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Reduced kallikrein excretion by liposome-encapsulated cyclosporin in the rat

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Abstract—Different phospholipids and methods of preparation were used to produce cyclosporin encapsulated in liposomes. The optimal formulation of cyclosporin-liposome was compared to the oily cyclosporin after intraperitoneal administration of 25 mg kg^{-1} body weight to rats. Urinary kallikrein excretion was significantly reduced with the liposomal form. The abrupt increase of kallikrein excretion after the tenth day with the control oil preparation suggests that cyclosporin toxicity could be present at the tubular level, and the encapsulation of cyclosporin in liposomes reduces tubular damage.

Aziz et al (1981) were the first to prepare cyclosporin liposomes in miglyol, showing the efficacy of parenterally administered liposomes in prolonging cardiac allograft survival. Gibson et al (1985) demonstrated that cyclosporin encapsulation in liposomes reduced its hepatotoxicity following intravenous administration in rats, but nephrotoxicity was not diminished. Other studies using the rat model showed that cyclosporin incorporation in liposomes decreased its nephrotoxicity while preserving immunosuppressive activity (Hsieh et al 1985; Smeesters et al 1988a, b). Recently, Venkataram et al (1990), in experiments on the pharmacokinetics of cyclosporin in liposomes and in intralipid forms, observed that the toxicity of the solubilizing agent of commercially-available cyclosporin could be eliminated, altering its nephrotoxicity and immunosuppression during intravenous therapy.

Renal kallikrein is a serine protease which releases kinins (potent vasodilators) from kininogen, their natural substrates (Adam et al 1989). It is the main enzyme of the renal kallikrein-kinin system, which is involved in blood pressure regulation, renal blood flow, and renal handling of salt and water (Mayfield & Margolius 1983). Renal kallikrein is localized and synthesized exclusively in the connecting tubule of both the human and rat distal nephron (Vio & Figueroa 1985) and is normally excreted in urine, where its activity can be measured (Adam & Damas 1988). It has been established that renal kallikrein has a rapid synthesis, turnover and secretion rate (Miller et al 1984), and any impairment of connecting tubular cells would affect its synthesis or its secretion. Martinez et al (1990) postulated that urinary kallikrein is a sensitive predictor of cyclosporin nephrotoxicity.

The purpose of this study was to demonstrate the effect of encapsulation of administered cyclosporin on the excretion of kallikrein in the urine.

Materials and methods

Materials. All chemicals, cholesterol and phospholipids, were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Solvents used for the chromatographic analysis of cyclosporin were HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sterile 0·9% NaCl (saline) was obtained from Abbott Laboratories (Montreal, Quebec, Canada).

Preparation of liposomes. To select the optimal formulation, different types of liposomes for encapsulation of cyclosporin were prepared. These formulations differ in phospholipids, cholesterol content, molecular ratio and method of preparation.

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The optimal formulation selected for this study was: dipalmitoyl phosphatidyl choline/dibehenoyl phosphatidyl choline/cholesterol/cyclosporin, 1·5:1·5:3:1 and was prepared using the thin film hydration method described previously (Akbarieh et al 1992). The liposomes thus prepared were extruded through a filter (1·0–0·1 μm) under pressures of 100–500 psi and flow rates of 20–60 mL min^{-1}, collected and re-injected. The size distribution of the liposome dispersions was determined as reported previously (Akbarieh et al 1991a), using a Nanosizer (N4SD, Coulter Electronics, Montreal, Quebec, Canada). Cyclosporin in liposomes was assayed by HPLC (Gilson Medical Electronics, Villiers-le-Bel, France), on a 15 cm × 4·6 mm Alltech column, Spherisorb CN 5 micron (Alltech, Deerfield, IL, USA), with 50% HPLC grade water and 50% acetonitrile (v/v) at a flow rate of 1·5 mL min^{-1}. The eluent was monitored at 210 nm.

Administration. Three groups of 7 female Sprague-Dawley rats (Charles River Canada, St Constant, Quebec, Canada), mean weight of 320 ± 15 g, were used. Two of these groups were given cyclosporin intraperitoneally (25 mg kg^{-1}); the first group received the commercially-available oil preparation (Sandimmune, Sandoz) and the second group received the optimal liposomal form. The third group served as control and received empty liposomes of the same formulation. Urine volume and osmolality were determined daily. The enzymatic activity of urinary kallikrein was measured by assay of liberated *p*-nitroaniline (absorption at 405 nm) synthetic substrate H-D-Val-Leu-Arg-*p*-nitroanilide (S2266 Kabi Diagnostica, Stockholm, Sweden) as described by Amundsen et al (1979).

Urinary sodium and potassium were measured by flame spectrophotometry. Creatinine and urea in urine were determined with a Hitachi 717 Analyzer (Boehringer Mannheim GmbH, Germany), the Jaffé reaction and urease coupled with glutamate dehydrogenase, respectively. These reactions were performed on urine diluted (1/20) in saline (v/v).

Results

Of the rats that received Sandimmune one died after 9 days, two after 10 days, one after 11 days and one after 12 days of treatment; only two rats survived after 15 days of Sandimmune administration. However, all rats receiving the cyclosporin encapsulated in liposomes survived and tolerated the liposomal form of cyclosporin. All control animals survived treatment with the empty liposomes.

Fig. 1 presents the analysis of urine for the three groups of rats. No significant difference could be found in daily urinary volume between the controls and rats receiving the liposomal form of cyclosporin (Fig. 1A). However, urine volume increased significantly after the third day in rats given Sandimmune. The decrease in osmolality was consistent with urinary volume augmentation after the third day of cyclosporin administration (Fig. 1B). After the eighth day of treatment until the end of the experiment, osmolality fluctuated between 0·8 and 0·2 Osm kg^{-1} water. These values were consistent with the decreased concentration of sodium ions. No change was found in the amount of urea and creatinine excreted.

Although kallikrein excretion in rats receiving the liposomal form was close to control values, it was significantly lower than

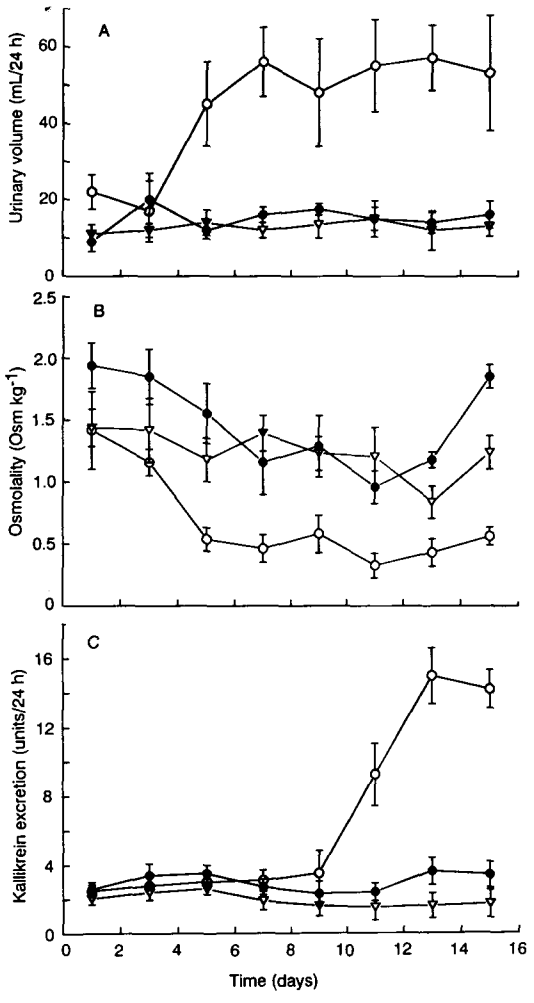

FIG. 1. Analysis of urine from rats receiving cyclosporin as Sandimmune (○) or encapsulated in liposomes (●), and from control rats receiving empty liposomes (▽). A. Urinary volume. B. Urine osmolality. C. Kallikrein excretion. All values are means ± s.e.m.

in the group given Sandimmune (Fig. 1C). There was an abrupt increase of enzyme activity after the tenth day in the Sandimmune group. Indeed, the evident rise in kallikrein excretion was the result of a real increment in the net concentration of urinary kallikrein in addition to the elevation of urinary volume.

Discussion

The choice of this particular formulation is based on considerations of vesicle size, lipid composition and lipid ratio, entrapment of cyclosporin and drug leakage (Akbarieh et al 1991b). Liposome clearance as well as the resulting biodistribution can be influenced by liposome size. Smaller vesicles are cleared more slowly than their larger counterparts (Cullis et al 1987). In this study, the size of cyclosporin-liposomes administered to rats was reduced by extrusion through a 1·0 μm polycarbonate filter. Although extrusion with a filter of 1 μm pore size produced homogeneous multilamellar liposomal dispersion with no significant drug release, dramatic drug leakage occurred when pore size filters smaller than 0·5 μm were used.

Gibson et al (1985) reported that following intravenous administration of cyclosporin in liposomal form to rats, hepatotoxicity was reduced but nephrotoxicity was not diminished. In that work the authors made liposomes from egg phosphatidyl choline but without cholesterol. It has been reported that, in the absence of cholesterol, liposomes usually leak substantially when introduced intravenously (Kirby et al 1980a; Scherphof & Morselt 1984). This leakage has been attributed to interactions of liposome phospholipids with plasma proteins and an exchange with lipoproteins (Kirby et al 1980b). Therefore, drug leakage can be significantly inhibited by the presence of cholesterol (Kirby et al 1980a, b). The results obtained in this study clearly showed that liposomal encapsulation of cyclosporin reduced its tubular damage which is consistent with the results reported by other investigators (Hsieh et al 1985; Smeesters et al 1988a, b).

The results obtained in this study clearly showed that liposomal encapsulation of cyclosporin reduced kallikrein excretion. The exact mechanism by which the encapsulation of cyclosporin reduces kallikrein excretion has not been clearly demonstrated. However, it has been reported (Guder & Hallbach 1988) that the tubule is responsible for the concentrating capacity of the nephron, and glandular kallikrein is synthesized as the proenzyme by distal connecting cells. The sudden increase of kallikrein excretion after the tenth day suggests that cyclosporin acts on the tubular level. The encapsulation of cyclosporin in liposomal form decreased kallikrein excretion by reducing tubular damage. If urinary kallikrein measurement is a valid biochemical parameter for assessing the level of nephrotoxicity, it would be possible to apply this parameter for future evaluation of new delivery systems for cyclosporin or other nephrotoxic drugs.

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Absence of desensitization to the relaxant activity of streptozotocin in isosorbide dinitrate-tolerant rat aorta

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Abstract—The effect of in-vitro isosorbide dinitrate (ISDN)-induced tolerance on the vasodilatory actions of streptozotocin, a nitric oxide containing compound, and papaverine was studied in rat aortic strips precontracted by phenylephrine. Aortas made tolerant to ISDN remained fully responsive to streptozotocin but exhibited a greater response to low concentrations of papaverine compared with control strips. Methylene blue produced parallel displacement to the right of the relaxant concentration-effect curves of both ISDN and streptozotocin, whereas responses to only low concentrations of papaverine were significantly antagonized. These results indicate that the relaxant activity of streptozotocin is due to the stimulation of guanylate cyclase and impaired activity of this enzyme is not likely to be the operating mechanism for nitrate tolerance. It is also suggested that the vasodilating action of papaverine is partly dependent on the tissue cGMP level.

Prolonged exposure of blood vessels to high levels of organic nitrates, in-vitro, induces tolerance against the vascular smooth muscle relaxing activity of these drugs. It is generally accepted that tolerance results from reduced biotransformation of organic nitrates (Brien et al 1986; Bennett et al 1989). However, marked desensitization to the vasodilating effect of sodium nitroprusside, an inorganic nitrovasodilator, observed in nitroglycerin-tolerant animals has led some authors to propose that reduced guanylate cyclase activity may also contribute to the development of tolerance (Molina et al 1987; Rapoport et al 1987). Sodium nitroprusside is known to contain a nitric oxide (NO) moiety in its structure which is not spontaneously released in aqueous solution in the absence of light (Marks et al 1991) and the guanylate cyclase-activating property of the drug is thought to result from generation of free NO (Ignarro et al 1981; Rinaldi & Cingolani 1983).

Recently, streptozotocin was shown to elicit relaxation of the rat aorta precontracted by phenylephrine, and this effect has been attributed to the presence of the NO group in the molecule (Thomas & Ramwell 1989). Therefore, it would be reasonable to expect that nitrate tolerance may alter the vasodilating activity of streptozotocin. To test this hypothesis, we conducted the present study by using isosorbide dinitrate (ISDN) as a tolerance-inducing agent in rat aorta. Papaverine was also used to assess the influence of nitrate tolerance on guanylate cyclase-independent vasodilation.

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Materials and methods

Male rats, 250–300 g, were killed by stunning and bleeding. The thoracic aortas were removed and dissected free of fat and connective tissue. Helically cut strips, approximately 2 mm wide and 25–30 mm long were suspended in 10 mL organ baths containing modified Krebs solution at 37°C and were aerated with 95% O_2–5% CO_2, The composition of the Krebs solution was (mM): NaCl 118·2, KCl 4·7, $MgSO_4$ 1·2, $CaCl_2$ 2·5, KH_2PO_4 1·2, $NaHCO_3$ 25, glucose 11·1. The aortic strips were attached to an Ugo Basile 7004 force-displacement transducer connected to an Ugo Basile 7070 recorder and were allowed to equilibrate under a resting tension of 1 g for 1·5 h, during which time the tissues were washed every 15 min. Concentration-response curves for relaxant drugs were constructed on strips precontracted with 10^{-7} M phenylephrine. In cumulative concentration-response curves on rat aortic strips, 10^{-7} M phenylephrine produced 60–80% of the maximum attainable contraction. The endothelium integrity was verified by testing the relaxant responses to 10^{-6} M acetylcholine. Strips which failed to respond to acetylcholine were discarded.

After obtaining preincubation concentration-response curves with the relaxant under examination, tolerance was induced by incubating the aortic strips with ISDN at a concentration of 6×10^{-4} M for 2 h. Control strips were incubated with vehicle. After exposure to the tolerance-inducing conditions, strips were washed repeatedly with Krebs solution for 15 min and then exposed to 10^{-7} M phenylephrine. A plateau level of contraction was obtained within 10 min and a single cumulative concentration-effect curve constructed for the vasodilator.