Inhibition of Rat Splenocyte Proliferation with Methylprednisolone: *In Vivo* Effect of Liposomal Formulation

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The effect of a liposomal formulation of methylprednisolone (MPL) on the inhibition of lymphocyte proliferation in spleen cells was investigated following IV dosing in rats. Liposomes do not alter the suppressive action of MPL when placed in lymphocyte culture. Rat splenocytes were found to have greater sensitivity to MPL (EC_{50} = 7.9 nM) than do human peripheral blood lymphocytes (EC_{50} = 28 nM). *In vivo* studies in rats utilized 2 mg/kg IV bolus doses of liposomal MPL compared to drug in solution. Animals were sacrificed at various times post-dosing until 120 h, spleen was excised and, after incubation of lymphocytes with PHA, splenocyte blastogenic responses were assessed by measuring cellular incorporation of ^{3}H-thymidine. The suppressive effect of liposomal MPL in comparison with free drug was significantly prolonged (>120 h vs < 18 h). Inhibition effects versus time were described by a pharmacodynamic model using MPL concentrations in plasma as an input function. A nonlinear relationship was found between suppression of splenocyte proliferation and the concentration of bound glucocorticoid receptors in spleen. Only partial receptor occupancy accompanied complete lymphocyte suppression. The suppression of endogenous corticosterone in plasma for both treatments was similar with values from L-MPL rats returning to baseline after 24 h. These results demonstrate enhanced efficacy of local immunosuppression by targeting spleen with liposomal MPL.

KEY WORDS: liposomes; methylprednisolone; pharmacodynamics; glucocorticoid receptors; immunosuppression; drug delivery.

INTRODUCTION

Corticosteroids are well known as immunosuppressive drugs affecting the activities of various types of circulating immunocompetent cells, namely T-lymphocytes (1,2). Increased production of T-cells occurs in many cases of immuno-modulated responses of animals and humans (3). Corticosteroid effects on lymphocytes are mediated by cytoplasmic receptors (4). The precise intracellular mechanism of interaction of corticosteroids with the immune system remains poorly understood. Their suppressive influence probably proceeds through a block at the transcriptional level of production of the cytokines IL2, IL4 or IL6 (5, 6) and the prevention of IL1 release at a posttranscriptional level (7). Corticosteroids have also been found to suppress lymphocyte proliferation by inhibition of mitogen-induced DNA synthesis (8).

Previously we developed a liposomal formulation of methylprednisolone (L-MPL) and showed that liposomes significantly prolonged blood circulation time of this steroid and altered organ distribution with preference for lymphatic tissues. Markedly extended glucocorticoid receptor occupancy was found in liver and spleen (9). Spleen is known to play a dominant role in lymphocyte homing and maturation (10) and, therefore, regulates the functions of the immune system. It was shown (11) that a liposomal formulation modestly increased the immunosuppression caused by cyclosporine measured as a splenocyte blastogenic response.

We investigated whether a liposomal formulation could enhance the immunosuppressive effect of methylprednisolone. Inhibition of rat splenocyte proliferation was measured both *in vitro* and *ex vivo* after 2 mg/kg IV doses of L-MPL compared to drug in solution.

EXPERIMENTAL

Materials.

L-α-lecitin (phosphatydylcholine) (PC) and L-α-phosphatydylglycerol (egg sodium salt) (PG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Methylprednisolone was obtained from Sigma (St. Louis, MO) and ^{3}H-thymidine (6.7 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY), phytohemagglutinin (PHA) was obtained from ICN Biochemicals (Cleveland, OH), and Ficoll-Paque was purchased from Pharmacia (Piscataway, NJ).

Liposomal Formulation.

Liposomes were freshly prepared as described previously (9). Briefly, lipids (PC:PG 9:1) and drug (5 mole %) 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 NaCL-HEPES buffer, pH 7.4. 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) with a Diaflo M_r 10000 cut-off ultrafilter. The dosage form was stored overnight at room temperature prior to the experiment.

Peripheral Blood Lymphocytes (PBL).

All procedures were conducted under aseptic conditions. Venous blood from healthy volunteers was diluted 1:1 with RPMI 1640, layered onto Ficoll-Paque 400 and centrifuged for 30 min at 1200 rpm. The interphase containing PBL was washed 3 times with RPMI 1640, the cells were resuspended in RPMI 1640 and supplemented with 2 mM L-glutamine, 20 mM HEPES, 15% human serum and 1% penicillin/streptomycin. The number of viable lymphocytes was determined by counting using 0.2% trypan blue. Viability was never <95%.

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PBL Proliferation.

PBL ($5 \cdot 10^4$ cells/well) in supplemented media were cultured in 96 well flat bottom microtiter plates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ). Methylprednisolone or its liposomal formulation in RPMI 1640 at a final concentration range of 10 – 1000 nM were added in triplicate and stimulated with PHA (50 – 250 μg/ml). The cells were incubated for 90 h at 37°C in a 5% CO_2 humidified atmosphere. ^{3}H-Thymidine (0.05 μCi/well) was added during the last 18 h of the culture period. After harvesting (Skatron Instruments Inc., Sterling, VA), proliferation was assessed by measuring cellular incorporation of ^{3}H-TdR using a Packard 1900CA Tri-Carb liquid scintillation counter (Downers Grove, IL). Correction for quenching was carried out using an external standard method.

Animals.

Male Sprague-Dawley rats, weighing 220–270 g, were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Animals were housed in a 12 h light/12 h 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 h 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. For the *in vitro* experiment, rats were used after a sham procedure. Rats (at least 3 per time interval) were sacrificed under light ether anesthesia by removal of blood from the abdominal aorta. Spleen was aseptically excised and immediately placed in RPMI 1640.

Splenocyte Proliferation.

All procedures were done on ice and in sterile conditions. Approximately 0.4 g of organ was placed in a glass tissue homogenizer containing media supplemented with 2 mM L-glutamine, 20 mM HEPES, 10% FCS, 1% penicillin/streptomycin, and 50 μM 2-mercaptoethanol and homogenized by hand. The resulting mixture was layered over Ficoll-Paque 400 and underwent the same procedure as for PBL. Splenocytes, $5 \cdot 10^5$ cells/well were cultured in 6 replicates with stimulation by 150 μg/ml PHA, incubated for a 66 h and pulsed with 2 μCi/well of ^{3}H-TdR 18 h before harvesting. For assessment of a possible influence by liposomes on lymphocyte proliferation, MPL, L-MPL and a mixture of liposomes without drug and MPL at a concentration range 1–10 nM were added. Liposomes were formed as described previously (9). The concentration of lipids in each well were the same and equal to the concentration of lipids in wells with 10 nM MPL in L-MPL. For the *in vitro* glucocorticoid inhibition study, MPL at a concentration range of 0.001–100 nM was added. The optimal incubation time, concentration of PHA, number of cells, and MPL concentrations were assessed in a series of preliminary experiments. Intraday and interday coefficients of variation of replicate samples were less than 8%.

Pharmacodynamic Analysis.

Data for suppression of splenocyte proliferation versus MPL concentration were analyzed using a nonlinear least-squares curve fitting program PCNONLIN (SCI Software, Lexington, KY). The Hill equation for sigmoidal inhibition with a baseline effect parameter was employed:

$$E = E_o - \frac{E_{max} \cdot C^\gamma}{C^\gamma + EC_{50}{}^\gamma} \quad (1)$$

where E is the effect (log(cpm)), E_{max} is maximal effect of cell proliferation with PHA, E_o is the effect at baseline, C is MPL concentration (nM), EC_{50} is the MPL concentration which produces 50% of maximum inhibition, and γ is a sigmoidal coefficient.

Data from our previous study (9) for corticosteroid receptor density versus time in spleen after doses of L-MPL or MPL were fitted to a PK/PD model by postulating a hypothetical "effect" compartment, i.e. drug in spleen which is available for receptor binding (12, 13). Under this interpretation, the effect compartment was modeled as an additional compartment linked to the plasma by a first-order process (k_d), and achieves a drug concentration, C_E. The equation is:

$$\frac{dC_E}{dt} = k_d \cdot (C_p - C_E) \quad (2)$$

Using C_E as a driving force to produce receptor binding, the pharmacodynamics of drug association (k_{on}) and dissociation (k_{off}) with receptors (R) were described using the law of mass action:

$$C_E + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C_ER \quad (3)$$

The data for free receptors (R) in splenic cytosol were fitted to differential equations (2), (4) and (5):

$$\frac{d(R)}{dt} = -k_{on} \cdot (R) + k_{off} \cdot (C_ER) \quad (4)$$

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

where C_ER is the concentration of MPL-receptor complex. The constants describing interaction of drug and receptor (k_{on} and k_{off}) and MPL equilibration with the effect site (k_d) were sought by least-squares fitting using PCNONLIN. It was assumed that drug in plasma controlled access to the effect compartment and C_p was described applying polyexponential equations:

$$C_p = \Sigma\, C_i \cdot e^{-\lambda_i \cdot t} \quad (6)$$

where i = 2 for free MPL and i = 3 for L-MPL studies (9).

Percent inhibition of lymphocyte proliferation (L) as a function of steroid receptor density in spleen for both lipo-

somal and free drug were fitted simultaneously to another 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, R_B is the bound receptor number as was defined above ($C_E R$), K_L is a concentration of bound receptors which is associated with 50% suppression of lymphocyte proliferation, and δ is a coefficient which reflects the steepness of the function.

Using the estimated parameters L_{max}, K_L, and δ we calculated the expected inhibition of splenocyte blastogenic response versus time based on plasma (C_p) and receptor (R_B) data for control and liposomal treatments separately.

Least-squares fittings were evaluated based on goodness-of-fit criteria which included visual inspection, examination of residuals, and correlation coefficients.

Drug Assay.

Plasma concentrations of corticosterone were determined by HPLC (14). Rat plasma was stripped with charcoal, spiked with MPL, corticosterone and dexamethasone (internal standard), and used to create standard curves.

Statistics.

The one way analysis of variance (ANOVA) was performed on the three groups of lymphocyte proliferation data for each concentration of MPL.

RESULTS

In vitro studies.

PBL proliferation.

Inhibition by MPL of human lymphocyte proliferation induced by mitogen is shown on Fig. 1. The primary proliferative response depends on the concentration of PHA. An increase of PHA concentration from 50 to 100 μg/ml increases the response, and a plateau is reached at 250 μg/ml PHA. Lymphocyte proliferation was strongly inhibited by MPL both in the absence and presence of its liposomal formulation. Differences between both treatment forms at drug concentrations of 10–1000 nM were not significant. Fitting the data to a simple sigmoid model ($\gamma = 1$) yields an EC_{50} = 27.7 ± 2.5 nM (curves are not shown). MPL at a concentration of 1000 nM suppressed proliferation so profoundly that it was not affected by the concentration of mitogen.

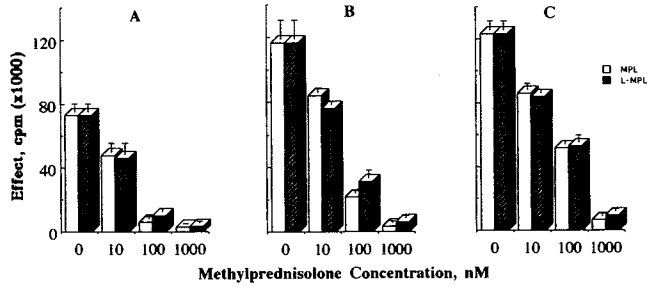

Fig. 1. Inhibition by methylprednisolone of human peripheral blood lymphocyte proliferation induced by PHA at concentrations of: a) 50; b) 100, and c) 250 μg/ml. N = 3 replicates/bar. Open bars—MPL, closed bars—L-MPL.

Splenocyte Proliferation.

Cells obtained from rat spleen were found to be more sensitive to MPL than human cells. Fig. 2 demonstrates the inhibition of splenocyte proliferation (induced by 150 μg/ml PHA) by MPL at the concentration range of 0.01–100 nM. Pronounced effects occur at a MPL concentration of 2 nM, and at 100 nM, proliferation was suppressed totally with ^{3}H-TdR incorporation found to be at the baseline level. The curve in Fig. 2 represents data fitted to the Hill equation (Eq. 1). Table I lists the parameters for the inhibition function for 5 rats; the EC_{50} averaged 7.9 ± 3.8 nM which was 4 times less than for human peripheral lymphocytes. The sigmoidal coefficient was found to average 1.5 ± 0.2.

Lymphocyte proliferation *in vitro* is not affected by the presence of liposomes. Fig. 3 presents a comparison of the treatments of spleen cells, induced by 150 μg/ml PHA, with free MPL, MPL incorporated with liposomes, and MPL added together with PC:PG liposomes formed without drug. The ANOVA performed for mean lymphocyte proliferation data for all groups (N = 6 in each group) showed no significant differences in the study. Neither the liposomes with drug, nor the empty liposomes altered the measured response in comparison with free drug.

In vivo studies.

Splenocyte proliferation.

The IV administration of MPL (control) and L-MPL at 2 mg/kg doses to rats caused a marked suppression of splenocyte proliferation. Fig. 4 shows a comparison of plasma and spleen levels of MPL, receptor density in spleen cytosol, and the proliferative response of splenocyte versus time after

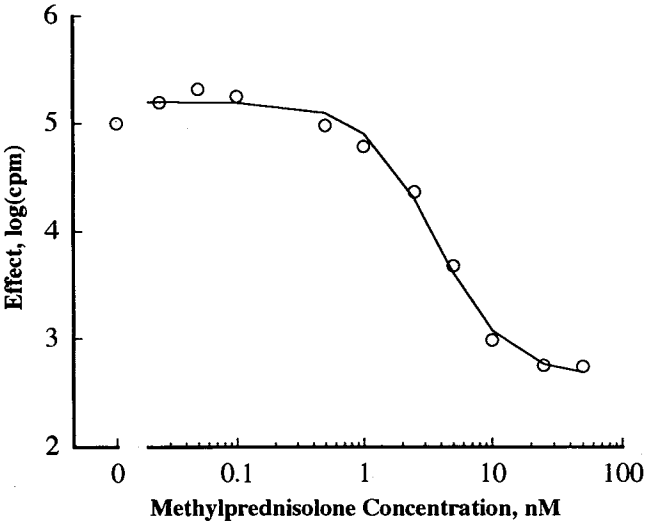

Fig. 2. Suppression of rat splenocyte blastogenic response *in vitro* stimulated by 150 μg/ml of PHA. Points are the mean values of 6 replicates for one animal. Curve represents fitting of data to Eq. 1.

TABLE I. Parameters of Inhibition of Splenocyte Proliferation *in vitro*.

Rat #	EC_{50}, nM	γ
1	5.49 (0.79)	1.19 (0.10)
2	11.88 (2.27)	1.34 (0.48)
3	3.68 (1.07)	1.45 (0.41)
4	6.38 (0.53)	1.73 (0.09)
5	11.83 (2.74)	1.65 (0.25)
Mean (SD)	7.85 (3.78)	1.47 (0.22)

a 2 mg/kg IV dose of free or liposomal drug. Each point represents data from an individual animal. The fitting of pharmacokinetic data for the biexponential and triexponential (L-MPL) models for plasma and spleen concentrations was done previously (9). After a dose of MPL in solution, plasma and spleen concentrations decline quickly with disappearance from plasma after 2.5 h and from spleen after 5 h. In these animals, concentration of free glucocorticoid receptors fell almost immediately to negligible values, and thereafter gradually increased to approach the baseline approximately 6 h after injection of drug. Following L-MPL, receptor occupancy was considerably prolonged.

Least-squares fitting of glucocorticoid receptor data to a partial PK/PD model included a hypothetical "effect compartment" to account for drug availability to receptors. Reasonable characterization of the receptor data was obtained as shown in Fig. 4. The estimated value of k_d was 1 h^{-1}. The apparent association (k_{on}) and dissociation (k_{off}) rate constants for the control data were 1.2 ml/ng·h and 25.6 h^{-1}. Immediately after administration of L-MPL, the plasma kinetics data showed a rapid release of sufficient amount of drug from the liposomes which fully occupies the receptors. At the early times receptor data were sparse. Thus it was necessary to set k_{on} as a constant assuming that the k_{on} value for both formulations was the same and depended on free drug concentration at the effect site. The estimated dissociation rate constant for L-MPL was 9.7 ± 1.7 h^{-1}, and the k_d for L-MPL was calculated as 0.14 ± 0.02 h^{-1}.

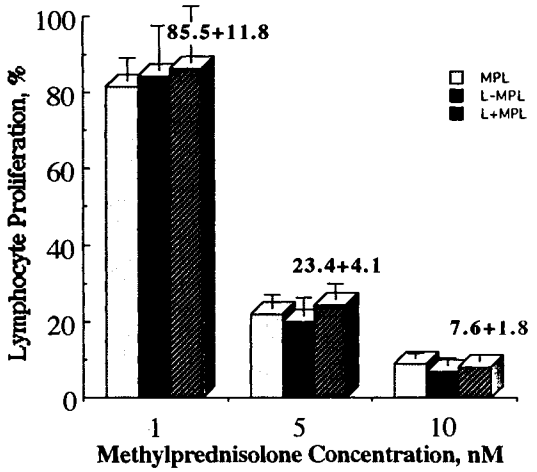

Fig. 3. Comparison of inhibition of splenocyte proliferation induced by 150 μg/ml PHA with free MPL (open bars), L-MPL (solid bars) and MPL added together with liposomes formed without drug (cross-hatched bars). Mean ± SD values for each group are shown.

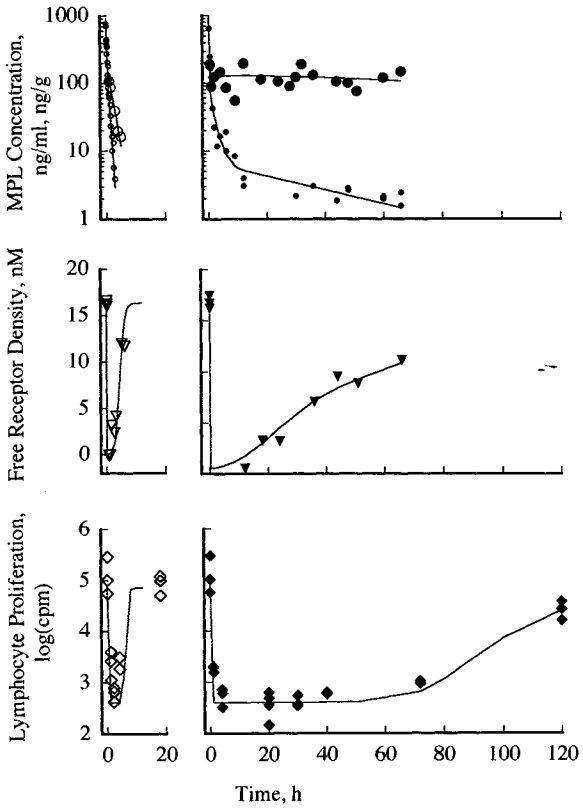

Fig. 4. Methylprednisolone concentrations as a function of time (upper panel) after 2 mg/kg IV dose in rat plasma (small circles) and spleen (large circles). Lines show fitting of plasma and spleen data as described previously (9). Glucocorticoid receptor density versus time in rat spleen (middle panel). Curves are the result of least-squares fitting using Eq. 2 and 4–6. Inhibition of rat splenocyte proliferation versus time (bottom panel). Lines represent a calculations form the fitted sigmoidal Hill function (Eq. 5 and 7). Open symbols—control; closed symbols—liposomal formulation.

Proliferation of splenocytes decreased with time reaching the maximum inhibition at 2 h. Thereafter, the response slowly returned to the baseline at 18 h. Following the dose of liposomal MPL, the level of drug in plasma initially shows a decline similar to that after the control dose, followed by a slow terminal phase. The concentrations of MPL in spleen persist at the level of 100 ng/g for a period of at least 66 h. Liposomes altered the dynamics of MPL: steroid receptors were completely occupied for 12 h post dosing, following by slow recycling over at least 66 h. Immunosuppression, measured as the splenocyte blastogenic response reached a maximum effect at 2 h for the control treatment and at 4 h for L-MPL. By 120 h after dosing with L-MPL, lymphocyte proliferation remained inhibited by 85%.

Fig. 4 also shows calculations of lymphocyte proliferation data using the sigmoidal inhibition function (Eq. 7). Parameters for this function were obtained by fitting the percent of lymphocyte response versus combined bound receptor data from both experiments with administration of free MPL and L-MPL, as shown in Fig. 5. The K_L was 1.9 ± 0.1 nM. Because the inhibition function had a very steep slope, δ was 24.3 ± 7.6. In general, the predicted lines describe the process of inhibition of lymphocyte blastogenic response

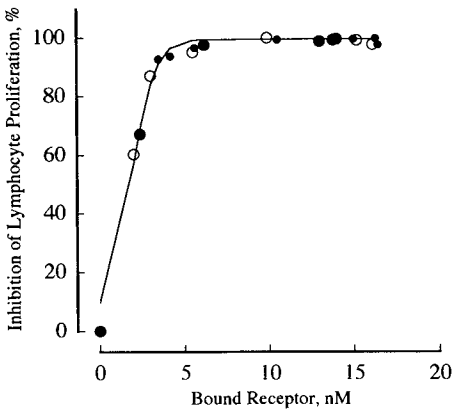

Fig. 5. Relation between receptor density and inhibition of lymphocyte proliferation. Open circles—free drug, closed circles—liposomal formulation. Curve represents fitting of combined data sets to Eq. 7.

very well, suggesting that the access and occupancy of receptors by steroid is associated with the immunosuppressive effect caused by free and liposomal MPL.

Inhibition of corticosterone

Fig. 6 presents the concentrations of corticosterone in rat plasma after a 2 mg/kg dose of L-MPL or drug in solution. The initial declines in concentrations of corticosterone were similar for both treatments. After the dose of L-MPL, the suppression of endogenous corticosterone lasts only for the first 24 h and then returns to baseline. The steroid concentrations in plasma in this case remain at low levels of 1–2 ng/ml until 120 h (Figure 4).

DISCUSSION

The liposomal formulation of methylprednisolone significantly prolongs the blood circulation time of steroid, alters organ distribution with preference to lymphatic tissues, and markedly extends corticosteroid receptor occupancy in liver and spleen (9). Determination of the lymphocyte blastogenic response upon stimulation with mitogen *in vitro* and *ex vivo* is a well established test to gain insight into the functional capacity of cellular immune reactivity (15). We have investigated the changes produced by MPL and L-MPL on lymphocyte blastogenesis *in vitro* and compared human lymphocytes to rat splenocytes.

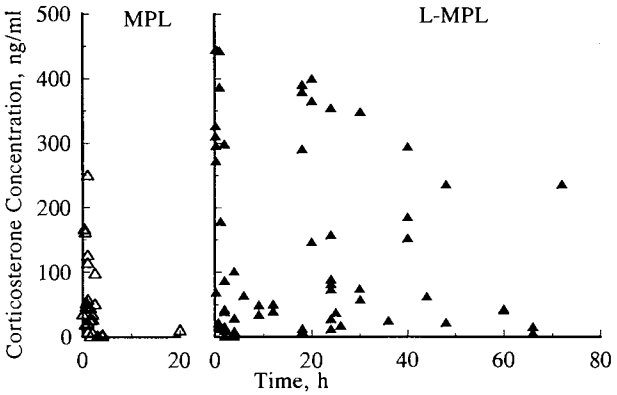

Fig. 6. Corticosterone concentrations in rat plasma versus time after 2 mg/kg IV dose of MPL (open triangles) and L-MPL (closed triangles).

A suppressive role of macrophages in the immune response is well documented (16). Macrophages act as a source of prostaglandins which are potential inhibitors of several lymphocyte functions such as mitogenesis, cytolysis, lymphokine production and proliferation. Liposomes are appreciably taken up and processed by macrophages, and we were interested to see if liposomes would interfere with the lymphocytic response to the steroid.

Inhibition of blastogenesis of human lymphocytes induced by the three concentrations of mitogen did not differ significantly for free and liposomal MPL over the chosen concentration range (Fig. 1). This is probably due to total efflux of drug from liposomes occurring during the incubation period and no biochemical interference from the PC and PG lipids. An optimal concentration of PHA of 150 μg/ml induces a significant proliferation response.

For the assessment of drug immunosuppressive activity, inhibition of blastogenesis of human lymphocytes is used in clinical studies (17,18); preclinical studies usually consider the same properties of lymphocytes obtained from spleen (19). We compared these two methods seeking to evaluate the differences in sensitivity of both sets of cells to MPL, to the liposomal formulation, and also to empty liposomes. Neither human lymphocyte nor splenocyte suppression data were described previously using an inhibition function (20,21).

Values for the EC_{50} and γ for rat spleen cells show that the affinity of drug to suppress proliferation was similar among animals. This is supportive of the use of population data for assessing the differences in responses between control and L-MPL-treated rats. Data for human lymphocytes were fitted to a simple inhibition function ($\gamma = 1$). Rat splenocytes have somewhat greater sensitivity to MPL, with EC_{50} values differing by about four-fold. This difference in sensitivity and in γ values for these two sets of cells might be due to either differences between species (3), source of cells, or media requirements. The spleen plays a major role in migration, maturation and homing of lymphocytes. Trafficking of lymphocytes through the compartments of the spleen can alter specific cell subsets (10) which might be another reason for the different sensitivity of cells between spleen and blood.

^{3}H-Thymidine uptake by splenocytes was not affected by liposomes added simultaneously with MPL to cell culture (Fig. 3), and liposomes alone did not alter the response obtained from control wells containing cells, media and PHA. This is probably due to the fact that during the prolonged culture conditions, all drug was released from the diluted liposomes. These data confirm that diminished lymphocyte reactivity depends only on the concentration of MPL alone.

Systematically administered immunosuppressive agents prevent transplant rejection at a cost of possible renal, hepatic, bone marrow and other organ dysfunction. Therapeutically accepted dosage schedules for corticosteroids promote the development of infections, diabetes and hypertension in allograft recipients. One of the major approaches toward reducing the drug-specific and general adverse consequences of systemic immunosuppression is utilization of

local drug administration to establish a more selective presence of the immunosuppressive agents with a concomitant reduction in systemic drug exposure. This has been accomplished previously for steroids by local administration using a mini pump (22, 23).

Liposome formulations have been attempted for preferential delivery of other immunosuppressive drugs such as cyclosporine (11, 24). Our formulation of corticosteroid directly targets the immune system (9). Methylprednisolone administered in liposomes was sequestered by lymphatic tissues; partitioning of steroid by spleen increased by 25-fold in comparison with drug injected in solution. After initial uptake of liposomes by the reticuloendothelial system, the latter may act as a reservoir of MPL, releasing it slowly back into the blood (Fig. 4). This process could probably explain the prolonged maintenance of drug in plasma at a low level (about 2–3 ng/ml).

The changes in distribution of liposomal MPL caused a markedly prolonged biological response for splenic glucocorticoid receptors. Our previously proposed partial PK/PD model has been extended by adding an effect compartment, connected with plasma by a first-order distribution rate constant. This led to excellent fittings of the data (Fig. 4). The general process involving receptors begins with drug-receptor association, activation, and nuclear translocation of steroid-receptor complexes. This process depends only on the concentration of free drug available to free receptors and we set the value for k_{on} from the control treatment as a constant when applied to the liposomal formulation. The next step in the receptor repertoire is the dissociation and recycling of free receptors from nucleus (25). Estimated values for k_{off} were within the same order of magnitude for both formulations. However, in the case of liposomal MPL, k_d was decreased by 7 times indicating that liposomes may slow the equilibration of free drug with the effect site; this may account for part of the prolonged suppression of the immune system.

Significantly extended immunosuppression caused by MPL and measured by the lymphocyte blastogenic response was found after IV administration of a 2 mg/kg dose of drug in liposomal formulation in comparison with the same dose of drug in solution. The relationship between the inhibition of lymphocyte proliferation and bound steroid receptors for both control and liposomal treatments has a nonlinear character. The good fitting which was obtained for the combined data to the inhibition function (Eq. 7) indicates that liposomes probably do not influence the internal cellular process which produces suppression of blastogenesis. This phenomenon simply depends on the concentration of free drug which binds with the receptors.

Previous studies have assessed relationships for indirect and direct effects of glucocorticoids (1, 2, 26). For prednisolone in rats it was shown that receptor-mediated induction of the liver enzyme tyrosine aminotransferase (TAT) required complete receptor occupancy for a full response (26). *In vivo* models for basophil (2) and for helper T lymphocyte (1) cell trafficking assume that physiologic responses to MPL occurs when all receptors are bound with drug. Our present finding that only partial receptor occupancy with steroid is related to substantial inhibition of lymphocyte proliferation was unexpected. It appears that the main processes which determine the prolongation of immunosuppression is the slow release of MPL from sequestered liposomes and the need for occupation of only part of the available receptors.

The suppression of the endogenous steroid, corticosterone, depends on the concentrations of MPL in rat plasma. The average plasma concentrations of corticosterone in naive rats was about 295 ± 177 ng/ml. The high variability is normal for non-adrenalectomized animals; it was previously reported as 378±211 ng/ml (27). Immediately after administration of free or liposomal MPL, corticosterone begins to decline and 20–22 h later returns to the baseline when drug is appreciably eliminated. Corticosterone was not affected by very low (about 1–2 ng/ml) concentrations of MPL which appeared in plasma due to slow release of drug from liposomes sequestered to liver and lymphatics. The suppression of cortisol (the endogenous steroid in man) produces a maximum effect at 6–8 h post-dosing with methylprednisolone (28). In our case, the dose of free drug provides total suppression of corticosterone at 4 h. Liposomal MPL did not inhibit secretion of corticosterone totally, perhaps because part of the drug circulating in plasma is incorporated with liposomes.

In summary, we previously demonstrated that our liposomal formulation of methylprednisolone increased retention of drug in spleen and markedly prolonged availability of glucocorticoid at the receptor. The present study shows that such preferential delivery of the drug to lymphatic tissues enhanced its *ex vivo* immunosuppressive activity in comparison with MPL administered in solution. Adrenal suppression was not unduly prolonged following L-MPL. These data suggest that selective targeting of MPL will increase its therapeutic index.

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