

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

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**The immunomodulatory effects of liposomal amphotericin B (LAMB), amphotericin B lipid complex, and amphotericin B colloidal dispersion (ABCD) on antifungal activity of human monocytes (MNCs), an important component of antifungal host defense, against *Aspergillus fumigatus* were compared to those of deoxycholate amphotericin B (DAMB). MNCs from healthy volunteers were incubated with 1 or 5  $\mu\text{g/ml}$  DAMB and 5 or 25  $\mu\text{g/ml}$  lipid formulations for 22 h. Drug-pretreated or untreated MNCs were then washed and assayed for the following: (i) activity against *A. fumigatus* hyphae by XTT assay at MNC:hypha ratios of 10:1 and 20:1; (ii) production of superoxide anion ( $\text{O}_2^-$ ) from MNCs in response to hyphae by cytochrome *c* reduction; (iii) production of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and  $\text{H}_2\text{O}_2$ -dependent intracellular intermediates (DIIs), such as  $\text{OH}^-$  and  $\text{HOCl}$ , from MNCs in response to *A. fumigatus* culture supernatant by flow cytometric measurement of dihydrorhodamine-1,2,3 oxidation. With the exception of 1  $\mu\text{g/ml}$  DAMB and 5  $\mu\text{g/ml}$  LAMB or ABCD at 10:1, all amphotericin B formulations at both concentrations and MNC:hypha ratios enhanced MNC-induced damage of *A. fumigatus* hyphae compared to results with untreated cells ( $P < 0.01$ ). While MNC  $\text{O}_2^-$  production upon hyphal challenge, an early event in oxidative burst, was not affected by the drugs, production of  $\text{H}_2\text{O}_2$  and DIIs, late events, were significantly increased by all four drugs ( $P < 0.01$ ). At clinically relevant concentrations, both conventional amphotericin B and its lipid formulations enhance antihyphal activity of MNCs against *A. fumigatus* in association with significant augmentation of  $\text{H}_2\text{O}_2$  and DIIs but not  $\text{O}_2^-$ , further demonstrating the immunomodulatory antifungal activities of these agents.**

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, NADP (NADPH)-dependent production of antifungal compounds, such as superoxide anion ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and  $\text{H}_2\text{O}_2$ -dependent intracellular intermediates (DIIs), contributes to phagocyte-induced damage of microorganisms including fungi (2, 8, 16). While  $\text{O}_2^-$  production is an early event of oxidative burst,  $\text{H}_2\text{O}_2$  and DIIs consisting of compounds such as hydroxyl radical and  $\text{HOCl}$  are produced at late steps and are even more powerful oxidizing species.

For decades, deoxycholate 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  $\text{O}_2^-$  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  $\text{O}_2^-$ , and production of  $\text{H}_2\text{O}_2$  as well as secondary DIIs, both in response to *A. fumigatus*.

### MATERIALS AND METHODS

**Drugs and reagents.** Deoxycholate 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

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at  $-20^{\circ}\text{C}$ . RPMI 1640 medium, fetal calf serum, penicillin, streptomycin, Hanks' balanced solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS $^{-}$ ) or with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS $^{+}$ ) and Ficoll (lymphocyte separation medium) were obtained from Gibco BRL, Life Technologies Ltd. (Paisley, Scotland). Phorbol myristate acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, MO) and DHR-123 from Lambda Fluoreszenztechnologie GmbH (Wien, Austria).

In all experiments, the working concentrations of DAMB were 1 and 5  $\mu\text{g}/\text{ml}$  and of LAMB, ABLC, and ABCD (each) were 5 and 25  $\mu\text{g}/\text{ml}$ . These drug concentrations were selected to be within a range of therapeutically achievable concentrations in plasma and tissue (3) (A. H. Groll, C. A. Lyman, R. Petraitiene, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., p. 474, 2003), as well as by preliminary experiments with different drug concentrations. Culture medium was prepared from RPMI 1640 without L-glutamine containing 10% heat inactivated fetal calf serum, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (fetal calf medium [FCM]). PMA at a final concentration of 2  $\mu\text{g}/\text{ml}$  was used as a control stimulus of antifungal activity of MNCs. DHR-123 was prepared and kept in the dark at  $-20^{\circ}\text{C}$  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  $-70^{\circ}\text{C}$ .

From suspensions containing  $10^5$  (for hyphal damage assay) or  $2 \times 10^6$  (for  $\text{O}_2^{-}$  production) conidia per milliliter in yeast nitrogen base (YNB) broth supplemented with 2% glucose, 200  $\mu\text{l}$  was plated in 96-flat-bottomed-well cell culture clusters (Corning, Inc., New York, NY) and incubated at  $32^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 18 h to become hyphae (approximately 150 to 200  $\mu\text{m}$ ). 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  $10^6$  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 a humidified  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$  for 2 h, yielding >85% monocyte enriched cell suspensions. Following adherence, the cells were washed with warm HBSS $^{-}$  and incubated in fresh FCM at  $37^{\circ}\text{C}$  for 24 h prior to incubation with the drugs in order to avoid cell activation due to handling. Cell viability of untreated and amphotericin B-treated MNCs for each experimental condition was checked by including additional control wells containing either untreated or treated cells and counting percentages of viable cells by trypan blue staining. The viability of the untreated cells and cells that had been treated with the concentrations of the drugs stated above was >95% in each experiment. MNCs were then incubated with 1 or 5  $\mu\text{g}/\text{ml}$  of DAMB and 5 or 25  $\mu\text{g}/\text{ml}$  of LAMB, ABLC, and ABCD, respectively at  $37^{\circ}\text{C}$  for 22 h in all the experiments.

**Hyphal damage assay.** Hyphal damage was assessed by a modified method of the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide (XTT) assay (13). The XTT assay is a colorimetric method using tetrazolium salts as an indicator. It is based on the property of these salts to be taken up by viable cells and to be reduced by mitochondrial dehydrogenase of the fungi to colored tetrazolium products that are determined spectrophotometrically. Pretreated MNCs were added to hyphae in HBSS $^{+}$  at effector:target (E:T) ratios of 10:1 and 20:1. After incubation at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 2 h, pretreated MNCs were lysed by washing with  $\text{H}_2\text{O}$  three times before adding 150  $\mu\text{l}$  of a solution containing 0.25 mg/ml XTT plus 40  $\mu\text{g}/\text{ml}$  coenzyme Q (both from Sigma). After incubation at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 1 h, 100- $\mu\text{l}$  aliquots were transferred to a new plate and the change in color (absorbance) was assessed on a spectrophotometer at 450 nm with a reference wavelength of 690 nm. Antihyphal activity was calculated according to the following formula: percent hyphal damage =  $(1 - X/C) \times 100$ , where  $X$  is the absorbance of experimental wells and  $C$  is the absorbance of control wells with hyphae only (6).

**Superoxide anion production.**  $\text{O}_2^{-}$  release was assessed by a modification of a previously described cytochrome  $c$  reduction method (10). Hyphae were prepared as described above. For opsonization, YNB broth was replaced by 100  $\mu\text{l}$  of 50% pooled human serum instead of HBSS $^{+}$  and incubated at  $37^{\circ}\text{C}$  for 30 min with rotation. After opsonization, serum was replaced by HBSS $^{+}$  and hyphae were thoroughly washed. PMA was added to control wells together with un-

treated MNCs to maximally stimulate them. MNCs that had been pretreated with drugs for 22 h or control (untreated) MNCs were added at an E:T ratio of 1:1. Cytochrome  $c$  (Sigma) was added to all wells at a final concentration 65  $\mu\text{M}$ , and plates were incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 1 h. Aliquots of 200  $\mu\text{l}$  were assessed for cytochrome  $c$  reduction colorimetrically at 550 nm with a reference wavelength of 690 nm.

**Production of  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{O}_2$ -dependent intracellular intermediates.** Secondary  $\text{H}_2\text{O}_2$  and DIIs were evaluated by flow cytometric measurement of dihydrorhodamine (DHR)-123 oxidation. This assay is based on the capacity of  $\text{H}_2\text{O}_2$  and DIIs in the presence of a metal catalyst to induce oxidation of DHR-123 to rhodamine 1,2,3 (R-123) (19). *A. fumigatus* conidia at  $10^7$  conidia/ml were grown in 2 ml YNB broth at  $32^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . After a 7-day growth period, the broth was centrifuged at 2,000 rpm for 15 min and the fungal supernatant was recovered. PMA was added to control wells (without hyphae) together with untreated MNCs to maximally stimulate them. MNCs that had been pretreated with drugs for 22 h or control (untreated) MNCs were incubated with fungal supernatant for 2 h. DHR-123 solution (20  $\mu\text{M}$ ) was added to the samples 1 h prior to termination of the 2-h incubation period with fungal supernatant to assess the oxidation of DHR-123 to R-123. The percentage of DHR-123-positive cells was calculated by flow cytometry (EPICS XL Flow Cytometer Coulter Beckman [Miami FL]) using an argon laser emitting 15 mV at 488 nm.

**Statistical analysis.** Each experiment was performed with cells of one donor and by use of duplicate wells for each condition. The average value of these duplicate wells was taken as the value for this particular donor/experiment. The total number of experiments performed was five for hyphal damage at each E:T ratio and for  $\text{O}_2^{-}$  production and six for oxidation of DHR-123. The averages of each experiment were then used to calculate the mean  $\pm$  standard error of the mean for each condition. The statistical program InStat (GraphPad, Inc., San Diego, CA) was used for the analysis. Comparisons between drug-treated and untreated MNCs were performed using one-way analysis of variance (ANOVA) with Dunnett as the post-test for multiple comparisons. A  $P$  value of <0.05 indicated statistical significance.

## RESULTS

**Hyphal damage.** Treatment of MNCs with the high concentrations of all amphotericin B formulations (5  $\mu\text{g}/\text{ml}$  of DAMB and 25  $\mu\text{g}/\text{ml}$  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 ABLC 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  $\mu\text{g}/\text{ml}$  of ABLC resulted in a relatively very high percentage of hyphal damage at an E:T ratio of 10:1 compared to results with MNCs alone ( $79.9\% \pm 6.4\%$  and  $91.2\% \pm 3.0\%$  versus  $17.1\% \pm 6.0\%$ , respectively;  $P < 0.001$ ). Similarly, at an E:T ratio of 20:1, MNCs pretreated with 5 or 25  $\mu\text{g}/\text{ml}$  of ABLC resulted in an extremely high percentage of hyphal damage as compared to results with MNCs alone ( $89.6\% \pm 3.8\%$  and  $95.2\% \pm 1.2\%$  versus  $16.1\% \pm 3.9\%$ , 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  $\mu\text{g}/\text{ml}$  of DAMB resulted in  $21.4\% \pm 5.4\%$  hyphal damage and in  $48.1\% \pm 8.1\%$  when treated with 5  $\mu\text{g}/\text{ml}$  ( $P = 0.025$ ). Similarly, MNCs treated with 5  $\mu\text{g}/\text{ml}$  of LAMB showed  $26.9\% \pm 4.3\%$  hyphal damage and  $42.5\% \pm 3.8\%$  when treated with 25  $\mu\text{g}/\text{ml}$  ( $P = 0.026$ ).

**Superoxide anion production.** When PMA was used as a control stimulus of MNC oxidative function, it considerably

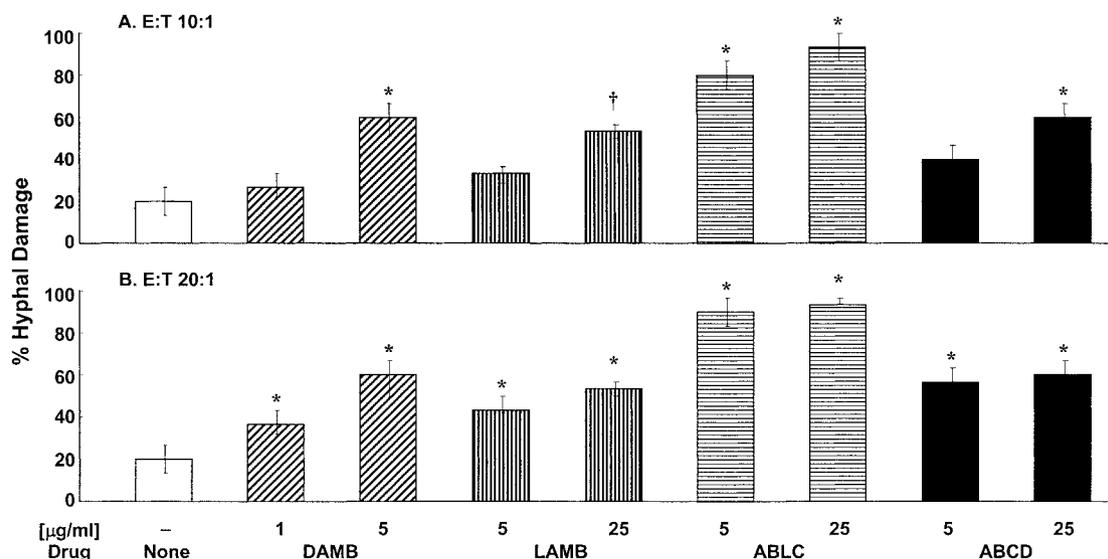


FIG. 1. Effects of different formulations of amphotericin B on human MNC-mediated hyphal damage of *A. fumigatus*, as determined by an XTT assay. MNCs were pretreated with the antifungal drugs DAMB (1 and 5 µg/ml; hatched bars) or LAMB (vertically striped bars), ABLC (horizontally striped bars), and ABCD (black bars) at 5 or 25 µg/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.

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 µg/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  $H_2O_2$  and  $H_2O_2$ -dependent intracellular intermediates.** When PMA was used as a positive control stim-

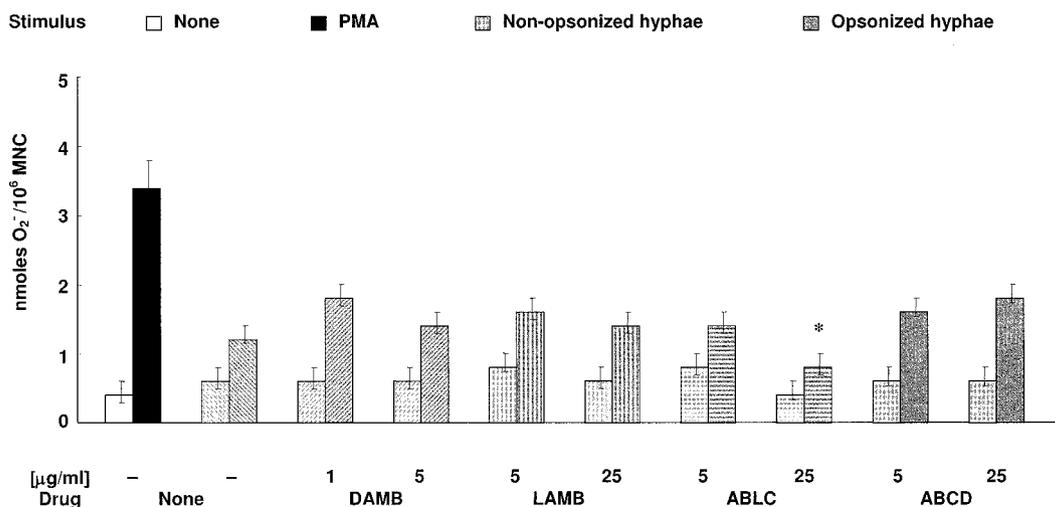


FIG. 2. Effects of different formulations of amphotericin B on superoxide anion ( $O_2^-$ ) produced by human MNCs in response to *A. fumigatus* hyphae. MNCs were preincubated with no drug, DAMB (1 and 5 µg/ml; hatched light or dark gray bars), or LAMB (vertically striped light or dark gray bars), ABLC (horizontally striped light or dark gray bars), and ABCD (light or dark gray bars) at 5 or 25 µg/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.

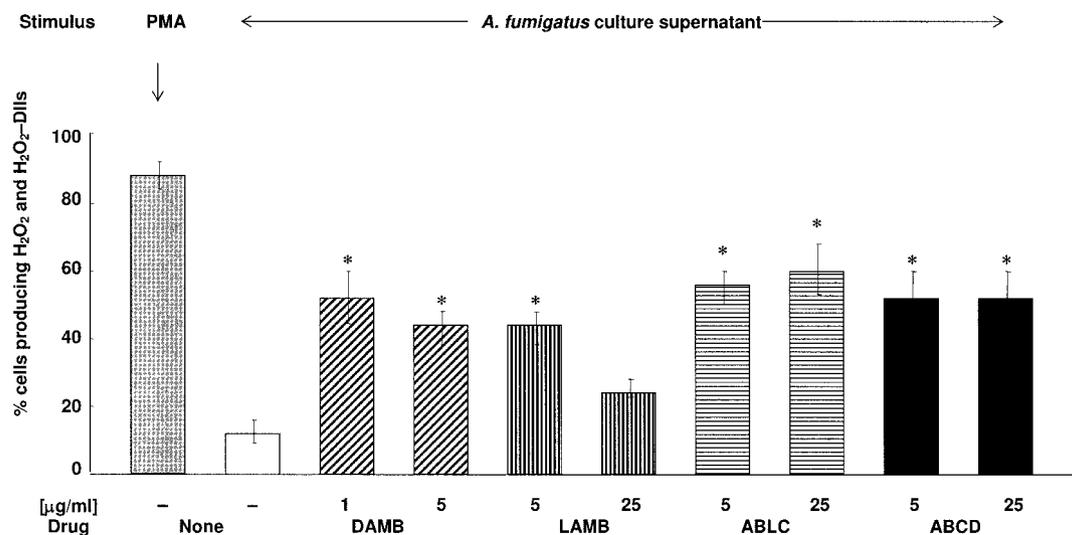


FIG. 3. Effects of different formulations of amphotericin B on oxidation of DHR-123 to R-123 of human MNCs in response to culture supernatant of *A. fumigatus*. MNCs were preincubated with no drug (open bar), DAMB (1 or 5 µg/ml; hatched bars), LAMB (vertically striped bars), ABLC (horizontally striped bars), or ABCD (black bars) at 5 or 25 µg/ml for 22 h. Drug-pretreated or untreated MNCs were then incubated with *A. fumigatus* for 2 h, and oxidation of DHR-123 to R-123 was assessed as a percentage of cells producing H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>-dependent intracellular intermediates. Data are presented as means ± standard errors derived from six donors/experiments. Comparisons between drug-treated cells and control cells were performed by ANOVA with Dunnett test for multiple comparisons. Statistically significant differences from control cells incubated with *A. fumigatus* only with *P* values of <0.01 are indicated by an asterisk.

ulus of oxidative burst of MNCs, it dramatically stimulated DHR-123 oxidation to R-123, with 86.1% ± 3.2% of cells being found positive for DHR-123 oxidation (Fig. 3). Supernatant of *A. fumigatus* culture stimulated DHR-123 oxidation to a much smaller degree, inducing a 7.2% ± 1.8% of cells to be positive for DHR-123 oxidation. MNCs treated with all lipid formulations of amphotericin B significantly increased the production of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>-dependent intracellular intermediates, assessed as oxidation of DHR-123 to R-123, compared to results with untreated MNCs (Fig. 3) (*P* < 0.01). In the case of pretreatment of MNCs with 25 µg/ml of LAMB, the trend of increase observed did not achieve significance (*P* = 0.13).

The highest degrees of DHR-123 oxidation were achieved by MNCs treated with 5 or 25 µg/ml of ABLC (50.7 ± 5.5% and 53.6 ± 8.4% versus 7.2 ± 1.8% of untreated cells with DHR-123 oxidation; *P* < 0.001). However, there was no significant difference between pretreatment with either low or high concentrations of amphotericin B formulations in percent MNCs with oxidation of DHR-123 to R-123 except for MNCs treated with LAMB. In the latter case, pretreatment with 25 µg/ml of LAMB induced a decrease in the percentage of cells positive for DHR-123 oxidation (22.5% ± 3.1%, compared to 42.0% ± 7.4% induced by 5 µg/ml of LAMB; *P* = 0.031).

## DISCUSSION

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 H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>-dependent intracellular intermediates but not of O<sub>2</sub><sup>-</sup> production. To our knowledge, despite the wide clinical use of amphotericin B, there are no reports on the comparative effects of the conven-

tional 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, O<sub>2</sub><sup>-</sup> constitutes the first released reactive oxygen intermediate followed by the production of H<sub>2</sub>O<sub>2</sub>. Subsequently, formation of even more powerful oxidizing species, such as the hydroxyl radical and HOCl, occurs. Secondary H<sub>2</sub>O<sub>2</sub> 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 (O<sub>2</sub><sup>-</sup> plus H<sub>2</sub>O<sub>2</sub> plus a transition metal [H<sub>2</sub>O<sub>2</sub>-Fe<sup>2+</sup>, H<sub>2</sub>O<sub>2</sub>-cytochrome c]), ferryl hemoproteins, or the toxin ONOO<sup>-</sup>/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 H<sub>2</sub>O<sub>2</sub> and secondary DIIs, during the short incubation time of pretreated MNCs with *A. fumigatus* supernatant.

While the reported effects of amphotericin B formulations on O<sub>2</sub><sup>-</sup> production by human phagocytes have been contradictory (18, 22, 25, 26), in the present study none of the amphotericin B formulations affected O<sub>2</sub><sup>-</sup> 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 O<sub>2</sub><sup>-</sup> 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 O<sub>2</sub><sup>-</sup> response, there was an increase in the production of H<sub>2</sub>O<sub>2</sub> and secondary DIIs from MNCs pre-

treated with all amphotericin B formulations, except for those treated with 25  $\mu\text{g/ml}$  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 ABLC effect on  $\text{O}_2^-$  production associated with increased levels of  $\text{H}_2\text{O}_2$  and secondary DIIs can be explained by the two following mechanisms. First, the enzyme-catalyzed reaction of  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  occurs at a near-diffusion-limited rate. As a result, the steady-state concentration of  $\text{O}_2^-$  is estimated to be about  $10^{-11}$  M (4). This suggests that  $\text{O}_2^-$  would have to act within a very short time, leading rapidly to the production of  $\text{H}_2\text{O}_2$  and secondary DIIs in high concentrations, explaining the previous paradox. Second, the dissociation of  $\text{O}_2^-$  production from  $\text{H}_2\text{O}_2$  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  $\text{O}_2^-$  and increased concentrations of  $\text{H}_2\text{O}_2$  as well as DIIs. Given the more potent oxidizing capacity of  $\text{OH}^-$  and  $\text{HOCl}$ , this shift toward DIIs may augment the host response. In addition, this negative result on the production of  $\text{O}_2^-$  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  $\text{H}_2\text{O}_2$  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 ABLC, and to the disk-like compound of ABCD by yet-undetermined mechanisms in the level of the cell membrane or immune stimulation 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  $\mu\text{g/ml}$  looking exactly like those of DAMB at 5  $\mu\text{g/ml}$  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 ABLC-treated MNCs have shown the highest level of antihyphal activity against *A. fumigatus*. The antifungal superiority of ABLC-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  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{O}_2$ -dependent intracellular intermediates but not of  $\text{O}_2^-$ . 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.

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