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In Vitro and In Vivo Comparisons of Amphotericin B and *N*-D-Ornithyl Amphotericin B Methyl Ester

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N-D-Ornithyl amphotericin B methyl ester (N-D-ornithyl AmE) has a lower toxicity for animals than does amphotericin B (AmB), and peak serum levels can be achieved that are fourfold higher than those obtained with an equivalent dose of AmB. However, N-D-ornithyl AmE has one-fourth the in vitro activity and between one-fifth and one-eighth the in vivo activity of AmB. N-D-ornithyl AmE and the corresponding lysyl derivative also lack the immunoadjuvant effects of AmB.

Amphotericin B (AmB) remains the drug of choice for most systemic mycoses. However, its toxicity and limited solubility in aqueous menstra complicate parenteral administration and have made it less than an ideal therapeutic agent. Consequently, there have been attempts to develop derivatives of AmB that are water soluble and less toxic than the parent compound, but which retain antifungal potency. Schaffner and Mechlinski (18) found that esterification of the free carboxyl group of AmB or its *N*-acetylation derivative increased its solubility in water. Lawrence and Hoeprich (8) reported that the methyl ester of AmB (AmE) was less toxic than AmB.

In addition to toxic effects on fungal and host cells, AmB and several of its derivatives can stimulate cells of the immune system and act as immunoadjuvants (2, 9, 15, 20, 21). These latter properties are of particular interest because they may augment host defenses against fungal infection and contribute to the therapeutic efficacy of the drug in vivo.

Recently, the *N*-aminoacyl derivatives of AmE, *N*-D-ornithyl amphotericin B methyl ester (*N*-D-ornithyl AmE) and *N*-D-lysyl amphotericin B methyl ester (*N*-D-lysyl AmE), have been synthesized (22). The acute toxicity of *N*-D-ornithyl AmE for mice was one-tenth that of AmB and the derivative provided six- to eightfold higher peak serum levels in dogs and cynomologous monkeys than those obtained with an equivalent dose of AmB (7). For this report, after determining the in vitro activity of both agents against two strains of *Histoplasma capsulatum*, we have compared the efficacy and toxicity of *N*-D-ornithyl AmE against AmB in a murine model of histoplasmosis and also have examined their respective potencies along with *N*-D-lysyl AmE in assays of immunostimulation.

MATERIALS AND METHODS

Compounds. *N*-D-Ornithyl AmE (Sch 28191; batch no. 15223-147-1), *N*-D-lysyl AmE, and AmB (Fungizone, control no. 2F739, E. R. Squibb & Sons, Princeton, N.J.) were kindly provided by David Lobenberg (Schering Corp., Bloomfield, N.J.). AmE (SQ 14,518; batch NN013ND) as the aspartate salt was supplied by W. E. Brown (E. R. Squibb & Sons). All compounds were suspended and diluted in 5% dextrose just before use.

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Animals. Six- to eight-week-old female AKR mice weighing between 20 and 24 g (average, 21.5 g) were purchased from the Animal Facilities, Division of Radiation Oncology, Washington University School of Medicine, St. Louis, Mo. All mice were housed according to the recommendations of the Department of Agriculture and fed Rodent Laboratory Chow 5001 (Ralston Purina Co., St. Louis, Mo.) and had unlimited access to water.

Organisms. *H. capsulatum* G217B and G186B were obtained from the American Type Culture Collection (Rockville, Md.). Both strains were kept in the yeast-phase morphology on 2% glucose–1% yeast extract broth incubated at 37°C with constant agitation. For maintenance of uniform populations of yeast cells, portions of late log-phase cells grown in glucose-yeast extract broth were mixed with an equal volume of glycerol and frozen as a slurry at −70°C. For preparation of inocula and suspensions for study, the frozen stock was inoculated into glucose-yeast extract broth, incubated on a rotary shaker at 37°C, and subcultured into fresh broth every 5 days. *Saccharomyces cerevisiae* (HLR) was used as a control organism in our susceptibility studies. It was originally obtained from Hoffman-LaRoche Inc. (Nutley, N.J.) and is part of the permanent culture collection of our laboratory (11).

Cell suspensions for inocula were quantitated by hemacytometer counts, and the numbers of viable units were determined by colony counts on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with growth factor and cysteine by the procedure of Burt et al. (3).

In vitro susceptibility studies. Broth dilution susceptibility studies for *H. capsulatum* G217B and G186B were conducted in the dark against AmB and *N*-D-ornithyl AmE by the method of Shadomy and Espinel-Ingroff (19). The MIC was defined as the lowest concentration of drug that inhibited multiplication of the yeasts as determined turbidometrically. Measurements were done when turbidity was noted in the control cultures (96 to 120 h).

Drug toxicity studies. Groups of 10 AKR mice were given intraperitoneal injections of either AmB or *N*-D-ornithyl AmE. The doses were adjusted according to the average weight of individual animals determined just before the start of the experiment. The drugs were administered every other day for a total of six doses. Deaths were recorded up to 14 days after the final injection. Animals were bled from the

retroorbital plexus 1 h after a 500-μg dose of either AmB or N-D-ornithyl AmE to determine whether detectable levels of AmB or N-D-ornithyl AmE were present in serum at termination of the experiment and then were necropsied. Blood levels were also determined on five uninfected mice in the toxicity studies after a 500-μg dose of either drug.

Serum levels of AmB and N-D-ornithyl AmE were determined by high-pressure liquid chromatography (7), using a μ-Bondapak C18 column (3.9 by 30 cm) in conjunction with the Waters model 6000A pump and 450 variable wavelength detector (Waters Associates, Inc., Milford, Mass.). The absorbance detector output was monitored at wavelengths of 388 and 405 nm with a 10-mV Linear model 261/MM recorder (Linear Instruments Corp., Irvine, Calif.), and detector sensitivity was 0.04 absorbance unit (full scale). Separation was accomplished with a solvent mixture consisting of 40 parts acetonitrile (CH_3CN) and 60 parts 0.01 M EDTA-dipotassium salt (pH 4.7) delivered at 1.5 ml/min (1,200 lb/in^2) at ambient temperature. The internal standard was *n*-acetyl AmB.

Polyene adjuvant potency and polyclonal B-cell activation assays. The methods used to compare the adjuvant potencies of AmB and the N-aminoacyl derivatives in vivo, as well as the intensity of polyclonal B-cell activation (PBA) induced by these compounds in culture, have been previously reported (9). In the adjuvant potency assay, mice were immunized by intraperitoneal injection of 100 μg of trinitrophenylated human serum albumin (TNP-HSA) in 0.2 ml of isotonic saline and then injected separately with AmB, AmE, or its N-aminoacyl derivative. An i.p. booster injection of 100 μg of TNP-HSA per mouse was given 28 days later. Mice were sacrificed 6 days after the secondary antigen injection. Spleen cell suspensions were prepared from individual mice and assayed for anti-TNP plaque forming cells (PFC) by the procedure of Yamada and Yamada (23), using trinitrophenylated sheep erythrocytes as target cells (17). Mice immunized with antigen (TNP-HSA) alone served as controls; these were assayed simultaneously.

To determine the degree of PBA produced by these polyenes (9), spleen cell suspensions were prepared from four to six normal mice and were pooled and incubated for 48 h in RPMI 1640, 10% fetal calf serum, and various concentrations of AmE or the N-aminoacyl derivatives. Each culture was then washed and scored for direct PFC, with trinitrophenylated sheep erythrocytes used as target cells (see above). Cell viability was measured by trypan blue exclusion and was routinely 35 to 65% after 48 h in culture in the presence or absence of polyene.

Experimental therapy. The lethality of *H. capsulatum* for AKR mice was determined by the number of deaths after tail vein injections of 0.2-ml suspensions of graded doses of viable yeast (10^5 to 10^7 organisms). There were 10 mice in each dose group. The lowest inoculum of *H. capsulatum* yeast cells which killed 100% of nontreated mice within a week of infection was determined (16). These experiments were repeated three times, and the variability among the experiments was less than 20%. This lowest lethal dose and twice this inoculum were used to infect mice in the therapy experiments. Treatment of AKR mice was begun 24 h after infection. Each group of 10 animals was treated intraperitoneally with different doses of either AmB (between 1 and 500 μg per mouse) or N-D-ornithyl AmE (between 10 and 1,000 μg per mouse) on alternate days after infection for a total of six doses.

TABLE 1. Fifty-percent protective doses (PD_{50}s) of AmB and N-D-ornithyl AmE for AKR mice infected with *H. capsulatum* G217B

Size of inoculum (no. of viable units per mouse)	Dosage regimen		Therapeutic result[b]	PD_{50} (mg/kg) ± 95% confidence limits
	Agent	Dose (mg/kg)[a]	No. survivors/no. inoculated	
8.0×10^5	None		0/20	
	AmB	0.37	0/10	1.52 ± 1.3
		0.74	4/10	
		2.98	6/10	
		5.95	10/10	
		11.90	10/10	
	N-D-ornithyl AmE	2.98	0/10	7.9 ± 5.3
		5.95	2/10	
		11.90	8/10	
		23.81	10/10	
1.6×10^6	None		0/20	
	AmB	0.74	0/10	3.2 ± 2.9
		2.98	4/10	
		5.95	8/10	
		11.90	10/10	
		23.81	10/10	
	N-D-ornithyl AmE	2.98	0/10	26.7 ± 9.3
		5.95	0/10	
		11.90	0/10	
		23.81	2/10	
		47.62	10/10	

[a] Administered on days 1, 3, 5, 7, 9, and 11 after inoculation.
[b] Determined 20 days after last dose of test agent.

RESULTS AND DISCUSSION

In vitro susceptibility studies. The MIC of AmB for the two strains of *H. capsulatum* (G217B and G186B) was 0.1 μg/ml; it was 0.4 μg/ml for *S. cerevisiae* (HLR). The MIC of N-D-ornithyl AmE for the *H. capsulatum* strains was 0.4 μg/ml; it was 1.6 μg/ml for *S. cerevisiae*. Therefore, AmB was fourfold more active against these organisms than was N-D-ornithyl AmE, but the MIC values were within the range of each drug achievable in serum.

Drug toxicity studies. The 50% lethal dose of AmB for AKR mice was 23.8 mg/kg with confidence limits of ±8.8 mg/kg. The 50% lethal dose N-D-ornithyl AmE was 238 mg/kg with confidence limits of ±38.8 mg/kg. The 50% lethal dose levels of N-D-ornithyl AmE were ca. 10-fold higher than those of AmB, which is similar to data already reported by others (7). There were no gross differences in the dose-response curves of the two agents.

Experimental therapy. Since each of the *H. capsulatum* strains had identical susceptibility levels to both polyenes, we used only one, G217B, in the therapy experiments. AKR mice infected intravenously with 8×10^5 viable G217B yeast cells had a mean survival of 4.5 days, and all were dead at 5 days. At this infecting dose, 1.52 ± 1.3 mg of AmB per kg was effective in protecting 50% of the mice, whereas 7.9 ± 5.3 mg of N-D-ornithyl AmE per kg was required to produce the same effect. When 1.6×10^6 viable yeast cells were used, the mean survival time of uninfected mice was 3.5 days and all mice were dead at 4 days. The 50% protective dose of AmB at this inoculum was 3.2 ± 2.9 mg/kg and for N-D-ornithyl AmE was 26.7 ± 9.3 mg/kg. All of the data on therapy are summarized in Table 1.

It is interesting that all of the deaths in the treatment groups occurred during the treatment period. Therefore, after the infection was initially controlled by therapy, no

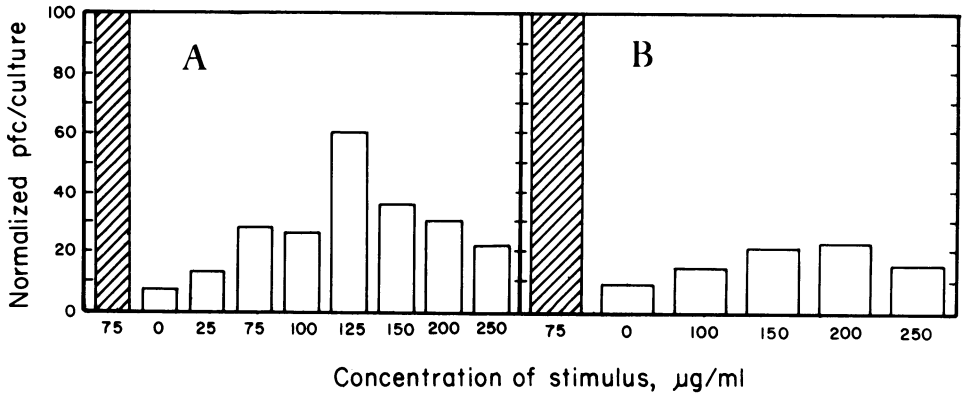

FIG. 1. Polyene-induced PBA in vitro. The mean values ± one standard deviation for cultures incubated in 75 μg of AmE per ml were (A) 370 ± 64 PFC per culture and (B) 327 ± 29 PFC per culture. These PFC values (which represent responses to the optimal concentration of AmE) were taken as 100% for purposes of normalizing the results obtained in the same experiment with the ornithyl and lysyl derivatives, respectively. The mean values of PFC per culture for AmE were significantly different from all other values shown ($P < 0.01$ by Student's t test). (A) PBA to *N*-D-ornithyl AmE; (B) PBA to *N*-D-lysyl AmE. The hatched bars are the responses to AmE.

further progression occurred even after the drugs were discontinued. The animals were not cured of their infection because viable *H. capsulatum* organisms were present in spleens removed from all of the surviving mice 4 weeks posttreatment. This implies that host factors played an important role in controlling the infection after therapy was completed.

Therefore, our studies indicate that AmB is a more effective therapeutic agent against murine histoplasmosis than is *N*-D-ornithyl AmE. The difference between the two drugs in vivo correlated reasonably well with the difference seen in vitro. *N*-D-ornithyl AmE had one-fourth the activity of AmB in vitro (MIC, 0.4 μg/ml for *N*-D-ornithyl AmE and 0.1 μg/ml for AmB) and between one-fifth and one-eighth of the activity in vivo (26.7 ± 9.3 mg/kg for *N*-D-ornithyl AmE and 3.2 ± 2.9 mg/kg for AmB at an infecting dose of 1.6×10^6 yeasts per mouse and 7.9 ± 5.3 mg/kg for *N*-D-ornithyl AmE and 1.52 ± 1.3 mg/kg for AmB when the infecting dose was 8×10^5 yeasts per mouse). We also confirmed the significant differences in blood levels of the two drugs reported previously by others (1, 5). The *N*-D-ornithyl AmE had a blood level of 11.2 μg/ml, and AmB had a level of 2.8 μg/ml 1 h after mice had received a 500-μg dose of each drug.

Immunoadjuvant effects of AmB and *N*-D-ornithyl AmE. Both amB and AmE are potent immunoadjuvants in mice and can stimulate humoral and cellular immunity to defined antigens (2, 20) and also increase resistance to bacterial infections (21) and even to transplantable and spontaneous tumors (12, 13).

Figure 1 shows the results of PBA assays with spleen cells from AKR mice. When AmB was used as the stimulus in these assays, much lower responses were observed due to antibiotic insolubility (data not shown). Therefore, the mitogenic effects of both *N*-D-ornithyl AmE (Fig. 1A) and *N*-D-lysyl AmE (Fig. 1B) were compared with those of AmE. Both *N*-D-ornithyl AmE and *N*-D-lysyl AmE were much less potent than AmE in PBA assays. Higher concentrations of *N*-D-ornithyl AmE and *N*-D-lysyl AmE, compared with AmE, were required to achieve maximal stimulation, and the maxima were significantly less with the aminoacylated derivatives.

Both AmB and AmE regularly induce a marked increase in immune responses in AKR mice (9, 20). *N*-D-Ornithyl AmE and *N*-D-lysyl AmE were much less potent than AmB in their immunoadjuvant effects. AmB treatment led to an approximately fourfold stimulation of immunoglobulin G (IgG) PFC in AKR mice, whereas exposure to the aminoacyl derivatives resulted in little or no stimulation (Table 2).

The advantages of *N*-D-ornithyl AmE over its parent compound AmB are: (i) it is a water-soluble salt at neutral pH in comparison with the highly aggregated state of AmB even in the presence of deoxycholate (22); (ii) it has a lower acute toxicity for animals (7); (iii) peak serum levels can be achieved that are five- to eightfold higher than those obtained with an equivalent dose of AmB (1, 5). The major disadvantages of the derivative are that it is 3- to 16-fold less active than AmB against various species of *Candida* (14) and ca. fourfold less than AmB against yeast isolates of *H. capsulatum*. Our in vivo results in AKR mice confirmed the

TABLE 2. Adjuvant effects of AmB and aminocylated AME derivatives in AKR mice[a]

Expt group no.	1° Stimulus	2° Stimulus	PFC per 10^6 spleen cells ± SD	
			IgM PFC	IgG PFC
1	TNP-HSA	TNP-HSA	6.2 ± 0.7	43.8 ± 25.3
2	TNP-HSA + 300 μg of AmB	TNP-HSA	9.3 ± 2.7	190.1 ± 9.8
3	TNP-HSA + 300 μg of L-AME[b]	TNP-HSA	9.8 ± 2.9	85.2 ± 2.8
4	TNP-HSA + 300 μg of O-AME[b]	TNP-HSA		55.8 ± 22.5

[a] Groups of three age-matched AKR female mice were immunized by two injections of 100 μg of TNP-HSA in phosphate-buffered saline. Polyene immunoadjuvants were injected intraperitoneally in 0.2 ml of 5% dextrose from a separate syringe at the same time as the 1° antigenic stimulus. No adjuvants were injected with the 2° antigenic stimulus. Spleen cell PFC assays were performed 6 days after the 2° stimulus. The IgG PFC mean value for experimental group 2 differed from that in all other groups, with $P < 0.05$ by Student's t-test.

[b] L-AME and O-AME refer to the *N*-D-lysyl and *N*-D-ornithyl derivatives, respectively, of AME.

lower acute toxicity of N-D-ornithyl AmE, and the comparative treatment studies showed that AmB was ca. five- to eightfold more active than N-D-ornithyl AmE in treating histoplasmosis in AKR mice. The aminoacylated derivatives had weaker immune effects than AmB and their parent compound AmE. We cannot judge from our data, however, the importance of the absence of the immune effects to the decreased efficacy of the aminoacyl derivatives. This question will require further study.

Despite its toxicity, AmB is a remarkably potent and effective drug. Attempts to decrease toxicity by synthesizing a methyl ester and now by preparing aminoacyl derivatives have led to decreased efficacy and to the possible introduction of other kinds of toxicity (4, 6). It is possible that the structure of AmB may be optimal for its therapeutic effects, and that any modification decreases efficacy.

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