Effects of Lipid Formulations of Amphotericin B on Activity of Human Monocytes against Aspergillus fumigatus

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Invasive aspergillosis is the most frequent opportunistic filamentous fungal infection causing excessive morbidity and mortality in immunocompromised patients (9, 12). Mononuclear phagocytes constitute a prominent component of the host defense against Aspergillus spp. (24). In particular, NADPH (NADP(H))-dependent production of antifungal compounds, such as superoxide anion (O$_{2^{-}}$), hydrogen peroxide (H$_{2}$O$_{2}$), and H$_{2}$O$_{2}$-dependent intracellular intermediates (DIIs), contributes to phagocyte-induced damage of microorganisms including fungi (2, 8, 16). While O$_{2^{-}}$ production is an early event of oxidative burst, H$_{2}$O$_{2}$ and DIIs consisting of compounds such as hydroxyl radical and HOCl are produced at late steps and are even more powerful oxidizing species.

For decades, deoxylcholate amphotericin B (DAMB) has been considered to be the cornerstone of antifungal therapy for fungal infections including invasive aspergillosis (1, 21). However, due to frequent infusion-related reactions and dose-limiting nephrotoxicity, less-toxic lipid-associated formulations, such as liposomal amphotericin B (LAMB), amphotericin B lipid complex (ABLC), and amphotericin B colloidal dispersion (ABCD), have been developed (5, 7, 15). These compounds appear to offer a better therapeutic index than DAMB, circumscribing excessive toxicity (5, 7). Although newer azoles and echinocandins have been added to the antifungal armamentarium, amphotericin B formulations remain important agents against invasive aspergillosis.

While lipid formulations of amphotericin B differ in their degree of induction of infusion-related reactions, little is known about their immunomodulatory effects when each of them is combined with phagocytes against Aspergillus fumigatus. Specifically, DAMB and ABLC were found to additively augment the fungicidal activity of pulmonary alveolar macrophages against conidia of A. fumigatus (17). However, the studies of the effects of DAMB on oxidative burst of phagocytes as evidenced by O$_{2^{-}}$ production have shown contradictory results (18, 22, 25, 26). To date, there are no reports published on the comparative effects of the four formulations on the antifungal activity of monocytes (MNCs) against A. fumigatus. We therefore investigated whether DAMB, LAMB, ABLC, and ABCD enhance the antifungal activities of monocytes against hyphae of A. fumigatus, as evidenced by monocyte-mediated hyphal damage, production of O$_{2^{-}}$, and production of H$_{2}$O$_{2}$ as well as secondary DIIs, both in response to A. fumigatus.

MATERIALS AND METHODS

Drugs and reagents. Deoxylcholate amphotericin B was purchased from Bristol-Myers Squibb (La Grande Nord, Paris, France). LAMB from Gilead Sciences (San Dimas, CA), ABLC from Enzon Pharmaceuticals (Piscataway, NJ), and ABCD from Sequus Pharmaceuticals (Menlo Park, CA). Since ABCD is a relatively unstable lipid formulation of amphotericin B, it was prepared fresh each day. LAMB and ABLC also were made fresh daily and stored at 4°C for no more than 24 h. DAMB at a concentration of 1 mg/ml in sterile water was stored
Pretreated MNCs were added to hyphae in HBSS cells and to be reduced by mitochondrial dehydrogenase of the fungi to indicator. It is based on the property of these salts to be taken up by viable assay (13). The XTT assay is a colorimetric method using tetrazolium salts as an 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide (XTT) respectively at 37°C for 22 h in all the experiments.

Pretreatment of MNCs was performed as described above. For opsonization, YNB broth was replaced by 100% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (fetal calf medium [FCM]). PMCA at a final concentration of 2 µg/ml was used as a control stimulus of antifungal activity of MNCs. DHR-123 was prepared and kept in the dark at −20°C as a 20 mM stock solution in nitrogen-purged dimethyl formamide.

Organism and preparation of hyphae. Strain AF 4215 of A. fumigatus, deposited in the American Type Culture Collection (Rockville, MD) (ATCC MYA 1163), isolated from a cancer patient with invasive pulmonary aspergillosis, and extensively characterized for antifungal drug susceptibility in vitro and in vivo, pathogenesis, and immune response (17) previously, was used in these studies. This strain was preserved on potato dextrose agar slants frozen at −70°C.

From suspensions containing 10⁶ (for hyphal damage assay) or 2 × 10⁷ (for O₂⁻ production) conidia per milliliter in yeast nitrogen base (YNB) broth supplemented with 2% glucose, 200 µl was plated in 96-flat-bottomed-well cell culture plates (Costar, Cambridge, Mass.), New York and incubated at 32°C with 5% CO₂ for 18 h to become hyphal (approximately 150 to 200 µm). Following centrifugation at 2,000 rpm for hyphal sedimentation, YNB broth was replaced with HBSS⁻.

Preparation of human monocyte-enriched mononuclear leukocytes. Human mononuclear cells were obtained from blood of healthy adult volunteers and separated by centrifugation over Ficoll, as previously described in detail (17). Briefly, the cells were washed and resuspended in HBSS⁻. They were counted on a hemocytometer by trypan blue staining, and percentages of monocytes over total number of mononuclear leukocytes were calculated after staining with May-Grunwald-Giemsa. Monocytes were adjusted to 10⁶ cells/ml in FCM. Monocyte-enriched cell populations (MNCs) were obtained from mononuclear leukocytes by adherence on plastic surfaces in 12-well plates during incubation in nitrogen-purged dimethyl formamide.

RESULTS

Hyphal damage. Treatment of MNCs with the high concentrations of all amphotericin B formulations (5 µg/ml of DAMB and 25 µg/ml of lipid formulations) resulted in a significant increase of MNC-induced hyphal damage compared to results with MNCs alone at an E:T ratio 10:1 (Fig. 1A). At 20:1, pretreatment of MNCs with either low or high concentrations of all amphotericin B formulations also had significant enhancing effects (Fig. 1B).

MNCs pretreated with ABL in both concentrations and incubated with hyphae at both E:T ratios resulted in the highest increases of hyphal damage compared to all other formulations of amphotericin B. For example, MNCs pretreated with 5 or 25 µg/ml of ABL induced a relatively very high percentage of hyphal damage at an E:T ratio of 10:1 compared to results with MNCs alone (79.9% ± 6.4% and 91.2% ± 3.0% versus 17.1% ± 6.0%, respectively; P < 0.001). Similarly, at an E:T ratio of 20:1, MNCs pretreated with 5 or 25 µg/ml of ABL resulted in an extremely high percentage of hyphal damage as compared to results with MNCs alone (89.6% ± 3.8% and 95.2% ± 1.2% versus 16.1% ± 3.9%, respectively; P < 0.001).

A significant difference also was observed between high and low concentrations of DAMB and LAMB in hyphal damage induced by pretreated MNCs at an E:T ratio of 10:1. In particular, treatment of MNCs with 1 µg/ml of DAMB resulted in 21.4% ± 5.4% hyphal damage and in 48.1% ± 8.1% when treated with 5 µg/ml (P = 0.025). Similarly, MNCs treated with 5 µg/ml of LAMB showed 26.9% ± 4.3% hyphal damage and 42.5% ± 3.8% when treated with 25 µg/ml (P = 0.026).
stimulated O$_2^-$ production compared to results with MNCs alone ($3.36 \pm 0.33$ versus $0.28 \pm 0.04$ nmol O$_2^-$/10$^6$ MNC/h, respectively; $P < 0.001$). Serum-opsonized and nonopsonized hyphae of A. fumigatus stimulated O$_2^-$ production to a smaller degree (Fig. 2). However, the amphotericin B formulations in general did not affect the production of O$_2^-$ by MNCs in response to either opsonized or nonopsonized hyphae of A. fumigatus. Furthermore, pretreatment of MNCs with 25 $\mu$g/ml of ABLC significantly reduced the production of O$_2^-$ in response to serum-opsonized hyphae of A. fumigatus compared to results with MNCs alone ($0.68 \pm 0.17$ versus $1.29 \pm 0.05$ nmol O$_2^-$/10$^6$ MNC/h, respectively; $P = 0.009$) (Fig. 2).

Production of H$_2$O$_2$ and H$_2$O$_2$-dependent intracellular intermediates. When PMA was used as a positive control stim-

FIG. 1. Effects of different formulations of amphotericin B on human MNC-mediated hyphal damage of A. fumigatus, as determined by an XTT assay. MNCs were pretreated with the antifungal drugs DAMB (1 and 5 $\mu$g/ml; hatched bars) or LAMB (vertically striped bars), ABLC (horizontally striped bars), and ABCD (black bars) at 5 or 25 $\mu$g/ml for 22 h. Drug-pretreated or untreated MNCs (open bars) were washed and incubated with A. fumigatus hyphae at an effector:target ratio of 10:1 (A) or 20:1 (B) for 2 h. Data are presented as means ± standard errors derived from five donors/experiments. Comparisons between drug-treated and control MNCs were performed by ANOVA with Dunnett test for multiple comparisons. Statistically significant differences from untreated control cells with $P$ values of <0.01 are indicated by an asterisk, and those with $P$ values of <0.05 are indicated by a dagger.

FIG. 2. Effects of different formulations of amphotericin B on superoxide anion (O$_2^-$) produced by human MNCs in response to A. fumigatus hyphae. MNCs were preincubated with no drug, DAMB (1 and 5 $\mu$g/ml; hatched light or dark gray bars), or LAMB (vertically striped light or dark gray bars), ABLC (horizontally striped light or dark gray bars), and ABCD (light or dark gray bars) at 5 or 25 $\mu$g/ml for 22 h. Drug-pretreated or untreated MNCs were incubated with nonopsonized or serum-opsonized hyphae of A. fumigatus and cytochrome c for 1 h. Data are presented as means ± standard error derived from five donors/experiments. Comparisons between drug-treated and control MNCs incubated with hyphae only were performed by ANOVA with Dunnett test for multiple comparisons. Statistically significant differences from control cells incubated with hyphae only with $P$ values of <0.01 are indicated by an asterisk.
A. fumigatus with bars), ABLC (horizontally striped bars), or ABCD (black bars) at 5 or 25 g/ml. There are no reports on the comparative effects of the conventional formulations of amphotericin B and its lipid formulations on the antifungal activity of MNCs against A. fumigatus.

In this study, we demonstrated that lipid formulations of amphotericin B augment antifungal activity of MNCs against A. fumigatus, as evidenced by MNC-induced damage of hyphae. This immunomodulatory effect was found to be associated with a similar enhancement of release of H2O2 and H2O2-dependent intracellular intermediates but not of O2·- production. To our knowledge, despite the wide clinical use of amphotericin B, there are no reports on the comparative effects of the conventional amphotericin B and its lipid formulations on the antifungal activity of MNCs against A. fumigatus.

During the oxidative burst in oxygen consumption originating with NADPH oxidase activation, O2·- constitutes the first released reactive oxygen intermediate followed by the production of H2O2. Subsequently, formation of even more powerful oxidizing species, such as the hydroxyl radical and HOCl, occurs. Secondary H2O2 and DIIs in the presence of a metal catalyst are responsible for the late DHR-123 oxidation (19). Specifically, it is noteworthy that only oxidants such as those derived from Fenton-type reactions (O2·- plus H2O2 plus a transition metal [H2O2-Fe2+2, H2O2-cytochrome c]), ferryl hemoproteins, or the toxin ONOO-/ONOOH are potent enough oxidizing agents as to be known to oxidize DHR-123 to R-123 (14, 19).

Our finding of increased oxidation of DHR-123 indicates an up-regulated production of reactive oxygen intermediates, mostly H2O2 and secondary DIIs, during the short incubation time of pretreated MNCs with A. fumigatus supernatant.

While the reported effects of amphotericin B formulations on O2·- production by human phagocytes have been contradictory (18, 22, 25, 26), in the present study none of the amphotericin B formulations affected O2·- production from pretreated MNCs in response to either nonopsonized or serum-opsonized A. fumigatus hyphae. The exception was the ABLC-treated MNCs, which at the high concentration of ABLC, 25 μg/ml, significantly reduced the production of O2·- in response to serum-opsonized A. fumigatus hyphae. This depressive effect may be due to the binding of ABLC to sterols of MNCs and subsequent cell membrane injury. The same depressive effect has been found to occur with high concentrations of DAMB in previous studies in response to different stimuli (11, 18).

In contrast to the O2·- response, there was an increase in the production of H2O2 and secondary DIIs from MNCs pre-
treated with all amphotericin B formulations, except for those treated with 25 μg/ml LAMB. Although in that case there was a tendency of increased oxidation of DHR-123 to R-123 compared with results for untreated MNCs, this did not reach statistical significance.

Various toxins released from *A. fumigatus* have been implicated in the pathogenesis of this fungus. These toxins may diffuse rapidly into the lung lining fluid, diminish macrophage function, and allow *A. fumigatus* to manifest its well known pathogenic effects (24). Our objective was to use an in vitro system where the experimental conditions would meet the in vivo environment of this fungus. For this reason, we used culture supernatants containing *A. fumigatus* virulence factors in order to investigate its effect on MNC antifungal function.

The apparent paradox of a depressive ABLCL effect on O$_2^-$ production associated with increased levels of H$_2$O$_2$ and secondary DIIs can be explained by the two following mechanisms. First, the enzyme-catalyzed reaction of O$_2^-$ to H$_2$O$_2$ occurs at a near-diffusion-limited rate. As a result, the steady-state concentration of O$_2^-$ is estimated to be about 10$^{-11}$ M (4). This suggests that O$_2^-$ would have to act within a very short time, leading rapidly to the production of H$_2$O$_2$ and secondary DIIs in high concentrations, explaining the previous paradox. Second, the dissociation of O$_2^-$ production from H$_2$O$_2$ levels may occur through differential expression of enzymes catalyzing the reactive oxygen intermediates. An up-regulation of superoxide dismutase and down-regulation of catalase may result in decreased concentrations of O$_2^-$ and increased concentrations of H$_2$O$_2$ as well as DIIs. Given the more potent oxidizing capacity of OH$^-$ and HOCl, this shift toward DIIs may augment the host response. In addition, this negative result on the production of O$_2^-$ may prevent host-destructive effects that this immune molecule has (23).

In another study performed by our group, we demonstrated that DAMB and lipid formulations of amphotericin B differentially affect gene expression and release of an array of proinflammatory and anti-inflammatory cytokines that potentially may explain the differences in infusion-related reactions, as well as the modulation of the host immune response (20). Taking together the results of that report and the present study, lipid formulations of amphotericin B appear to variably affect MNC gene expression and release of cytokines but also hyphal damage and production of H$_2$O$_2$ and secondary DIIs.

There are considerable differences in the hyphal damage induced by MNCs treated with the four amphotericin B formulations. This may have resulted from the various effects that the different structures of lipid formulations of amphotericin B may have on MNC response and activation. In particular, MNCs may respond differently to the small spherical liposome of LAMB, to the large complex ribbon-like structures of ABLCL, and to the disk-like compound of ABCD by yet-unidentified mechanisms in the level of the cell membrane or immune activation compared to the response to the DAMB molecule. The result may be either a further activation of MNCs with a subsequent increase in their antifungal functions or the opposite. Further studies are warranted to investigate in more detail the signal transduction pathways and regulatory cytokine profiles of amphotericin B and its lipid formulations.

Differential stability of the lipid forms may have influenced their effects in comparison to those of DAMB, especially those of the most unstable form, ABCD. However, during the study very much attention was given to the preparation of fresh drugs as described in Methods. Thus, the results with the ABCD at 5 μg/ml looking exactly like those of DAMB at 5 μg/ml and do not seem to be due to the instability of the ABCD and release of amphotericin B from the cholesteryl sulfate disks, although this hypothesis cannot be entirely excluded. In addition, the effects of empty liposomes, lipid ribbons, or cholesteryl sulfate on human MNCs and how particle size or physical nature is responsible for the immunomodulatory activities rather than amphotericin B need further evaluation.

It should be stressed that ABLCL-treated MNCs have shown the highest level of antihyphal activity against *A. fumigatus*. The antifungal superiority of ABLCL-treated MNCs may be based on different mechanisms. The complex ribbons may exert a direct effect on MNC antifungal function by either enhanced expression of a combination of immunoenhancing cytokines, which activate them further, or by higher intracellular concentrations of the drug and subsequent release to the microenvironment of MNC-hyphae, which increases the antifungal action (6).

In conclusion, at a clinically relevant range of concentrations, DAMB and lipid formulations of amphotericin B enhance antihyphal activity of human MNCs against *A. fumigatus*. This up-regulatory activity of amphotericin B formulations is associated with significantly augmented production of H$_2$O$_2$ and H$_2$O$_2$-dependent intracellular intermediates but not of O$_2^-$.

While further study on the molecular mechanisms of these interactions is required immunomodulatory effects of lipid formulations of amphotericin B on the antifungal functions of host phagocytes may be clinically relevant in contributing to the optimal host defense activation and better management of invasive aspergillosis.

**REFERENCES**


