Liposomal Amphotericin B Inhibits In Vitro T-Lymphocyte Response to Antigen

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The effects of free amphotericin B (as Fungizone) and amphotericin B (AMB) incorporated into liposomes on the proliferation of lymphocytes were determined. Freshly obtained guinea pig and rat antigen-specific lymphocytes were compared with rat T-lymphocyte cell lines cultured for a long period of time. Incorporation of AMB into multilayered vesicles significantly reduced its effect relative to that of Fungizone on cultured T-cell lines, as reported by others for mammalian cells. In contrast, the effects on freshly obtained antigen-specific lymphocytes were different. Fungizone inhibited proliferation of antigen-specific lymph node cells freshly obtained from immunized guinea pigs at fungicidal concentrations, and incorporation into multilayered lipid vesicles did not have much of a protective effect. Higher concentrations of Fungizone were required to inhibit proliferation of fresh rat lymph node cells, but incorporation into multilayered lipid vesicles still did not have much of a protective effect. Some T lymphocytes in the peripheral circulation of guinea pigs and in the lymph nodes of rats were more resistant to liposomal AMB than another more sensitive T-lymphocyte population was. Proliferation of lymphocytes in response to mitogens was inhibited less than that in response to specific antigen was. Thus, sensitivity to AMB depended on the species, the strength of the stimulus used to activate the lymphocytes, and on some other property of the lymphocytes, possibly their state of differentiation. Regardless of the reason for the difference in effects on freshly obtained lymph node lymphocytes and cultured line cells, the former may be more relevant to effects in vivo and should be considered in a complete evaluation of the in vivo toxicity of these forms of the drug. Incorporation into sonicated unilamellar vesicles had more of a protective effect, while equimolar drug-lipid complexes had even more of a protective effect. These forms of AMB might have less of an immunosuppressive potential than multilayered vesicles containing low amounts of AMB do.

The polyene antibiotic amphotericin B (AMB) is the drug of choice for many systemic fungal infections. However, its usefulness in its usual dosage form in deoxycholate is limited by its renal, liver, hematologic, and central nervous system toxicity. Encapsulation in liposomes has recently been found to reduce its in vivo host toxicity while retaining its therapeutic effect against parasitic or fungal infections in animals and humans (8, 15, 16, 25). AMB encapsulated in multilayered vesicles of dimyristoylphosphatidylcholine (DMPC)-dimyristoylphosphatidylglycerol (DMPG) (7:3 [mol/mol]) or in small unilamellar vesicles of egg phosphatidylcholine (PC)-cholesterol-stearylamine (4:3:1 [mol/mol]) has been tried in patients with systemic fungal infections and was found to be effective and well tolerated (8, 15). Consistent with this, in vitro studies have shown that liposomal AMB is as effective on fungal cells as AMB in deoxycholate (Fungizone) or in dimethyl sulfoxide is, but it is much less toxic to mammalian cells such as erythrocytes, macrophages, fibroblasts, and lymphocytes (11, 17, 18, 23). Janoff et al. (10) have recently reported a new formulation of AMB at high AMB-to-lipid mole ratios, consisting of nearly equimolar AMB-lipid complexes, which is even less toxic to animals and mammalian cells but equally effective on fungal cells (10).

If encapsulation of AMB in liposomes protects mammalian cells from its toxic effects, AMB might be a useful drug for targeting to specific mammalian cells in liposomes. Binding of liposomes to specific target cells might allow the AMB to interact with the target cells and leave nontarget cells unaffected. We are interested in targeting drugs to antigen-specific clones of T lymphocytes (5). In preparation for this, we investigated the effect of Fungizone and untargeted liposomal AMB on the in vitro proliferation of T lymphocytes. Consistent with other reports on mammalian cells such as erythrocytes, we found that encapsulation of AMB in liposomes had a large protective effect for antigen-specific T-cell lines stimulated with antigen. However, it had only a small protective effect for lymph node T lymphocytes freshly obtained from immunized animals and stimulated to proliferate with specific antigen. Proliferation of these cells was inhibited by liposomal AMB at concentrations that are used in vivo to treat fungal infections. This immunosuppressive effect of liposomal AMB on fresh antigen-specific lymphocytes may be more relevant to the effects in vivo than are its effects on cultured cells or erythrocytes and should be considered in a complete evaluation of the in vivo utility and toxicity of this dosage form.

MATERIALS AND METHODS

Animals. Adult female strain 13 guinea pigs (weight, 400 to 500 g) were supplied by Crest Caviary (Raymond, Calif.). Adult Lewis rats (weight, 200 g) were supplied by Charles River (Montreal, Quebec, Canada) and were kept in pathogen-free microisolator containers.

Materials. Pure (deoxycholate-free) AMB was purchased from Sigma Chemical Co. (St. Louis, Mo.) and was dis-

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solved in methanol (200 μg/ml). AMB was tested for endotoxin by the *Limulus* amebocyte lysate assay by using an E-Toxate kit from Sigma. It contained less than 0.3 endotoxin units/mg of AMB; no positive response was detected in 10 μg of AMB. Fungizone containing 41 mg of deoxycholate per 50 mg of AMB was obtained from Squibb Canada Inc. (Montreal, Quebec, Canada) and was reconstituted by adding 10 ml of sterile water. Egg PC, dioleoylphosphatidylcholine (DOPC), DMPC, DMPG, bovine brain sphingomyelin, phosphatidylethanolamine (PE) prepared from egg PC by transphosphatidylation, and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, Ala.) and were chromatographically pure. Myelin basic protein (MBP) was prepared from guinea pig brains obtained frozen from Pel-Freez (Rogers, Ark.) by a modification of the method of Deibler et al. (9) and Cheifetz and Moscarello (7). The peptide containing residues 113 to 122 of human MBP (MBP113–122) was synthesized by the Hospital for Sick Children Biotechnology Centre (Toronto, Ontario, Canada) by using the Merrifield solid-phase method. Keyhole limpet hemocyanin (KLH) was from Sigma. Phytohemagglutinin (PHA) was from ICN Immunobiologicals (Lisle, Ill.), and conconavalin A (ConA) was from Sigma. Complete Freund adjuvant was obtained from Difco Laboratories (Detroit, Mich.). [³H]thymidine (specific activity, 0.05 Ci/mol) was from Dupont-NEN Products, Lachine, Quebec, Canada. RPMI 1640 medium was from GIBCO Canada Inc. (Burlington, Ontario, Canada).

Preparation of liposomal AMB. A chloroform solution of the lipid and a methanol solution of AMB were combined in weight ratios of lipid to AMB of 50:1 (1.6 mol%). The solvent was evaporated under a stream of nitrogen followed by evacuation in a lyophilizer at 0.1 torr for 2 h. To 2.5 mg of dried lipid was added 0.5 ml of phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 at pH 7.4. The lipid was dispersed by vigorous mixing at a temperature above the lipid-phase transition temperature for 5 min, resulting in multilamellar vesicles (MLVs) of lipid-AMB. Sonicated unilamellar vesicles (SUVs) were prepared by sonication of the MLVs under nitrogen in a bath sonicator until the suspension was opalescent. Lipid-AMB complexes in an equimolar ratio of lipid to AMB were prepared similarly by bath sonication (10).

Immunization of animals and isolation of lymphocytes. Guinea pigs were immunized with intradermal injections of MBP113–122 or KLH by using 100 μg of antigen per 0.2 ml of phosphate-buffered saline emulsified with 0.2 ml of complete Freund adjuvant (supplemented with 10 mg of mycobacteria per ml) given at several sites in the nuchal area. Rats were immunized similarly with guinea pig MBP in the footpads. The guinea pigs were sacrificed on days 12 to 20, and the draining superficial dorsal cervical lymph nodes were excised. Rats were sacrificed on day 9, and the popliteal lymph nodes were removed. The lymph nodes were dissociated into single-cell suspensions with a wire mesh. The lymph node cells were washed with culture medium and adjusted to 2×10^5 cells per ml of culture medium. The culture medium for guinea pig lymphocytes was composed of RPMI 1640 medium supplemented with 2-mercaptoethanol (5×10^{-5} M), penicillin-streptomycin (100 U/ml), L-glutamine (2 mM), and 1.25% guinea pig serum. For Lewis rats, it also contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), buffer, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1% rat serum instead of guinea pig serum. Peripheral blood lymphocytes

(PBLs) were isolated from guinea pig blood as described previously (5).

Preparation of T-cell lines. Anti-MBP and anti-KLH T-lymphocyte lines were raised from Lewis rats and maintained as described previously (3). The rat lymph node cells were suspended at 5×10^6 cells per ml in stimulation medium consisting of RPMI 1640 medium with antibiotics, glutamine, 2-mercaptoethanol, and autologous serum as described above. Six milliliters of the cell suspension was incubated for 3 days with antigen, 50 μg of guinea pig MBP per ml or 5 μg of KLH per ml, in a 25-cm² tissue culture flask incubated at 37°C in 5% CO_2. Blasts were separated on a Ficoll density gradient and propagated further in growth medium containing 10% heat-inactivated horse serum, antibiotics, glutamine, 2-mercaptoethanol, nonessential amino acids, and sodium pyruvate, as described above, and 10% T-cell growth factor. The latter was prepared from Lewis rat spleen cells suspended at 5×10^6 cells per ml in stimulation medium with 5 μg of ConA per ml and 5% heat-inactivated horse serum. After 45 h, the supernatant was collected and α-methyl mannoside (15 mg/ml) was added. It was filtered under sterile conditions and stored at $-20°C$. The T-cell lines were maintained in growth medium for 4 days and were then restimulated with antigen and X-irradiated thymocytes (2,500 rads) at 2×10^5 T-cell line cells per ml and 10^7 thymocytes per ml in stimulation medium containing 1% fresh syngeneic normal rat serum and 50 μg of antigen per ml for 3 days. The T-cell lines were alternately stimulated and propagated until proliferation was restricted to their respective antigens.

T-lymphocyte proliferation assay. Guinea pig or rat lymph node cells were plated with 180 μl containing 0.5×10^6 cells per well in 96-well microtiter plates (Flow Laboratories, Inc., McLean, Va.). A total of 20 μl of the antigen or mitogen, 0.5 mg of MBP113–122 per ml, 5 μg of KLH per ml, 2 μg of PHA per ml, or 2.5 μg of ConA per ml, and 20 μl of Fungizone or liposomal AMB were added to designated wells in triplicate. The cells were incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere. A total of 20 μl containing 1 μCi of tritiated thymidine was added during the last 18 h of culture. The cells were harvested on filter mats (Skatron Inc., Sterling, Va.) by using a Skatron cell harvester. They were counted in a beta counter by using Beckman Ready Value liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, Calif.). The proliferation response of the T-cell lines was measured similarly by culture of 2×10^4 T-cell line cells with 0.5×10^6 X-irradiated thymocytes per well in stimulation medium. The percent proliferation in the presence of AMB was determined relative to the percent proliferation in the absence of AMB.

Most experiments to test different preparations of liposomes or different types of cells were repeated on several different occasions. The number of times each experiment was repeated is given in the tables. The concentration of AMB which inhibited proliferation by 50% (ED_{50}), relative to that in the absence of AMB, was determined in each experiment. The mean ED_{50}s $\pm$ the standard deviation of the number of different experiments is given in the tables.

RESULTS

The effects of Fungizone and MLVs of DOPC containing AMB at a 1:0.016 mole ratio on the in vitro proliferation of guinea pig lymph node T lymphocytes (LNCs) freshly obtained from an animal immunized with KLH and stimulated in vitro with KLH are compared in Fig. 1. Similar results
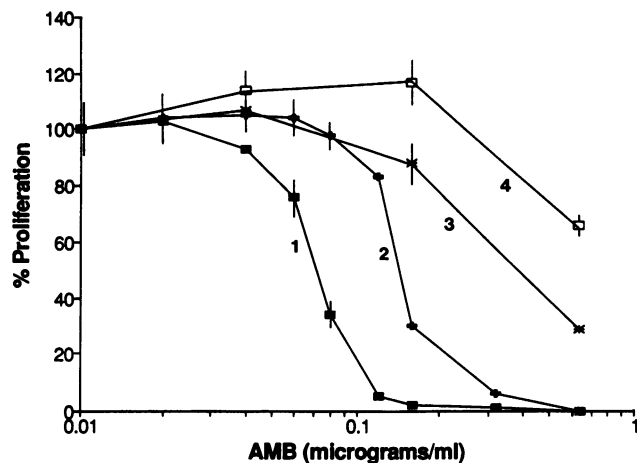
FIG. 1. Comparison of the effects of Fungizone and liposomal AMB on the proliferation of freshly obtained lymphocytes expressed as a percentage of proliferation of cells in the absence of AMB. Effect of Fungizone (■) (curve 1) and DOPC-AMB MLVs (+) (curve 2) on proliferation of guinea pig LNCs in response to KLH. Effect of Fungizone (*) (curve 3) and DMPC-AMB MLVs (□) (curve 4) on proliferation of guinea pig PBLs in response to PHA. Data from representative experiments are shown. Each point is the mean of triplicate cultures ± standard deviations. Error bars of less than ±3% are not shown. The mean $ED_{50}s$ ± standard deviation of a number of such experiments on LNCs is given in Table 1. Proliferation in the absence of AMB to KLH was 25,000 cpm, and that for PHA was 16,000 cpm.
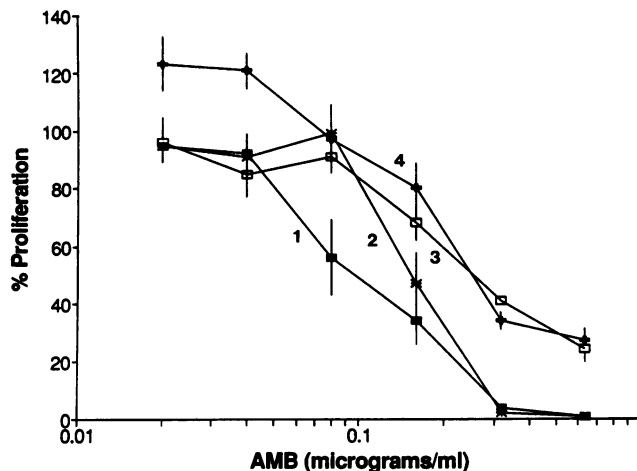
FIG. 2. Comparison of the effect of liposomal AMB on the proliferation of freshly obtained guinea pig LNCs and PBLs in response to specific antigen expressed as a percentage of proliferation of cells in the absence of AMB. DMPC-AMB MLVs on LNCs (■) (curve 1), egg PC-AMB MLVs on LNCs (*) (curve 2), egg PC-AMB MLVs on PBLs (□) (curve 3), and DMPC-AMB MLVs on PBLs (+) (curve 4) are shown. Cells responding to MBP113–122 and KLH behaved similarly. Data from representative experiments are shown. Each point is the mean of triplicate cultures ± standard deviations. Error bars of less than ±3% are not shown. The mean $ED_{50}s$ of a number of such experiments are given in Tables 1 and 2. Proliferation in the absence of AMB ranged from 15,000 to 48,000 cpm. The stimulation of proliferation at low AMB concentrations shown for one curve (+) was occasionally but not routinely observed. It was also observed occasionally with liposomes or detergent in the absence of AMB.

were obtained on T cells specific for and stimulated by MBP113–122. Fungizone inhibited the proliferation of these cells at low, less than fungicidal concentrations. Fungizone or free AMB in dimethyl sulfoxide is fungicidal at concentrations of 0.18 to 1.8 µg/ml, depending on the fungus strain (12, 17, 23). Although DOPC-AMB MLVs were somewhat less toxic than Fungizone, they also inhibited proliferation at less than fungicidal concentrations. Incorporation of AMB into a number of different phospholipids, DMPC, egg PC, and PE-PS, gave similar results; AMB in MLVs of these lipids inhibited proliferation by 50% (ED_{50}) at 0.15 µg/ml,

TABLE 1. Comparison of ability of different forms of AMB to inhibit proliferation of freshly obtained antigen-specific lymph node lymphocytes in response to antigen

Form of AMB[a]	n[b]	ED_{50} (µg of AMB/ml)[c]
Fungizone	4	0.07 ± 0.04
DMPC-AMB (1:0.016) MLVs	4	0.13 ± 0.02
DOPC-AMB (1:0.016) MLVs	8	0.15 ± 0.03
Egg PC-AMB (1:0.016) MLVs	4	0.14 ± 0.03
PE-PS-AMB (0.5:0.5:0.016) MLVs	2	0.16 ± 0.01
SM-AMB (1:0.016) MLVs	1	>2.6
Egg PC-AMB (1:0.016) SUVs	1	0.32
DOPC-AMB (1:1 complex)	2	0.64 ± 0.02
DMPC-DMPG-AMB (0.7:0.3:1 complex	4	1.4 ± 0.4

[a] Numbers in parentheses indicate mole ratios.

[b] n, Number of experiments done.

[c] All dose-response curves looked like those shown in Fig. 1 for LNCs. The $ED_{50}s$ were determined from curves such as those shown in Fig. 1. The $ED_{50}s$ from n such curves, from experiments done on different occasions, were averaged. The values shown are the mean ± standard deviations of these n experiments.

only a two times higher concentration than the ED_{50} for Fungizone (Table 1). The only exception was sphingomyelin (SM); AMB in this lipid did not inhibit proliferation at concentrations up to 2.6 µg/ml, the highest concentration tried. None of these lipids had any significant effect on T-cell proliferation in the absence of AMB at the highest lipid concentrations used (up to 64 µg/ml for egg PC, DOPC, and PE-PS and 500 µg/ml for DMPC). Sodium deoxycholate also had no significant effect at the highest concentrations used.

However, if AMB was incorporated into egg PC SUVs at a 1:0.016 mole ratio, it required a four to five times higher concentration to inhibit proliferation than that required for Fungizone (Table 1). It was even less inhibitory if it was bound at a 1:1 mole ratio of lipid to drug to DOPC or DMPC-DMPG (0.7:0.3 [mol/mol]). At this high drug-to-lipid ratio, unusual ribbon-like structures rather than vesicles are formed (10). In this form, a 10 to 20 times higher concentration of AMB than Fungizone was required to inhibit proliferation (Table 1). MLVs of DMPC-DMPG-AMB (0.7:0.3: 0.016 [mole ratio]) were not tried on these cells.

Lipid-AMB MLVs were less inhibitory of proliferation of PBLs freshly obtained from an immunized animal than they were of LNCs, as shown in Fig. 2 and Table 2. Although the lipid-AMB MLVs had some effect on PBLs at low AMB concentrations (0.2 µg/ml), they did not completely inhibit proliferation even at 0.64 µg of AMB per ml. A plateau was generally seen at about 60 to 70% inhibition of proliferation, and no further inhibition occurred at higher AMB concentrations (up to 0.64 µg/ml), suggesting that a heterogeneous population of antigen-specific T cells with various sensitivi-

TABLE 2. Comparison of the abilities of Fungizone and liposomal AMB to inhibit proliferation of lymphocytes from different sources in response to different stimuli

Lymphocytes	Stimulus	n^a	ED_{50} (μg of AMB/ml)[b]	
			Fungizone	Liposomal AMB[c]
Guinea pig LNC	Antigen	4	0.07 ± 0.04	0.13 ± 0.02
Guinea pig PBL	Antigen	2		0.27 ± 0.1^d
Guinea pig PBL	PHA	1	0.4	>0.64
Rat T-cell lines	Antigen	3^e	0.64–2	>10
Rat LNC	Antigen	3	1.4 ± 0.5	2.5 ± 0.1^f
Rat LNC	ConA	2	>10	>10

[a] n, Number of experiments.
[b] The ED_{50}s shown are the means $\pm$ standard deviations of n different experiments.
[c] DMPC-AMB (1:0.016) MLVs.
[d] Inhibition was not complete even at 0.64 μg/ml (see Fig. 2).
[e] Results of all three experiments resembled those shown in Fig. 4. However, the concentration of Fungizone causing inhibition of proliferation by 50% was not determined accurately within the range of 0.64 to 2 μg/ml. The highest concentration tested was 10 μg/ml.
[f] Inhibition was not complete even at 10 μg/ml (see text).

ties to liposomal AMB are present in the blood. This effect was rarely seen with guinea pig LNCs.

Higher concentrations of AMB were required to inhibit proliferation of guinea pig PBLs in response to PHA than were required when PBLs were stimulated by antigen (Fig. 1 and Table 2). However, the difference in inhibitory concentrations between Fungizone and DMPC-AMB-MLVs was still only about twofold, as found for the antigen-specific cells.

In the experiments described above, lipid-AMB MLVs were added at the beginning of the culture, followed by a 4-day incubation. If the lipid-AMB MLVs were added to guinea pig LNCs after culturing of the cells for 3 days, 3 h before adding [3H]thymidine for a further 18 h of incubation, a less inhibitory effect was observed. Indeed, the results resembled those on PBLs when they were added at the beginning of the culture. As shown in Fig. 3, the lipid-AMB
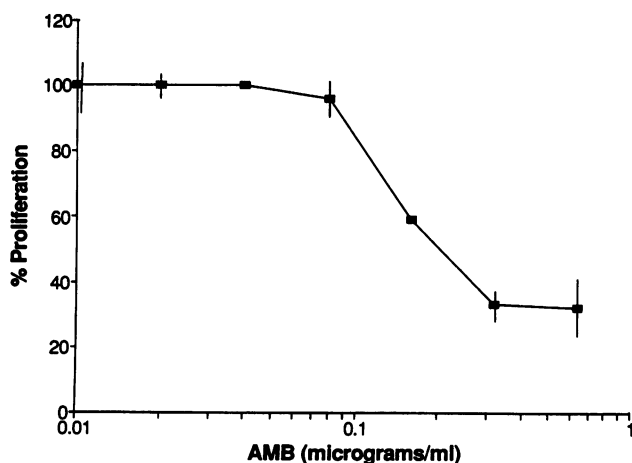

FIG. 3. Effects of egg PC-AMB MLVs on proliferation of guinea pig LNCs in response to KLH when added after 3 days of culture and 3 h before the addition of tritiated thymidine, rather than at the beginning of the culture. Each point is the mean of triplicate cultures $\pm$ standard deviations. Error bars of $\pm$3% are not shown. Proliferation in the absence of AMB was 55,000 cpm.
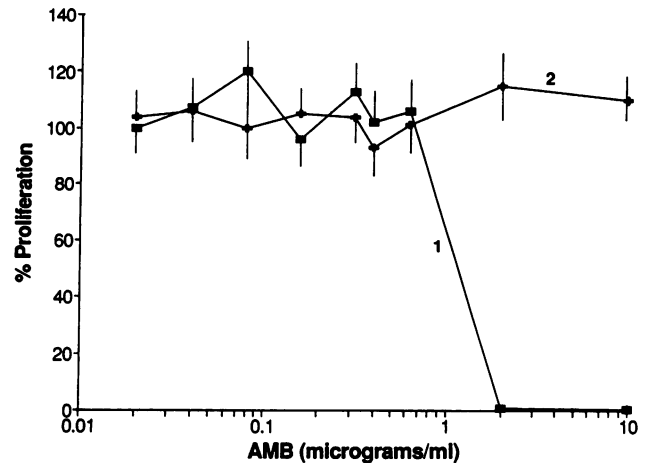

FIG. 4. Comparison of the effects of Fungizone and liposomal AMB on the proliferation of rat anti-MBP line cells in response to MBP expressed as a percentage of proliferation of cells in the absence of AMB. ■, Fungizone (curve 1); +, DMPC-AMB MLVs (curve 2). Data from a representative experiment are shown. Each point is the mean of triplicate cultures $\pm$ standard deviations. In some cases, only half of the error bar is shown for clarity. Error bars of less than $\pm$3% are not shown. Proliferation in the absence of AMB was 60,000 cpm.

MLVs had a significant effect at low concentrations (0.15 μg/ml) but inhibited proliferation by only 60 to 70% even at high concentrations (up to 0.64 μg/ml), suggesting that some of the lymphocytes become resistant after stimulation, culture, or both for several days.

In contrast to the inhibitory effects of low concentrations of AMB on proliferation of guinea pig LNCs freshly obtained from an immunized animal, much higher concentrations of AMB, whether as Fungizone or in lipid MLVs, were required to inhibit the proliferation of rat antigen-specific T-cell lines cultured for long periods of time. Furthermore, incorporation of AMB into liposomes had a large protective effect. As shown in Fig. 4 and Table 2, Fungizone did not inhibit proliferation until the concentration was above 0.64 μg/ml. When incorporated into DMPC MLVs, it did not inhibit proliferation at all up to 10 μg/ml (the highest concentration used). Similar results were obtained for an anti-KLH cell line and an anti-MBP cell line. These concentrations were similar to those of Fungizone and liposomal AMB (MLVs and SUVs) required to increase cation release from erythrocytes, affect macrophage properties, and inhibit proliferation of a myeloma cell line and of lymphocytes in response to mitogens (6, 11, 12, 18, 19, 21, 22, 24, 27).

In order to determine whether this difference in effect on rat line cells and guinea pig LNCs was a species difference, the effect of AMB on rat LNCs was tested. Rat anti-MBP or anti-KLH LNCs freshly obtained from an immunized animal were inhibited by 50% by Fungizone at 1.4 μg/ml and by DMPC-AMB MLVs at 2.5 μg/ml (Fig. 5 and Table 2). However, there was no further inhibitory effect of DMPC-AMB MLVs on rat LNCs from 2.5 to 10 μg/ml, while Fungizone completely inhibited proliferation at 10 μg/ml. Thus, rat LNCs are less sensitive to AMB than guinea pig LNCs are, but they are more sensitive than rat line cells are, particularly to liposomal AMB. Like guinea pig PBLs (Fig. 2), there is a population in the rat LNCs which is nearly as sensitive to liposomal AMB as it is to Fungizone and there is
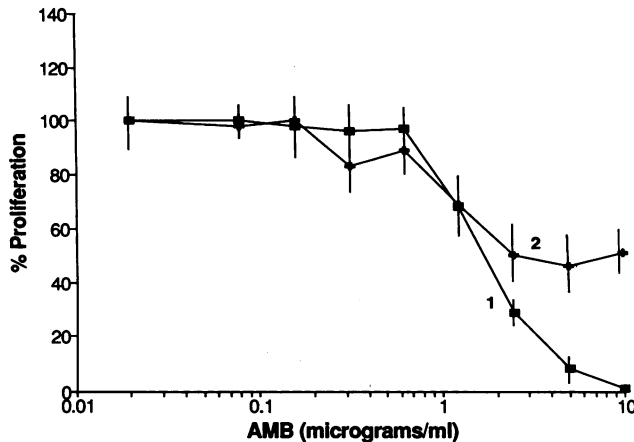
FIG. 5. Comparison of the effects of Fungizone and liposomal AMB on the proliferation of freshly obtained rat LNCs in response to KLH expressed as a percentage of proliferation of cells in the absence of AMB. ■, Fungizone (curve 1); +, DMPC-AMB MLVs (curve 2). Data from a representative experiment are shown. Each point is the mean of triplicate cultures ± minus standard deviations. Rat LNCs proliferating in response to MBP behaved similarly. Proliferation in the absence of AMB was 18,000 cpm.

another population which is resistant to liposomal AMB. Rat LNCs stimulated by ConA proliferated strongly and were not inhibited by either Fungizone or DMPC-AMB MLVs until the AMB concentration was above 5 µg/ml (Table 2). DMPC-DMPG-AMB MLVs (0.7:0.3:0.016) were also tried on rat LNCs and were more toxic to them than DMPC-AMB MLVs were; however, this was because the DMPC-DMPG MLVs without AMB were also toxic at lipid concentrations above 16 µg/ml (data not shown).

DISCUSSION

The results reported here indicate that sensitivity to AMB depends on the species, the strength of the stimulus given to activate proliferation, and some property distinguishing different populations of lymphocytes, possibly their state of differentiation. Thus, guinea pig cells were more sensitive than
rat cells; LNCs were more sensitive than PBLs; cells stimulated by antigen were more sensitive than cells stimulated by mitogen; and freshly obtained lymphocytes were more sensitive than cell lines cultured for long periods of time. Even more importantly, these results indicate that encapsulation of AMB in liposomes does not have the same protective effect for the proliferation of some populations of antigen-stimulated fresh lymphocytes as it does for cell lines cultured for long periods of time and for the effects on other mammalian cells, as reported in the literature (11, 17, 18, 23).

Species differences in sensitivity to AMB have not been reported before, but Little and Shore (14) found strain differences in mice and attributed them to differences in the serum lipoprotein composition. Stewart et al. (21) noted an effect of the strength of the stimulus given to activate proliferation. The reason for the much greater sensitivity to Fungizone or liposomal AMB of the antigen-specific fresh lymphocytes stimulated by specific antigen compared with those of antigen-specific line cells and the greater sensitivities of LNCs compared with those of PBLs is not known. Others have also reported some differences in the effect of Fungizone on cells of the immune system, depending on what organ they are isolated from or on their state of activation. Thymus cells are more sensitive than spleen cells (4), activated macrophages are more sensitive than normal macrophages (6), and spleen B cells are more sensitive than T cells (27). Roselle and Kauffman (19) found that human antigen-specific T cells stimulated by antigen were more sensitive to Fungizone than lymphocytes stimulated by mitogen were, although much higher concentrations were required to inhibit proliferation of both than were required in our study. However, Tarnvik and Ansehn (24) found that the same concentration of Fungizone was required to inhibit proliferation of lymphocytes in response to either mitogen or specific antigen. The effects of Fungizone and liposomal AMB on human lymphocytes stimulated with specific antigen have not been compared.

Inhibition of proliferation of lymphocytes may be caused by the effects of AMB on the lymphocyte, such as leakage of cations, or by effects on macrophages, which affect antigen presentation. However, antigen presentation by macrophages or other antigen-presenting cells is required for both fresh lymphocytes and line cells. The antigen-presenting cells for the fresh LNCs come from the lymph nodes, while those for the line cells come from the thymus.

The effects of AMB on different cell lines have been found to increase as the cholesterol/phospholipid ratio decreases (13). Although AMB is thought to interact with the cholesterol in the membrane, increasing permeability, an increase in the amount of cholesterol may stabilize the membrane against this effect. Possibly, fresh lymphocytes have a different cholesterol/lipid ratio from line cells cultured for long periods of time. PBLs have been shown to have a higher cholesterol/lipid ratio than those of LNCs (26). Smith et al. (20) have reported that the plasma membranes from lymphocytes (thymus or spleen) isolated from immunized animals had a lower cholesterol/phospholipid ratio than those from unimmunized animals.

Although fresh rat LNCs were less sensitive to AMB than fresh guinea pig LNCs were, encapsulation in liposomes did not have much of a protective effect for either, in contrast to the effect for cultured line cells. Encapsulation in liposomes is also not protective for fungal cells, in contrast to mammalian cells such as erythrocytes. Julien et al. (12) have suggested that one reason for the lack of protective effect of incorporation of AMB in SUVs for fungal cells in contrast to its protective effect for erythrocytes is that it is actually free AMB that acts on the cells. Since erythrocytes require a higher concentration of free AMB than fungal cells do, sufficient AMB does not dissociate from the liposomes to affect them, while there is enough free AMB to affect the more sensitive fungal cells. This may partly explain the relatively similar effects of liposomal AMB and Fungizone that we observed on freshly obtained guinea pig LNCs, which are affected at low concentrations, and the dissimilar effects on line cells, which require higher concentrations. However, it does not explain the relatively similar effects of liposomal AMB and Fungizone on fresh rat LNCs, which also require a higher concentration. Furthermore, in contrast to MLVs, liposomal AMB SUVs or 1:1 lipid-AMB complexes had much less of an effect on freshly obtained guinea pig LNCs than Fungizone did, even though these forms of AMB have effects on fungal cells similar to those of Fungi-

zone (10). Thus, dissociation of AMB from liposomes or 1:1 lipid-AMB complexes cannot explain the relatively similar effect of Fungizone and liposomal AMB on LNCs.

It could be argued that the greater effect of MLVs on lymphocytes relative to those of SUVs or 1:1 complexes is due to sedimentation on top of the cells in the microtiter plate, increasing the probability of diffusion of AMB from the liposomes to the cells, while the SUVs and 1:1 complexes remain suspended in solution. However, sedimentation of MLVs also occurs with line cells and yet they are not affected by AMB MLVs.

Regardless of the reason for the differences in the effects on the fresh lymphocytes stimulated by specific antigen and the line cells, the former may be more relevant to the immunosuppressive effects in vivo. The fact that Fungizone and liposomal AMB MLVs inhibited antigen-specific proliferation of fresh lymphocytes at such low concentrations and that liposomes were not protective should be considered in an evaluation of the toxic effects of these forms of AMB used in vivo. Liposomal AMB should be tested on human lymphocytes stimulated with specific antigens rather than mitogens. The concentrations achieved in serum when Fungizone is used to treat fungal infections are 0.4 to 2 µg/ml (8, 24), and thus, they may be high enough to cause inhibition of antigen-specific responses. When liposomal AMB is used to treat fungal infections in vivo, concentrations of 10 to 20 µg of AMB per ml in serum are reached. Thus, although incorporation of AMB into liposomes may decrease the toxic effects on many cells and tissues, it may not prevent immunosuppression by AMB. Indeed, Fungizone and liposomal AMB were found to inhibit antibody production at doses of 1 µg/g when given in vivo after immunization with antigen (18). This is greater than the dose required to alleviate fungal infection in vivo (0.25 µg/g) (1). However, we found that when liposomal AMB (egg PC-AMB MLVs) is given subcutaneously in several doses of 0.1 to 0.2 µg/g several days after immunization with MBP in complete Freund adjuvant, it prevents development of experimental allergic encephalomyelitis, a T-cell-mediated disease, in guinea pigs (2). In vitro proliferation of T cells obtained from these treated animals in response to MBP is significantly reduced. Since 1:1 complexes of lipid-AMB were much less inhibitory of proliferation of antigen-specific fresh lymphocytes than AMB MLVs were, this might be a less immunosuppressive form of the drug for use in the treatment of fungal infections.

ACKNOWLEDGMENTS

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