug (p < 0·001 and p < 0·03, respectively). The distribution of survival times between the LAMB- and the LAMB-Ab-treated groups of mice was also significant (p < 0·007). Additional mice, which were infected and treated parallel to this study, were killed at days 7 and 14 of the infection. At day 7, there were no recoverable cfu from the spleens or livers of all three groups of treated mice (fAMB, LAMB, and LAMB-Ab); all had similar recovery values of $10^4$ cfu in their kidneys. Day 7 colony counts in the untreated mice are incorporated in the data presented in fig. 1. Fourteen days after infection, LAMB-treated mice had $10^7$ cfu in their kidneys and $10^5$ cfu in their livers. At this time, in mice treated with LAMB-Ab, $10^7$ cfu and $10^4$ cfu were recovered from their kidneys and livers, respectively. Because of the survival rates of the untreated and fAMB groups (fig. 2), mice from these groups did not survive to be sampled at day 14.

Surviving mice from one 0·6-mg of AMB/kg study were examined at day 42 for residual candidal infection. Of this group, which consisted of 1 fAMB-, 2 LAMB-, and 5 LAMB-Ab-treated mice, all the organs gave negative culture results except for two of the LAMB-Ab-treated mice. In these two mice, $10^3$ cfu were grown from the livers and $10^6$ cfu from the kidneys.

Experimental therapy studies at the dose of 1·2 mg of AMB/kg showed, as previously seen in our peritoneal murine candidiasis model (Hosenthal et al., 1989), little separation of the results of the two liposomal compounds. The 21-day survival
of mice at this dose was 64.7% for LAMB-Ab-treated and 61.1% for LAMB-treated mice. The survival of the LAMB-Ab- and LAMB-treated groups did prove to be better than that of the fAMB-treated groups (p < 0.02 and p < 0.03, respectively).

**Discussion**

Liposomal amphotericin B which bears antican-didal antibody (LAMB-Ab) was shown to produce increased survival in mice infected with *C. albicans* at a dose (0.6 mg of AMB/kg) that is below the maximum tolerated dose of prescription amphotericin B in these mice. Our formulation of LAMB at this dose also produced a greater survival rate than the free drug (fAMB), though much less than that of the LAMB-Ab treatment. Murine survival studies with these liposomal amphotericin B compounds at a dosage of 1.2 mg of AMB/kg also show these compounds to be more effective than fAMB. LAMB at this dose increases the survival rate of mice to that of LAMB-Ab. LAMB-Ab at this dose shows no change in survival rate over that which it produces at 0.6 mg of AMB/kg. It is suggested that this may be due to problems associated with the 90% nonspecific protein content of the antisera used in the preparation of LAMB-Ab. Future studies employing a monoclonal antican-didal antibody will be necessary to explore this further.

The organ homogenisation study with this model of systemic candidiasis shows that at the time when untreated mice begin to die from this infection, 6 days after infection, they usually have 10⁵ cfu in their livers and 10⁶ cfu in their kidneys. The normal survival of these mice is 6–17 days after infection. Mice killed in the 0.6 mg of AMB/kg LAMB and LAMB-Ab groups at 14 days produced cfu counts which averaged 10 times greater than those produced by the untreated control. This may indicate that mice surviving longer, may be more resistant to the infection, and yet may harbour large numbers of the yeast.

The encapsulation of amphotericin B in liposomes has been shown to reduce the toxic effect of this antifungal drug in numerous studies (Mehta et al., 1984; Shirkhoda et al., 1986; Juliano et al., 1987; Szoka et al., 1987). In these studies, this reduction in toxicity enabled increasingly larger doses of AMB to be employed, and thus improved its clinical efficacy. In these studies, our formulation, LAMB-Ab, increased the survival of mice with disseminated candidiasis in comparison with fAMB when administered in single, low dosages. In previous studies we have shown that this compound is less toxic than fAMB when administered to non-infected mice or incubated with human red blood cells. Dissemination of fungal cells which are susceptible to AMB is thought to be largely via the circulatory system. If our compound, or other preparations, can produce antibody-antigen targeting of drugs to infectious agents, then it may be possible to employ lower dosages of drugs with toxic side effects successfully in the treatment of these diseases. The combination of this targeting and the reduction in toxicity that liposomes provide could improve the efficacy of many toxic compounds, including that of amphotericin B in the treatment of life-threatening mycoses.

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