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Biodistribution of Cyclosporin Encapsulated in Liposomes Modified with Bioadhesive Polymer

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Abstract

The purpose of this investigation was to study the possibility of renewing the immunosuppressive activity of cyclosporin by formulating the compound in liposomes modified with bioadhesive polymers. The liposomes prepared were evaluated both pharmacokinetically and pharmacodynamically. Tissue distribution and plasma pharmacokinetics of cyclosporin and model dye, sudan black, which is as hydrophobic as cyclosporin, were studied in rats after intravenous infusion (10 mg kg^{-1}). The immunosuppressive efficacy of liposomal cyclosporin preparations was studied in the allogenic rat-heart-transplantation model, where cyclosporin therapy (10 mg kg^{-1}) continued for one week.

The entrapment of sudan black in liposomes modified with bioadhesive polymers resulted in higher sudan black delivery to the spleen and the liver than with standard sudan-black-loaded liposomes. Among the modified liposomes, those modified with carbopol 941 showed the most remarkable enhancing effect on the delivery of sudan black to these organs and total plasma clearance of sudan black decreased to $38{\cdot}6 \pm 7{\cdot}8$ mL h^{-1} kg^{-1} (standard liposomes, $58{\cdot}9 \pm 6{\cdot}4$ mL h^{-1} kg^{-1}). Delivery of cyclosporin to the spleen and the liver was increased approximately twofold by modifying the liposomes with carbopol 941. In the preliminary study on the allogenic rat-heart-transplantation model, the mean survival days of the graft were $18{\cdot}8 \pm 2{\cdot}9$ days for the group receiving cyclosporin liposomes modified with carbopol 941, $14{\cdot}2 \pm 4{\cdot}4$ days for the group receiving standard cyclosporin liposomes and $7{\cdot}6 \pm 0{\cdot}5$ days for the group receiving cyclosporin solution.

The encapsulation of cyclosporin in liposomes modified with bioadhesive polymer enhanced the residence time of cyclosporin in the systemic circulation, resulting in approximately twofold greater delivery of cyclosporin to the spleen and liver. However, in the allogenic rat-heart-transplantation model no significant difference was detected between the immunosuppressive efficacy of cyclosporin encapsulated in bioadhesive polymer-modified liposomes and that encapsulated in standard liposomes.

Strong immunosuppressants such as cyclosporin and tacrolimus (FK506) are administered in two dosage forms, oral preparation and intravenous solution (Kahan 1989; Venkataram et al 1990). Intravenous solutions are used during pre- and post-operational hospital periods in organ transplantation (Thomson 1989) and thereafter oral immunosuppressive therapy continues. However, immunosuppressant use has been hampered by serious toxicity to the kidney, liver and central nervous system (Fahr 1993) because of the non-selective distribution of these immunosuppressants to these organs (Awni 1992).

The pharmacological mechanism of both immunosuppressants is interference with the process of T-cell activation by blocking transcription initiation of interleukin-2 (Kronke et al 1984; Tocci et al 1989). The target cell of these immunosuppressants is thought to be T cells, especially helper T cells (Baumann et al 1992; Sigal & Dumont 1992). As these cells exist at high density in the thoracic lymph duct, local immunosuppression was attempted with a cyclosporin implant directly applied on to the thoracic lymph duct (Katayama et al 1994, 1995). However, satisfactory immunosuppressive activity was not obtained. From this study, it was postulated that the direct delivery of immunosuppressant to the lymphocyte-rich organs would elucidate stronger immunosuppressive efficacy than conventional therapy. Gorecki et al (1991) used liposomes as cyclosporin carrier for delivery to organs rich in reticuloendothelial cells. However, the liposomal cyclosporin used enhanced the delivery of cyclosporin to the spleen only, and not to the liver or kidney, the target organs of routine clinical transplantation.

Recently, Yerushalmi & Margalit (1994) suggested the usefulness of bioadhesive, collagen-modified liposomes for binding drugs to the viable cells engaged in the healing process. In the healing process many lymphocytes are present at the site of inflammation. Therefore, there is a possibility that a polymer having a much stronger bioadhesive nature might enhance the delivery of cyclosporin to the lymphocyte-rich organs. On the basis of this concept, we initially prepared bioadhesive liposomes containing sudan black, a model of a highly lipophilic neutral drug such as cyclosporin which was also used in our previous study on the implantable slow-release delivery system applied to the thoracic lymph duct (Katayama et al 1995). On the basis of the results of that preliminary study with sudan black, bioadhesive liposomes containing cyclosporin were prepared and the distribution characteristics of these liposomes to lymphocyte-rich organs have been studied.

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Materials and Methods

Materials

Cyclosporin powder was a gift from Sandoz (Basel, Switzerland). Sudan black as a model drug was obtained from Tokyo Chemicals Industry (Tokyo, Japan). Egg phosphatidylcholine, β-1,3-glucan and *t*-butanol were obtained from Nacalai Tesque (Kyoto, Japan). Poly(acrylic acid) (carbopol 934P, 940, 941 and 1342) was obtained from B. F. Goodrich (Cleveland, Ohio) through Chugai Boueki (Tokyo, Japan). Sodium alginate (100–150 centipoise (cp) and 1000 cp) and propylene glycol were obtained from Wako Pure Chemical Industry (Osaka, Japan). All other materials were of reagent grade and were used as received.

Male Wistar rats for tissue distribution and plasma pharmacokinetic studies were obtained from SLC (Hamamatsu, Japan). Inbred Lewis (LEW/SEA) and Fisher rats (F344/N) were obtained from Seiwa Experimental Animals (Fukuoka, Japan) and SLC, respectively.

Preparation of liposomes

Liposomes were prepared according to the method of Vadiei et al (1989). Sudan black powder or cyclosporin powder or both (35 mg) and egg phosphatidylcholine powder (502 mg) (molar ratios 1:8 and 1:22, respectively) were mixed in *t*-butanol (10 mL) at 37°C with vigorous stirring. The mixture was pipetted into glass vials, frozen rapidly and then lyophilized overnight to remove the solvent. Drug-encapsulated liposomes were prepared by adding phosphate buffered saline (PBS, pH 7·4; 17·5 mL) to a final drug concentration of 2·0 mg mL^{-1}. This liposome preparation was incubated for 1 h at 37°C, to enable hydration of the liposomes, then sonicated for 10 min at 100 W in water at 37°C with an ultrasonicator (Sonifier Model 250, Branson, Danburg, USA). The size of the liposomes was adjusted by passing the freshly prepared liposomes through microfilters with pore sizes of 1·2 and 0·4 μm (Millipore Cat. No. RTTP02500 and HTTP02500, Bedford, MA). The liposomes were separated from excess unencapsulated drug by ultra-high-speed centrifugation at 250 000 *g* and 4°C for 1 h. After removal of the supernatant the pellets were resuspended in PBS. The suspension (2 mL) was lyophilized overnight and the entrapment efficiencies of sudan black and cyclosporin were measured spectrophotometrically and by HPLC, respectively, after dissolution in *t*-butanol.

Liposome modification

The modification of liposomes with adhesive polymer was performed by the methods of Dong & Rogers (1993) and Yerushalmi & Margalit (1994). To the hydrated liposomes, the same volume of polymer solution (1 mg mL^{-1}) was added. The mixture was incubated at 23°C for 1 h. The sizing of the liposomes was then performed as described above. Thereafter, the liposomes were separated from excess reagents and by-products by ultracentrifugation (as described above) and repeated washing. The entrapment efficiencies of sudan black and cyclosporin were also measured as described above.

Tissue distribution study

Experiments were performed on groups of 3 or 4 male Wistar rats, 200–225 g. The rats were fasted overnight but had free access to water. Under anaesthesia by intraperitoneal injection of sodium pentobarbital, 45 mg kg^{-1}, a polyethylene tube (i.d. 0·5 mm, o.d. 0·8 mm; Dual Plastics, Australia) was cannulated into the left femoral vein. Test solution (10 mg kg^{-1}) was infused through the cannula by means of a variable-speed infusion pump (model SP220i, World Precision Instrument, Sarasota, FL); the infusion speed was 1·5 mL h^{-1}. The infusion period was 80 min for cyclosporin and 90 min for sudan black. The rats were killed at the end of the infusion by collecting blood from the carotid artery. The plasma was obtained by centrifugation at 37°C and was stored under deep refrigeration (−80°C) until analysis. Six organs (liver, heart, kidney, lung, lymph nodes (neck, axillary and mesenteric) and spleen) were excised. Each organ, 1–5 g, was cut and quickly rinsed with ice-cold saline and weighed. The organ (1 g) was homogenized with PBS (2·0, 6·0, 3·0, 6·0, 2·0 and 10 mL for liver, heart, kidney, lung, lymph nodes and spleen, respectively) in a glass tissue-homogenizer. After centrifugation at 1000 g for 20 min, samples of the supernatant from the tissue homogenates were taken and stored for up to two weeks under deep refrigeration (−80°C) until assay for sudan black and cyclosporin.

Plasma pharmacokinetic study

A polyethylene cannula was surgically introduced into the left femoral vein of another group of 3 or 4 male Wistar rats for administration of the test solution; the dose and the infusion speed were the same as used for the tissue distribution experiment. Samples of blood (0·3 mL) were collected from the right femoral vein of rats that received the sudan black preparation (10 mg kg^{-1}) at 0, 30, 60 and 90 min during the infusion period and up to 240 min post-infusion at 30, 60, 90 and 150 min. For the cyclosporin (10 mg kg^{-1}) study, the same amount of blood was collected throughout the infusion period at 0, 30 and 80 min, and up to 240 min post-infusion at 10, 40, 70, 100 and 160 min. The plasma was immediately obtained by centrifugation at 37°C and was stored under deep refrigeration (−80°C) until analysis.

Analytical methods for drugs

Sudan black in plasma. Methanol (1 mL) was mixed well with rat plasma (100 μL) and the supernatant was obtained by centrifugation for 10 min at 1000 *g*. The sudan black content was determined by measuring the optical density at 660 nm. The standard curves of sudan black added to rat plasma samples were linear over the range 0·5–50 μg mL^{-1} and passed through the origin.

Sudan black in organs. Methanol (5 mL) was added to organ homogenate (1 mL) to extract the sudan black; the mixture was shaken for 15 min and then centrifuged for 20 min at 1000 *g*. The resulting supernatant fluid was used for assay of sudan black content as described above. The standard curves of sudan black added to rat plasma samples were linear over the range of 1–100 μg mL^{-1} and passed through the origin.

Cyclosporin in plasma and organ homogenate. The concentration of cyclosporin in plasma and organ homogenates was measured according to the method reported previously (Takada et al 1985). Extraction from plasma (100 μL) or organ homogenates (0·5 mL) was performed with organic solvent. After extraction the resulting residue was dissolved in the mobile

phase (200 μL) and 100 μL was then injected into the HPLC system (Shimadzu LC-10A, Kyoto, Japan). The analytical column (25 × 0·46 cm × 5 μm LiChrosorb Si-60 (Chemco Scientific, Osaka, Japan); Shibata et al 1987) was maintained at 60°C with a column heater. The mobile phase was *n*-hexane–ethanol (85:15) at a flow rate of 1·0 mL min^{-1} (pressure 50 kg cm^{-2}). Under these conditions, the retention time of cyclosporin was 7·5 min. Cyclosporin was detected at 205 nm with a Shimadzu SPD-10A UV-detector. No interfering peak was detected in the plasma and organ homogenate samples used as blanks or in those from rats given cyclosporin. The concentrations of cyclosporin in the plasma and organ homogenate were determined from calibration curves. The calibration curves of cyclosporin added to rat plasma and organ samples were linear over the range 0·5–10 g mL^{-1} for rat plasma and 1·0–100 g mL^{-1} for rat organ homogenates; both passed through the origin.

Allogenic rat heart transplantation

Allogenic rat heart transplantation was performed using a cuff technique as described previously (Katayama et al 1995). Briefly, male Fisher rats, 250–300 g, and male Lewis rats, 200–250 g, were used as recipients and donors, respectively. The donor heart was obtained by midline incision from the anaesthetized rats. After ligatures had been performed around the superior and inferior vena cava, the pulmonary and the thoracic aortae were transected 3–5 mm distal to their origins and the removed heart was placed in ice-cold saline. Next, the external jugular vein and the common carotid artery of the recipient rat were dissected. The cuffs consisted of 3·0 mm of cuff body and 1·0 mm of cuff extension, both made of Teflon intravenous cannula, for the carotid artery (cuff body) and the external jugular vein (cuff extension). The donor heart was transferred to the neck of the recipient. The pulmonary artery was drawn over the jugular vein cuff and the thoracic aorta was also anastomosed to the carotid artery. After suture of the incision, the recipient rat was maintained in the cage. Four groups of five heart-transplanted rats were prepared. Groups 1–4 were control group, cyclosporin solution group, standard liposome group and carbopol 941-modified liposome group, respectively. The cyclosporin dose was 10 mg kg^{-1} day^{-1} for each group and therapy was performed for one week by an intravenous injection of each cyclosporin test preparation into the tail vein. For the control group liposomal preparation containing no cyclosporin was injected. The survival of the transplanted heart was monitored every day by measuring the heart beat. When a heart beat was not detected, the rat was killed and the heart was isolated. Graft heart was placed in buffered formalin for 24 h, then serially sectioned in wax blocks and stained with haematoxylin and eosin for microscopic histological examination.

Data analysis

A non-compartmental pharmacokinetic analysis was applied to the plasma sudan black and cyclosporin concentration–time data. The terminal elimination rate constant, λ_z, was determined by linear regression of at least three data points from the terminal portion of the plasma concentration–time plots. The area under the plasma concentration–time curve, AUC, after intravenous infusion was calculated using the linear trapezoidal rule. The area under the first moment curve, AUMC, after intravenous administration was also calculated using the linear trapezoidal rule. The terminal elimination half-life, $t_{1/2}$, was determined by dividing ln2 by λ_z. The total plasma clearance, CL_{tot}, was determined by dividing the intravenous dose by the AUC. The volume of distribution at steady state, Vd_{ss}, was calculated from the AUC and AUMC by use of the equation $Vd_{ss} = \text{dose} \times \text{AUMC} \times \text{AUC}^{-2}$. The mean residence time, MRT, was determined by dividing AUMC by AUC.

Statistics

All the values are expressed as mean ± s.e.m. unless otherwise noted. Statistical differences were assumed to be reproducible when $P < 0.05$ (two-tailed *t*-test). Wilcoxon's rank test was used to assess the significance of differences among heart-transplanted rats in each group.

Results

Liposome characteristics

After preparation, standard and modified liposomes were assessed under light microscopy for morphological evaluation. Neither clumping nor crystals were observed in the two liposomes. The mean entrapment efficiencies of standard liposomes were 65% for sudan black and 72% for cyclosporin; those of modified liposomes were 64% for sudan black and 73% for cyclosporin; s.e.m. values were less than 2%.

Effect of liposome modification on the tissue distribution of sudan black in rats

Levels of sudan black in various tissues at the end of the intravenous infusion period (i.e. 90 min after the start of the infusion) for each test liposomal preparation (dose of sudan black, 10 mg kg^{-1}) are shown in Table 1, in which these results can be compared with those obtained by infusion of a solution of sudan black in propylene glycol (sudan black is insoluble in water). Table 1 also shows tissue levels of sudan black at the end of infusion normalized with the plasma levels of sudan black at the same time, i.e. tissue-to-plasma concentration ratios for sudan black at 90 min were calculated for each test sudan black preparation. Initially the organ distribution characteristics of sudan black were compared for sudan black-loaded standard liposomal preparation and modified liposomes with natural bioadhesive polymers (two kinds of sodium alginate and β-1,3-glucan) where sudan black solution was used as a reference. The sodium alginate (100–150 cp)-modified liposomes showed higher liver/plasma sudan black concentration ratio than standard liposomes. However, the concentration ratio in the spleen was lower than that obtained by use of standard liposomes. Even for β-1,3-glucan-modified liposomes the liver/plasma concentration ratio did not increase as compared with that obtained with sodium alginate (100–150 cp)-modified liposomes, although the spleen/plasma concentration ratio was increased. However, the spleen/plasma concentration ratio was lower than that obtained with standard liposomes. As the spleen and the liver are lymphocyte-rich organs, these two organs were considered to be the target organ of cyclosporin in this study. To discover a liposomal preparation delivering more sudan black to the liver and the spleen, liposomes were modified with the synthetic bioadhesive polymer, carbopol; the results are also shown in Table 1. Homogeneous organ distribution characteristics were not

Table 1. Sudan black tissue/plasma concentration ratios after 90-min infusions of various formulations to rats.

Formulation	Modification material	Tissue				
		Liver	Heart	Kidney	Lung	Spleen
Solution	–	0·17 ± 0·04	0·27 ± 0·07	0·07 ± 0·01	1·26 ± 0·25	0·95 ± 0·16*
Standard liposomes	–	0·27 ± 0·03	0·23 ± 0·01	0·11 ± 0·03	1·21 ± 0·21	8·66 ± 2·04*
Modified liposomes	100-150 cp Sodium alginate	0·62 ± 0·18	0·46 ± 0·13	N.D.	0·18 ± 0·09	3·80 ± 1·59
	1000 cp Sodium alginate	0·24 ± 0·08	0·11 ± 0·01	0·09 ± 0·02	0·54 ± 0·12	5·00 ± 1·09*
	β-1,3-Glucan	0·41 ± 0·04	0·11 ± 0·01	0·06 ± 0·01	0·99 ± 0·10	6·80 ± 0·48*
	Carbopol 941	1·39 ± 0·08	0·28 ± 0·02	0·01 ± 0·01	0·87 ± 0·13	10·19 ± 0·18*
	Carbopol 940	0·38 ± 0·13	0·18 ± 0·01	0·05 ± 0·03	0·75 ± 0·13	3·74 ± 0·56*
	Carbopol 1342	0·85 ± 0·01	1·80 ± 0·70	0·70 ± 0·13	7·11 ± 1·87	1·98 ± 0·31*
	Carbopol 934P	1·11 ± 0·16	1·31 ± 0·29	0·69 ± 0·04	4·14 ± 0·23	3·04 ± 0·38*

All data are mean ± s.e.m. *$P < 0·05$, significantly different from the liver/plasma concentration ratio. N.D. not detected.

obtained for sudan black after intravenous infusion of several carbopol-modified liposomes. Carbopol 940-modified liposomes reduced the spleen/plasma concentration ratio of sudan black. Carbopol 934P-modified liposomes also reduced the spleen/plasma concentration ratio but increased the heart/plasma and lung/plasma concentration ratios. Carbopol 1342-modified liposomes increased the liver/plasma, heart/plasma, kidney/plasma and lung/plasma concentration ratios, though the spleen/plasma concentration ratio decreased. On the other hand, carbopol 941-modified liposomes increased the liver/plasma and spleen/plasma concentration ratios by approximately fourfold and by 20%, respectively, compared with standard liposomes. In contrast, the kidney/plasma concentration ratio decreased to one-tenth that obtained from standard liposomes. This is a great advantage of the carbopol 941-modified liposomes, because the kidney is the main organ on which cyclosporin exerts a toxic effect. Because the concentration of sudan black in the lymph nodes was below 1·0 $\mu g\ mL^{-1}$, the lymph nodes/plasma concentration ratio could not be determined.

Effect of liposomes and carbopol-modified liposomes on the pharmacokinetics of sudan black

Fig. 1 shows plasma sudan black concentration–time profiles after intravenous infusion of sudan black solution, standard liposomal preparation and carbopol 941-modified liposomes to rats at a dose of sudan black of 10 mg kg^{-1}. The plasma sudan black concentration–time profiles varied among the preparations. The lowest plasma sudan black levels were obtained from the solution, the highest from carbopol 941-modified liposomes. Pharmacokinetic analysis was performed on these plasma sudan black concentration–time profile data; the results are shown in Table 2, in which the pharmacokinetic parameters after intravenous infusion of sudan black solution are also presented for reference. The AUC for carbopol-modified liposomes was larger than that for sudan black solution, i.e. the total plasma clearance of sudan black entrapped in carbopol-modified liposomes was less than that for sudan black solution.

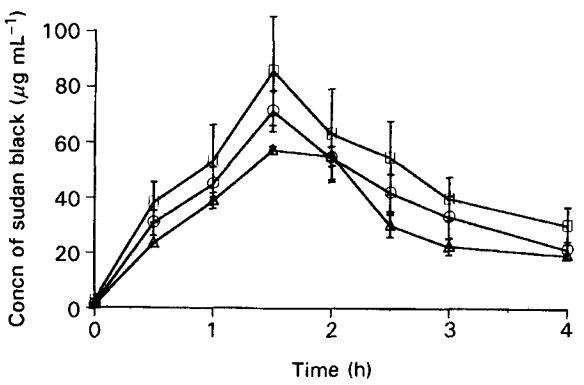

FIG. 1. Plasma sudan black concentration–time curves during and after the intravenous infusion of solution (△), standard liposomes (○) and carbopol 941-modified liposomes (□) to rats; sudan black dose, 10 mg kg^{-1}. Each point represents the mean ± s.e.m. from three rats.

Effect of liposomes and carbopol 941-modified liposomes on plasma pharmacokinetics and tissue distribution of cyclosporin

Because of the results obtained in the sudan black study, liposomes modified with carbopol 941 were selected as a carrier for delivery of cyclosporin to lymphocyte-rich organs, the spleen and the liver. Three kinds of cyclosporin preparation, solution, standard liposomes and modified liposomes, were used in the study. As for sudan black, cyclosporin solution was prepared by dissolving cyclosporin in propylene glycol. After intravenous infusion of cyclosporin, 10 mg kg^{-1}, plasma cyclosporin concentration increased gradually and reached a maximum at the end of the infusion period, as shown in Fig. 2. Thereafter, plasma cyclosporin concentration decreased with the mean terminal half-life of 3·03 ± 0·90 h. When cyclosporin in standard liposomes was administered in the same manner, the plasma cyclosporin concentration–time profile did not differ from that obtained with cyclosporin solution. However, the plasma cyclosporin concentration was increased by administering cyclosporin in carbopol 941-modified liposomes. The values of the pharmacokinetic parameters for cyclosporin were also determined and are shown in Table 3. As had been observed with sudan black, the AUC obtained for standard liposomes was no higher than that obtained for cyclosporin solution whereas the AUC obtained with carbopol-modified liposomes was greater. Therefore, total plasma clearance of cyclosporin was reduced and the MRT prolonged when cyclosporin was administered in carbopol-modified liposomes.

The tissue-distribution study was performed with another group of rats; in this study, preparations containing cyclosporin were infused for the same period of time and tissue cyclosporin levels were determined at the end of the infusion period. Table 4 shows the result as tissue-to-plasma cyclosporin-concentra-

Table 2. Pharmacokinetic parameters of sudan black after intravenous infusion (10 mg kg^{-1}) to rats.

Pharmacokinetic parameter	Solution	Standard liposomes	Carbopol 941-modified liposomes
Area under the plasma concentration–time curve from time 0 to infinity (g h mL^{-1})	17 ± 418	222 ± 33	277 ± 47
Mean residence time (h)	2·28 ± 0·29	2·48 ± 0·23	2·73 ± 0·38
Volume of distribution at steady-state (mL kg^{-1})	132 ± 4	116 ± 19	111 ± 39
Total plasma clearance (mL h^{-1} kg^{-1})	58·9 ± 6·4	46·8 ± 6·0	38·6 ± 7·8
Terminal elimination half-life (h)	1·88 ± 0·16	1·92 ± 0·23	2·08 ± 0·30

Each value represents the mean ± s.e.m. from three rats.

tion ratios. The standard liposomal preparation enhanced the delivery of cyclosporin to the lung but there was no significant differences in delivery to the other organs. However, carbopol 941-modified liposomes increased the delivery of cyclosporin to both the liver and the spleen compared with the standard liposomal preparation. As the cyclosporin concentration in the lymph nodes was lower than 1·0 μg mL^{-1}, the lymph nodes/plasma concentration ratio could not be determined.

Pharmacodynamic study of liposomal cyclosporin preparations

The immunosuppressive activity of liposomal cyclosporin preparations was studied by means of an allograft rat-heart-transplantation model; the survival times are summarized in Table 5. Recipients in groups 3 and 4 survived longer than those in group 1, the control group. None of the transplanted hearts in group 1 survived longer than 10 days, the mean survival time was 6·4 ± 1·9 days. In group 2 rats, the mean survival time was 7·6 ± 0·5 days. In group 3, three of five transplanted hearts stopped within two weeks, and the remaining two recipient's hearts stopped on days 21 and 23 (the mean survival time was 14·2 days). In group 4, the carbopol 941-modified liposomes group, the mean survival time was 18·8 ± 2·9 days. There was no significant prolongation of the survival time between groups 1 and 2, because the cyclosporin dose was low, 10 mg kg^{-1}. Furthermore, there was also no significant difference between survival time in groups 3 and 4.

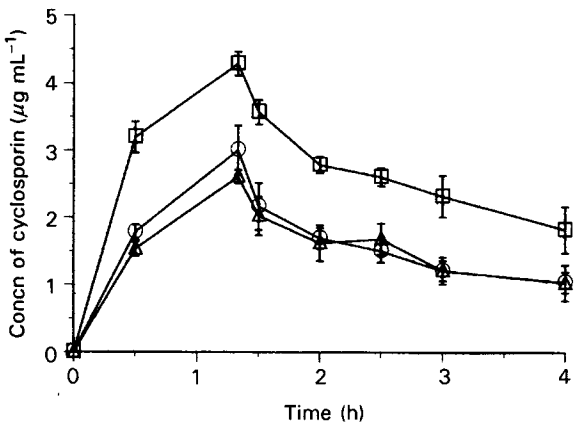

FIG. 2. Plasma cyclosporin concentration–time curves during and after the intravenous infusion of solution (△), standard liposomes (○) and carbopol 941-modified liposomes (□) to rats; cyclosporin dose, 10 mg kg^{-1}. Each point represents the mean ± s.e.m. from three rats.

Discussion

Because cyclosporin is a neutral, extremely hydrophobic cyclic peptide composed of 11 amino acid residues and with a molecular weight of 1202, it has been reported to be suitable for encapsulation in liposomes (Aziz et al 1981; Merion et al 1985; Gruber et al 1989; Venkataram et al 1990; Gorecki et al 1991; Vadiei et al 1991; Fahr 1993). However, they reported that liposomal preparations reduced the residence time of cyclosporin in the systemic circulation and increased the delivery to the spleen by twofold at best. From the standpoint of biopharmaceutics, a more efficient and safe delivery system is needed for immunosuppressive therapy in organ transplantation. To improve cyclosporin preparations we have been performing both pharmacokinetic and biopharmaceutical studies (Takada et al 1985, 1986, 1988, 1989; Nakaji et al 1988). During these studies sudan black was found to be a model neutral dye with the same hydrophobicity as cyclosporin; it has been used in this study also. Throughout the explorative study using sudan black, carbopol 941-modified liposomes were selected as the carrier delivering more cyclosporin to the spleen and the liver. Comparison of Tables 1 and 4 shows that modifying the liposomes with various bioadhesive polymers had almost the same effects in terms of increasing or reducing the liver/plasma, heart/plasma, kidney/plasma or spleen/plasma concentration ratios for sudan black or cyclosporin. However, for the delivery of cyclosporin to the lung, increased delivery was obtained by encapsulating cyclosporin with either standard liposomes or carbopol 941-modified liposomes, although the opposite results were obtained for sudan black. As both sudan black and cyclosporin are neutral compounds, it is inconceivable that they should affect the surface charge of the liposomes. The most probable cause is the difference between the molar ratio of sudan black and cyclosporin to phosphatidylcholine. For sudan black, the molar ratio was 1:8 whereas that for cyclosporin was 1:22. There was also a difference between the plasma kinetics for sudan black and cyclosporin after intravenous infusion to rats. Most biopharmaceutical studies on liposomes have been performed by administering liposomal preparations by intravenous bolus injection. In these studies, the increased AUC and prolonged MRT were obtained by administering the drugs in liposomes. With such a mode of administration the uptake of liposomes to the reticu-

Table 3. Pharmacokinetic parameters of cyclosporin after intravenous infusion (10 mg kg^{-1}) to rats.

Pharmacokinetic parameter	Solution	Standard liposomes	Carbopol 941-modified liposomes
Area under the plasma concentration–time curve from time 0 to infinity (g h mL^{-1})	12·8 ± 3·2	12·3 ± 1·8	23·8 ± 4·3
Mean residence time (h)	4·22 ± 1·32	4·53 ± 0·42	5·63 ± 1·27
Volume of distribution at steady-state (mL kg^{-1})	3210 ± 260	3760 ± 240	2330 ± 120
Total plasma clearance (mL h^{-1} kg^{-1})	876 ± 198	852 ± 138	451 ± 87
Terminal elimination half-life (h)	3·03 ± 0·90	3·60 ± 0·28	4·32 ± 0·90

Each value represents the mean ± s.e.m. from three rats.

Table 4. Cyclosporin tissue/plasma concentration ratios after 80-min infusions of various formulations to rats.

Formulation	Liver	Heart	Kidney	Lung	Spleen
Solution	36·7 ± 10·76	7·95 ± 0·46	17·03 ± 0·64	11·94 ± 0·97	11·12 ± 0·52*
Standard liposome	39·1 ± 61·08	10·4 ± 41·40	13·18 ± 2·45	24·38 ± 4·50	15·44 ± 1·42*
Carbopol 941-modified liposome	86·7 ± 34·60	13·9 ± 31·48	19·64 ± 5·37	28·31 ± 4·52	25·17 ± 4·55*

All data are mean ± s.e.m. *$P < 0·05$, significantly different from the liver/plasma concentration ratio.

Table 5. Pharmacological effect of cyclosporin on the cardiac allograft survival time in rats.

Group	Formulation	Survival time (days)	Mean survival time (days)	Statistical test		
				Group 1	Group 2	Group 3
1	Standard liposome	3, 4, 5, 10, 10	6·4 ± 1·9	–	–	–
2	Cyclosporin solution, 10 mg kg^{-1}	7, 7, 7, 8, 9	7·6 ± 0·5			
3	Cyclosporin, 10 mg kg^{-1}, entrapped in standard liposomes	5, 10, 12, 21, 23	14·2 ± 4·4	*	–	–
4	Cyclosporin, 10 mg kg^{-1}, entrapped in carbopol 941-modified liposomes	11, 17, 20, 22, 24	18·8 ± 2·9	*	†	–

Values are mean ± s.e.m. *$P < 0·05$, significantly different from group 1. †$P < 0·05$, significantly different from group 2.

loendothelial system is likely to be saturated, and so an increased AUC would be obtained, as suggested by Harashima et al (1995). Because test preparations were administered to rats by intravenous infusion, however, a predominant increase in AUC was not obtained in this study.

A recent report by Fahr et al (1995) showed that the distribution of cyclosporin to the liver, kidney, spleen, lung and brain increased in accordance with the formulated amount of lipid in the liposomes. However, this effect was not a result of specific enhancement of the cyclosporin delivery to these organs, because the residence of cyclosporin in the blood circulation was prolonged. On the other hand, two groups (Lee et al 1995; Ko et al 1994, 1995) studied the liposomal preparation of tacrolimus, another strong immunosuppressant, and reported that spleen tacrolimus levels increased approximately twofold compared with the standard intravenous formulation. However, the groups obtained different results for the pharmacological activity of the liposomal preparation. Lee et al (1995) evaluated the pharmacodynamics of tacrolimus by measuring the extent of inhibition of splenocyte proliferation and obtained no increase. Ko et al (1995), on the other hand, demonstrated the enhanced immunosuppressive efficacy of liposomal tacrolimus in the canine orthotropic liver transplantation model. A different liposome formulation was used by the groups. Unlike Lee et al (1995), Ko et al (1995) used cholesterol to prepare liposomes. Cholesterol-poor liposomes are mainly taken up by Kupffer cells (liver macrophages) and cholesterol-rich liposomes by spleen macrophages (Moghimi & Davis 1994). As Moghimi & Davis (1994) suggested, the liver tacrolimus level was increased by adding cholesterol to the liposomes (Ko et al 1994), although the increase was only observed 10 min after administration. Thereafter, liver tacrolimus levels were lower than those obtained by administration of intravenous solution until 24 h. Therefore, we studied another technology to strengthen the activity of immunosuppressant-modification of the liposomes with bioadhesive polymer. The bioadhesive polymers used were two types of sodium alginate (100–150 cp and 1000 cp), β-1,3-glucan and four kinds of carbopol. Although sodium alginate and β-1,3-glucan are natural products, carbopols are synthetic bioadhesive polymers; the viscosities of the neutralized 0·5% aqueous dispersion of the polymers are 30 500–39 400 cp for carbopol 934P, 40 000–60 000 cp for carbopol 940, 4000–11 000 cp for carbopol 941 and 4200–13 000 cp for carbopol 1342. There was no relationship between the viscosity of the bioadhesive polymers used and the organ distribution pattern of sudan black. Among the carbopols, carbopol 941, with the lowest viscosity, had the most efficient effect on the delivery of cyclosporin to the spleen and liver and so a preliminary comparative pharmacodynamic study was performed. However, enhancement was not obtained by modifying the cyclosporin-loaded liposomes with carbopol 941 in our allo-

genic rat-heart-transplantation model. As a result of this we might have to consider the homing process of the lymphocytes. Certainly the spleen is the single, most important organ in lymphocyte recirculation (Pabst & Binns 1989). However, T cell differentiation occurs in secondary lymphoid tissues such as lymph nodes, because the thymus is essentially non-functional owing to inherent age-dependent atrophy and the toxic effects of the radiotherapy or chemotherapy, or both, of the preparative regimen (Sackstein 1993). Therefore, we tried to measure the sudan black and cyclosporin concentrations in the lymph nodes. However, because of limitations in the sensitivity of our assay, sudan black and cyclosporin were not detected in the lymph nodes even when encapsulated in liposomes.

Yerushalmi & Margalit (1994) suggested the use of bioadhesive liposomes as sustained-release carriers for anti-inflammatory and antibiotic drugs, though they performed an in-vitro experiment only. Certainly, in our study, carbopol 941-modified cyclosporin liposomes led to a prolonged MRT for sudan black compared with standard liposomes. In general, the advantages of the use of liposomes are reduced toxicity—especially, for immunosuppressants, nephrotoxicity—and an increase or decrease of the residence time of drugs in the blood circulation. As cyclosporin is an extremely hydrophobic compound, conventional parenteral solution contains much surfactant, which is suspected of causing nephrotoxicity. However, liposomal preparations do not contain surfactant. Therefore, the benefit of reducing nephrotoxicity by use of liposomal preparation is beneficial. If reduced delivery of cyclosporin to the kidney was achieved by use of the modified liposomal preparation, the efficiency of this new preparation would be more emphasized. Therefore, the merit of cyclosporin liposomal preparation is thought to be the avoidance of the addition of surfactant to the preparation.

Much effort has been focused on an attempt to increase the delivery of immunosuppressant to the spleen and the liver in this study. However, most studies on liposomal encapsulation have been performed with antibacterial, antitumour and antifungal drugs (Dean 1993; Mori et al 1993; Bergers et al 1995). In these fields, clinicians want to reduce the delivery of these drugs to endoplasmic-cell-rich organs. As the carbopol 941-modified liposomes reduced sudan black delivery to the spleen and the liver compared with standard liposomes, carbopol-modified liposomes might contribute to advances in these fields.

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