Liposomal Methylprednisolone in Rats: Dose-Proportionality and Chronic-Dose Pharmacokinetics/Pharmacodynamics

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Purpose. Methylprednisolone (MPL) encapsulated in liposomes (L-MPL) targets the immune system and enhances immunosuppressive activity of the steroid. We performed dose-dependent and chronic dose studies of L-MPL versus MPL.

Methods. Male Lewis rats received 10 mg/kg IV bolus doses of L-MPL (Solu-Medrol). Plasma samples were obtained over an 8 day period and MPL concentrations were assayed by HPLC. Immunosuppressive effects were measured as inhibition of *ex vivo* splenocyte proliferation induced with PHA.

Results. Drug concentrations declined in a similar manner over the first few hours following MPL or L-MPL. Free MPL was cleared from plasma by 6 hr, while the same dose of L-MPL resulted in persistance over an 8-day period. Dose-dependent changes in pharmacokinetic parameters were observed for both free and liposomal drug. Increasing the dose from 2 to 10 mg/kg led to increased clearance from 5.9 to 10.5 (MPL) and from 1.8 to 2.3 L/hr/kg (L-MPL). Blastogenesis was suppressed over 5 days with return to the baseline at day 8 (L-MPL); free MPL produced immunosuppression only over 10 hr. Multiple 2 mg/kg IV doses of L-MPL versus MPL twice a week produce plasma drug profiles similar to those obtained after single doses, indicating that neither free nor liposomal steroid accumulates in tissues. Liposomes without drug simultaneously administered with MPL caused partial prolongation of plasma steroid half-life (8.4 hr).

Conclusions. These studies clarify factors causing prolonged drug persistence and immunosuppression with L-MPL. Nonlinear disposition, irregular pharmacokinetics, and secondary effects of the liposomes are complicating factors in use of L-MPL.

KEY WORDS: liposomes; methylprednisolone; pharmacokinetics; dose dependence; multiple doses; pharmacodynamics.

INTRODUCTION

In order to improve the therapeutic index and to reduce toxicity of corticosteroids, an approach of targeted drug delivery has been successfully applied[1]. One such system is drug entrapment in liposomes. Liposomes have advantages of biodegradability, ease of preparation, and lack of toxicity. Recently we developed liposomal methylprednisolone (L-MPL) with PK/PD properties significantly different from the parent drug. The formulation markedly prolonged plasma circulation time of steroid, sequestered drug in the lymphatic tissues, extended glucocorticoid receptor occupancy[2], enhanced the immunosuppressive effect of MPL measured as lymphocyte blastogenic response, and markedly increased the duration of heart allograft transplantation in a rat model[3-5].

It is conventional to examine the kinetic properties of drugs at various dose levels[6]. Free MPL undergoes nonlinear disposition in the rat[7]. In the case of MPL, this also occurs pharmacodynamically because of the limited concentration of receptors[2,3]. Furthermore, liposome uptake by macrophages of the RES might become saturable at a high dose of lipids due to binding site limitations[8,9].

Our previous studies[2,3] were conducted using a single dose of encapsulated steroid. For lipophilic drugs, incorporation into liposomes depends on the aqueous to lipid partition coefficient. MPL rapidly leaks from liposomes after an IV bolus dose due perhaps to dilution. Alterations in PK/PD could be caused not only by sustained release from liposomes but also by the *in vivo* effects of the carrier itself. Therefore, we studied the effects of coadministration of empty liposomes and free drug and performed dose-dependence and chronic dose studies of L-MPL versus MPL.

EXPERIMENTAL

Materials, Liposomal Formulation, and Animals

These were used as described previously[3].

Experimental Design

The liposomal formulation of methylprednisolone or the free drug (Solu-Medrol) in NaCl - HEPES buffer, pH 7.4, was administered via cannula over 1 min as 2 or 10 mg/kg doses. Blood samples were taken at various time up to 168 hr (less than 7 samples per rat). These were replaced with a citrate-dextran anticoagulant solution[10].

The multiple doses of L-MPL and MPL were administered as 8 IV bolus doses of 2 mg/kg twice a week via the penile vein under light ether anesthesia. One day prior to the last dose the cannula was placed in the right jugular vein. Blood samples were obtained before the last dose and later according to the previous protocol. Rats were sacrificed at various time points for up to 8 days (two animals per time point) under light ether anesthesia by removal of blood from the abdominal aorta. The spleen was aseptically excised and immediately placed in RPMI 1640.

Also, 2 mg/kg doses of MPL (Solu-Medrol) were administered via cannula immediately after the dose of empty liposomes (prepared by the same method of L-MPL but without drug). Blood samples were taken for up to 48 hr.

Splenocyte Proliferation

The assay was performed by methods described previously[3]. Intraday and interday coefficients of variation (CV) of replicate samples were less than 8%.

Drug Assay

Plasma concentrations of methylprednisolone were determined by HPLC[11]. The calculated limit of quantification

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for plasma was 8 ng/ml. The (CV) were less than 10% (intra-day and inter-day).

Pharmacokinetics

Plasma data from individual animals were pooled and fitted together. Most plasma methylprednisolone concentration-versus-time data (C_p) were described by polyexponential equations:

$$C_p = \Sigma C_i \cdot e^{-\lambda_i \cdot t} \quad (1)$$

where i = 2 for the MPL and i = 3 for L-MPL studies. To characterize the plasma kinetics after the 10 mg/kg dose of L-MPL, we used a bioexponential equation with an absorption phase (k_a) and lag-time (t_{lag}).

$$C_p = C_1 \cdot e^{-\lambda_1 \cdot t} + C_2 \cdot e^{-\lambda_2 \cdot t} + C_3 \cdot k_a \cdot [e^{-k_a \cdot (t - t_{lag})} - e^{-\lambda_2 \cdot (t - t_{lag})}]/(\lambda_2 - k_a) \quad (2)$$

The intercept coefficients (C_i), slopes (λ_i), k_a, and t_{lag} were estimated by least-squares fitting using the PCNONLIN computer program (SCI, Software Inc., Lexington, Kentucky). The area under the concentration-versus-time curve (AUC = $\Sigma\ C_i/\lambda_i$) and area under the first moment curve (AUMC = $\Sigma\ C_i/\lambda_i^2$) for Eq. 1 and [AUC = $C_1/\lambda_1 + (C_2 + C_3)/\lambda_2$] for Eq. 2 were calculated from the slopes and coefficients. The mean residence time (MRT) was determined as AUMC/AUC. The apparent clearance (CL) was obtained as CL = Dose/AUC. The central (V_c) and total (V_{ss}) volumes of distribution were calculated as V_c = Dose $/\Sigma\ C_i$ and V_{ss} = CL · MRT.

Pharmacodynamics

Data for suppression of splenocyte proliferation *ex vivo* after the multiple 2 mg/kg doses of MPL or L-MPL were analyzed as described previously[3]. The PK/PD model is shown in Figure 1. Plasma is linked to an effect compartment (C_E) by a first-order rate constant (k_d).

$$\frac{dC_E}{dt} = k_d \cdot (C_p - C_E) \quad (3)$$

Receptor binding with C_E is described using the law of mass action.

$$C_E + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C_ER \quad (4)$$

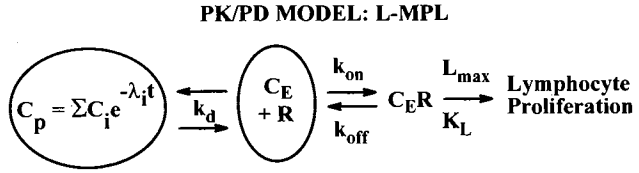

Fig. 1. PK/PD model scheme: plasma compartment (C_p) links to the effect compartment (C_E) with rate constant k_d; the C_E concentration in spleen is drug available for interaction with receptors (rate constants k_{on} and k_{off}); the concentration of drug-receptor complex (C_ER) determines the suppression of lymphocyte proliferation (parameters L_{max} and K_L).

$$\frac{d(R)}{dt} = -k_{on} \cdot (C_E) \cdot (R) + k_{off} \cdot (C_ER) \quad (5)$$

$$\frac{d(C_ER)}{dt} = k_{on} \cdot (C_E) \cdot (R) - k_{off} \cdot (C_ER) \quad (6)$$

The rate constants describing drug/receptor interaction (k_{on} and k_{off}) and MPL equilibration with the effect site (k_d) for each formulation were obtained previously[2], and C_ER is the concentration of MPL - receptor complex. Receptor binding parameters for splenic cytosol were obtained by simulations using the polyexponential coefficients (Eq. 1) estimated for MPL and L-MPL. The inhibition of lymphocyte proliferation (L) versus concentration of bound receptor (R_B or C_ER) data were pooled for both formulations and fitted to the Hill equation:

$$L = \frac{L_{max} \cdot R_B^{\delta}}{K_L^{\delta} + R_B^{\delta}} \quad (7)$$

where L_{max} is effect at baseline, K_L is the concentration of bound receptors which is associated with 50% suppression of lymphocyte proliferation, and the δ coefficient reflects the steepness of the function. The L_{max}, K_L, and δ parameters were used for prediction of expected inhibition of splenocyte blastogenesis versus time for control and liposomal treatments separately.

Statistics

A one-way analysis of variance or t-test was performed on all groups of pharmacokinetic parameters.

RESULTS

Dose-Dependence of MPL versus L-MPL

Figure 2 shows the total methylprednisolone concentrations in rat plasma versus time after 10 mg/kg IV bolus doses of MPL and L-MPL. In all experiments we used freshly prepared L-MPL, and the actual steroid content was determined later. The high dose of methylprednisolone averaged

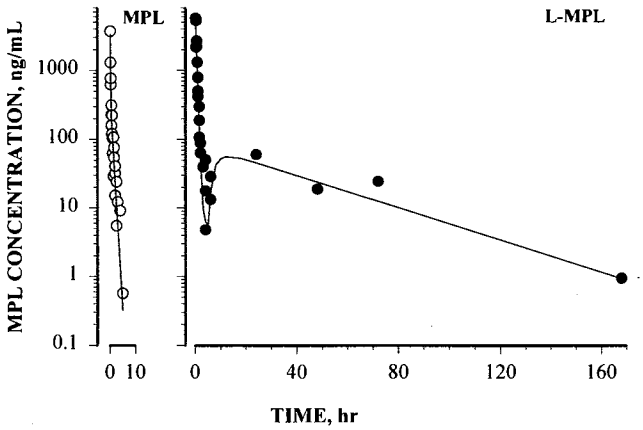

Fig. 2. Plasma concentrations versus time of MPL after a 10 mg/kg dose of free (open circles) and liposomal (closed circles) steroid. The lines represent least-squares fitting of data to Eq. 1 (i = 2) and Eq. 2.

11.8 ± 0.90 mg/kg. After the MPL dose in solution, the drug was eliminated rapidly and could be detected only until 5 hr, whereas with the same dose of L-MPL drug was found in plasma up to 7 days. Figure 2 also shows the fitting of pharmacokinetic data for the biexponential model for MPL (Eq. 1, i = 2) and L-MPL (Eq. 2). At early time points, the kinetic profiles were similar. For MPL, the distribution phase was followed by an elimination phase. For L-MPL, after a short distribution phase, a secondary peak was found. The latter exhibited a lag-time of 5.4 hr, an absorption rate constant of 0.35 hr^{-1}, and attained a maximum at about 10 hr followed by a decline.

The polyexponential coefficients obtained by computer fitting are listed in Table I and pharmacokinetic parameters are shown in Figure 3. The $t_{1/2}$ values for both 2 and 10 mg/kg doses of free drug were similar (0.48 and 0.49 hr), but the increased dose of L-MPL led to a reduced $t_{1/2}$ (from 30.1 to 25.7 hr, p = 0.098). The AUC values increased 2.8-fold (MPL) and 5-fold (L-MPL) for high versus low doses. The CL or MPL after increased from 5.9 to 10.5 L/hr/kg (p = 0.004), but changed marginally in case of L-MPL (1.83 and 2.26 L/hr/kg, p = 0.040) over the 2 to 10 mg/kg dosages. The V_c diminished slightly (from 1.39 to 1.19 L/kg, p = 0.240) for MPL and increased from 0.39 to 1.77 L/kg (p < 0.0001) following the low and high doses of L-MPL. The MRT of MPL decreased (from 0.41 to 0.26 hr, p = 0.003) and increased for L-MPL from 11.9 to 18.6 hr (p = 0.052). The V_{ss} were similar for MPL at both doses (2.42 and 2.72 L/kg) and increased for L-MPL (from 21.9 to 42.0 L/kg).

Multiple Doses of MPL versus L-MPL

After the 8 2 mg/kg IV bolus doses the decline in plasma steroid concentrations was biexponential for MPL and triexponential for L-MPL (Table I). The trough concentrations of methylprednisolone in both cases were very low (2.5 for MPL and 8.5 ng/ml for L-MPL) and could be detected only upon pooling samples from several animals. After the distribution phase, C_1 and λ_1 values after single and multiple doses were comparable (but the latter were larger) for both MPL and L-MPL. The λ_2 was not influenced significantly by multiple injections of MPL ($t_{1/2}$ = 0.38 hr), but decreased in case of L-MPL: the $t_{1/2}$ value increased from 30.1 to 49.5 hr (p < 0.0001). The V_c for free drug was 2-fold smaller after the single versus multiple doses (1.39 and 0.75 L/kg, p = 0.004)), but similar after L-MPL: 0.39 and 0.33 L/kg (p = 0.128). The CL for MPL increased from 5.90 (single dose) to 9.14 L/hr/kg (multiple dose), p = 0.004, and did not depend on the number of doses for L-MPL (1.83 and 1.75 L/hr/kg, p = 0.628). After chronic dosing, the MRT for MPL decreased from 0.41 to 0.22 hr (p = 0.0005), while MRT for L-MPL increased from 11.9 to 18.9 hr (p = 0.263). The V_{ss} for free drug was larger after single versus multiple doses (2.42 and 2.01 L/kg, p = 0.225), while for L-MPL chronic dosing caused of V_{ss} to increase from 21.9 to 33.2 L/kg, p = 0.244.

Pharmacodynamics

The administration of MPL or L-MPL to rats produced marked immunosuppression. The inhibition of splenocyte blastogenesis estimated *ex vivo* after multiple 2 mg/kg IV doses of MPL vs L-MPL is shown in Figure 4. Each data point represents an individual animal. After the last dose of MPL, splenocyte proliferation decreased and then returned towards the baseline by 10 hr. Following chronic dosing of L-MPL, immunosuppression remained pronounced (>95%) over 5 days and returned to baseline by day 8. The lines in Figure 4 are the results of prediction of inhibition of splenocyte proliferation using Eq. 7. Parameters for this function were estimated by fitting the percent of lymphocyte response versus bound receptor data obtained for both experiments using polyexponential coefficients for C_p for each treatment (MPL and L-MPL). The K_L was 2.59 ± 1.34 nM and δ was 13.0 ± 2.5. The lines in Fig. 4 describe the immunosuppression data well.

Coadministration of Methylprednisolone and Liposomes

Figure 5 shows MPL concentrations versus time in plasma after a 2 mg/kg dose of Solu-Medrol following injec-

Table I. Polyexponential Parameters of Methylprednisolone after Free MPL, Mixture of MPL and Liposomes, and L-MPL

Parameter	MPL		MPL + LIP		L-MPL		
Dose, mg/kg(*)	2 (s)[a]	10 (S)[a]	2 (m)[b]	2 (s)[a]	2 (s)[a]	10 (s)[c]	2 (m)[b]
# of animals	8	5	4	4	10	12	12
C_1, (SD) ng/mL	1202 (320)	8000 (1250)	2500 (310)	3300 (780)	5000 (600)	6700 (1800)	7000 (2470)
C_2, (SD) ng/mL	236 (61)	387 (37)	134 (59)	250 (202)	80 (58)	6.62 (1.26)	411 (130)
C_3, (SD) ng/mL	NA	NA	NA	10.4 (8.5)	6.63 (6.20)	64.5 (101.0)	5.15 (3.97)
λ_1, (SD) hr^{-1}	6.72 (1.62)	11.73 (2.16)	17.84 (1.66)	11.32 (3.47)	7.62 (0.86)	2.5 (1.2)	9.67 (2.01)
λ_2, (SD) hr^{-1}	1.44 (0.15)	1.42 (0.34)	1.83 (0.33)	1.69 (1.00)	0.55 (0.31)	NA	1.36 (0.28)
λ_3, (SD) hr^{-1}	NA	NA	NA	0.083 (0.053)	0.023 (0.015)	0.027 (0.002)	0.014 (0.012)

[a] s = single dose.
[b] m = multiple dose study.
[c] Also, k_a, hr^{-1} = 0.54; t_{lag}, hr = 5.4; NA: not applicable.

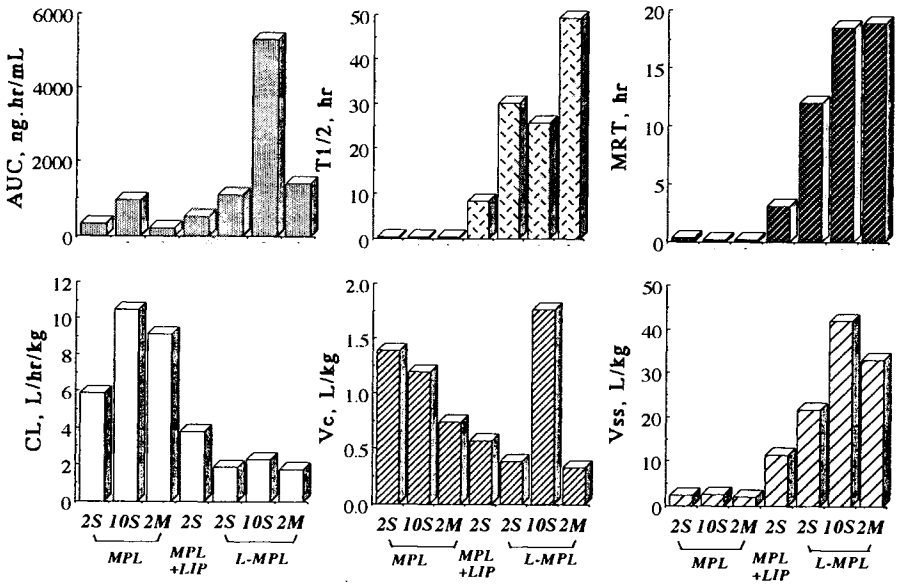

Fig. 3. Pharmacokinetic parameters of MPL after 2 and 10 mg/kg doses of free drug (MPL), coadministration with liposomes (MPL + LIP), or in liposomal formulation (L-MPL). Key: S = Single, M = Multiple doses.

tion of liposomes prepared under similar conditions as L-MPL but without steroid. The initial phases are similar but the terminal phase shows partial prolongation of plasma methylprednisolone in comparison with that of free drug; however, this effect is less than in case of L-MPL. After 24 hr, the MPL concentrations fell below the detection limit.

The pharmacokinetic parameters are presented in Table I and Fig. 3. The V_c was comparable with L-MPL (0.57 and 0.39 L/kg, p = 0.002). The AUC value increased from 339 (free drug) to 526 ng.hr/mL. The CL (3.80 L/hr/kg) was smaller than found for free steroid, but twice larger than for L-MPL ($p < 0.01$). The $t_{1/2}$ was calculated as 8.35 hr, while for free drug $t_{1/2}$ was 0.48 hr and for L-MPL was 30.1 hr ($p < 0.01$). The MRT (3.04 hr) also fell between values for free and liposomal steroid. The V_{ss} calculated for MPL administered with empty liposomes was 4-fold larger than for free drug and 2-fold smaller than for L-MPL.

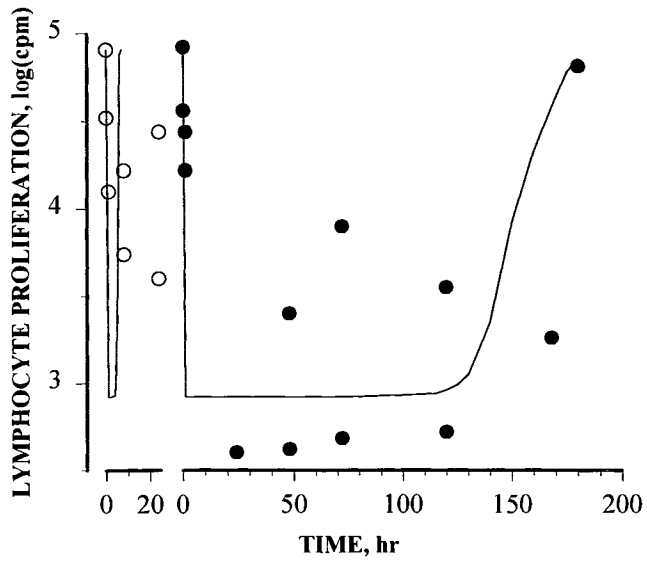

Fig. 4. Inhibition of splenocyte proliferation versus time after multiple 2 mg/kg doses of MPL (open circles) and L-MPL (closed circles). The curves represent simulations made separately for free and liposomal steroid based on Eq. 7.

DISCUSSION

In the present study, AUC values were nonlinear for free drug and the apparent CL value increased 1.7-fold with the 5-fold increase of dose, MRT values diminished about 1.6 times for the high dose. Therefore, a 2 mg/kg dose is a borderline dose where the kinetics of MPL may be linear. Nonlinearities in V_c and V_{ss} were not found.

The encapsulation of MPL into liposomes altered the pharmacokinetics of drug at 2 mg/kg dose: plasma circula-

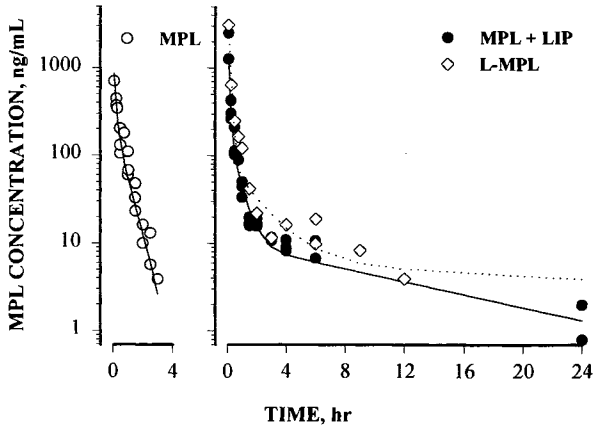

Fig. 5. Plasma concentrations of MPL after the single 2 mg/kg dose of free steroid (open circles), coadministration of MPL and empty liposomes (closed circles), and L-MPL (open diamonds). The lines show least-squares fittings to Eq. 1, i = 2 (left panel), i = 3 (right panel, solid line is MPL + liposomes, dashed line is L-MPL).

tion time was markedly prolonged. The 10 mg/kg dose of L-MPL demonstrates a substantially different profile from the low dose. The triexponential model with lag-time and k_a values fits the data very well. At early time points, MPL concentrations decline in a manner similar to free drug; however, free drug is rapidly eliminated, while liposomes loaded with MPL were sequestered by the lymphatic tissues[2]. When the macrophages of the RES are saturated with liposomes, release of drug may occur and MPL plasma concentrations thus increase. Plasma concentrations reach a maximum at 10 hr and thereafter decline with an elimination rate similar to that which was found for the 2 mg/kg dose. This observation is not in accord with observations for large multilamellar particles[9] where the efflux of liposomes from liver and spleen is negligible for at least 8 hr. This may be because this process is highly dependent on the size and structure of liposomes. Small liposomes are most likely trapped in the first capillary network which they encounter, thus accounting for the initial rate of liposomes deposition in organs[12]. On the other hand, the uptake of liposomes by peripheral macrophages may be inhibited at high concentrations. Release of drug from macrophages could depend on liposome degradation. This involves two processes: phagocytosis or pinocytosis[13]. The first mechanism proceeds much faster ($t_{1/2}$ 13 min) and produces fast efflux of free steroid to plasma.

The AUC values were dose-proportional and CL was similar for both doses of L-MPL. The two-fold increase in V_{ss} indicates nonlinearities in distribution. This may reflect retention of steroid in tissues.

The main reason to investigate chronic dosing of L-MPL comes from concern for accumulation or toxicity related to the prolonged terminal phase of disposition. We thus studied the PK of MPL in plasma after eight 2 mg/kg doses of L-MPL versus MPL administered twice weekly. This dosage regiment of liposomal steroid maintains continuous immunosuppression as measured by lymphocyte blastogenesis[3] which was essential for prolonged allograft function in a rat cardiac transplantation model[4,5]. Slight changes occur in distribution profiles obtained for free MPL after single versus multiple doses. This may be related to the increased CL found with the larger dose.

Chronic dosing of L-MPL caused prolongation of the $t_{1/2}$ (Figure 3). Multiple doses also produced larger V_{ss} values which may reflect deposition of liposomes in macrophages and tissues. The negligible trough concentrations of MPL and L-MPL indicate that steroid accumulation in the body was low.

The inhibition of lymphocyte proliferation is a well established test which reflects cellular immune reactivity. L-MPL administered in multiple doses previously[4] caused marked immunosuppression in rats which underwent cardiac transplantation. The present study indicates that in intact rats after 8 doses of L-MPL, proliferative responses of splenocytes were completely suppressed, while MPL produced similar effects for single and multiple doses. Administration of L-MPL twice a week at low doses did not cause adrenal suppression[3,4] but inhibited splenocyte function due to direct targeting of immune organs[2]. Our PK/PD model reasonably predicted the lymphocyte proliferation data. This suggests that free drug access to glucocorticoid receptors controls the dynamics of such effects.

Simultaneous administration of two compounds may produce drug interactions. Liposomes themselves appear to alter the pharmacokinetics of simultaneous MPL. The changes in plasma profiles were similar but less marked than for L-MPL (Figure 5). This effect might be explained by interaction of drug with liposomal particles. MPL is moderately lipophilic and could partially dissolve in the lipid bilayer of circulating liposomes. The retention of MPL in liposomes depends on its lipid : water partition coefficient and the concentration of lipids. Pretreatment with empty liposomes was found to produce no effect on antipyrine disposition probably due to the low dose of liposomes and the particular mechanism of drug entrapment[14]. The intrinsic properties and the liposome dose coadministered with MPL may be important factors for our steroid.

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