Activity of Liposomal Amphotericin B with Prolonged Circulation in Blood versus Those of AmBisome and Fungizone against Intracellular Candida albicans in Murine Peritoneal Macrophages

ELS W. M. VAN ETTEN,* WIM VAN VIANEN, JANNEKE HAK, AND IRMA A. J. M. BAKKER-WOUDENBERG

Department of Medical Microbiology and Infectious Diseases, Erasmus University Rotterdam, Rotterdam, The Netherlands

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Activity against intracellular Candida albicans was assessed in C. albicans-infected murine peritoneal macrophages exposed to long-circulating pegylated amphotericin B liposomes (PEG-AMB-LIP), AmBisome, or Fungizone. The level of antifungal activity of Fungizone is much higher than that of AmBisome or PEG-AMB-LIP, while PEG-AMB-LIP and AmBisome show equivalent activity levels. Previous exposure of uninfected macrophages to PEG-AMB-LIP or AmBisome is advantageous for intracellular antifungal activity.

At our laboratory a new type of liposomal amphotericin B (AMB), in which AMB is complexed to a hydrophilic phospholipid derivative of polyethylene glycol 1900 (PEG), was prepared and designated PEG-AMB-LIP. Incorporation of PEG-derivatized distearoylphosphatidylethanolamine (PEG-DSPE) results in a hydrophilic PEG coating on the surface of the liposomes, by which the binding of blood proteins is substantially reduced. As a result, uptake of liposomes by the phagocytic cells of the mononuclear phagocyte system (MPS) is substantially avoided. A relatively long blood residence time of intact liposomes is obtained (14); this may be important for increased accumulation of liposomal AMB at sites of fungal infection outside the MPS, such as the kidney and lung (2, 3).

The PEG-AMB-LIP formulation shows three characteristics that are expected to be important for improved antifungal efficacy (9, 10): low toxicity, high direct antifungal activity, and prolonged circulation time of intact liposomes in the blood. It was shown in our model of severe invasive Candida albicans infection that treatment with PEG-AMB-LIP resulted in a rapid decrease in the number of viable C. albicans organisms in the kidney within a short period after C. albicans infection. This effect could not be achieved with AmBisome at the same dosage. An almost sixfold-higher dosage of AmBisome was as effective as PEG-AMB-LIP (10).

C. albicans can be considered a facultatively intracellular pathogen, as it is able to survive within macrophages and to grow out of these cells by forming germ tubes and hyphae (7, 12). The question arises whether, due to low uptake of PEG-AMB-LIP by macrophages, the intracellular activity of PEG-AMB-LIP against C. albicans is relatively low. From a therapeutically relevant point of view, it is therefore relevant to investigate the activity of PEG-AMB-LIP against intracellular C. albicans in macrophages. In the present study, the activity of PEG-AMB-LIP against intracellular C. albicans in murine peritoneal macrophages is compared with those of AmBisome and Fungizone.

AMB and Fungizone were kindly provided by Bristol Myers-Squibb, Woerden, The Netherlands. AmBisome was obtained from NeXstar Pharmaceuticals, Inc. (San Dimas, Calif.). Hydrogenated soybean phosphatidyicholine (HSPC) and PEG-DSPE were obtained from Avanti Polar Lipids, Inc. (Alabaster, Ala.). Cholesterol (Chol) was obtained from Sigma (St. Louis, Mo.). PEG-DSPE–HSPC-Chol–AMB in a molar ratio of 0.21:1.79:1.0:0.32 (PEG-AMB-LIP) and placebo liposomes (devoid of AMB) were prepared as described previously (9). AmBisome, consisting of HSPC-Chol-distearoylphosphatidylglycerol (DSPG)-AMB in a molar ratio of 2:1:0.8:0.4, was provided as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions. C. albicans ATCC 44858 (7, 8), for which the MIC and minimal fungicidal concentration of AMB are 0.1 and 3.2 mg/liter, respectively (7), was used.

Activity against intracellular C. albicans was assessed as previously described (11). Briefly, peritoneal macrophages were obtained from 12- to 14-week-old specified-pathogen-free female BALB/c mice (Iffa Credo, L’Arbresle, France), cultured, and infected with C. albicans. The macrophage monolayers were reincubated for 24 h in the presence of twofold-increasing concentrations of PEG-AMB-LIP or AmBisome (3.2 to 102.4 mg of AMB/liter, equivalent to 28 to 896 mg of lipid/liter) or Fungizone (0.1 to 1.6 mg of AMB/liter), or with placebo liposomes or the solvent of the antifungal agent in the appropriate dilution. At time zero (directly after C. albicans ingestion) and at 4 and 24 h of incubation, the monolayers were prepared for microscopic examination. For each incubation condition three experiments were performed; in each individual experiment, a total of 150 macrophages were scanned. The results were expressed as the percentage of total macrophages in each of four categories: (i) uninfected macrophages, (ii) macrophages infected with C. albicans blastospores, (iii) macrophages infected both with C. albicans blastospores and with germ tubes and hyphae, and (iv) macrophages infected with C. albicans germ tubes and hyphae. Furthermore, the effect of previous exposure of macrophages during 6 or 24 h to PEG-AMB-LIP, AmBisome, or Fungizone on intracellular antifungal activity was studied, as previously described (11). In Table 1 the various experimental conditions are summarized.
The intracellular growth of *C. albicans* in monolayers of murine peritoneal macrophages in the absence of antifungal agents is presented in Table 2. Immediately after the 30-min *C. albicans* infection period (time zero), 67% of total macrophages were infected with *C. albicans* blastospores. We used the method of microscopic examination, and no discrimination was made between *C. albicans* organisms attached to macrophages and those interiorized by the macrophages. It has been previously demonstrated (11) that under similar experimental conditions, *C. albicans* was actually interiorized in more than 95% of the infected macrophages. After 4 h of incubation, the majority of intracellular *C. albicans* organisms had formed germ tubes or hyphae. After 24 h of incubation, massive hyphal growth of *C. albicans* was observed within a heavily disrupted monolayer.

The activities of PEG-AMB-LIP, AmBisome, and Fungizone against intracellular *C. albicans* after 4 and 24 h of incubation are presented in Table 3. No cellular toxicity of the AMB formulations was observed at the concentrations used, as intact monolayers of viable macrophages were still obtained after exposure. The viability of the macrophages was determined by trypan blue exclusion. Antifungal activity was evaluated in terms of either stabilization of the state of *C. albicans* infection or eradication of *C. albicans* from infected macrophages. Since about 67% of total macrophages were infected with *C. albicans* blastospores immediately after *C. albicans* ingestion, this state was considered stabilized when the percentage of macrophages infected did not exceed 67% and the percentage of macrophages containing germ tubes or hyphae was less than 10%. *C. albicans* was considered eradicated when the percentage of macrophages infected was reduced from 67% to less than 10%.

The intracellular activity of Fungizone is much higher than that of AmBisome. Previous exposure of uninfected macrophages to AmBisome is advantageous for the antifungal activity of AmBisome against intracellular *C. albicans*. The results for AmBisome and for Fungizone are largely confirmatory in comparison with the data from our previous study on the interactions of AmBisome with extracellular and intracellular *C. albicans* (11). For the interpretation of the results, it is important that in previous studies on the in vitro activity of AmBisome and Fungizone during short-term exposure of extracellular *C. albicans* (5, 8, 10, 11), it was clearly shown that for AmBisome the reduction of AMB’s toxicity following liposomal encapsulation seemed to be associated with a substantial reduction of AMB’s direct antifungal activity. In contrast, with the PEG-AMB-LIP formulation, the toxicity of AMB was substantially reduced without reduction of its direct antifungal activity (10). With respect to activity against intracellular *C. albicans*, it is now demonstrated that the antifungal activity of Fungizone is much higher than that of PEG-AMB-LIP. The activity of PEG-AMB-LIP against intracellular *C. albicans* shows great similarity to that of AmBisome. It is also demonstrated that, as for AmBisome, prolonged exposure of macrophages to PEG-AMB-LIP is advantageous for antifungal activity.

We do not yet know the mechanism by which each liposomal AMB formulation exerts its intracellular antifungal activity. As it has been previously shown that AMB is tightly associated on June 22, 2017 by guest http://aac.asm.org/ Downloaded from
PEG-AMB-LIP it has been reported previously that in vivo uptake by macrophages in the liver and spleen is relatively low, which results in a prolonged residence time of intact liposomes in blood (9). At present no data are available on the in vitro uptake and intracellular degradation of PEG-AMB-LIP in macrophage monolayers. For other types of PEG-containing liposomes, the in vitro uptake by macrophages has been studied quantitatively (1, 13, 15). In these studies it is clearly demonstrated that the uptake of PEG-containing liposomes is reduced compared with that of non-PEG-containing liposomes with similar particle sizes and lipid compositions.

On the basis of the data from these previous studies (1, 4, 9, 13, 15) it is assumed that only a small percentage of AmBisome or PEG-AMB-LIP is taken up by the peritoneal macrophages in the present study. Unfortunately, due to these low uptake levels, it is not possible to determine differences in liposomal AMB uptake accurately in the culture system that was used in the present study. Still, we expect a lower level of uptake of AMB-LIP than of AmBisome in the murine peritoneal macrophage monolayers. For other types of PEG-containing liposomes, the in vitro uptake by macrophages has been studied quantitatively (1, 13, 15). In these studies it is clearly demonstrated that the uptake of PEG-containing liposomes is reduced compared with that of AmBisome.

REFERENCES