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Research Article

Enhancement of Tissue Delivery and Receptor Occupancy of Methylprednisolone in Rats by a Liposomal Formulation

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A liposomal formulation of methylprednisolone (L-MPL) was developed to improve localization of this immunosuppressant in lymphatic tissues. Liposomes containing MPL were formulated from a mixture of phosphatydylcholine and phosphatydylglycerol (molar ratio, 9:1) and sized by extrusion through a 0.1-μm membrane. Male Sprague–Dawley rats received a bolus dose of 2 mg/kg of L-MPL or free MPL in solution (control). Samples of blood, spleen, liver, thymus, and bone marrow were collected at intervals over a 66-hr period. Concentrations of MPL in plasma and organs and free cytosolic glucocorticoid receptors (GCR) in spleen and liver were determined. The plasma MPL profiles for free and L-MPL were bi- and triexponential. Although the alpha phase kinetics of both dosage forms were similar, L-MPL showed a substantially slower elimination phase than did free drug. Incorporation of MPL into liposomes caused the following increases: terminal half-life, from 0.48 (MPL) to 30.13 hr (L-MPL); MRT, from 0.42 to 11.95 hr, V_{ss}, from 2.10 to 21.87 L/kg; and AUC, from 339 to 1093 ng · hr/mL. Uptake of liposomes enhanced significantly the delivery of drug to lymphatic tissues and liver; AUC tissue:plasma ratios for spleen increased 77-fold; for liver, 9-fold; and for thymus, 27-fold. The duration of GCR occupancy was extended 10-fold in spleen and 13-fold in liver by the liposomal formulation. Lymphatic tissue selectivity and extended receptor binding of liposome-delivered MPL offer promise for enhanced immunosuppression.

KEY WORDS: liposomes; methylprednisolone; pharmacokinetics; tissue distribution; pharmacodynamics; glucocorticoid receptors; drug delivery.

INTRODUCTION

Glucocorticoids have substantial and diverse effects on various cellular and metabolic processes which play an important role in inflammatory and immune responses (1). The major problem limiting the therapeutic application of these drugs is the considerable toxicity which often occurs with chronic administration even when dosages are moderate (2). The known side effects of these drugs include Cushing syndrome, osteoporosis, psychosis, hyperglycemia, and muscle wasting. Upon intravenous or oral dosing, the glucocorticoids generally distribute rapidly to all body tissues by passive diffusion and are extensively metabolized by liver and kidney. The synthetic corticosteroids prednisolone and methylprednisolone exhibit a mono- or biexponential disposition, with terminal half-lives of 2–4 hr in most humans.

Liposomes have been used to reduce toxicity associated with many compounds, including antitumor agents (3), antibiotics (4), and immunosuppressive drugs (5). The major therapeutic advantage of liposomes lies in controlling the bioavailability of their pharmaceutical payload, either spatially by altering tissue distribution or temporally by offering prolonged release of drug within the body (6). After administration, liposomes are intensively taken up by phagocytic elements of the reticuloendothelial system (7–9), including hepatic Kupffer cells and fixed macrophages in the spleen, thymus, and bone marrow. We sought to take advantage of this property to deliver corticosteroid (methylprednisolone) to the immune system.

Initial attempts to develop liposomal formulations of corticosteroids were for local use (10,11) as antiinflammatory drugs injected directly into joints (12), in the eye (13), and for topical routes of administration (14). Enhanced efficacy of drug has been demonstrated. Intramuscular injection of ^{3}H-prednisolone in a lipid emulsion increased the uptake of steroid by organs (15). However, only radioactivity was assayed and no responses were measured.

Most of the known effects of glucocorticoids are moderated by intracellular receptors which act as regulators of gene transcription (16). Previous studies describe receptor gene-mediated pharmacodynamics of prednisolone (17) and methylprednisolone (18) in rats, depict the binding and the replenishment of free hepatic cytosolic receptors after doses of steroid, and describe the cascade of events controlling corticosteroid responses. Glucocorticoid receptor occupancy has been shown to directly govern the net biological response.

The purpose of our study was to develop a liposomal formulation of methylprednisolone that could enhance the delivery of drug to lymphatic tissues, producing enhanced

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immunosuppressive activity. Glucocorticoid receptor density in spleen and liver was used as a response measure.

EXPERIMENTAL

Materials

L-α-Lecitin (phosphatydylcholine) (PC), L-α-phosphatydylglycerol (egg sodium salt) (PG), and hydrogenated soy phosphatydylinositol (PI) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All solvents used for the chromatographic analysis of methylprednisolone in plasma and tissues were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI). Methylprednisolone and dexamethasone were obtained from Sigma (St. Louis, MO), and ^{3}H-dexamethasone was obtained from Amersham (Arlington Heights, IL).

Liposomal Formulation

Liposomes were freshly prepared as follows: lipids (PC:PG, 9:1) and drug (5 mol%) were dissolved in chloroform and the organic solvent was evaporated at 37°C under argon at a reduced pressure. The dried film was suspended in buffer containing 143 mmol of NaCl and 4 mmol of HEPES, pH 7.4, by gentle vortex mixing for 5 min. Liposomes were extruded to a uniform diameter by repeated passage through 0.1-μm polycarbonate filters using a low-pressure device (Liposofast, Avestin Inc., Ottawa, Canada). Liposomes containing methylprednisolone were separated from free drug by gel-permeation chromatography using a Sephadex G-75 column and concentrated immediately with an Amicon concentration unit (W. R. Grace, Beverly, MA) and a Diaflo M_r 10,000 cutoff ultrafilter. The dosage form was stored overnight at room temperature prior to the experiment.

Methylprednisolone encapsulation (E) in liposomes was calculated as

$$E = \frac{\text{amount of MPL (mol)}}{\text{amount of lipids (mol)}} \cdot 100\% \qquad (1)$$

where the amount of MPL was determined by HPLC analysis after extraction of an aliquot of liposomes as described under Drug Assay; phospholipid concentration was measured by a combined method of sulfuric acid digestion, oxidation to phosphate by H_2O_2, and colorimetric reaction with molybdate (19).

Animals

Male Sprague–Dawley rats, weighting 220–270 g, were purchased from Harlan Sprague–Dawley Inc. (Indianapolis, IN). Animals were housed in a 12-hr light/12-hr dark, constant-temperature (22°C) environment with free access to rat chow (Agway RMH 1000) and drinking water. Animals were acclimatized to this environment for at least 1 week. One day prior to the study, rats were subjected to right external jugular vein cannulation under light ether anesthesia. Cannula patency was maintained with sterile 0.9% NaCl. Food was removed 14 hr before each experiment but water was allowed.

Animal Procedure

The liposomal formulation of methylprednisolone or the free drug in NaCl–HEPES buffer, pH 7.4, was administered via cannula over 1 min as a 2 mg/kg dose. Blood samples were taken at a various times postdosing until 66 hr. At each sampling time, the removed blood volume was replaced with a citrate–dextran anticoagulant solution (20). Rats were sacrificed under light ether anesthesia and blood was drained from the abdominal aorta into a heparinized syringe. Blood was immediately centrifuged, and plasma was harvested and frozen at −20°C until assayed. Following blood removal, liver, spleen, thymus, and femurs were excised. Bone marrow was flushed from femurs using saline. Spleen and liver were placed immediately in ice-cold buffer prior to measurement of cytosolic glucocorticoid receptor content. A portion of excised tissue was flash-frozen using liquid nitrogen and stored at −80°C until HPLC analysis for methylprednisolone.

Drug Assay

Samples were thawed at room temperature. Aliquots (0.3–1.0 mL) of rat plasma were extracted with methylene chloride. The organic layer was washed with 0.1 N sodium hydroxide followed by distilled water. Plasma concentrations of methylprednisolone were determined by HPLC (21). Spleen, liver, and thymus were homogenized in PBS (pH 7.4) before extraction. A cell count was performed for the bone marrow suspension prior to HPLC analysis. The calculated limit of quantitation was 8 ng/mL for plasma and 50 ng/g for tissues. The coefficients of variation were less than 10% (intraday and interday).

Cytosolic Glucocorticoid Receptors

For the determination of splenic glucocorticoid receptors, we used a modified method of Miller *et al.* (22). Hepatic glucocorticoid receptors were measured as described previously (17). Briefly, spleen was homogenized in ice-cold buffer containing Tris (10 mM). EDTA (1 mM), dithiotreitol (5 mM), glycerol (10%), and disodium molybdate (20 mM) and liver in buffer, containing Tris (50 mM), molybdic acid (10 mM), and EDTA (2.5 mM) (pH 7.4) followed by centrifugation at high speed to obtain a clear supernatant. Cytosol was treated with dextran-coated charcoal to remove endogenous glucocorticoids and incubated at 4°C for 18–22 hr with ^{3}H-dexamethasone (44 Ci/mmol; concentration ranges, 5–200 nM) in the presence and absence of a 500-fold excess unlabeled dexamethasone. Aliquots of cytosol were counted to determine total binding (D_T) and nonspecific binding (D_{NS}) of dexamethasone. Based on data obtained for total bound, nonspecific bound, and free dexamethasone (D_F), the glucocorticoid receptor density (B_{max}), dissociation constant (K_D), and nonspecific binding constant (K_{NS}) were estimated using PCNONLIN (SCI, Lexington, KY) by solving Eqs. (2) and (3) simultaneously:

$$D_T = D_{NS} + \frac{D_F \cdot B_{max}}{D_F + K_D} \qquad (2)$$

$$D_{NS} = K_{NS} \cdot D_F \qquad (3)$$

Pharmacokinetic Analysis

Plasma methylprednisolone concentration-versus-time data were described by polyexponential equations:

$$C_{MPL} = \Sigma\, C_i \cdot e^{-\lambda_i \cdot t} \quad (4)$$

where $i = 2$ for control and $i = 3$ for L-MPL studies.

The intercept coefficients (C_i) and slopes (λ_i) were estimated by least-squares fitting using the PCNONLIN computer program. The area under the methylprednisolone concentration–time curve (AUC = $\Sigma\, C_i/\lambda_i$) and area under the first moment curve (AUMC = $\Sigma\, C_i/\lambda_i^2$) were calculated from the slopes and coefficients. The mean residence time (MRT) was determined as a ratio of AUMC/AUC. The apparent systemic clearance (CL) of methylprednisolone was obtained as CL = Dose/AUC. The central (V_c) and total (V_{ss}) volumes of distribution were determined as V_c = Dose/$\Sigma\, C_i$ and V_{ss} = CL · MRT (17,18). The mean residence time for tissues (MRT_T) was calculated as $MRT_T = (V_{ss} - V_c)/CL$ (23). Distribution clearance (CL_D) (24) was obtained by applying

$$CL_D = D \cdot \left[\frac{\Sigma\,(\lambda_i \cdot C_i)}{(\Sigma\, C_i)^2} - \frac{1}{AUC}\right] \quad (5)$$

The AUC for methylprednisolone in tissues were calculated over 0–6 hr (control) and 0–66 hr (liposomal formulation) by polynomial interpolation using the LAGRAN program (25) with extrapolation to infinity using the terminal slope from the plasma data.

Tissue Distribution

A semiphysiologic approach to quantitation of tissue drug concentrations C_T was based on an individual tissue distribution clearance (CL_d) equation:

$$V_t \cdot \frac{dC_T}{dt} = CL_d \cdot \left(C_p - \frac{C_T}{R}\right) \quad (6)$$

where V_t is tissue mass and R is the equilibrium tissue: plasma distribution ratio of methylprednisolone. The consideration of liver as an eliminating organ with intrinsic clearance (CL_{int}) transforms Eq. (6) to

$$V_t \cdot \frac{dC_L}{dt} = CL_d \cdot \left(C_p - \frac{C_L}{R}\right) - CL_{int} \cdot \frac{C_L}{R} \quad (7)$$

where C_L is the concentration of drug in liver. Substitution of $k_t = CL_d/V_t$, $k_m = CL_{int}/V_t$, and joint solution of Eqs. (4) and (6) or (7) yields

$$C_T = \sum \frac{k_t \cdot C_i}{(k_t/R) - \lambda_i} \cdot (e^{-\lambda_i \cdot t} - e^{-k_t \cdot t/R}) \quad (8)$$

and

$$C_L = \sum \frac{k_t \cdot C_i}{[(k_t + k_m)/R] - \lambda_i} \cdot (e^{-\lambda_i \cdot t} - e^{-(k_t + k_m) \cdot t/R}) \quad (9)$$

to describe C_T as a function of time. Equation (8) or (9) was fitted to the tissue data using PCNONLIN and using C_i and λ_i values from the plasma curves to generate least-squares values of k_t and R for each tissue. Areas under concentrations in tissue–time curves AUC_T were calculated from slopes, coefficients, and parameters using Eq. (10):

$$AUC_T = \sum \frac{k_t \cdot C_i}{(k_t/R) - \lambda_i} \cdot \left(\frac{1}{\lambda_i} - \frac{R}{k_t}\right) \quad (10)$$

Pharmacodynamic Analysis

The pharmacological effects of corticosteroids occur due to sequential reactions following dosing (1). After passive diffusion of unbound steroid into cells, binding occurs to intracellular receptors with considerable specificity and affinity. After binding, the steroid–receptor complex undergoes activation and this complex is rapidly translocated into the nucleus. These steps proceed very quickly and lead to a decrease in free cytosolic receptor concentrations. Cytosolic glucocorticoid receptor concentrations are later restored principally by dissociation of the nuclear-bound steroid–receptor complex. The interaction of glucocorticoid receptor with the cell DNA leads to transcription, and translation, and protein synthesis.

To characterize the fall and return of glucocorticoid receptor concentrations in splenic and hepatic cytosol after administration of MPL, we used a partial PK/PD model based on the law of mass action:

$$D + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} DR \quad (11)$$

The free cytosolic receptor (R) data were fitted to differential equations (12) and (13)

$$\frac{d(R)}{dt} = -k_{on} \cdot (D) \cdot (R) + k_{off} \cdot (DR) \quad (12)$$

$$\frac{d(DR)}{dt} = k_{on} \cdot (D) \cdot (R) - k_{off} \cdot (DR) \quad (13)$$

where D are concentrations of methylprednisolone, R is unbound receptor concentration in splenic or hepatic cytosol, and DR is drug–receptor complex concentration. The apparent constants for association (k_{on}) and dissociation (k_{off}) of D and R were sought by least-squares fitting using PCNONLIN. For free methylprednisolone it was assumed that drug in plasma controlled access to free receptors and D was thus described with Eq. (4), $i = 2$.

Results were evaluated based on goodness-of-fit criteria which included visual inspection, examination of residuals, and correlation coefficients. The area under the curves 0–6 hr (control) and 0–66 hr (liposomes) of total receptor (R_{tot}) and free receptor (R_{free}) were calculated by polynomial interpolation using the LAGRAN program (25). The AUC of bound receptor (R_B) was represented by the difference between AUC of R_{tot} and free receptor.

RESULTS

Liposomal Formulation

The optimal composition of lipids and drug was sought to obtain the highest incorporation of MPL. In choosing the lipid ratio we considered that MPL is a neutral compound

and has better solubility in neutral lipids such as phosphatyldylcholine. It is known that slightly charged liposomes prevent an aggregation of particles in suspension, improve stability (6), and can increase the rate of elimination from the blood and uptake by the reticuloendothelial system (9). By modifying the fraction of charged lipids (PG or PI), we found that 10% of negative charge is the optimal condition for retention of MPL. The molar ratio of drug/lipid depends on the solubility of drug in water and in lipids. The structure of the MPL molecule is similar to cholesterol, which is known to mix well with lipids, but we found that using more than 5 mol% of drug leads to precipitation of steroid. This was determined by HPLC after separation of liposomes and free drug by gel-permeation chromatography.

We varied the ratio of neutral (PC) and negatively charged lipids (PG, PI) over a range of 0–10%; the range of MPL:lipid was 5–40 mol% and it was found that the optimal liposomal formulation consisted of PC:PG (9:1) and 5 mol% of MPL. Liposomes of a large size (>500 nm) have a strong preference for uptake by liver macrophages, while small liposomes (50–60 nm) can circulate in blood longer and may be taken up by hepatocytes (9). Liposomes <100 nm were chosen in an effort to obtain longer circulation time, and to produce a larger lipid surface to carry drug.

The concentration of MPL in the liposome suspension was 0.7 mg/mL, although the drug is probably contained in membrane. This incorporation yields a final molar ratio of 9.4:0.6 (lipid:drug) in liposomes. This result is in agreement with previous incorporation studies of fluorinated steroids in liposomes (26). Our formulation could allow doses of up to 250 mg of methylprednisolone in humans without exceeding 10 g of lipids, which can be given safely to humans (27,28).

After storage overnight at room temperature, the steroid:lipid ratio was the same as that determined 1 hr after preparation, suggesting no significant leakage. However, drug loss from liposomes of approximately 70% occurs after 1 week of storage.

Pharmacokinetics

Figure 1 shows the total MPL concentrations in rat plasma versus time after 2 mg/kg iv bolus doses for control and liposomal formulations. After the dose of free MPL, the drug was eliminated rapidly and could be detected only to 2.5 hr, whereas with the same dose of L-MPL drug was found in plasma until 66 hr. Points on the plot which show concentrations of MPL below 8 ng/mL were obtained by parallel extraction of several portions of plasma and quantitation of the combined extracts by HPLC. Figure 1 also provides the fitting of pharmacokinetic data for the biexponential (control) and triexponential model (L-MPL). The declines of the α-phase in both cases do not differ significantly, while the terminal slope for liposomal formulation is considerably prolonged. Table I lists the polyexponential parameters obtained by computer fitting.

The pharmacokinetic parameters calculated using area–moment analysis demonstrate marked differences in the disposition of MPL between the two formulations. The AUC of MPL after the free drug was 339 ng · hr/mL, in comparison with 1093 ng · hr/mL for L-MPL, producing apparent CL of 5.04 and 1.83 L/hr/kg. The CL_D values were 2.24 and 67.08 L/hr/kg. The terminal half-life increased dramatically [from 0.48 (control) to 30.13 hr (L-MPL)], as did the MRT (from 0.42 to 11.95 hr) and MRT_T (from 0.14 to 11.73 hr). The volume of central compartment V_c decreased from 1.39 to 0.39 L/kg, while the apparent V_{ss} was enlarged more than 10-fold: from 2.10 to 21.87 L/kg. A comparison of these parameters and their ratios is shown in Fig. 2.

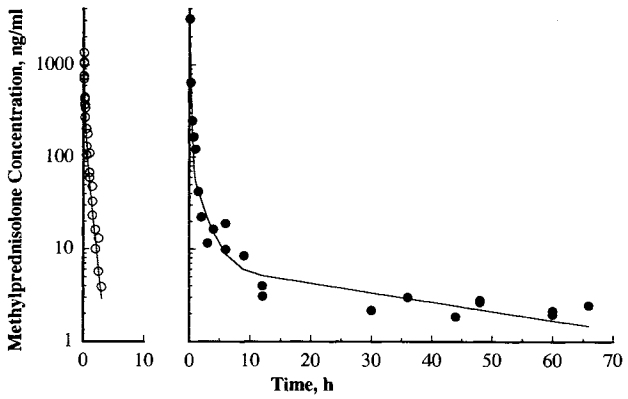

Fig. 1. Plasma methylprednisolone concentrations after single intravenous doses of 2 mg/kg in groups of rats. Lines represent fitting of data to Eq. (4). Open circles, control; filled circles, liposomal formulation.

Tissue Distribution

Distribution of MPL in spleen, liver, and thymus after control and liposomal doses is presented in Fig. 3. Each point represents an individual animal. In the control experiment, the decline of MPL concentrations in tissues reaches the detection limit by 6 hr. However, following L-MPL administration, concentrations of drug in spleen and thymus indicate very consistent levels over 66 hr; liver demonstrates a very slow decline. Figure 3 also shows the nonlinear least-squares fitting of MPL data to the venous equilibrium model as described by Eqs. (8) and (9). Data from both formulations were fitted very well for all organs and all fitted curves for tissue distribution were parallel to the terminal slopes of drug concentrations in plasma.

Table II summarizes the results of the tissue distribution study. Partition coefficients (R), relating drug concentration in tissue to drug concentration in plasma at equilibrium, increased for L-MPL in comparison with free drug by 24.6-fold

Table I. Pharmacokinetic Parameters Describing the Disposition of Methylprednisolone After a Single Intravenous Dose of Free Drug and Its Liposomal Formulation

	Formulation	
Parameter	MPL (biexponential fitting)	L-MPL (triexponential fitting)
C_1, ng/mL	1202.1 (322.5)	5000.0 (594.1)
C_2, ng/mL	235.5 (61.3)	80.00 (49.5)
C_3, ng/mL	—	6.63 (7.32)
λ_1, hr^{-1}	6.72 (1.62)	7.62 (0.86)
λ_2, hr^{-1}	1.44 (0.15)	0.55 (0.41)
λ_3, hr^{-1}	—	0.023 (0.031)

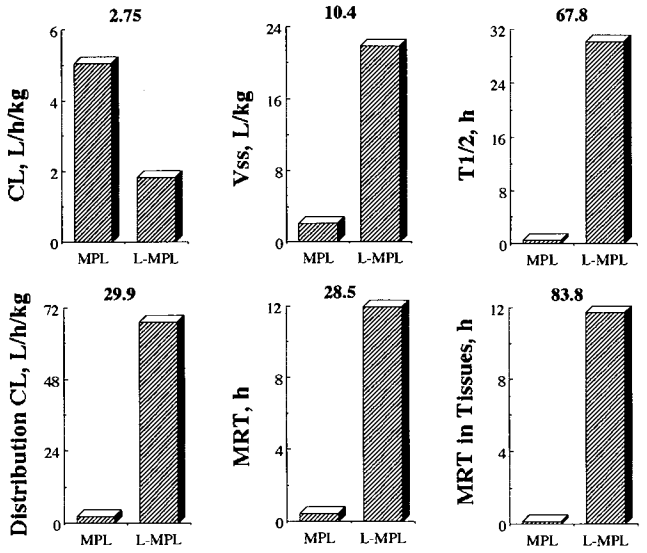

Fig. 2. Clearance (CL), volume of distribution at steady state (V_{SS}), half-ife ($t_{1/2}$), distribution clearance (CL_D), mean residence time (MRT), and mean residence time in tissues (MRT_T) of free methylprednisolone (MPL) or its liposomal formulation (L-MPL) in rat plasma after intravenous doses 2 of mg/kg.

in spleen, by 7.9-fold in thymus, and by 2.7-fold in liver. At the same time, distribution clearances of liposomal steroid diminished in spleen, thymus and liver by 3.5, 14.5, and 15.3 times.

Estimated parameters for liver using Eq. (8) represent apparent values. The consideration of the liver as an eliminating organ by applying Eq. (9) yields more correct values, in theory, for k_t, R, and CL_d. The intrinsic clearance for liver in control animals (CL_{int} = 145 mL/min) was adapted from a previous study of isolated rat liver perfusion of methylprednisolone (29). For L-MPL we assumed that CL_{int} for the liposomal formulation decreased in the same proportion as the apparent CL for free drug. The fitted curves for liver were identical using both Eq. (8) and Eq. (9). CL_d values do not show significant differences, but calculated R values for L-MPL were enlarged by 100-fold and the ratio of $R_{L\text{-}MPL}$:R_{MPL} increased about 5-fold using Eq. (9) vs Eq. (8)

A comparison of tissue distribution using AUC values is shown in Fig. 4. The absolute values of the uptake of MPL by organs increased by 77.4 times for spleen. 26.9 times for thymus, and 2.8 times for liver using liposomes. Relative distribution was altered with a marked preference of the liposomal formulation for lymphatic tissues. The calculation of tissue:plasma ratios of AUC for spleen, thymus, and liver demonstrates that administration of L-MPL increased this ratios of drug in spleen from 0.9 to 21.7 (24.1-fold), in thymus from 1.9 to 15.8 (8.3-fold), and in liver from 12.4 to 34.2 μg · hr/mL (2.8-fold). Neither free drug nor L-MPL at this low dose produced detectable concentrations of MPL in bone marrow.

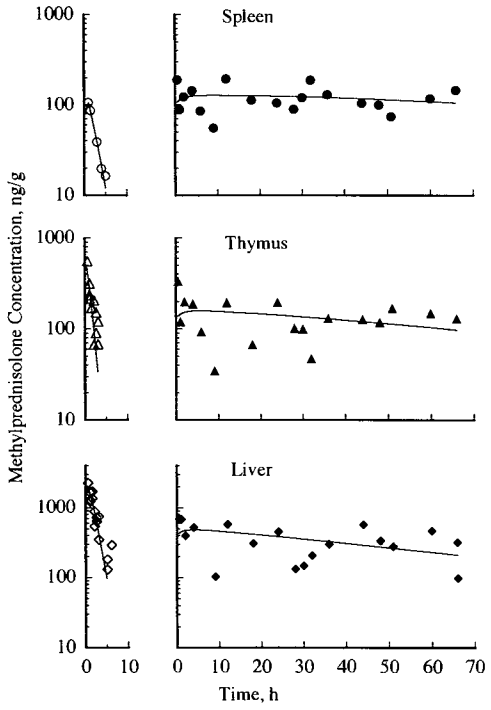

Fig. 3. Methylprednisolone concentrations in rat liver, spleen, and thymus after single intravenous doses of 2 mg/kg. Lines represent fitting of data to Eqs. (8) and (9). Open symbols, control; filled symbols, liposomal formulation.

Pharmacodynamics

Figure 5 shows the competitive binding of ^{3}H-dexamethasone with glucocorticoid splenic cytosol receptors at 4°C in the presence and absence of unlabeled drug. At baseline, the spleen has a lower density of receptor binding sites than liver, but liver data were similar otherwise. Free splenic cytosolic receptor content averaged 16.7 ± 0.8 nM at time 0 and was in agreement with previously obtained data (22). The K_D values at baseline were 25.64 nM for spleen and 19.61 nM for liver. Similar values were obtained at other times during the studies. In hepatic cytosol, baseline free receptor density averaged 35.4 ± 0.7 nM, which was lower than for adrenalectomized rats (18).

Figure 6 demonstrates the free receptor concentrations over time in splenic and hepatic cytosol after administration of MPL or its liposomal formulation. Almost immediately after drug administration, the amount of free receptors decreased to negligible values. Thereafter, free receptor concentrations gradually increased to reach an apparent new baseline, which may be caused by receptor down-regulation (18) approximately 6 hr after control MPL injection. Following liposomal MPL, glucocorticoid receptors were completely occupied for 12 hr postinjection in spleen and for 18 hr in liver. For L-MPL, slow recycling of free receptors to reach the new baseline occurred until 66 hr.

The control hepatic GR cytosol data were obtained from a previous study after a 5 mg/kg dose of MPL (18); the simulation of these data was performed for a 2 mg/kg dose. Figure 6 shows the results of least-squares fitting of the control data using the partial PK/PD model described by Eqs. (12) and (13). The apparent association (k_{on}) and dissociation (k_{off}) rate constants for control data in splenic cytosol were calculated as 0.064 (0.041) mL/ng · hr and 0.445 (0.112) hr^{-1}. These parameters were used to simulate the receptor occupancy for liposomal formulation assuming that all drug

Table II. Distribution Parameters of Methylprednisolone in Tissues

Tissue	Formulation	k_t (hr^{-1})	R^a	$R_{L\text{-}MPL}/R_{MPL}$	CL_d (mL/hr)	$CL_{d\ L\text{-}MPL}/CL_{d\ MPL}$
Spleen	MPL	0.550 (0.022)	0.89 (0.02)	24.57	0.440 (0.018)	0.289
	L-MPL	0.159 (0.022)	21.85 (12.16)		0.127 (0.018)	
Thymus	MPL	2.996 (0.716)	2.01 (0.21)	7.87	0.539 (0.129)	0.069
	L-MPL	0.204 (0.039)	15.79 (7.40)		0.037 (0.007)	
Liver[b]	MPL	9.850 (1.325)	12.18 (1.01)	2.71	76.83 (10.34)	0.065
	L-MPL	0.643 (0.114)	32.94 (9.34)		5.015 (0.889)	
Liver[c]	MPL	9.838 (1.323)	1390 (234)	14.0	69.34 (9.34)	0.072
	L-MPL	0.642 (0.114)	20912 (8136)		5.01 (0.89)	

[a] Tissue/plasma distribution ratio.
[b] Model without elimination.
[c] Model included intrinsic clearance.

in plasma is available to receptors (i.e., values of D were taken from Table I for the liposomal formulation). These curves underestimated receptor binding as shown in Fig. 6, suggesting that the liposomes enhance steroid occupancy of receptors to an extent greater than predicted by plasma concentrations (Fig. 1).

A comparison of the AUC of bound receptor for both formulations is shown in Fig. 7. By using the liposomal methylprednisolone, receptor occupancy was extended 10-fold in spleen (from 74 to 733 nM · hr) and 13-fold in liver (from 138 to 1857 nM · hr) compared to the steroid given in solution.

DISCUSSION

Liposomal encapsulation markedly altered the pharmacokinetics of methylprednisolone in plasma. Best fittings were obtained using a biexponential decay curve for free drug and a triexponential function of L-MPL. The rate of declines in MPL concentrations were similar for both formulations during the early phase ($\alpha = 6.72 \pm 1.62\ hr^{-1}$, control; and $7.62 \pm 0.86\ hr^{-1}$, L-MPL), but CL_D values were dissimilar, with a large increase in CL_D for L-MPL (Fig. 2). These properties reflect rapid plasma clearance and distribution into peripheral compartments, which include tissues such as liver, spleen, and thymus. Similarities in the α-phage might be explained by the appearance of free drug following L-MPL dosing due to partial leakage immediately after dilution of liposomes in blood (6). This factor plays a positive role in establishment of a loading dose of drug. The increase of initial plasma concentrations observed after L-MPL administration (C_o = 5000 ng/mL) in comparison with free drug (C_o = 1202 ng/mL) may be due to inclusion of both free and liposomal MPL. Such an observation was made previously for dexamethasone given as a lipid emulsion (12).

For the free drug the estimated half-life (0.48 hr) is similar to that previously obtained for MPL (0.51 hr) at a 10 mg/kg dose (18). No metabolite (methylprednisone) was found after injection at this low dose. The present calculations assume that the contribution of reversible metabolism is negligible (8) and that the kinetics are linear at this low steroid dose.

The triexponential model for L-MPL describes both the disposition of steroid in liposomes and the distribution of drug into peripheral compartments which include the lymphatic tissues. The terminal slope of L-MPL is strikingly prolonged, by 62.8-fold, with a half-life of 30.13 hr.

Area/moment analysis of both kinetic profiles revealed an increase in AUC, together with a decrease in V_c and a decrease in CL for liposomal encapsulated MPL, all by approximately threefold (Fig. 2). The considerable increase in MRT (22-fold) and MRT_T (84-fold) for liposome-associated MPL results primarily from the 10-fold enlargement of the V_T, the latter reflecting sequestration of drug in tissues. A previous study (29) indicated that the use of a large iv dose of MPL in solution (50 mg/kg) has no influence on the half-life of steroid and increases its MRT only by 1.5 times. The prolonged maintenance of low MPL plasma concentrations from the liposomal formulation occurs because of the slow

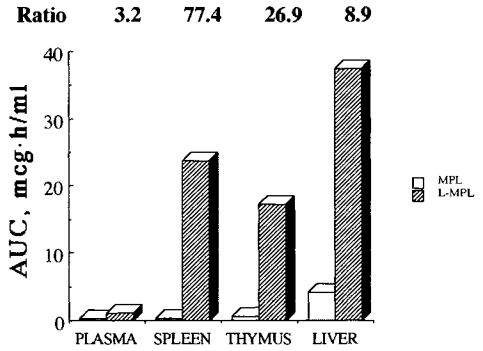

Fig. 4. Comparative distribution (AUC) of methylprednisolone in rat plasma, spleen, thymus, and liver. Open bars, control; filled bars, liposomal formulation. Ratio values reflect liposomal:control values.

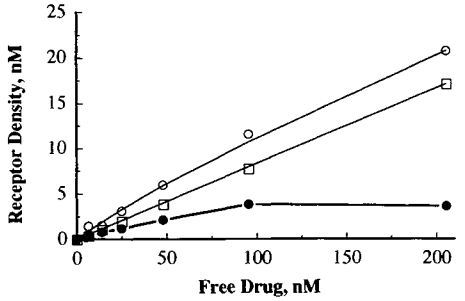

Fig. 5. Receptor binding of ^{3}H-dexamethasone in the homogenate (dilution, 1:4) of splenic cytosol at 4°C. Total binding, open circles; nonspecific binding, open squares; and specific binding, filled circles.

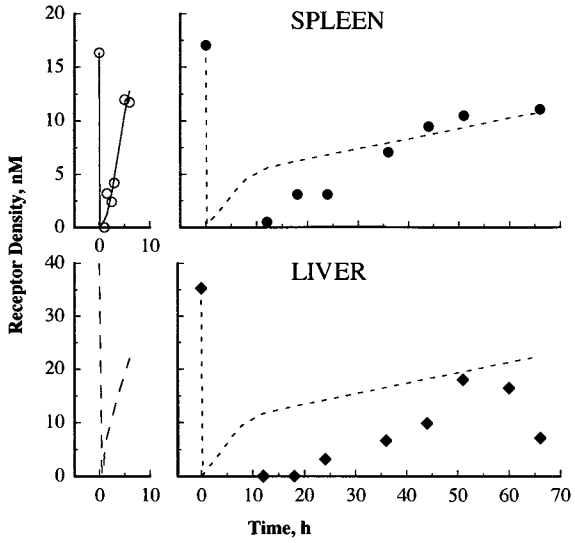

Fig. 6. Glucocorticoid receptor density as a function of time in rat spleen (top) and liver (bottom). Open circles, free drug; filled circles or diamonds, liposomal formulation. Data points are measured values and curves are the results of least-squares fitting (solid line) or the simulations (dashed lines) using Eqs. (4), (12), and (13). Broken line is the simulation after a 2 mg/kg dose of MPL based on data from Ref. 18.

release of steroid from tissues and subsequent return of drug to the systemic circulation.

Most conventional liposomes are usually taken up by macrophages located in the liver and spleen, which can be disadvantageous for drug targeting to other sites. In utilizing glucocorticoids as immunosuppressive agents, this unique property of liposomes can be used for direct delivery to the immune system.

We determined drug concentrations in spleen, liver, thymus, and bone marrow at various postdosing times (Fig. 3) in order to assess relative drug targeting. In general, the highest concentrations of MPL were found in liver, followed by thymus and spleen. The initial concentrations of MPL in spleen and thymus after control doses were similar (100 and 300 ng/g) compared to L-MPL (150 and 250 ng/g) a few hours after injection. These values in liver were higher for the free drug (2500 ng/g) than for liposomal drug (800 ng/g). In control experiments steroid was eliminated from organs rapidly, and after 6 hr, concentrations of drug fell below the detection

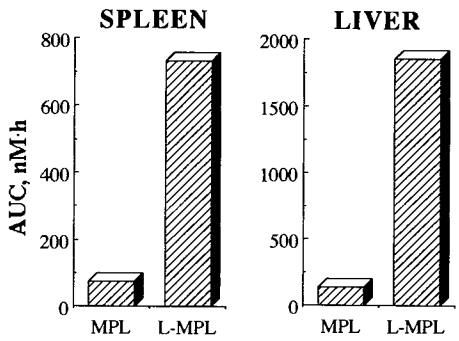

Fig. 7. Net glucocorticoid receptor occupancy in rat spleen and liver after iv doses of methylprednisolone (MPL) or its liposomal formulation (L-MPL).

limit. Passive diffusion and tissue binding appear to be the main controlling factors for tissue distribution of methylprednisolone as was shown to be the case for prednisolone (30).

In control experiments, appreciable drug was taken up by liver: R values of steroid in liver were the highest (12.18), followed by thymus (2.01) and spleen (0.89); also, specific tissue CL_d values for liver were approximately 160-fold higher than for other organs (Table II). This is probably due to the high blood flow to the liver, which is typically 20.7 mL/min, compared to 1.7 mL/min to the spleen (31). The same dose of liposomal steroid exhibited different tissue distribution: spleen and thymus levels of MPL persisted and in liver showed a slow decline during 66 hr. After L-MPL, drug was sequestered by lymphatic tissues: partitioning by spleen increased significantly (by 25-fold) with deceleration of CL_d by three times. The low apparent values of R for liver obtained using Eq. (8) were converted to extremely large values by consideration of liver as eliminating organ. However, the use of AUC values (Fig. 4) indicated that L-MPL increased net AUC values in spleen > thymus > liver.

Steroid-loaded liposomes probably were taken up by macrophages and monocytes of the RES of the spleen, thymus and liver. Extensive RES uptake of drug-carrying liposomes may reduce the systemic exposure of non-RES tissues to the steroid. This could improve significantly the safety of MPL by reducing drug accumulation in nontarget organs, thereby avoiding toxicity. Bone is one of the major organs damaged by steroids, but no MPL was found in bone marrow for either formulation. After initial uptake of liposome-encapsulated drug, the RES may act as a reservoir of MPL, releasing it slowly back to the blood. The absolute values of uptake of MPL by spleen and thymus increased, while liver gained relatively less drug than spleen and thymus. Liver is especially important in liposome clearance due to its substantial blood flow and the accessibility of Kupffer cells. Nonphagocytic cells, such as hepatic parenchymal cells, may also internalize small liposomes by pinocytosis (8). The enhancement of the delivery of MPL to spleen by 24.6 times and to thymus by 7.9 times may be beneficial for diseases where suppression of a strong immune response is required.

The altered distribution of MPL administered in liposomes caused a markedly prolonged biological response for splenic and hepatic glucocorticoid receptors. We described the behavior of GCR by applying a partial PD model (17,18), which simplified the complex molecular events which occur within the cell (1), using concentrations of MPL in plasma as the input. The general processes involving receptors are association and nuclear translocation of steroid–receptor complexes and dissociation and recycling from the nucleus of free receptors. The calculated values of k_{on} and k_{off} for free methylprednisolone–receptor interaction were similar to previous values (18). Attempts to use actual drug concentrations in plasma (C_p) and free drug in tissue (i.e., C_T/R) as an input function for the PD model did not result in good fittings for the liposomal formulation. Simulations based on k_{on} and k_{off} obtained for free drug show that the rate of receptor recycling for L-MPL was slower than predicted (Fig. 6). Receptor occupancy was enlarged 10-fold in spleen and 13-fold in liver. Without liposomes, analogous results could be

achieved by increasing the dose or by infusing the free drug for an extended period.

The present study provides the most promising results to date using liposomes for systemic administration of immunosuppressive drugs. Shinozawa *et al.* (15) administered prednisolone in the lipid layer of positively charged liposomes to rats and found that this formulation increased spleen levels by about 4-fold at 24 hr and maintained plasma concentrations 8 to 24 higher over 0.5 to 24 hr. No response measurements were made. Vadiei *et al.* (5) also used positively charged liposomes containing cyclosporine and found a modest increase in AUC of drug in spleen but a threefold decrease in the *ex vivo* lymphocyte blastogenic response to PHA. Hsieh *et al.* (32) used three formulations of liposomal cyclosporine in rats but found no changes in immunosuppressive effect as measured by lymphocyte transformation tests. Glucocorticoid receptor binding has been shown to correlate directly with splenocyte proliferation (22). The success with our L-MPL formulation may depend on the particular physicochemical properties of the liposomes (lipid composition, size, and charge), but these variables have not yet been explored in our system.

In conclusion, the liposomal formulation of MPH significantly prolonged the blood circulation time of steroid and altered its organ distribution with preference to lymphatic tissues. Such improved pharmacokinetics, along with the markedly enlarged receptor occupancy, might be applicable for improving use of the drug as an immunosuppressive agent.

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