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In vitro evaluation of liposomal cyclosporine

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Summary

The commercially available intravenous formulation of cyclosporine (CSA) is associated with acute hemodynamic changes, resulting in significant renal dysfunction. The present study investigated the properties of two liposomal CSA formulations: dimyristoylphosphatidylcholine (DMPC) : stearylamine 7 : 1 (A) and DMPC : dimyristoylphosphatidylglycerol (DMPG) 4 : 1 molar ratio (B) developed as an alternative for the i.v. CSA. The optimal drug:lipid weight ratio was 1 : 20 for both formulations. The liposomal suspensions were obtained by rehydrating the lyophilized powder containing CSA and the lipids with saline. CSA entrapment was 90–95% for both formulations. The encapsulation efficiency of the lyophilized powder stored at 2–5 °C was 95% at 24 h and not significantly different with either formulation. The encapsulation efficiency of liposomal formulations A and B in saline was 99 ± 1 and 50 ± 1%, respectively, after 5 days. In 50% serum, the encapsulation efficiency measured as percentage drug remaining entrapped at different time points was 90 ± 3 and 53 ± 4% for A and B, respectively, at 24 h. In vitro activity of both liposomal formulations demonstrated greater potency compared to the commercially available CSA i.v. formulation (A, 69.3 ± 14.8%; B, 50.8 ± 11.5%; i.v., 27.4 ± 20.6% inhibition of T-lymphocyte proliferation; $p < 0.05$ at 10^{-3} mM). The present study shows that liposomes can be used as a drug delivery system for CSA and result in an enhanced in vitro immunosuppressive potency compared with the i.v. formulation.

Introduction

Liposomes are phospholipid vesicles with a hydrophilic compartment and a hydrophobic compartment, allowing binding and delivery of both hydrophilic and lipophilic drugs (Juliano, 1980; Gregoriadis and Allison, 1980). Liposomes target drugs preferentially to organs rich in reticuloendothelial system (RES) cells with sinusoidal capillaries. This altered drug distribution can be used to decrease certain target organ toxicities such as adriamycin cardiotoxicity (Juliano and Lopez-Berestein, 1985). Many properties of liposomes such as low intrinsic toxicity, biodegradability and lack of immunogenicity make them very attractive as drug carrier systems.

Cyclosporine (CSA), a potent T-cell-specific immunosuppressant, is extensively used in the prevention of tissue rejection following kidney, liver, skin, pancreatic and bone marrow transplantation (Miach, 1986). Its use however, has been hampered by serious toxicities to the kidney, liver and central nervous system (Matzke and Luke, 1988). A fur-

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ther limitation of CSA is its insolubility in biological fluids. Cremophor, a polyoxyethylated castor oil used as vehicle in the commercially-available intravenous formulation, is associated with acute renal failure and anaphylactic shock (Luke et al., 1987; Thiel et al., 1986). Toxicity appears to be dose- and rate-limited, with constant intravenous infusions being associated with a lesser degree of renal dysfunction.

Liposomes offer several advantages as a drug carrier system for CSA. By targeting the RES, serious organ toxicities may be avoided while immunosuppression is maintained or enhanced. The objective of the present study was to develop a liposomal formulation of CSA. Two liposomal formulations of CSA with a high encapsulation efficiency were developed. The in vitro activities of these formulations were compared with the commercially available intravenous formulation.

Materials and Methods

Materials

CSA powder and commercially available intravenous solution (50 mg/ml) were gifts from the Sandoz Research Institute, East Hanover, NJ. Chromatographically pure dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids, Birmingham, AL. Methanol, chloroform, stearylamine (SA) and *t*-butanol were purchased from Fisher Scientific, Fairlawn, NJ. T-lymphocytes were obtained and isolated from fresh human blood of healthy volunteers. Ficoll-Hypaque (Sigma, St. Louis, MO), phytohemagglutinin (PHA; Gibco, Grand Island, NY), fetal calf serum (Hazelton Labs., Lenexa, KS) and RPMI-1640 growth medium (Mediatech Corp., Herndon, VA) were prepared fresh and used within 2 days.

Preparation of liposomes

Solutions of CSA (1 mg/ml) and SA (1 mg/ml) were prepared in methanol and stored at $-20\,^{\circ}C$ until use. DMPC and DMPG were available as 25 mg/ml solutions in chloroform. DMPC, DMPG and SA were mixed at the desired molar ratio with a precalculated quantity of CSA and subsequently the organic solvent was evaporated under vacuum using a rotary evaporator. The dried lipid-drug film obtained was dissolved in *t*-butanol and frozen in a dry ice-acetone bath. The mixture was then lyophilized for 12 h. To form the liposome suspension, the lyophilized powder was reconstituted with normal saline solution (to a CSA concentration of 1 mg/ml) in a water bath ($35\,^{\circ}C$) for 30 min, shaken at 80 rpm, and then centrifuged at $100\,000 \times g$ for 50 min. The pellets were resuspended in saline to a resultant concentration of 2 mg/ml.

Liposome characterization

To assess the entrapment efficiency, aliquots (50 μl) of the initial suspension and the resuspended pellet were dissolved in 950 μl methanol and the absorbances read at a wavelength of 240 nm on a Beckman UV scanning spectrophotometer.

Liposome encapsulation was measured in saline and 50% serum at 0, 1, 2, 6 and 24 h at room temperature ($25\,^{\circ}C$) and at $37\,^{\circ}C$, respectively. The entrapment efficiency of the lyophilized powder of drug-lipid mixture was also assessed stored for different periods of time at $4\,^{\circ}C$. Samples were removed at the specific time and the suspension centrifuged as described above. The pellet was resuspended and CSA entrapment was determined by spectrophotometry.

Entrapment efficiency (%EE) was calculated according to:

$$\%EE = \frac{[CSA] \text{ in pellet}}{[CSA] \text{ in liposome suspension}} \times 100$$

In vitro immunosuppressive activity

Heparinized whole blood (5 ml) was mixed with an equal volume of phosphate-buffered saline (PBS, pH 7.4). This mixture was slowly poured over 5 ml Ficoll-Hypaque solution and the tubes were centrifuged at 1400 rpm for 30 min. The middle layer containing the white mononuclear cells was removed, placed in a clean polypropylene tube, washed with 5 ml PBS, and again centrifuged for 10 min. The supernatant was dis-

carded and the wash step was repeated. The remaining pellet of cells was resuspended in 2 ml growth medium (RPMI 1640 with added glutamine, 10% v/v fetal calf serum and streptomycin). Cell concentration was assessed with a Coulter cell counter. An aliquot (0.25 ml of 10^6 cells/ml) of each cell suspension was placed in 96-well microtiter plates (Falcon, Oxnard, CA) and 0.02 ml of a 1:20 dilution of stock PHA added. 50 μl of previously prepared solutions of IV, A, B and drug-free liposomes were added to each well such that the final concentration of CSA in the wells ranged from 1×10^{-1} to 1×10^{-9} mM. The wells were incubated at 37°C in a 5% CO_2/95% O_2 humidified incubator for 4 days. Following the incubation period, all wells were pulsed with 0.02 ml [^{3}H]thymidine (1.0 μCi/ml, ICN Radiochemicals, Irvine, CA) and allowed to incorporate for 6 h. Cells were harvested and counted in a liquid scintillation counter (Beckman LSC) to determine [^{3}H]thymidine uptake. T-lymphocytes were counted by the direct correlation with [^{3}H]thymidine cpm corrected to 10^6 cells.

The percent inhibition of T-lymphocyte proliferation was calculated by the equation:

$$\% \text{ inhibition} = (\text{cpm}_0 - \text{cpm}_{CSA})/\text{cpm}_0 \cdot 100$$

where cpm_0 and cpm_{CSA} denote the values corresponding to drug-free and CSA-treated cells, respectively. The ID_{50} was calculated from the concentration of CSA required to inhibit T-lymphocyte proliferation by 50%.

Statistical analysis

%EE between the two liposomal formulations was compared by Student's *t*-test. Comparisons of percent inhibition between each liposomal formulation and intravenous product were made by Student's *t*-test. Differences in %EE longitudinally from time 0 to the end of study period were performed by ANOVA with repeat measures. A difference was considered significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). All data are presented as means ± standard deviation ($X \pm$ S.D.).

Results

Liposome characteristics: morphology

Both liposomal formulations were assessed on several occasions under light microscopy for morphological evaluation. Neither clumping nor crystals were observed in the 2 liposomal formulations. Size distribution, determined with a Coulter counter, ranged from 1 to 5 μm, with the median size at 3 μm.

Liposome characteristics: encapsulation efficiency

The percent encapsulation efficiencies of both liposomal preparations A and B were 97 ± 1 and 96 ± 2% ($N = 6$ each), respectively. The entrapment efficiency following reconstitution with physiological saline did not change significantly after storing the lyophilized powder preparations of formulations A and B up to 14 days at 4°C (95 ± 3 and 98 ± 2%, respectively, Table 1). However, entrapment efficiency of both formulations of the lyophilized powder was significantly lower after 30 days of storage (82.3 ± 2.3 and 79 ± 6.1%; $p < 0.05$).

The long-term encapsulation efficiency of the liposomal suspension was also assessed at room temperature. Formulation A was stable for up to 5 days at room temperature, while formulation B was only stable for 24 h. The encapsulation efficiency of formulation A in 50% fetal calf serum was 90 ± 3% at the 24 h period, that of formulation B being 53 ± 4% (Table 2).

TABLE 1

Encapsulation efficiency of DMPC:SA:CSA and DMPC:DMPG:CSA as the lyophilized powder over 30 days (N = 3 of each) (X ± S.D.)

	Day				
	1	2	6	14	30
DMPC:SA:CSA	94.3 (3.2)	96.7 (0.6)	94.7 (3.8)	95.0 (3.0)	82.3 [a] (2.3)
DMPC:DMPG:CSA	91.7 (1.2)	98.0 (1.0)	95.7 (4.9)	98.0 (1.7)	79.0 [a] (6.1)

[a] $p < 0.05$ from day 1.

TABLE 2

Effects of saline and serum on encapsulation efficiency DMPC:SA:CSA and DMPC:DMPG:CSA (N = 3). (X ± S.D.)

	Time (h)				
	0	2	6	14	120
Saline					
DMPC:SA:CSA	98.3	96.3	98.3	97.7	98.7 [b]
	(1.5)	(2.9)	(1.2)	(2.3)	(0.6)
DMPC:DMPG:CSA	97.0	96.3	97.0	98.3	50.3 [a]
	(2.7)	(0.6)	(2.0)	(1.2)	(0.6)

50% serum	Time (h)				
	0	1	2	6	24
DMPC:SA:CSA	88.1	89.1 [b]	85.3 [b]	87.1 [b]	89.8 [b]
	(5.5)	(2.2)	(3.4)	(2.7)	(2.6)
DMPC:DMPG:CSA	88.1	83.3	78.5 [a]	76.5 [a]	52.9 [a]
	(3.1)	(3.0)	(2.8)	(0.9)	(3.9)

[a] $p < 0.05$ from start of study.
[b] $p < 0.05$ from B formulation.

In vitro immunosuppressive activity

The immunosuppressive activity of CSA incorporated in liposomal formulations A and B was assessed by in vitro T-lymphocyte proliferation and compared to the intravenous product as well as drug-free liposomes. At 10^{-1} mM, all samples including drug-free liposomes inhibited T-cell growth. Conversely, all formulations containing less than 10^{-6} mM CSA allowed T-lymphocyte proliferation. The ID_{50} values of liposomal formulations A and B were $10^{-3.8 \pm 0.5}$ mM and $10^{-3.1 \pm 0.5}$ mM, respectively, compared to IV CSA ($10^{-3.2 \pm 1.0}$ mM; Fig. 1).

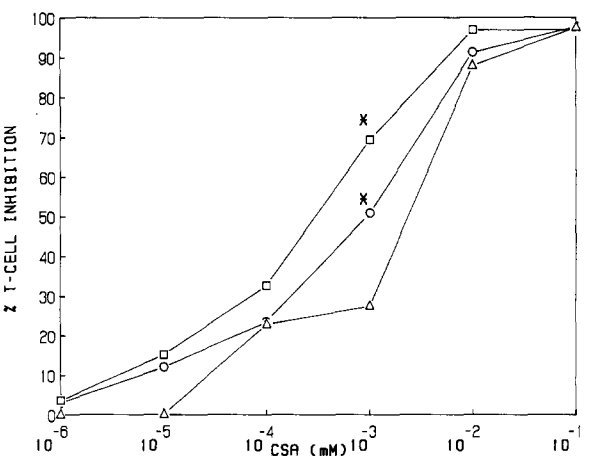

Fig. 1. Percent T-cell inhibition with CSA IV (triangle), DMPC:SA:CSA (square), and DMPC:DMPG:CSA (circle). $N = 3$ for each triplicate treatment concentration. * $p < 0.05$ from IV.

Discussion

The present study investigated the feasibility of liposomes as an alternative vehicle for the commercially available intravenous formulation of CSA. Two liposomal formulations with an encapsulation efficiency greater than 90% were developed. Furthermore, formulation A was highly stable in serum, saline and in the form of lyophilized powder with minimal loss of entrapment over 24 h, 5 and 30 days, respectively. In vitro immunosuppressive activity of CSA entrapped in liposomes was at least equivalent to that observed with the intravenous formulation. Although in vitro activity may not necessarily translate to in vivo efficacy, the results justify the need for in vivo studies of these formulations.

Liposomes are lipid vesicles that can be used as drug delivery systems for many pharmaceuticals (Gregoriadis, 1983). Furthermore, liposomes have been useful in the modification of pharmacokinetics and biological activity, whether toxic or beneficial, of a number of drugs including a cisplatin analog (Perez-Soler et al., 1986) and antifungal agents (Lopez-Berestein and Juliano, 1987). Depending on the size of vesicles, drug pharmacokinetics may vary greatly. For example, elimination half-life and volume of distribution of drug incorporated in multilamellar vesicles (MLV) are markedly greater than drug encapsulated in small unilamellar vesicles (SUV). This is due to increased uptake of larger liposomes by the liver and to some extent the spleen (Allen and Everest, 1983). Other variables such as the inclusion of cholesterol may stabilize the liposome bilayer against the disruptive effects of biological fluids and decrease the liposomal permeability to the entrapped compound (Scherphof, 1978). Posi-

tively- or negatively-charged molecules added to the liposomal formulation provide surface charge to prevent aggregation of the liposomes, facilitate rehydration, easy removal from the side of the container and increase the MLV spacing which in turn will increase the entrapment capability of the liposomes (Szoka and Papahadjopoulos, 1983).

There are a number of procedures for preparation of liposomes. In the present study, liposomes were initially prepared by direct hydration of the lipid film following solvent evaporation. However, these liposomes were inconsistent with slow hydration of the lipid layer, resulting in poor encapsulation efficiency and size distribution. To the contrary, hydration of the lyophilized powder was complete with formation of multilamellar vesicles while the preparation was absent of clumping and crystals under light microscopy. Further, distribution of liposomes was homogeneous ranging from 1 to 5 μm, with the greatest proportion being 3 μm in size.

One of the principal criteria for commercial applicability of liposomal formulations is encapsulation efficiency as the non-hydrated lyophilized powder, as well as following hydration in saline in vitro and in the presence of serum components in vivo. The lyophilized powder of each formulation was stable at 4°C for a minimum of 14 days. Samples analyzed on day 30 demonstrated significant loss in encapsulation efficiency from day 1, although approx. 80% remained entrapped. Reasons for the loss most likely include lipid/drug degradation secondary to entrapped moisture. Although not evaluated, it is possible that storage of the lyophilized powder at −20°C would extend the encapsulation efficiency. However, it is unclear whether freezing would have a detrimental effect on the immunosuppression activity of CSA.

Of importance is the assessment of encapsulation efficiency of liposomes in biologic fluids. Multilamellar vesicles have shown relative encapsulation efficiency on storage and in the presence of blood components (Scherphof et al., 1980). Changes in pH and osmolality also determine the encapsulation efficiency of liposomes. Formulation A was stable in 50% fetal calf serum for 24 h; in contrast, encapsulation efficiency of drug in formulation B was approx. 50% following the 24 h period. Similarly, the encapsulation efficiency of formulation A in saline was 2-fold greater than formulation B after 5 days. Since both formulations were prepared under similar conditions, the difference in their encapsulation efficiency most likely is due to the lipid content of the liposome vesicles.

Mean ID_{50} (50% inhibition of in vitro T-cell proliferation) was not significantly different between i.v. CSA and the 2 liposomal preparations. Drug-free liposomes exhibited moderate immunosuppressive activity at the high concentrated levels, most likely due to physical interference of cellular growth. It is interesting to note that drug encapsulated in either formulation A or B demonstrated increased inhibitory effect compared to i.v. CSA at 10^{-3} mM. Due to the surface charge and lipoidal nature of liposomes, the membrane interaction between liposomes and lymphocytes may lead to inhibition of cellular growth (Hsieh et al., 1985). It is tempting to speculate that in vivo effect of liposomal CSA would be greater than the commercially-available intravenous formulation on an equimolar basis. Further studies are warranted on the in vivo inhibitory effects of liposomal CSA.

In summary, both formulations demonstrated superior encapsulation efficiency data vs. previous preparations (Stuhne-Sekalec et al., 1986a,b), exceeding 90% entrapment. Furthermore, formulation A was stable for a longer period of time compared to formulation B. Although other investigators did not describe encapsulation efficiency or encapsulation efficiency over time (Luke et al., 1987; Smeesters et al., 1988a,b), drug-associated toxicity was absent with their formulations. Studies are presently ongoing evaluating the efficacy and toxicity of our formulations in whole animal models.

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