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An *in vitro* study of liposomal curcumin: Stability, toxicity and biological activity in human lymphocytes and Epstein-Barr virus-transformed human B-cells

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This work is dedicated to and in memory of Dr. Thomas G. Burke, who passed away before this manuscript was completed. He was a beloved mentor and an invaluable collaborator in this project.

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ABSTRACT

Curcumin is a multi-functional and pharmacologically safe natural agent. Used as a food additive for centuries, it also has anti-inflammatory, anti-virus and anti-tumor properties. We previously found that it is a potent inhibitor of cyclosporin A (CsA)-resistant T-cell co-stimulation pathway. It inhibits mitogen-stimulated lymphocyte proliferation, NFκB activation and IL-2 signaling. In spite of its safety and efficacy, the *in vivo* bioavailability of curcumin is poor, and this may be a major obstacle to its utility as a therapeutic agent. Liposomes are known to be excellent carriers for drug delivery. In this *in vitro* study, we report the effects of different liposome formulations on curcumin stability in phosphate buffered saline (PBS), human blood, plasma and culture medium RPMI-1640 + 10% FBS (pH 7.4, 37 °C). Liposomal curcumin had higher stability than free curcumin in PBS. Liposomal and free curcumin had similar stability in human blood, plasma and RPMI-1640 + 10% FBS. We looked at the toxicity of non-drug-containing liposomes on ^{3}H-thymidine incorporation by concanavalin A (Con A)-stimulated human lymphocytes, splenocytes and Epstein-Barr virus (EBV)-transformed human B-cell lymphoblastoid cell line (LCL). We found that dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were toxic to the tested cells. However, addition of cholesterol to the lipids at DMPC:DMPG:cholesterol = 7:1:8 (molar ratio) almost completely eliminated the lipid toxicity to these cells. Liposomal curcumin had similar or even stronger inhibitory effects on Con A-stimulated human lymphocyte, splenocyte and LCL proliferation. We conclude that liposomal curcumin may be useful for intravenous administration to improve the bioavailability and efficacy, facilitating *in vivo* studies that could ultimately lead to clinical application of curcumin.

1. Introduction

Curcumin, a hydrophobic polyphenol, is a principal component of turmeric, used for centuries in Asian countries as a spice and also an herbal anti-inflammatory agent. Curcumin has been shown to have anti-oxidant, anti-inflammatory and anti-mutagenic properties (Ammon et al., 1993; Rao et al., 1995). In our laboratory, we have reported that curcumin has potent immunosuppressive properties, as it blocks cyclosporin A (CsA)-resistant CD28 co-stimulatory pathway in T-cell activation (Ranjan et al., 1998a) and mitogen-stimulated lymphocyte proliferation, NFκB activation and IL-2 signaling in isolated human splenocytes (Ranjan et al., 2004). We have also found that curcumin inhibits Epstein-Barr virus (EBV)-transformed human B-cell proliferation (Ranjan et al., 1999). Due to its pharmacological efficacy and safety, curcumin has been investigated in a wide range of research areas *in vitro* and *in vivo*, in animal and human studies (Anand et al., 2007). However, a major hurdle for curcumin's utility and testing as a therapeutic agent is its poor bioavailability (Anand et al., 2007). To make curcumin amenable to intravenous administration and to overcome the problem of poor bioavailability of free curcumin, Li et al. investigated the effect of liposome-encapsulated curcumin on pancreatic carcinoma cell growth *in vitro* and the anti-tumor and anti-angiogenesis effects in an animal model (Li et al., 2005, 2007). However, a comparison of the stability of free curcumin and liposomal curcumin in a buffer system, culture medium, or human serum, plasma and whole blood and the biological effects of free curcumin and liposomal curcumin *in vitro* and *in vivo* has not been reported (Anand et al., 2007). In addition, the toxicity of non-drug-containing liposomes (Mayhew et al., 1987; Campbell, 1983) used for curcumin encapsulation has not been reported. In this paper we aim to address the above-mentioned points and propose a non-toxic liposomal formulation

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for encapsulation of curcumin. We also report the effects of liposomal curcumin on our laboratory models of immunosuppression and EBV-transformed human B-cell proliferation *in vitro*.

2. Materials and methods

2.1. Chemicals

Curcumin, methanol, chloroform, citric acid, dimethylsulfoxide (DMSO), ethyl acetate, 1-propanol, concanavalin A (Con A), cholesterol, and tetrahydrofuran (THF) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). ^{3}H-thymidine (2 Ci/mmol) was from ICN (Costa Mesa, CA). Culture medium RPMI-1640, fetal bovine serum (FBS) and antibiotic/antimycotic were obtained from Invitrogen (Carlsbad, CA). HPLC-purified curcumin (~99% pure) was a generous gift from the National Cancer Institute (NCI) to TGB.

2.2. Liposome preparation

Liposomes with three different lipid and curcumin compositions were prepared in molar ratios as follows:

1 Lipo-curcumin—DMPC:DMPG:cholesterol:curcumin = 7:1:8:0.5.
2 High-lipo-curcumin—DMPC:DMPG:cholesterol:curcumin = 70:10:80:0.5.
3 High-lipo-high-curcumin—DMPC:DMPG:cholesterol:curcumin = 70:10:80:1.5.

Non-drug-containing liposomes were also prepared for toxicity tests in cultured cells:

DMPC:DMPG = 7:1
DMPC:DMPG:cholesterol = 7:1:8 (molar ratio).

The method for preparing liposomes was according to published methods, with modifications (Sampedro et al., 1994). Lipids, cholesterol and curcumin were dissolved in methanol (2 volume) and chloroform (4 volume) to a final curcumin concentration of 1.35–2.7 mM. The solution was transferred to a sterile flask and dried in a rotary evaporator, then lyophilized overnight. The thin film on the inner surface of the flask was hydrated and re-suspended in 3 mL of RPMI-1640 + 10% FBS with 1% antibiotic/antimycotic for 20 min at 30 °C (above the gel-to-liquid-crystalline phase transition temperature, Tm, of the lipids). The multilamellar liposomes were vortexed for 15 min and were used immediately or stored at −20 °C. The size of the liposomes was not measured. No further treatments, such as bath-type sonicating or extruding through a polycarbonate membrane, were applied (Smistad et al., 2007). The curcumin concentration in the liposomes was assayed by high-performance liquid chromatography (HPLC) with a known concentration of free curcumin as a standard.

2.3. Analysis of curcumin by HPLC

HPLC was performed with a Waters HPLC system (Milford, MA) containing the following instruments: 501 HPLC pumps A and B, 717 plus auto-sampler, 486 PRD272 UV detector, 470 scanning fluorescence detector, in-line degasser with water pump control module, C-18 column (150 mm × 3.9 mm, 5 μm particle size), a pre-column filter and a guard column. The Millennium software and computer were used for data processing. The HPLC procedures were according to published methods with minor modifications (Wang et al., 1997). Briefly, to determine liposome–curcumin concentration, standard free curcumin was prepared in DMSO to 5 mM; 1.6 μL of 5 mM standard free curcumin and the unknown concentration of liposomal curcumin were added to 798 μL of PBS (pH 7.4) to make 10 μM standard free curcumin with the unknown diluted concentration of liposomal curcumin, soon after preparation, 150 μL of the sample was added to 600 μL of chilled methanol (−20 °C) in an autosample vial; the vial was vortexed for 30 s, then run on the HPLC system immediately with a mobile phase of 40% THF and 60% H_2O and 1% citric acid (pH 3.0). The UV detector was set at 420 nm for curcumin, and the fluorescence detector was set at 365 nm for excitation and 512 nm for emission. Peak areas (from the chromatogram of the fluorescence detector) were used for calculating the curcumin concentrations. The flow rate was at 1 mL/min. The retention time for curcumin typically ranged from 5.5 to 6.5 min. Total running time was set for 10 min.

2.4. Liposomal curcumin and free curcumin stability assay

To check the stability of free curcumin and liposomal curcumin in PBS (pH 7.4), both were added to 2 mL PBS (pH 7.4) to achieve a final concentration of 10 μM. Since the stock samples (free and liposomal curcumin) were diluted 400–500 times in PBS, culture medium + 10% FBS or human blood and plasma, the previous presence of 10% FBS with 1% antibiotic/antimycotic in the liposomal and free curcumin stocks were negligible. The samples were incubated in a water bath at 37 °C for 1–180 min; at each incubation time point, 150 μL of the samples was added to 600 μL of chilled methanol (−20 °C), and the procedures described above were followed. To check the stability of free and liposomal curcumin in culture medium RPMI-1640 + 10% FBS, human whole blood and plasma, the medium, blood and plasma were incubated in a water bath for 30 min at 37 °C, and the pH was adjusted to 7.4 with 0.1 M KOH. Free curcumin and liposomal curcumin were added to the culture medium, blood or plasma as described above to reach a

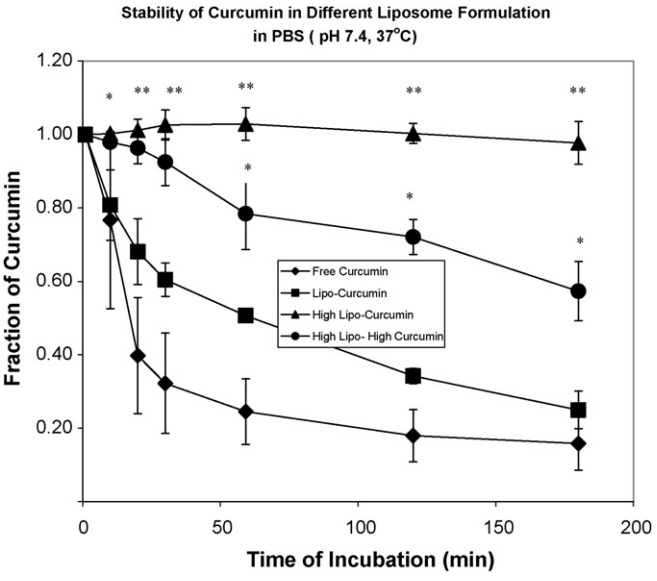

Fig. 1. Stability of free and liposomal curcumin in PBS (pH 7.4, 37 °C). Curcumin obtained from Sigma Chemical was used in this experiment. Liposomal curcumin formulations are described in Section 2. Mean ± S.D. (n = 3), $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to free curcumin.

final concentration of 10 μM. Sample incubation times (1–180 min) and temperature (37 °C) were as described above. After addition of 150 μL of sample to 600 μL of chilled methanol and vortexing, the samples were placed in a table-top centrifuge at 8000 rpm for 45 s then in the auto-sampler for HPLC. In the stability assay, the amount of curcumin at 1 min incubation time was considered 100%, which allowed the amounts of curcumin at 10, 20, 30, 60, 120 and 180 min to be normalized when expressed as fractions of the amount of curcumin at 1 min. The experiment was repeated three times for each time point ($n = 3$).

2.5. Cells and cell culture

Human whole blood was obtained from healthy volunteers. The use of human lymph nodes and human spleen tissue from cadaveric donors was approved by our Institutional Review Board. The methods to isolate lymphocytes and splenocytes have been previously published (Ranjan et al., 2004). The EBV-transformed human B-cell line (LCL) was generated in our laboratory, also described previously (Ranjan et al., 1999). Primary lymphocytes and splenocytes were cultured in RPMI-1640 + 10% FBS with 1%

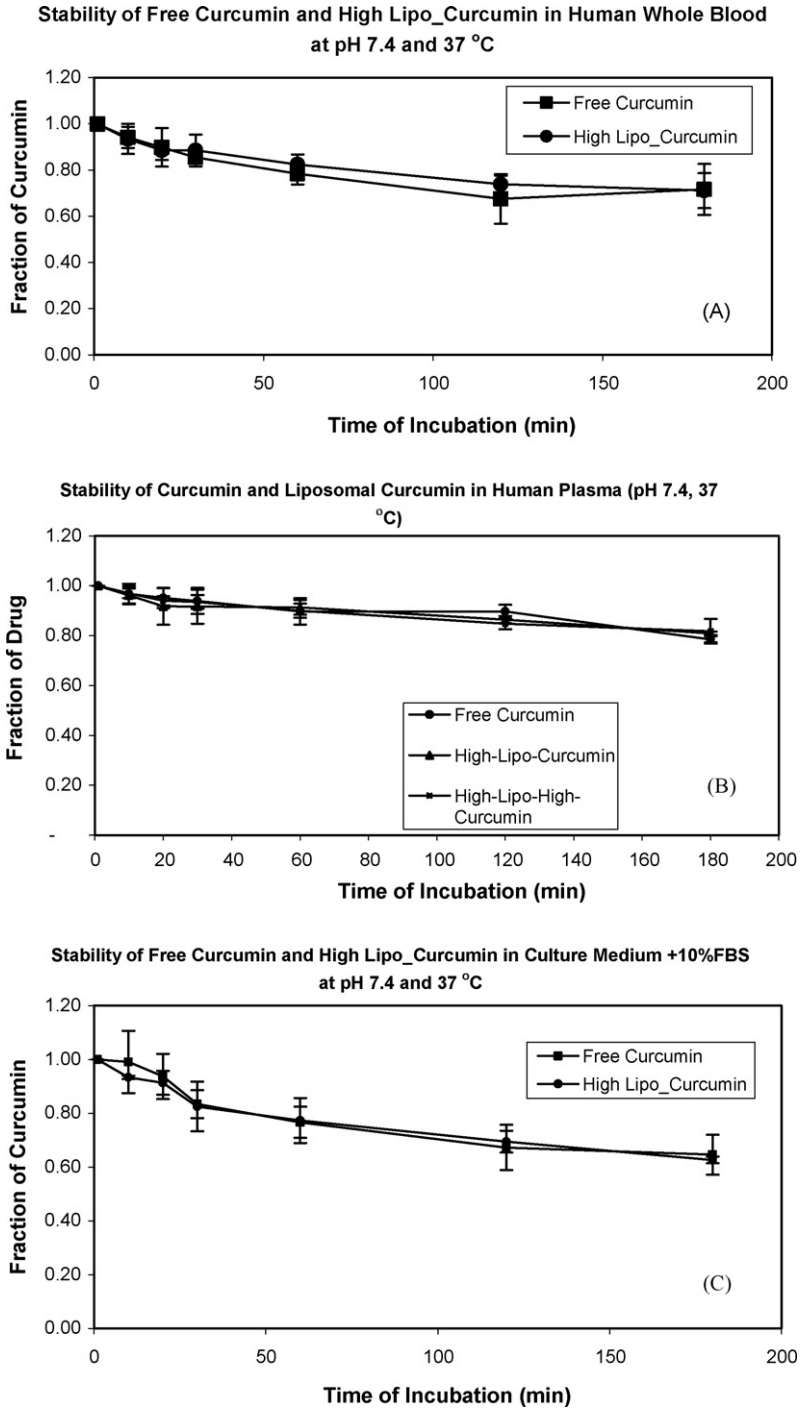

Fig. 2. (A–C) Stability of free and liposomal curcumin in human whole blood, plasma and RPMI-1640 + 10% FBS. No significant difference in stability was found between free curcumin and liposomal curcumin in blood, plasma and culture medium + 10% FBS. Mean ± S.D. ($n = 3$).

antibiotic/antimycotic and with 2 μg/mL Con A for mitogen stimulation. LCL cells were cultured in RPMI-1640 + 10% FBS with 1% antibiotic/antimycotic. The cells were cultured in 96-well plates at 0.1 million cells/(well/0.2 mL) (LCL) or at 0.2 million cells/(well/0.2 mL) (with 2 μg/mL Con A, lymphocytes) with three to six repeats. Free curcumin, liposomal curcumin or drug-free liposome was added to the designated wells as shown in the figures. The control wells were added with carrier (culture medium or DMSO at a final concentration of 0.1%). Cells were cultured for 72–96 h (without removing added curcumin or free curcumin) in a humidified CO_2 incubator at 37 °C and 5% CO_2 in the air. Sixteen hours before cell harvesting, 1 μCi/well of ^{3}H-thymidine (2 Ci/mmol) was added to each well. ^{3}H-thymidine incorporation was determined with a Matrix-96 beta counter.

2.6. ^{3}H-thymidine incorporation assay

The non-drug-containing liposome toxicity on target cell growth was assayed by ^{3}H-thymidine incorporation as described by Campbell and by our published method (Campbell, 1983; Ranjan et al., 2004). The effect of free and liposomal curcumin on Con A-stimulated lymphocyte and splenocyte proliferation and on LCL proliferation was assayed by ^{3}H-thymidine incorporation (Ranjan et al., 2004).

2.7. Cell viability assay

Cell viability was checked in 0.2% Trypan blue solution under a phase contrast microscope. The Trypan blue-excluded cells were considered viable (Fimognari et al., 2002).

2.8. Statistics

Data were analyzed using SPSS 15.0 (Chicago, IL). Data were analyzed by one-way ANOVA followed by Bonferroni/Tukey multiple range test. All data are presented as mean ± S.D. with statistical significance defined as $P \leq 0.05$.

3. Results

3.1. Liposome encapsulation enhanced curcumin stability in PBS (pH 7.4)

Free curcumin has been reported not to be stable in PBS (pH 7.2–8.0) (Wang et al., 1997). At pH 7.2 in PBS, curcumin degraded more than 85% within 30 min incubation. We checked and compared different liposome formulations on the stability of curcumin in PBS at pH 7.4 after incubation for 1–180 min at 37 °C. We found that free curcumin in PBS degraded gradually in PBS and only 16% was left after 180 min incubation when compared to its amount at 1 min incubation (set as 100%). However, liposomes with different formulations enhanced curcumin stability in PBS, the most effective being the high-lipo-curcumin formulation (DMPC:DMPG:cholesterol:curcumin = 70:10:80:0.5, molar ratio). The high-lipo-curcumin formulation protected curcumin 100% after incubation in PBS (pH 7.4) at 37 °C for 180 min. The other two liposome formulations also enhanced curcumin's stability in PBS but to a lesser degree (Fig. 1).

3.2. Stability of free and liposomal curcumin in human whole blood, plasma and RPMI-1640 + 10% FBS

We compared the stability of free curcumin to high-lipo-curcumin in human whole blood. Free curcumin in whole blood (pH 7.4, 37 °C) was more stable than in PBS (pH 7.4); it degraded about 25% after 180 min incubation. High-lipo-curcumin formulation neither enhanced nor decreased curcumin stability in whole blood after 180 min incubation. Similar results were found in plasma and in RPMI-1640 + 10% FBS (Fig. 2).

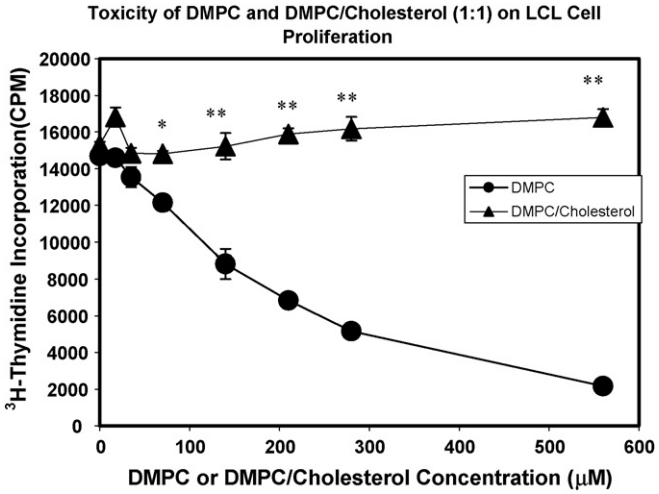

Fig. 3. Toxicity of DMPC and DMPC/cholesterol (1:1) non-curcumin-containing liposomes on LCL cell proliferation. LCL cells were plated in 96-well plates at 0.1 million cells/well in 0.2 mL of RPMI-1640 + 10% FBS with 1% antibiotic/antimycotic. DMPC and DMPC/cholesterol liposomes were added to the designated wells to the indicated lipid concentrations. Cells were cultured for 72 h in a humidified CO_2 incubator at 37 °C and 5% CO_2 in the air. Sixteen hours before cell harvesting, 1 μCi/well of ^{3}H-thymidine (2 Ci/mmol) was added to each well. ^{3}H-thymidine incorporation was determined with a Matrix-96 beta counter. Mean ± S.D. ($n = 3$), $^*P < 0.005$ and $^{**}P < 0.001$.

3.3. Toxicity of non-drug-containing liposomes and formulation of non-toxic liposomes

The toxicity of liposomes to various cell lines has been reported in several studies (Mayhew et al., 1987; Smistad et al., 2007; Berrocal et al., 2000). We investigated whether the non-drug-containing liposome formulations, we used were toxic to the human lymphocytes, splenocytes and LCL cell line. We found that DMPC dose-dependently inhibited ^{3}H-thymidine incorporation, and that the DMPC concentration for 50% inhibition (ID_{50},

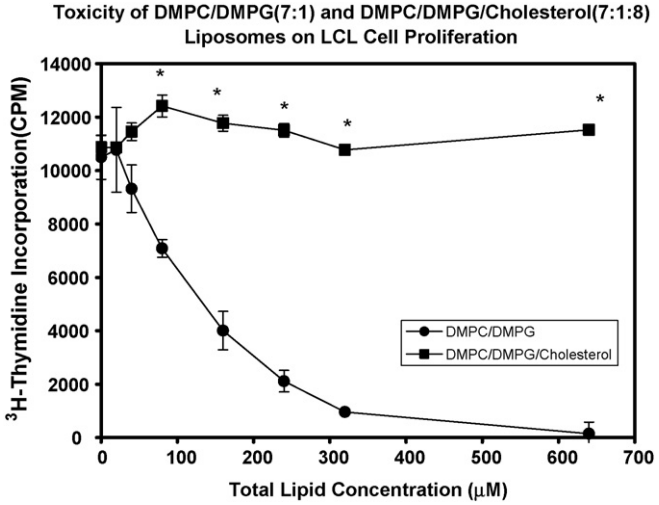

Fig. 4. Toxicity of DMPC/DMPG (7:1) and DMPC/DMPG/cholesterol (7:1:8) non-curcumin-containing liposomes on LCL cell proliferation. Experimental conditions as described in Fig. 3; $^*P < 0.002$.

determined from the concentration curve, Fig. 3) is ~200 μM. Formulation of DMPC with cholesterol at a 1:1 molar ratio completely reduced DMPC toxicity in LCL cells (Fig. 3). The Trypan blue exclusion assay indicated that DMPC decreased cell viability at 560 μM and 88 h of incubation, LCL cell viability decreased from control (no addition of DMPC liposome) of 96 ± 2% to 75 ± 3% (n = 3). In contrast, DMPC/cholesterol liposomes at 560 μM and 88 h incubation did not decrease LCL cell viability, with control (no addition of DMPC/cholesterol) at 96 ± 1% and the 560 μM DMPC/cholesterol treated cells at 95 ± 2% (n = 3) viability.

We investigated the toxicity of DMPC/DMPG (7:1) and DMPC/DMPG/cholesterol (7:1:8, molar ratio) formulation on ^{3}H-thymidine incorporation in LCL cells. We found that DMPC/DMPG was slightly more toxic than DMPC alone and that the ID_{50} value (determined from the concentration curve, Fig. 4) of DMPC/DMPG was ~143 μM (total lipid concentration), compared to the ID_{50} of DMPC which was ~200 μM. Interestingly, adding cholesterol to DMPC/DMPG at 7:1:8 molar ratio completely reduced DMPC/DMPG toxicity to LCL cells (Fig. 4). Trypan blue exclusion assay indicated that DMPC/DMPG decreased cell viability, similar to DMPC as described above. In contrast, DMPC/DMPG/cholesterol did not decrease LCL cell viability (data not shown).

We also looked at the toxicity of DMPC/DMPG (7:1) and DMPC/DMPG/cholesterol (7:1:8) liposomes on ^{3}H-thymidine incorporation in Con A-stimulated human lymphocytes. Similar to our previous findings, DMPC/DMPG dose-dependently inhibited ^{3}H-thymidine incorporation in human lymphocytes. Addition of cholesterol to the liposomes at DMPC/DMPG/cholesterol (7:1:8) completely eliminated DMPC/DMPG toxicity to human lymphocytes (Fig. 5A) and significantly reduced DMPC/DMPG toxicity to splenocytes (Fig. 5B). DMPC/DMPG formulation at 640 μM (total lipid concentration) decreased viability of splenocytes from a control value of 84 ± 1% to 64 ± 4% (n = 3); however, DMPC/DMPG/cholesterol formulation had no significant toxic effect on cell viability. Similar cell viability results were found in lymphocytes after treatment with DMPC/DMPG liposomes and with DMPC/DMPG/cholesterol liposomes (data not shown).

3.4. Comparison of NCI purified curcumin, Sigma curcumin and liposomal curcumin on Con A-stimulated human splenocyte and lymphocyte proliferation

The curcumin obtained from Sigma Chemical (Cat#C7227) was ≥80% curcumin and ≥94% curcuminoid content. We wanted to assess how the purity of curcumin affected the observations in the studied cell populations. Therefore we compared the effects of HPLC-purified curcumin (99% pure), Sigma curcumin and liposomal curcumin (NCI) on Con A-stimulated splenocyte proliferation. Since both free curcumin and liposomal curcumin are relatively stable in RPMI-1640 + 10% FBS, blood and plasma, and considering the toxicity of liposomes, we used the lipo-curcumin formulation of DMPC:DMPG:cholesterol:curcumin = 7:1:8:0.5 (molar ratio) in all cell culture experiments (Figs. 6–8). We found that Sigma free curcumin was as potent as HPLC-purified free curcumin on Con A-

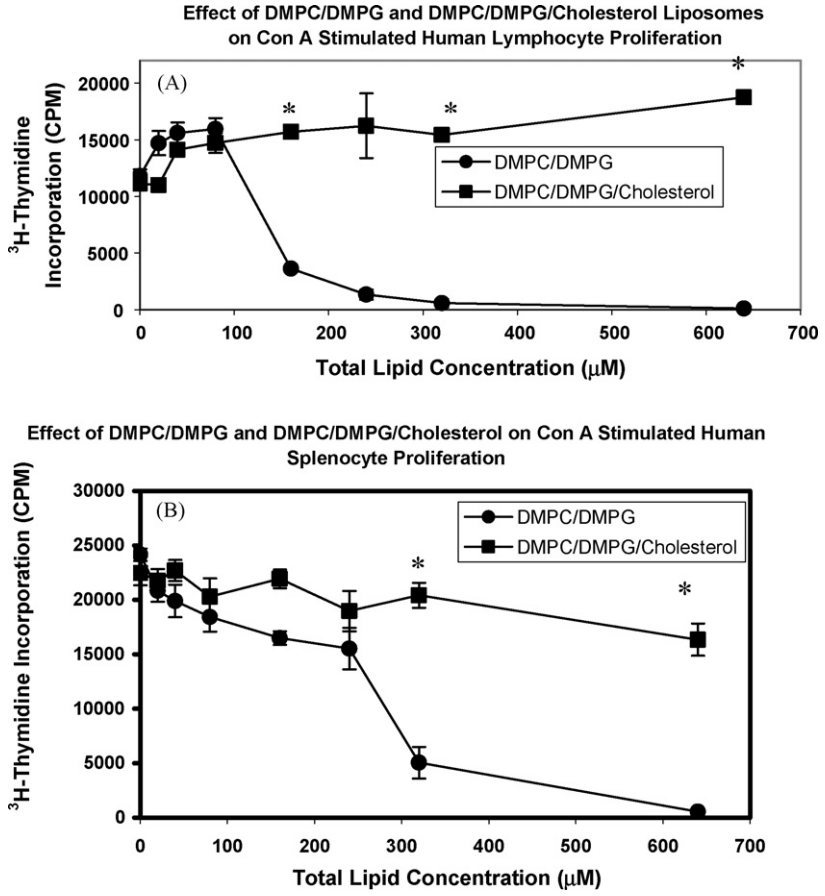

Fig. 5. Toxicity of DMPC/DMPG (7:1) and DMPC/DMPG/cholesterol (7:1:8) non-curcumin-containing liposomes on Con A-stimulated (A) human lymphocyte and (B) splenocyte proliferation. Isolated human lymphocytes and splenocytes were plated in 96-well plates at 0.2 million cells/well in 0.2 mL RPMI-1640 + 10% FBS with 1% antibiotic/antimycotic and 2 μg/mL of Con A. The DMPC/DMPG and DMPC/DMPG/cholesterol (7:1:8) non-curcumin-containing liposomes were added to the designated wells to the final indicated concentrations. Cells were cultured for 96 h in a humidified CO_2 incubator at 37 °C and 5% CO_2 in the air. Sixteen hours before cell harvesting, 1 μCi/well of ^{3}H-thymidine (2 Ci/mmol) was added to each well. ^{3}H-thymidine incorporation was determined with a Matrix-96 beta counter. Mean ± S.D. (n = 3), *P < 0.005.

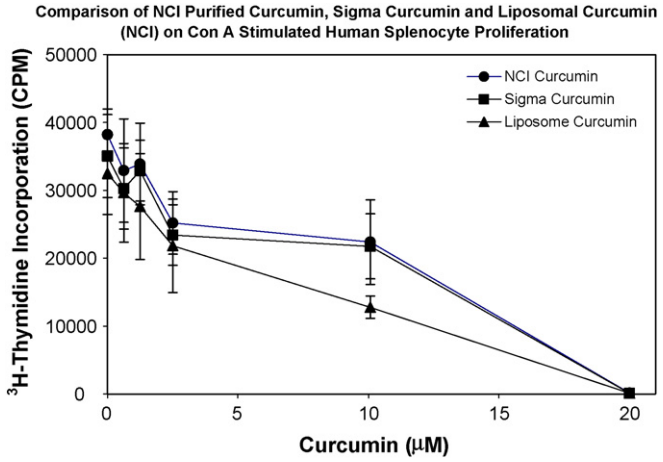

Fig. 6. Comparison of HPLC-purified curcumin (NCI), Sigma curcumin and liposomal curcumin (NCI) on Con A-stimulated human splenocyte proliferation. The isolated human splenocytes were plated in 96-well plates at 0.2 million cells/well in 0.2 mL RPMI-1640 + 10% FBS with 1% antibiotic/antimycotic and 2 μg/mL of Con A. Free curcumin (from either NCI or Sigma) was added to the indicated concentrations and with final carrier DMSO concentration of 0.1%. Liposomal curcumin (DMPC:DMPG:cholesterol:curcumin (NCI) = 7:1:8:0.5) was added to the designated wells to the indicated concentrations without DMSO. Cells were cultured for 96 h in a humidified CO_2 incubator at 37 °C and 5% CO_2 in the air. Sixteen hours before cell harvesting, 1 μCi/well of ^{3}H-thymidine (2 Ci/mmol) was added to each well. ^{3}H-thymidine incorporation was determined with a Matrix-96 beta counter. Mean ± S.D. (n = 3 for each time point). There was no significant difference in potency between Sigma curcumin, HPLC-purified curcumin (NCI) and liposomal curcumin.

stimulated splenocyte proliferation. Liposomal curcumin (NCI) was slightly more potent than free curcumin (NCI) and Sigma (Fig. 6). Liposomal curcumin (NCI) also dose-dependently inhibited Con A-stimulated lymphocyte and splenocyte ^{3}H-thymidine incorporation with an ID_{50} (determined from the concentration curve, Fig. 7) of ~3 μM.

3.5. Effect of free and liposomal curcumin on EBV-transformed LCL cell proliferation

We found that free curcumin and liposomal curcumin at low concentration (0–5 μM) had similar potency inhibiting ^{3}H-thymidine uptake in LCL cells. However at higher concentration (10–15 μM), liposomal curcumin was more potent than free curcumin on LCL cell proliferation (Fig. 8). Free curcumin from Sigma, purified curcumin (NCI), and liposomal curcumin (NCI) at the concentrations shown in Figs. 6–8 did not significantly affect human lymphocytes, splenocytes or LCL viability (data not shown).

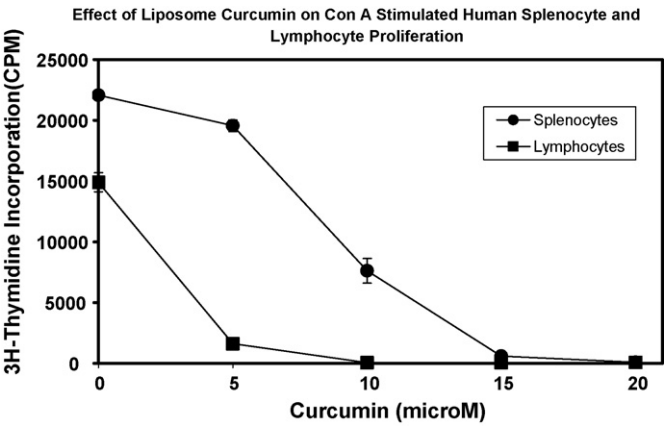

Fig. 7. Effects of liposomal curcumin on Con A-stimulated human lymphocyte and splenocyte proliferation. HPLC-purified curcumin (NCI) was used in this experiment. Liposomal curcumin was formulated as DMPC:DMPG:cholesterol:curcumin = 7:1:8:0.5. Experimental conditions as described in Figs. 5 and 6. Mean ± S.D. (n = 3). Liposomal curcumin at 10, 15 and 20 μM significantly inhibited both lymphocyte and splenocyte proliferation, $P < 0.05$.

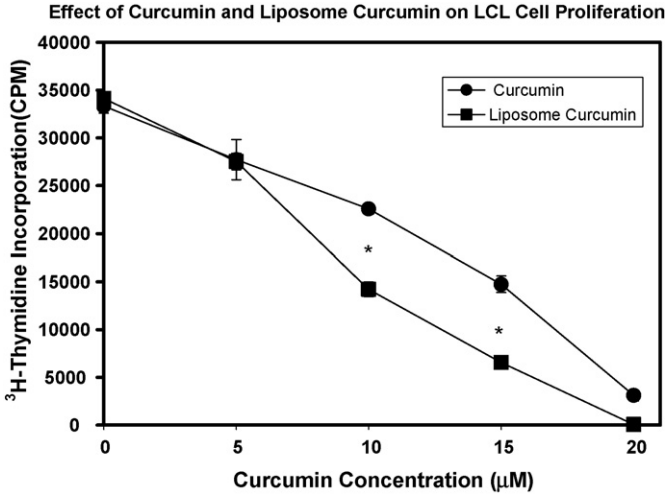

Fig. 8. Curcumin and liposomal curcumin inhibit LCL proliferation. HPLC-purified curcumin (NCI) was used in this experiment. The formulation of liposomal curcumin was DMPC:DMPG:cholesterol:curcumin = 7:1:8:0.5. Experimental conditions as described in Figs. 3 and 7. Mean ± S.D. (n = 6 for each time point), *$P < 0.05$.

4. Discussion

Curcumin has been used safely as a food additive for centuries without reports of significant toxicity. In humans, 6 g oral uptake daily for 3 months did not show side effects (Chueh et al., 2003). In another study, curcumin was safe even at a high dose of 12 g/day in phase I clinical trials (Anand et al., 2007). In addition, curcumin has many attractive properties for use in transplant immunology. It has potent immunosuppression properties as it inhibits CsA-resistant T-cell co-stimulation pathway (Ranjan et al., 1998a) and enhances CsA immunosuppression activity in rat cardiac allografts (Chueh et al., 2003) by inhibiting NFκB activation, mixed lymphocyte reaction and IL-2 signaling (Chueh et al., 2003; Ranjan et al., 2004). Curcumin also inhibits EBV-transformed B-cell proliferation and reduces the rate of EBV infection of human B-cells (Ranjan et al., 1998b). The combination of immunosuppressive properties and anti-post-transplant lymphoproliferative disorder effects make curcumin an attractive compound to study in transplant immunology. However, implementation in *in vivo* studies is hindered by its poor bioavailability.

We chose to use PBS at a pH of 7.4 and whole human blood and plasma (also at pH 7.4) to study the stability of liposomal curcumin under physiological conditions. The results obtained under these conditions hopefully provide some guidance for future *in vivo* study. We selected DMPC and DMPG for use in liposome lipid formulation for two main reasons. First, the addition of negatively charged DMPG to neutral DMPC can increase liposome stability and prevent aggregation, which can occur with liposomes using DMPC alone (Sampedro et al., 1994). Second, the DMPC/DMPG formulation has been used successfully to encapsulate curcumin (Li et al., 2005).

Our study demonstrated that curcumin is not stable in PBS at pH 7.4 and 37 °C, but it is relatively stable in human blood, plasma and culture medium RPMI-1640 + 10% FBS (pH 7.4, 37 °C), in agreement with a previous report (Wang et al., 1997). Encap-

sulation of curcumin with various liposome formulations resulted improved curcumin stability in PBS depending on the formulations. The highest stability in PBS was found with the high-lipo-curcumin formulation, indicating high efficiency of curcumin encapsulation and better physical construction of the multil-amellar vesicles. However, we did not observe enhanced stability in the liposome-encapsulated curcumin compared to free curcumin in human blood, plasma and RPMI-1640 + 10% FBS over the studied time period (1–180 min). Prolonged *in vitro* observation (24–48 h) or *in vivo* animal model would be of interest for future studies.

Non-drug-containing liposomes are known to be toxic to several cell types (Mayhew et al., 1987; Campbell, 1983; Smistad et al., 2007; Kuhn et al., 1983); however, the liposome toxicity that we observed in our formulations to human lymphocytes, splenocytes and EBV-transformed B-cells has not been previously reported. We found that both DMPC and DMPC/DMPG (7:1) are toxic to human lymphocytes, splenocytes and EBV-transformed B-cells. It is not entirely clear why the DMPC/DMPG (7:1) formulation is slightly more toxic than liposomes with DMPC alone. It is possible that the increased stability and decreased aggregation (Sampedro et al., 1994) of the DMPC/DMPG liposomes may increase cell uptake of the liposomes and cause more toxicity in the cells. Interestingly, the addition of cholesterol to DMPC or DMPC/DMPG at a lipid to cholesterol = 1:1 molar ratio nearly eliminated the previously observed toxicity in these cells. Previously, cholesterol in the PG/PC/cholesterol formulation and at lipid:cholesterol = 1:1 molar ratio was found to reduce liposome toxicity dramatically in several human cell lines, including human colon adenocarcinoma HT29, rhabdomyosarcoma A204 and the "normal" diploid fibroblast cell line MLD (Mayhew et al., 1987). It is possible that cholesterol's reduction of non-drug-containing liposome toxicity is related to both lipid composition and cell type. In urinary bladder carcinoma cell line RT-4, addition of cholesterol to PG/PC liposomes had only minor change in the ID_{50} value (Mayhew et al., 1987). The mechanism behind cholesterol's effect is not clear. It has been reported that addition of cholesterol tends to improve the quality of liposome preparations (Sampedro et al., 1994). Cholesterol in the lipid bilayer may have a stabilizing and protective effect by decreasing lipid bilayer hydration (Samuni and Barenholz, 2004). The major biological effect of cholesterol is presumably to reduce either the direct effects of liposomes at the cell surface or the secondary effects after the liposomes have undergone endocytosis (Mayhew et al., 1987).

Liposomal curcumin can inhibit Con A-stimulated human lymphocyte and splenocyte proliferation similar to or even stronger than free curcumin, indicating that liposomal curcumin maintains or even enhances the immunosuppression property of free curcumin. Liposomal curcumin inhibits EBV-transformed human B-cell proliferation similar to or stronger than free curcumin, indicating that liposomal curcumin maintains or even enhances free curcumin's property as an anti-tumor agent.

It should be noted that the stability of liposomal curcumin at 3 h showed ~25% degradation in the culture medium + 10%FBS, and the cells were cultured for 72–96 h. Although ~60% of curcumin in the culture medium + 10% FBS remained after 8 h of incubation (Wang et al., 1997), the exact effects of liposomal curcumin, free curcumin and curcumin degradation products on cell proliferation could not be determined. Our comparison of the liposomal curcumin and free curcumin on cell proliferation was performed without removing the added liposomal curcumin or free curcumin in cell culture during the culture period (72–96 h). Treating for 3 h, removing the liposomal and free curcumin by washing, re-plating the cells to 96-well plates, and culturing for an additional 70–80 h may be worthwhile to perform in the future.

Liposomes are excellent carrier systems for both hydrophilic and hydrophobic drugs, which gives them an advantage as a blood dosing and delivery method (Li et al., 2005). Previously, liposome-encapsulated curcumin was shown to inhibit human pancreatic carcinoma and colorectal cancer cell lines and inhibit NFκB activation and COX2 expression *in vitro*, and was also found to inhibit tumor growth in animal model. Interestingly, the tumor suppression efficacy of the liposome-encapsulated curcumin *in vivo* was even higher than that of the standard chemotherapy for malignancy drug, oxaliplatin (Li et al., 2005, 2007).

5. Conclusion

Our *in vitro* study has found a proper formulation of non-drug-containing liposomes that demonstrates very low toxicity to human lymphocytes, splenocytes and EBV-transformed human B-cells. Our study has also found that liposomal curcumin has equal or enhanced immunosuppression efficacy and enhanced anti-EBV-lymphoma cell line efficacy. Future *in vivo* study using an animal model with comparing liposomal and free curcumin in terms of bioavailability and stability may generate promising results for future clinical applications of curcumin.

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