Dextran-Methylprednisolone Succinate as a Prodrug of Methylprednisolone: Immunosuppressive Effects After *In Vivo* Administration to Rats

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Purpose. To study the immunosuppressive activities of a macromolecular prodrug of methylprednisolone (MP), dextran-methylprednisolone succinate (DEX-MPS), in rats.

Methods. Single 5 mg/kg (MP equivalent) doses of MP or DEX-MPS were administered intravenously to rats, and blood and spleen samples were collected over 96 h. The immunosuppressive activity was determined by the effects of the free or dextran-conjugated drug on the mitogen-stimulated spleen lymphocyte proliferation. Additionally, the number of lymphocytes in the spleen cell suspensions was estimated. Further, the plasma and spleen concentrations of the conjugated and free MP were determined using size-exclusion and reversed-phase chromatographic methods, respectively.

Results. Both MP and DEX-MPS injections resulted in the inhibition of the spleen lymphocyte proliferation. However, the maximal effect of DEX-MPS was significantly ($P < 0.003$) more intense (~100% inhibition) and delayed (24 h) relative to that of MP (~50% inhibition at 2 h). The DEX-MPS injection also resulted in a significantly ($P < 0.0001$) higher decline in the estimated number of spleen lymphocytes (~80% at 24 h), compared with the MP injection (~30% at 2hr). Whereas the plasma and spleen concentrations of MP could not be measured at ≥2 h after the drug injection, relatively high concentrations of DEX-MPS persisted in plasma and spleen for 24 h and 96 h, respectively.

Conclusion. Dextran-methylprednisolone conjugate can effectively deliver the corticosteroid to its site of action for immunosuppression, resulting in more intense and sustained effects when compared with the free drug administration.

KEY WORDS: methylprednisolone; dextran-methylprednisolone conjugate; immunosuppression; lymphocyte proliferation; splenocytes; targeted delivery.

INTRODUCTION

With the introduction of new and potent immunosuppressive drugs, solid organ transplantation has advanced dramatically during the recent years (1). However, this advancement has been at the expense of significant morbidity and mortality due to the high toxicity of immunosuppressants (2,3). Although some of the toxic effects of immunosuppressants are related to their indiscriminate suppression of the immune system, other toxic effects (4), such as nephrotoxicity and neurotoxicity observed with cyclosporine and tacrolimus, are unrelated to the immune suppression (2). However, in most cases, these non-immune related toxicities are serious enough that they would limit the administration of sufficient doses of immunosuppressants (1). Therefore, it is necessary to search for new strategies to improve immunosuppressive protocols while reducing the toxicity of immunosuppressants (3).

One of the strategies to improve the efficacy of immunosuppressants and/or reduce their side effects is to deliver these drugs selectively to their site of action, which is the reticuloendothelial system such as the spleen and the liver. Indeed, other investigators (5–7) have shown the advantages of targeting the immunosuppressive drugs to the reticuloendothelial system in experimental organ transplantation.

Through a series of systematic studies (8,9), we recently investigated the tissue distribution of dextran macromolecules with Mws ranging from 4 kD to 150 kD in rats. These studies revealed that neutral dextrans of high Mw (e.g. 70 kD) slowly, but substantially (up to 70% of the administered dose), accumulated in the liver and spleen, whereas lower Mw dextrans (20 kD or less) were rapidly excreted into urine (8,9). Therefore, high Mw neutral dextrans may be suitable for targeting immunosuppressants to these organs.

Methylprednisolone (MP) is a corticosteroid which has routinely been used for the induction and maintenance of immunosuppression (10) and treatment of acute rejections (11) in organ transplantation. Additionally, recent studies (5,12,13) have shown promising results with a liposomal formulation of the drug which targeted the steroid to the spleen. We selected MP as a model drug to test the use of dextrans for targeted immunosuppression into the reticuloendothelial system. Earlier studies (14) on a conjugate of dextrans with corticosteroids, for local delivery of the steroids to the colon, had demonstrated the feasibility of the conjugate synthesis. Additionally, our preliminary data (15) on a conjugate of dextran 70 kD with MP succinate (DEX-MPS) suggested appropriate pharmacokinetic characteristics for the conjugate. In this article, the immunosuppressive activities of DEX-MPS and free MP after the *in vivo* administration of the conjugated and free drug are reported.

MATERIALS AND METHODS

Chemicals

Dextran with an average Mw of 73 kD and polydispersity of <2, 6α-methylprednisolone (MP), RPMI 1640 (HEPES modification with 1-glutamine), concanavalin A (Con-A), Histopaque lymphocyte separation medium, penicillin/streptomycin, and 2-mercaptoethanol were obtained from Sigma Chemical (St. Louis, MO). 6α-Methylprednisolone 21-hemisuccinate (MPS) was purchased from Steraloids (Wilton, NH). [Methyl-^{3}H]-thymidine (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). For chromatography, HPLC grade acetonitrile (Mallinckrodt Chromar HPLC) was obtained from VWR Scientific (Minneapolis, MN). All other reagents were analytical grade and obtained through commercial sources.

Dextran-methylprednisolone succinate (DEX-MPS) (Fig. 1) was synthesized, purified, and characterized by modification of a previously reported method (14) as described before (16). Briefly, 0.3 g of MPS was dissolved in 3 ml di-

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methylsulfoxide (DMSO), and 360 mg 1,1′-carbonyldidmidazole was added and allowed to react with MPS at room temperature for 30 min. Next, 40 ml of a 5% (w/v) solution of dextran in DMSO and 3.5 mL of triethylamine were added, and the mixture was left at room temperature for 24 hr. This procedure results in a conjugate of dextran and MP with succinic acid as a linker (Fig. 1). The DEX-MPS conjugate was then purified (14) and stored at −20°C as a powder. The purity and degree of substitution of DMP conjugate were then determined using a size-exclusion chromatographic method described before (16). The MP and MPS impurities in the conjugate powder were less than 0.1% (w/w) and the degree of substitution of the powder was 8 mg of MP per 100 mg of the powder.

Animals

All the procedures involving animals used in this study were consistent with the guidelines set by the National Institutes of Health (NIH publication #85-23, revised 1985) and approved by our institutional animal committees. Adult, male Sprague-Dawley rats were purchased from a commercial source and housed in a light- and humidity-controlled animal facility at least 2 days prior to the experiments. The animals had free access to water and food. A total of 75 animals were divided into 3 groups of control, MP injection, and DEX-MPS injection. The mean ± SD of the body weights of rats were 239 ± 24, 234 ± 25, and 238 ± 22 g for the control, MP, and DEX-MPS groups, respectively. Single 5 mg/kg (MP equivalent) doses of MP or DEX-MPS were injected into the tail veins of MP or DEX-MPS groups, respectively. At 10 min and at 2, 24, 48, and 96 h after the drug injection, animals (n = 5 for each group and each time point) were euthanized by means of carbon dioxide, and spleen was harvested. Additionally, a blood sample was collected into a heparinized syringe by cardiac puncture, and plasma was separated. The spleen was divided into two equal parts, and one part was used immediately for the lymphocyte proliferation assay described below. The other half of the spleen and the plasma samples were stored at −80°C for analysis of their contents of conjugated and/or free MP. Each individual experiment consisted of a control, a MP, and a DEX-MPS animal.

Lymphocyte Proliferation Assay

The immunosuppressive activity of MP or DEX-MPS was determined using the spleen lymphocyte proliferation assay (17). Briefly, after gently pushing the spleen through a stainless steel mesh screen, the cells were suspended in an RPMI solution in a sterile petri dish. An aliquot (8 ml) of the cell suspension was then layered on top of a Histopaque solution (3 ml) in a sterile conical tube. After centrifugation (400 g for 30 min), the lymphocyte band was taken and washed with RPMI via an additional centrifugation. The final cell pellet was suspended in RPMI complete medium, containing penicillin (100 IU/ml), streptomycin (100 μg/ml), heat-inactivated rat serum (2.5%), and 2-mercaptoethanol (5×10^{-5} M). The cells were then counted using a hemocytometer.

To each well in a 96-well microplate were added 100 μl of the cell suspension and 100 μl of a Con-A solution in RPMI complete to make a final cell count of 1×10^6 cells/ml and a Con-A concentration of 2.5 μg/ml. Control wells were prepared similarly without Con-A. All samples were prepared in triplicates. The plates were then incubated at 37°C in a humidified incubator containing air: CO_2 (95:5). After 48 h of incubation, 2 μCi of tritiated thymidine was added to each well, and the plate was incubated for an additional 18 h. Finally, the cells were harvested on a glass fiber paper using a Skatron Cell Harvester (Sterling, VA), and the papers were soaked in 3 ml of a Cytoscint liquid (ICN, Costa Mesa, CA) for counting on a Beckman LS 1800 counter.

The optimum concentration of Con-A for stimulation of lymphocytes was determined in a preliminary study with the Con-A concentrations of 0, 1.25, 2.5, 5.0, 10, or 25 μg/ml in the wells.

The viability of the spleen cells was >90%, as determined microscopically by the trypan blue exclusion test.

Lymphocyte Concentrations of the Conjugate

In addition to the above experiments, two separate groups of rats (n = 3/group) were injected with single doses of 5 mg/kg (MP equivalent) of DEX-MPS, and the spleens were collected at 24 or 48 h. The spleen lymphocytes were then harvested as described above and stored at −80°C for the analysis of their contents of DEX-MPS and free MP.

Analytical Methods

Spleen was homogenized in 3 volumes of 2% (v/v) acetic acid, and the resultant homogenate was used for the assay of DEX-MPS and that of free MP and MPS.

The concentrations of free MP and MPS in plasma were determined using a reversed-phase HPLC method described recently (18). The assay has a limit of quantitation of 0.1 μg/ml for both MP and MPS. For determination of MP and MPS in the spleen, the plasma method with a slightly modified mobile phase was used; the mobile phase for the spleen samples contained 0.1 M phosphate buffer (pH 5.8), instead of 0.1 M acetate buffer (pH 5.6) which was used for plasma.

The concentrations of DEX-MPS in plasma or spleen homogenates were measured directly using a modified version of a size-exclusion chromatographic method reported before (19). Briefly, after the addition of 50 μl of 0.4 M phosphate buffer (pH 7.0) and 50 μl of methanol to 100 μl of the sample, the proteins were precipitated by the addition of 20 μl of a 20% (v/v) perchloric acid solution. The conjugate in the supernatant was then separated from the endogenous peaks using a mobile phase of water: acetonitrile: acetic acid (75:25:

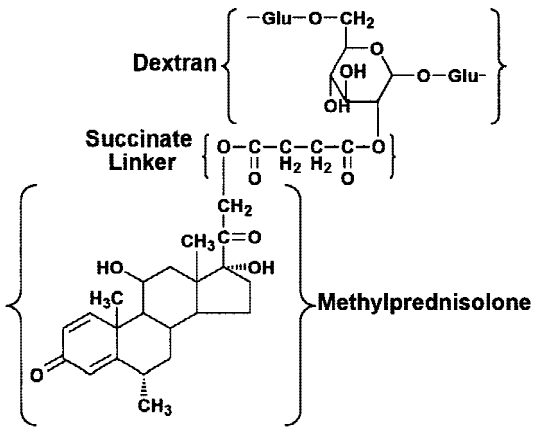

Fig. 1. Chemical structure of dextran-methylprednisolone conjugate.

0.2) for plasma or 0.1 M KH_2PO_4: acetonitrile (75:25) for spleen samples. The sensitivity of this assay was ~1 μg/ml (MP equivalent).

Analysis of Data

The animals were injected in groups of 3, each group consisting of a control (drug free), MP injected, and DEX-MPS injected rats. The count per minute (CPM) for each drug (MP or DEX-MPS)-injected rat were expressed as a percentage of CPM for the control rat used in that group. The differences between the MP and DEX-MPS groups were determined using a two-factor ANOVA at a significance level of 0.05, with time and drug treatments as the factors. The data are presented as mean ± SD.

RESULTS

Preliminary studies indicated that the maximum lymphocyte proliferation is observed at a mitogen concentration of 2.5 μg/ml in the well. Therefore, a mitogen concentration of 2.5 μg/ml was used in all the subsequent studies.

The immunosuppressive activities of MP and DEX-MPS at different times after the drug injections are presented in Fig. 2. Methylprednisolone injection resulted in a decrease in the lymphocyte proliferation starting at 10 min after the drug injection and reaching a maximum effect (~50% decline in CPM) at 2 h (Fig. 2). Thereafter, the effect gradually returned to control values. For DEX-MPS, the effect also appeared at 10 min after the macromolecular injection. However, the maximum effect, which was observed at 24 h after the prodrug injection, resulted in an almost complete inhibition of the lymphocyte proliferation (Fig. 2). Thereafter, the CPM values gradually increased, reaching control values within 96 h. The differences between the effects of MP and DEX-MPS on the lymphocyte proliferation were statistically significant ($P < 0.003$).

The effects of MP and DEX-MPS on the estimated number of lymphocytes harvested from the spleen of rats are shown in Fig. 3 for different times after the drug injection. Generally, there was a significant difference between the two treatments ($P < 0.0001$). For MP, the trend in the number of harvested lymphocytes (Fig. 3) was similar to that for the lymphocyte proliferation assay (Fig. 2); a maximum decrease of ~30% was observed at 2 h, and the levels returned to normal values at 24 h. On the other hand, after an initial rise in the number of cells at 2 h, the number of lymphocytes drastically decreased by 80% at 24 h after the injection of DEX-MPS (Fig. 3). Additionally, in contrast to the lymphocyte proliferation data (Fig. 2), the significant decrease in the number of spleen lymphocytes as a result of DEX-MPS injection persisted during the entire study period (96 h) (Fig. 3).

The plasma and spleen concentrations of DEX-MPS and/or MP after the administration of MP or DEX-MPS are presented in Table 1. After the DEX-MPS administration, relatively high concentrations of DEX-MPS were observed in plasma, whereas the plasma concentrations of free MP were below the limit of quantitation of the assay (<0.1 μg/ml) at all the time points. In the spleen, the concentrations of DEX-MPS were high and persistent (Table 1). Additionally, the spleen concentrations of free MP were measurable up to 48 h after the injection of DEX-MPS (Table 1). No measurable concentrations of MPS were found in the plasma or spleen samples after the administration of DEX-MPS. After the injection of MP, the plasma and spleen concentrations of the drug were measurable only for the 10-min samples (Table 1).

The concentration-effect relationships after the administration of DEX-MPS are depicted in Fig. 4 for both the prodrug and the released MP. A counterclockwise hysteresis was observed when the lymphocyte proliferation was plotted against the prodrug concentrations in spleen. However, when the spleen concentrations of the free drug were used, the hysteresis collapsed (Fig. 4).

The concentrations of DEX-MPS in the lymphocyte suspensions at 24 and 48 h after the injection of DEX-MPS were below the sensitivity of the DEX-MPS assay (1 μg/ml). Therefore, DEX-MPS was hydrolyzed by 0.1 M NaOH (16), and the released MP was quantitated using the reversed-phase assay with a limit of quantitation of 0.1 μg/ml. The MP assay revealed a DEX-MPS concentration of 0.305 ± 0.146 μg/ml for the lymphocyte suspensions harvested 24 h after the injection of DEX-MPS. The concentrations of free MP in the lymphocyte suspensions were below the detection limit. Further, at 48 h after the conjugate injection, no free or conjugated MP was detected in the spleen lymphocytes.

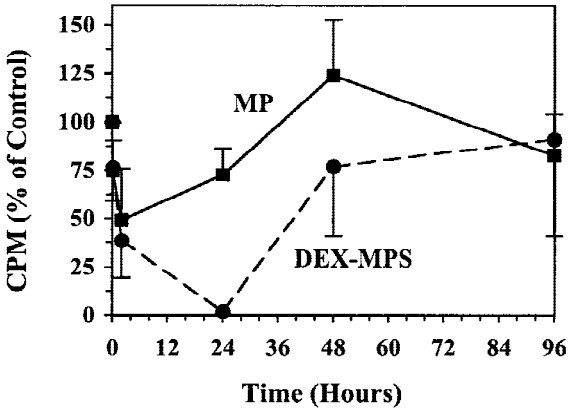

Fig. 2. The effects of a single iv dose (5mg/kg; MP equivalent) of DEX-MPS (●) or MP (■) on the spleen lymphocyte proliferation in the presence of 2.5 mg/ml of concanavalin A (n = 5). Data are presented as mean and standard deviations.

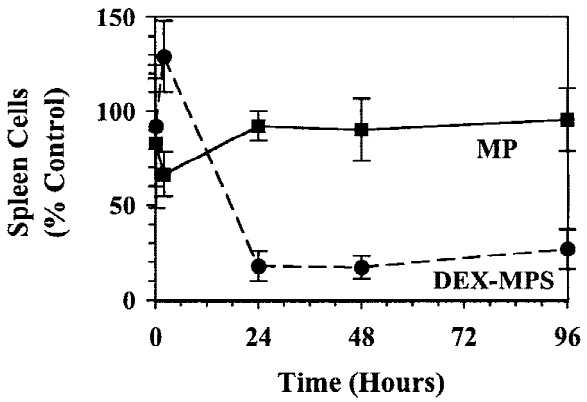

Fig. 3. The effects of a single iv dose (5mg/kg; MP equivalent) of DEX-MPS (●) or MP (■) on the estimated number of cells harvested from the spleen of rats at different times after the drug injection (n = 5). Data are presented as mean and standard deviations.

Table 1. Mean ± SD of Plasma and Spleen Concentrations of DEX-MPS and/or MP after Single IV Doses of 5 mg/kg MP as Free Drug or in the Form of DEX-MPS (n = 5)

	DEX-MPS Injection				MP Injection	
	Plasma conc. (μg/ml)[a]	Spleen conc. (μg/g)			Plasma conc. (μg/ml)	Spleen conc. (μg/g)
Time (h)	DEX-MPS	DEX-MPS	MP	Ratio[d]	MP	MP
0.167	140 ± 10.3	—[b]	0.235 ± 0.071		1.81 ± 0.479	4.44 ± 0.237
2	12.8 ± 3.55	54.9 ± 14.3	0.692 ± 0.264	86.3 ± 27.2	—[c]	—[c]
24	1.67 ± 0.537	179 ± 17.1	1.23 ± 0.224	150 ± 29.2	—[c]	—[c]
48	—[b]	123 ± 10.6	0.284 ± 0.041	448 ± 84.5	—[c]	—[c]
96	—[b]	84.2 ± 17.4	—[c]		—[c]	—[c]

[a] No measurable concentrations of MP or MPS were found in plasma after DEX-MPS injection.
[b] Below the sensitivity of the size-exclusion assay for DEX-MPS (1 μg/ml).
[c] Below the sensitivity of the reversed-phase assay for MP (0.1 μg/ml).
[d] DEX-MPS:MP concentration ratio.

DISCUSSION

Previous studies (5–7,12,13,20–22) have used liposomes for altering the pharmacokinetics and pharmacodynamics of different immunosuppressant, such as cyclosporine (6,20,21), tacrolimus (7,22), and MP (5,12,13). While some of these studies (5–7) showed an improved efficacy and/or reduced toxicity with the liposomal formulations, others showed no change (20) or a reduction (21) in the effects of the liposome encapsulated agent. This is because the pharmacokinetics of the liposomal agents are substantially affected by the composition of the liposomes (20) which may be different in different studies. Additionally, liposomal formulations may cause irregular pharmacokinetics and secondary effects which may complicate their use (23). Other complicating issues such as relatively short (<1 week) stability during storage (6,12) and the potential *in vivo* adverse effects of the carries (e.g. blood clotting, capillary blocking, and lipid side effects) (24) should also be considered before clinical application of liposomal formulations.

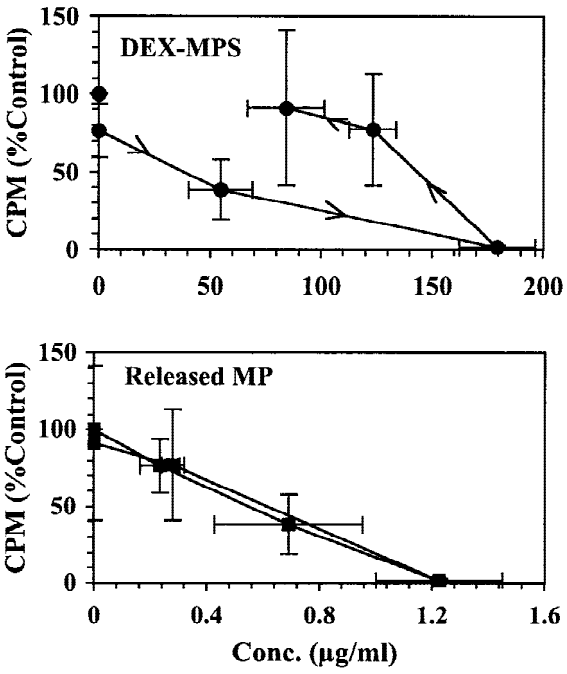

Fig. 4. The concentration-effect relationships after a single iv dose (5mg/kg; MP equivalent) of DEX-MPS when the effect is plotted against DEX-MPS (top) or released MP (bottom) (n = 5). Symbols represent the means and vertical and horizontal bars indicate standard deviations for the effect and concentrations, respectively.

We proposed (15) to use an alternative approach in targeted delivery of immunosuppressants to the reticuloendothelial organs by using a macromolecular delivery system based on dextrans which are glucose polymers with predominantly α-1,6-linkage (25). Dextrans have been investigated as macromolecular carriers for delivery of various drugs, including anticancer agents (25). Our previous studies with dextrans (8,9) indicated that dextran with a Mw of 70 kD substantially accumulates in the reticuloendothelial organs such as the spleen. Therefore, we hypothesized that the conjugation of MP to dextran 70 kD would result in a favorable alteration of the pharmacokinetics of MP for immunosuppression. Indeed, the concentration-time data reported in Table 1 indicate that the conjugation of MP with dextran 70 kD substantially altered the plasma and spleen pharmacokinetics of the drug. Overall, high concentrations of the conjugate in the spleen persisted for the entire duration of the study (96 h), whereas after the injection of the free drug, the spleen concentrations of the drug could not be measured at ≥2 h after the drug injection (Table 1).

The corticosteroids may affect the immune response in several ways, including a reduction in the number of circulating lymphocytes and an alteration in the immune response of lymphocytes (26). The significant (~80%) and persistent (96 h) reduction in the number of spleen lymphocytes after the injection of DEX-MPS (Fig. 3), compared with free MP injection, is consistent with the substantial and sustained concentrations of DEX-MPS in the spleen (Table I). However, the inhibition of the spleen lymphocyte proliferation after DEX-MPS injection (Fig. 2), although substantial (~100%) and significantly larger than that after the MP injection, is not sustained beyond 48 h. Therefore, a plot of lymphocyte proliferation effect against the concentrations of DEX-MPS showed a counter clockwise hysteresis (Fig. 4), indicating reduced response to the same spleen concentration of DEX-MPS at later times. However, when this effect was plotted against the free MP released from DEX-MPS in the spleen, the hysteresis collapsed (Fig. 4). These data suggest that the inhibition of lymphocyte proliferation after DEX-MPS is re-

lated to the released MP. Indeed, *in vitro* data (unpublished data) on the spleen lymphocytes have shown that DEX-MPS has minimal immunosuppressive activities by itself; the *in vitro* concentrations producing half of maximum effects for inhibition of spleen lymphocytes were 3.1 and 52 nM for the free and dextran conjugated MP, respectively.

The lack of a substantial inhibition of lymphocyte proliferation at 48 and 96 h after the injection of DEX-MPS (Fig. 2), despite high spleen concentrations of the conjugate at these times (Table I), may be due to a time-dependent accumulation of DEX-MPS in different cells of the spleen (lymphocytes and macrophages). Between 24 and 48 h after the DEX-MPS injection, the whole spleen concentrations of the conjugate declined by only 30% (from 179 to 123 μg/g; Table I). However, the concentrations of DEX-MPS in the lymphocyte suspensions declined substantially faster (from 0.305 ± 0.146 μg/g at 24 h to significantly below the limit of quantitation at 48 h), suggesting that DEX-MPS found in the spleen at later hours (48 and 96 h) mostly resides in non-lymphocyte cells (i.e., macrophages).

Cell-dependent dextran concentration-time course has also been reported for the liver cells in a histochemical study (27). Dextran-like material was found in the parenchymal cells as soon as 2 h after injection and the dextran contents of these cells declined by 24 h. However, for Kupffer cells, dextran-like material appeared at 4 h after the injection, reached a maximum at 24 h, and significant amounts were still present at 96 h (27). Our limited data in spleen are consistent with these findings in the liver.

Previous *in vitro* studies in our laboratory (28) indicated that a very slow hydrolysis DEX-MPS to MPS or MP in both blood and liver lysosomes occurs mostly via a first order chemical hydrolysis. On the other hand, the conversion of MPS to MP is mediated by esterases (28). Our concentration-time data in spleen (Table 1) indicate a non-linear relationship between the DEX-MPS and free MP concentrations; with time the DEX-MPS:MP concentration ratio significantly increased (Table 1). The reason(s) for the change in this ratio is(are) not clear from the data presented here. However, a possible mechanism could be an incomplete conversion of MPS to MP at later times after the conjugate administration. This could be due to a time-dependent depletion of cellular esterases and/or a shift in the distribution of DEX-MPS to different spleen cells with lower esterase reserves.

A very recent study (29) on the disposition of a conjugate of dextran with tacrolimus reported a 2000-fold increase in the plasma AUC of the radioactive conjugate, compared with the AUC after the administration of the free drug. In contrast to our study, however, the spleen concentrations of the radioactivity were not substantially increased by conjugation (29). It should be noted that these investigators (29) used a negatively charged dextran (carboxy-pentyl dextran) in their studies, whereas dextran used in our study was neutral. Previous studies (30) have shown that electric charge would substantially affect the tissue distribution of dextrans, with negatively charged dextrans having lower tissue uptake and higher plasma concentrations. Nevertheless, it remains to be seen whether such a dextran-tacrolimus conjugate will have immunosuppressive activity superior to that of free tacrolimus after *in vivo* administration.

In conclusion, it was shown that a macromolecular prodrug of methylprednisolone increases the intensity and duration of the immunosuppressive activity of the corticosteroid after its *in vivo* administration to rats. The increased activity is apparently related to the macromolecule-induced significant alterations in the concentrations of the steroid in the spleen.

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