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NOTES

Pretreatment with Empty Liposomes Attenuates the Immunopathology of Invasive Pulmonary Aspergillosis in Corticosteroid-Immunosuppressed Mice▽

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In a nonneutropenic murine model of invasive pulmonary aspergillosis, pretreatment with empty liposomes (E-lipo) was nearly as effective as 10 mg/kg of body weight liposomal amphotericin B and superior to 1 mg/kg amphotericin B deoxycholate. The beneficial immunomodulatory properties of E-lipo appear to compensate for their lack of direct antifungal activity.

The pathology of invasive pulmonary aspergillosis (IPA) varies considerably between neutropenic and nonneutropenic hosts (2, 5, 9, 16). Experimental models of IPA have demonstrated that, in the setting of persistent neutropenia, damage to the lung is mediated by angioinvasive growth of hyphae leading to hemorrhage, thrombosis, and tissue necrosis with eventual fungal dissemination (2, 9). Histologically, minimal inflammatory exudates are observed in infected animals despite extensive fungal burden; however, high concentrations of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) can be detected in the bronchial alveolar lavage fluid (BALF) (2). Antifungals such as amphotericin B (AMB), which effectively inhibit hyphal growth, can significantly prolong the survival of neutropenic animals with IPA (2, 5). In contrast, the pathogenesis of IPA in corticosteroid-immunosuppressed animals without neutropenia is characterized by an exuberant, dysregulated polymorphonuclear leukocyte (PMN)-mediated inflammatory response in the lung, low or undetectable concentrations of TNF-α or IL-10 in the BALF, few angioinvasive hyphae, and surprisingly minimal efficacy of AMB treatment (2, 5, 9). Consequently, control of a dysregulated exuberant host inflammatory response may be as critical as antifungal activity for animal survival in the setting of intensive corticosteroid immunosuppression (2, 8, 9).

Beyond their useful role as drug carriers, liposomes are known to have potent immunomodulating effects in phagocytic cells (3, 10, 14). Specifically, exposure to liposomes ex vivo can stimulate nonoxidative killing mechanisms in PMNs required for damaging *Aspergillus* sp. hyphae while minimizing release of reactive oxygen species and collagenases that damage the lung (3, 4, 6, 11). Hence, incorporation of AMB into a liposome may reduce the collateral inflammatory pathology of the drug while maintaining its antifungal efficacy. We hypothesized that the immunomodulatory properties of empty liposomes alone (without drug) may be sufficient to control the immunopathogenesis of IPA in corticosteroid-immunosuppressed mice with IPA. To test this hypothesis, we compared patterns of fungal burden clearance and inflammation, animal survival, and effectiveness of ex vivo PMN hyphal killing in corticosteroid-immunosuppressed mice treated with AMB-deoxycholate (AMB-d), liposomal amphotericin B (L-AMB), or empty liposomes.

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Eight-week-old female BALB/c mice (18 to 25 g) (Charles River Laboratories) were immunosuppressed with 10-mg intraperitoneal injections of cortisone acetate (Sigma, St. Louis, MO) suspended in phosphate-buffered saline (PBS) plus 0.5% Tween 20 at 4 days, 1 day prior to inoculation. Mice were then inoculated intranasally with a 50-μl droplet containing 2.5×10^6 *A. fumigatus* 293 conidia suspended in PBS under nebulized 6% isoflurane for oxygen anesthesia as previously described (13, 19).

To ensure that the drug was available in the lung tissue at the time of infection, antifungal therapy was administered 72 h prior to inoculation and continued daily until day +3 after inoculation. AMB-d (1 mg/kg of body weight) (Amerisource-Bergan, Chesterbrook, PA), L-AMB (10 mg/kg) (Ambisome; Gilead Sciences, Inc., Foster City, CA), empty liposomes (Gilead Sciences) diluted in sterile 5% dextrose water (D5W), or D5W alone (control) was administered once daily by lateral tail vein injection. The final concentration of empty liposomes administered to mice was similar to the liposome concentration in the L-AMB injection.

At consecutive time points after inoculation (baseline, 24 h, and 72 h), five infected mice were euthanized by CO_2 narcosis. Blood was immediately collected (1 ml) by cardiac puncture into heparinized syringes for isolation of PMNs, and lungs were harvested for determination of *Aspergillus fumigatus* fungal burden by quantitative real-time PCR as previously de-

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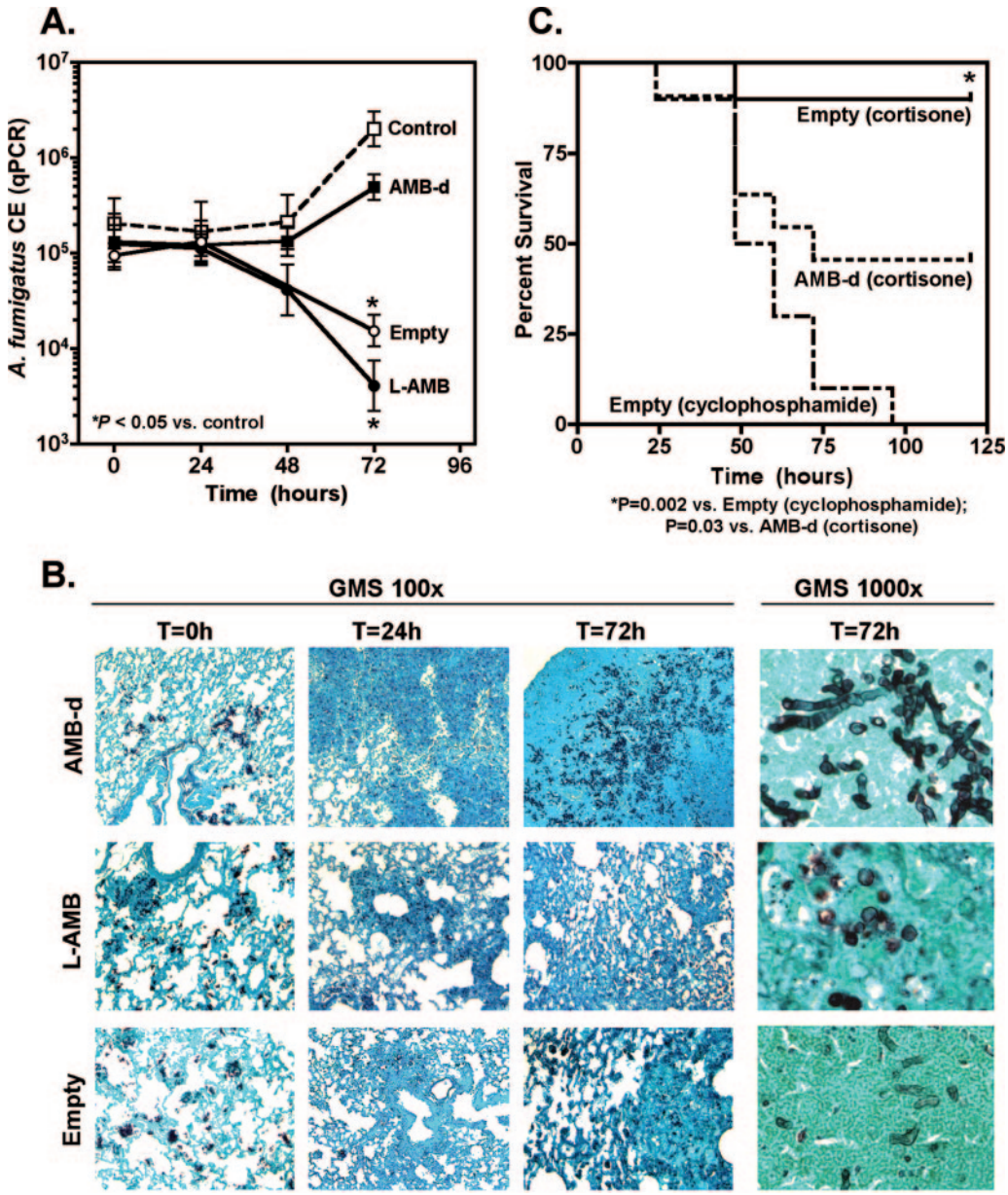

FIG. 1. Pretreatment with L-AMB or empty liposomes, but not AMB-d, is associated with enhanced fungal clearance, survival, and reduced lung injury in corticosteroid-immunosuppressed mice with IPA. Control, 5% dextrose water; empty, empty liposomes. AMB-d was given at 1 mg/kg, and L-AMB was given at 10 mg/kg. A) Lung fungal burden versus time. Each datum point plus error bar represents the mean plus standard error of the mean of the *A. fumigatus* lung fungal burden of five mice versus time (hours) determined by real-time quantitative PCR (qPCR). Fungal burden is reported in *A. fumigatus* conidial equivalents (CE) of DNA. Fungal burdens at 72 h in each treatment group were compared by analysis of variance with Tukey's post hoc comparison. B) Lung histopathology. Shown are Grocott's methamine silver nitrate stains of lungs harvested at baseline and at 24 and 72 h after inoculation. Baseline (time zero) conidia are visible in the lung in all treatment groups. AMB-d-treated animals exhibit bronchiolitis with onset of conidium germination (24 h) that progresses to diffuse pneumonia at 72 h and extensive hyphal invasion despite treatment with AMB-d. L-AMB-treated animals display focal bronchiolitis, without substantial hyphal invasion at 72 h. Animals treated with empty liposomes exhibit focal bronchiolitis and evidence of containment of *A. fumigatus* hyphal invasion within infiltrates at 72 h. C) Survival curves of empty-liposome- and AMB-d-pretreated mice. Groups of 10 mice were immunosuppressed with either cortisone alone or cortisone plus cyclophosphamide and inoculated with 2.5×10^6 *A. fumigatus* conidia. Data are presented as Kaplan-Meier survival curves and were compared by means of log rank.

scribed (7, 18). We observed clear differences in the pattern of lung fungal burden between the treatment groups during the course of infection (Fig. 1A). In contrast to the control and AMD-d-treated animals, decreases in *A. fumigatus* lung fungal burden were observed only in the L-AMB- and empty-liposome-treated animals, with maximal -1.6 and -0.8 $\log_{10}$ changes in lung fungal burden, respectively, 72 h after inoculation. Differences in fungal clearance between the three treatment groups were confirmed by histology (Fig. 1B). The lungs of cortisone-immunosuppressed mice treated with AMB-d

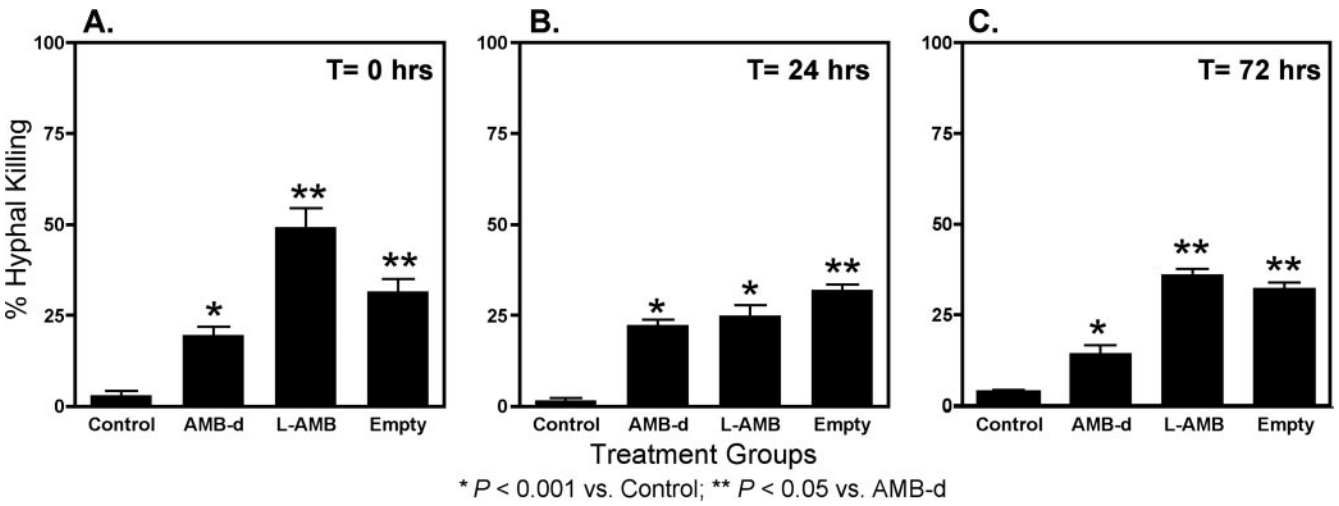

FIG. 2. Ex vivo peripheral PMN killing activity against *A. fumigatus* hyphae is enhanced by AMB-d, L-AMB, and empty-liposome pretreatment. Shown are baseline data (A) and data obtained 24 h (B) and 72 h (C) following intranasal inoculation with 2.5×10^6 *A. fumigatus* conidia in cortisone-immunosuppressed BALB/c mice. The degree of hyphal killing was determined by the XTT assay. Control, 5% dextrose water; empty, empty liposomes. AMB-d was given at 1 mg/kg, and L-AMB was given at 10 mg/kg. Percent killing is determined in relation to parallel *A. fumigatus* controls not exposed to PMN. Each bar represents the mean ± standard deviation of PMN killing results from three different mice.

demonstrated progression to large foci of pneumonia with hyphal growth that was not observed to the same degree in L-AMB- and empty-liposome-pretreated animals.

In "proof-of-principle" survival experiments, groups of 10 mice were immunosuppressed and inoculated as described above and pretreated with either empty liposomes or AMB-d. A third group of animals were administered intraperitoneal cyclophosphamide at 100 mg/kg in conjunction with cortisone at day 4 and day 1 during the pretreatment phase with empty liposomes. Pretreatment with empty liposomes significantly prolonged the survival of cortisone-immunosuppressed mice but not mice immunosuppressed with cortisone plus cyclophosphamide until 5 days after inoculation (hazard ratio [HR], 0.06; 95% confidence interval [CI], 0.015 to 0.23; $P = 0.0024$) (Fig. 1C). Survival was significantly improved by empty-liposome pretreatment in comparison with AMB-d pretreatment in corticosteroid-immunosuppressed mice with IPA (HR, 0.036; 95% CI, 0.15 to 0.89; $P = 0.035$).

Peripheral PMNs from each mouse were isolated from heparinized blood by dextran sedimentation (Amersham Biosciences, Uppsala, Sweden) and centrifugation over a Ficoll-Hypaque gradient (Sigma) to test their capacity to damage unopsonized *A. fumigatus* 293 hyphae (17). Hyphae were generated from cultures of 1×10^5 *A. fumigatus* 293 conidia grown for 16 h in endotoxin-free RPMI 1640 plus 10% fetal calf serum. After resuspension in fresh culture medium, hyphae were exposed to PMNs (1:1 ratio) for 60 min at 37°C in 5% CO_2. PMNs were then hypotonically lysed, and hyphal viability was determined by the 2,3-bis {2-methyloxy-4-nitro-5-[(sulfenylamino)carbonyl]-2*H*-tetrazolium-5-carboxanilide} (XTT) assay (1). PMNs harvested from control, immunosuppressed mice demonstrated minimal capacity (≤5%) to damage *A. fumigatus* hyphae (Fig. 2). However, pretreatment with AMB-d, L-AMB, or empty liposomes enhanced baseline PMN ability to damage *A. fumigatus* hyphae (18.7% ± 4%, 44.7% ± 4%, and 32% ± 3%, respectively; $P = 0.03$ for L-AMB versus control), and this enhancement was maintained in L-AMB- or empty-liposome-treated animals for 72 h.

In conclusion, we demonstrated that pretreatment of intensively corticosteroid-immunosuppressed mice with empty liposomes could positively influence the immunopathogenesis of IPA by enhanced fungal clearance, reduced lung injury, and improved survival. Direct modulation of key innate immune effector cells (i.e., PMNs), possibly through intracellular pattern recognition receptors (i.e., Toll-like receptor 4) (3, 4, 12, 15), may account for the efficacy of the empty liposomes despite their inherent lack of antifungal activity.

Animals used in this study were cared for in accordance with the highest standards for humane and ethical care as approved by the institutional animal care and use committee.

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