Amphotericin B Formulations Exert Additive Antifungal Activity in Combination with Pulmonary Alveolar Macrophages and Polymorphonuclear Leukocytes against *Aspergillus fumigatus*

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Deoxycholate amphotericin B (DAMB) and amphotericin B lipid complex (ABLC) additively augmented the fungicidal activity of pulmonary alveolar macrophages against the conidia of *Aspergillus fumigatus*. DAMB, ABLC, and liposomal amphotericin B similarly displayed additive effects with polymorphonuclear leukocytes in damaging the hyphal elements of *A. fumigatus*.

During the past two decades, invasive pulmonary aspergillosis (IPA) has emerged as an important life-threatening opportunistic fungal infection in immunocompromised hosts (6, 9, 17, 22, 24, 26). The conidia of *Aspergillus fumigatus* enter the respiratory tract, swell, germinate, and invade pulmonary tissue as hyphae. The predominant host defenses against *A. fumigatus* in the lungs are pulmonary alveolar macrophages (PAMs) and peripheral blood polymorphonuclear leukocytes (PMNs). PAMs ingest inhaled *Aspergillus* conidia and inhibit their intracellular germination (7, 16, 23). PMNs defend the host against *A. fumigatus* by mediating damage to invading hyphae through the release of microbicidal metabolites (2, 7, 16).

Conventional deoxycholate amphotericin B (DAMB) and newer lipid formulations of amphotericin B are standard antifungal agents used in the management of IPA. Whether these compounds have a potentially beneficial additive antifungal effect in combination with host phagocytic defenses is not well understood. We therefore investigated the potential additive effects between PAMs or PMNs and conventional or lipid formulations of amphotericin B against *A. fumigatus* conidia or hyphae.

PAMs were obtained by bronchoalveolar lavage from 22 pathogen-free female New Zealand White rabbits (Hazleton, Rockville, Md.), as described previously (3). PAMs were incubated at a concentration of 10⁶/ml in RPMI 1640 containing 10% fetal bovine serum (Gibco), 100 U of penicillin per ml, and 100 µg of streptomycin per ml (complete medium) at 37°C in 5% CO₂ for 2 days before the conidiocidal assay was performed (see below).

Whole blood was obtained from healthy young adult volunteers. PMNs were isolated by dextran sedimentation and Ficoll centrifugation as reported previously (15).

Strain 4215 (MYA-1163; American Type Culture Collection, Manassas, Va.), a well-characterized isolate of *A. fumigatus*, was stored, cultured, and processed for generation of conidial suspensions (12).

The amphotericin B formulations, DAMB (Bristol-Myers Squibb, Paris, France), amphotericin B lipid complex (ABLC; The Liposome Company, Princeton, N.J.), and liposomal amphotericin B (LAMB; Gilead Nextra, San Dimas, Calif.), were used at concentrations of 0.062, 0.125, and 2.5 µg/ml, respectively, in combination with PAMs and at concentrations of 0.062, 0.125, and 0.625 µg/ml, respectively, in combination with PMNs. The higher concentration of LAMB (2.5 µg/ml) was necessary in order to assess conidiocidal activity. These concentrations were selected as the most appropriate, as determined from separate dose-response conidiocidal assays and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2 *H*-tetratiazolium-5-carboxaniilde (XTT) experiments. In these experiments, different concentrations of drugs were mixed with the fungal targets (conidia or hyphae) and were incubated by the methods used for the assay of conidiocidal activity and the XTT assay described below. The concentrations of drugs chosen to be used in combination with phagocytes were those that achieved ≥50% activity against the fungal targets (conidia or hyphae) when they were used alone. DAMB and LAMB were provided in the form of powders and were dissolved in distilled H₂O, while ABLC was provided in aqueous solution. The antifungal drugs were added to the phagocytes simultaneously with the conidia or the hyphae.

A modified CFU assay was used to assess the conidiocidal activities of the PAMs (12). PAMs were mixed with 10⁶ conidia at a 1:1 effector cell-to-target cell ratio with and without antifungal agents in a final volume of 1 ml in complete medium in polypropylene tubes. Tubes were rotated at 37°C for 6 h, PAMs were lysed with sterile water, and serial dilutions were plated as previously described (12). Colonies were counted and conidiocidal activity was calculated by the following formula: percent killing = (1 – X/C) × 100, where X is the number of CFU with PAMs, antifungals, or combinations thereof at 6 h, and C is the number of CFU of conidia only at 6 h.

PMN-induced hyphal damage was assessed by use of the XTT (Sigma, St. Louis, Mo.) colorimetric metabolic assay.
with coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) (8).

Results are shown as the means ± standard error of means (SEMs). The antifungal activities of the combinations of drugs and phagocytes were compared to the activities of each of the components alone (drug- or phagocyte-induced antifungal activities), and the resulting differences were evaluated by repeated-measures analysis of variance (ANOVA) followed by Dunnett’s correction for multiple comparisons. In the case of unpaired experiments, one-way ANOVA followed by Dunnett’s test was used. A two-sided P value of <0.05 indicated statistical significance.

DAMB and ABLC, but not LAMB, produced additive conidiocidal effects in combination with PAMs (Table 1). PAMs alone killed 17.9% ± 5.5% of the conidia, and DAMB alone killed 14.1% ± 13.9% of the conidia. The combination of DAMB with PAMs increased the proportion of conidia killed to 48.9% ± 9.6% (P < 0.05). Similarly, ABLC exhibited an additive effect on the conidiocidal activity, from 21.7% ± 6.6% and 22.2% ± 9.7% for PAMs and ABLC alone, respectively, to 58.3% ± 5.5% for their combination (P < 0.01). By comparison, LAMB did not exert any suppressive or additive effects on PAMs.

All of the amphotericin B formulations resulted in additive antifungal effects against A. fumigatus hyphae when they were used in combination with PMNs (Table 1). PMNs alone damaged 28.2% ± 8.0% of the hyphae, and DAMB alone damaged 26.7 ± 8.0% of the hyphae; the combination damaged 45.7% ± 7.0% of the hyphae (P < 0.01). ABLC alone damaged 49.1% ± 5.7% of the hyphae, and its combination with PMNs resulted in 60.7% ± 5.4% hyphal damage (P < 0.05). Likewise, the levels of hyphal damage produced by LAMB alone and by its combination with PMNs were 44.4% ± 7.3% and 61.3% ± 4.5%, respectively (P < 0.05).

This study found that DAMB and ABLC enhanced the fungicidal activities of PAMs against A. fumigatus conidia. Similarly, all three formulations of amphotericin B examined displayed additive effects with PMNs against the hyphae of A. fumigatus.

Previous studies have shown that voriconazole may have additive or synergistic effects with host phagocytes against A. fumigatus (21). However, little has been known about the interactions of amphotericin B and its lipid formulations with phagocytic host defense cells against this pathogen.

Pulmonary host defenses against A. fumigatus consist of PAMs, which ingest and destroy conidia, and PMNs, which damage hyphae through extracellular microbial mechanisms (13). Cytokines such as macrophage colony-stimulating factor, tumor necrosis factor alpha, and gamma interferon may further augment pulmonary phagocytic host defenses (3, 12, 13, 14, 27; E. Rolides, C. A. Lyman, T. Sein, and T. Walsh, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother, abstr. 700, p. 555, 1999). Antifungal drugs that are administered to patients with IPA must act in collaboration with host phagocytes in the microenvironment of infected tissues. This collaboration must occur either extracellularly or intracellularly (within the phagocytes) in the case of conidia.

The proposed mechanisms of action of amphotericin B include the formation of transmembrane pores, induction of lipoperoxidation, inhibition of membrane enzymes, blockade of endocytosis, and stimulation of phagocytic cells (1, 5, 28). Amphotericin B in the form of DAMB may increase the killing of phagocytosed A. fumigatus conidia by macrophages (4), inhibit PMN migration and chemotaxis (15, 19, 29), enhance PMN adherence, and reduce PMN viability (15, 18, 20, 29). At the concentrations at which they were used in the present study, none of the antifungal agents exerted deleterious effects on the antifungal activities of PAMs or PMNs against conidia or hyphae of A. fumigatus, respectively.

Table 2 shows several potential mechanisms for the enhanced antifungal activities of amphotericin B formulations used in combination with PAMs or PMNs observed in this study. First, amphotericin B may induce an increase in fungal membrane permeability to PMN microbicidal products such as oxidative burst metabolites or nonoxidative products. Second, amphotericin B may induce the secretion of oxidative and nonoxidative metabolites by PMNs. In this regard, amphotericin B is known to induce secretion of immunoenhancing cytokines such as interleukin-1 and tumor necrosis factor alpha by

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<th>Compound</th>
<th>% Conidiocidal activity</th>
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<tr>
<td></td>
<td>PAMs alone</td>
</tr>
<tr>
<td>DAMB</td>
<td>17.9 ± 5.5</td>
</tr>
<tr>
<td>ABLC</td>
<td>21.7 ± 6.6</td>
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<tr>
<td>LAMB</td>
<td>29.4 ± 5.8</td>
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<sup>a</sup> The results are shown as means ± SEMs of percent conidiocidal activity for three to eight experiments for each antifungal compound.

<sup>b</sup> P < 0.05.

<sup>c</sup> P < 0.01.

<sup>d</sup> P > 0.05.

<sup>e</sup> Comparisons of antifungal compounds or PAMs and their combination were analyzed.
phagocytic cells (11). Increased expression of tumor necrosis factor alpha further enhances the host response to Aspergillus fumigatus (12). Third, the enhanced conidoidal activities of PAMs against A. fumigatus conidia induced by DAMB and ABLC may be due to enhanced phagocytosis of conidia or enhanced oxidative and nonoxidative mechanisms (25, 27).

LAMB appears to interact with host phagocytes differently from the other amphotericin B formulations by acting synergistically only with PMNs against A. fumigatus hyphae. A different configuration of amphotericin B in the liposomes of LAMB may be responsible for the lack of additive effects of the latter when it is used in combination with PAMs. In the case of ABLC, amphoterocin B is concealed within ribbonlike structures. The release of lipases by the fungus breaks down the ribbonlike structures, thus releasing drug (10) and increasing the sensitivity of conidia to PMN fungicidal products. LAMB is a small (80-nm) negatively charged liposome, while ABLC is a large (256-nm) negatively charged liposome. The release of lipases by the fungus breaks down the fungal membrane to PMN fungicidal products. LAMB is a ribbonlike structures, thus releasing drug (10) and increasing the permeability of the fungal membrane to PMN fungicidal products. LAMB is a small (80-nm) negatively charged liposome, while ABLC is a large (256-nm) negatively charged liposome.

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