Dextran-Methylprednisolone Succinate as a Prodrug of Methylprednisolone: Local Immunosuppressive Effects in Liver after Systemic Administration to Rats

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Purpose. The purpose of this work was to study the local immunosuppressive effects of systemically administered methylprednisolone (MP) and its prodrug, dextran-methylprednisolone (DMP), in rat livers.

Methods. Single 5 mg/kg (MP equivalent) doses of MP or DMP were injected intravenously to rats, and livers were isolated at different time points (0–72 h; n = 4/time point). Isolated livers were stimulated *ex vivo* with bacterial lipopolysaccharide, and outlet perfusate and bile samples were analyzed for their concentrations of tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay. The area under the perfusate TNF-α concentration-time curve (AUC) was used as a measure of immune response. Hepatic concentrations of MP and DMP were also measured by high-performance liquid chromatography.

Results. Both MP and DMP resulted in a decrease in lipopolysaccharide-induced increase in TNF-α AUC. MP injection 8 h before liver isolation resulted in a maximum of 50% decrease in TNF-α AUC. Compared with MP, the maximum effect of the prodrug (DMP) was both more intense (~80% reduction in TNF-α AUC) and delayed (maximum inhibition at 24 h). Overall, the area under the effect (% inhibition of TNF-α)-time (%inhibition·h) for DMP (3680 ± 406) was approximately four times more than that for the parent drug (846 ± 114). Whereas the MP concentrations in the liver were not quantifiable after the injection of the parent drug, relatively large concentrations of DMP and regenerated MP were found in the liver of DMP-injected rats.

Conclusions. After systemic administration to rats, both MP and DMP exhibit local immunosuppressive effects in the liver. The local effects of the prodrug (DMP), however, appear to be more intense and sustained than those of the parent drug (MP).

KEY WORDS: methylprednisolone; dextran prodrugs; local immunosuppression; liver perfusion; hepatic delivery.

INTRODUCTION

Immunosuppressive drugs form the mainstay in the therapy of orthotopic liver transplantation. The discovery of newer and more potent drugs has significantly improved the outcome of liver transplantation (1,2). However, many of these agents cause significant toxicities in patients, thus limiting their use (2). Some of these toxic effects, such as cardiotoxicity, nephrotoxicity, and neurotoxicity, are caused by the nonspecific actions of these agents at the extra-hepatic sites of the body (3). These extra-hepatic toxicities seriously limit the optimum use of immunosuppressants in various therapeutic protocols. Therefore, there is a need for alternate approaches to increase the efficacy and/or decrease the toxicity of immunosuppressants.

It has been proposed that targeting of immunosuppressive drugs to the transplanted organ would increase the efficacy and improve the toxicity profiles of this class of drugs (4). Additionally, local immunosuppressive action at the site of transplantation has been found to be the main determinant of graft viability (4). Indeed, the strategy of local immunosuppression at the site of transplantation has been shown by many investigators to be superior to nonspecific systemic immunosuppression (5–7). Therefore, targeting of immunosuppressants to the liver might increase graft acceptance while reducing the morbidity and mortality associated with the use of these drugs in liver transplantation.

We have proposed the use of dextrans as macromolecular carriers to target immunosuppressive drugs to the reticuloendothelial tissue, like liver and spleen (8). To demonstrate the feasibility of this approach, the corticosteroid methylprednisolone (MP) was used as a model immunosuppressive drug attached to a 70-kDa dextran macromolecule. Previous studies (9) in our laboratory showed that compared with the parent drug, the dextran conjugate of MP, dextran-methylprednisolone succinate (DMP), preferentially accumulates in the liver and spleen of rats. The active drug (MP) is then slowly released from DMP, with area under the tissue concentration-time curves (AUCs) of the regenerated MP in the spleen and liver being 55- and 4.8-fold, respectively, larger than those after the administration of the equimolar doses of the parent drug (9). Furthermore, pharmacodynamic studies (10) showed that the systemic immunosuppressive activity of the conjugate is both more intense and sustained than that of the parent drug. Because a significant portion of the DMP dose is delivered to the liver (9), here we hypothesized that DMP would also exhibit higher local immunosuppression in the liver compared with the administration of the equimolar doses of the parent drug. Higher local immunosuppression in the liver would be especially advantageous in liver transplantation. Studies reported here are designed to test this hypothesis in a recently developed isolated perfused rat liver model (11) that can be used for delineation of the local immunosuppressive effects in the liver following systemic administration of drugs to rats.

MATERIALS AND METHODS

Chemicals

Dextran with an average molecular weight of 73 kDa and polydispersity of <2, 6α–methylprednisolone (MP), *Escherichia coli* (Serotype 0111:B4) lipopolysaccharide (LPS), sodium taurocholate, and transaminases kit for measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6α-Methylprednisolone 21-hemisuccinate was obtained from Steraloids (Wilton, NH, USA). Enzyme-linked immunosorbent assay (ELISA) kits for measurement of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were purchased from Biosource International, Inc (Camarillo, CA, USA). Xylazine and ketamine sterile solutions

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for anesthesia were from Lloyd Laboratories (Shenandoah, IA, USA) and Fort Dodge Animal Health (Fort Dodge, IA, USA), respectively. For chromatography, high-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from EM Science (Gibbstown, NJ, USA). All other reagents were analytical grade and obtained through commercial sources.

Dextran–methylprednisolone succinate (DMP) was synthesized, purified, and characterized as described before (12). The MP and 6α-methylprednisolone 21-hemisuccinate 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 powder.

The dosing solution of MP was prepared in a mixture of water:ethanol:PEG400 according to a previously described method (13), whereas DMP dosing solution was prepared in distilled water.

Animals

All the procedures involving animals used in this study were consistent with the guidelines set by the National Institute of Health (NIH publication #85-23, revised 1985) and approved by our Institutional Animal Care and Use Committee. 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 before experiments. The animals had free access to food and water.

A total of 56 rats were divided into 14 groups, each consisting of four rats. Five groups were used for determination of the time courses of the effects of each of the two drugs (MP or DMP), and the remaining four groups were used as negative control, positive control, MP vehicle, and DMP vehicle groups. The MP and DMP groups received single 5-mg/kg doses (MP equivalent) of MP or DMP, respectively, into their tail veins. At 2 (MP-2 h), 5 (MP-5 h), 8 (MP-8 h), 12 (MP-12 h), and 24 (MP-24 h) h after the injection of MP and at 5 (DMP-5 h), 12 (DMP-12 h), 24 (DMP-24 h), 48 (DMP-48 h), and 72 (DMP-72 h) h after the injection of DMP, rats were anesthetized and livers were isolated and stimulated with LPS as described below. The negative and positive control groups did not receive any drug treatments before liver isolation. However, the livers isolated from the positive control group were subjected to LPS stimulation *ex vivo*, whereas negative controls did not receive LPS. Because the dosing vehicles were different for MP and DMP, two additional groups only received MP or DMP vehicles, and the livers were isolated at a time corresponding to the maximum effects for MP (8 h) or DMP (24 h). The mean ± SD of the body weight of the rats were 257 ± 6, 248 ± 20, 252 ± 14, 252 ± 19, 249 ± 24, and 243 ± 6 g for the negative control, positive control, MP injection, DMP injection, MP vehicle, and DMP vehicle, respectively.

Isolated Liver Perfusion Model

The local immunosuppressive activity of MP or DMP after systemic administration was determined using an isolated perfused rat liver model, which was developed recently in our laboratory (11). In this model, livers are isolated from rats and stimulated *ex vivo* with LPS to release cytokines such as TNF-α in the outlet perfusate. The model allows the delineation of the local effects of systemically-administered immunosuppressants in the liver.

The techniques used for isolation and cannulation of the livers have been reported by us before (14,15). Briefly, rats were anesthetized by an intramuscular injection of a ketamine:xylazine mixture (80:12 mg/kg), and bile duct, the hepatic portal vein (inlet), and the thoracic inferior vena cava (outlet) were cannulated. The liver was then excised and transferred to a temperature (37°C)-controlled perfusion tray. The livers were perfused for 120 min in a single-pass manner using a water-jacketed glass apparatus. The perfusate was Krebs-Henseleit bicarbonate buffer (pH 7.4) fortified with 1.2 g/L glucose and 75 mg/L of sodium taurocholate and was oxygenated with a 95:5 oxygen:carbon dioxide mixture. The perfusate flow rate was 30 mL/min (3–4 mL/min/g liver weight).

After isolation, all livers were allowed to stabilize for ~10 min before the start of the experiments. Except for the negative control group, all livers were infused with a 300 μg/mL solution of LPS at a rate of 50 μL/min for the initial 20 min (a total of 300 μg) to stimulate the Kupffer cells (11).

Liver viability was tested as described before (11,14,15) through macroscopic examination of the liver, relatively high and constant bile flow rates, low levels of AST and ALT at the beginning and end of perfusion, and wet liver weight of <4% of the body weight at the end of perfusion.

Sample Collection

Samples of the perfusate (1 mL) were taken from the outlet at 0, 15, 30, 45, 60, 80, 100, and 120 min. Additional samples were taken from the outlet at the start (time zero) and the end (120 min) of perfusion and stored at 4°C for measurement of liver transaminases (AST and ALT) within 1 week. Bile samples were also collected into preweighed microcentrifuge tubes at 30-min intervals. Perfusate and bile samples were stored at –80°C until further analysis. At the end of perfusion, the livers were blotted dry and stored at –80°C for analysis of DMP and/or free MP concentrations.

Sample Analysis

The concentrations of TNF-α, IL-1β, and IL-6 in the outlet perfusate and bile were quantitated using commercial ELISA kits. The TNF-α assay uses a 50-μL sample and has a minimum detectable concentration limit of 4 pg/mL and intra- and inter-assay precision coefficient of variation of <4%. The IL-1β and IL-6 assays each use a 100-μL sample and have minimum detectable concentration limits of 3 and 8 pg/mL, respectively. The intra- and inter-run precision coefficients of variation of the IL-1β and IL-6 assays are <9 and <6%, respectively. Liver transaminases (ALT and AST) in the perfusate were quantitated based on a colorimetric method using a commercial kit from Sigma.

The livers were homogenized with 3 volumes of 2% (v/v) acetic acid, and the resultant homogenate was used for the HPLC analysis of DMP and/or free MP. The concentrations of free MP were determined using a reversed-phase HPLC method (16) modified for measurement of MP in tissue homogenates (9). The assay had a limit of quantitation of 0.1 μg/mL of homogenate based on a 0.5-mL homogenate sample. For measurement of DMP in the liver homogenate, a size-exclusion assay described before (9) was used with a mi-

nor modification; instead of dissolving the residue after precipitation of DMP in 200 μL of 0.1 M KH_2PO_4:acetonitrile (65:35), samples were first dissolved in 120 μL of 0.1 M KH_2PO_4. Then, 80 μL of acetonitrile was added and, after a brief vortex mixing and centrifugation, 100 μL was injected into the HPLC. The lower limit of quantitation of the assay was 1 μg/mL of homogenate based on a 100-μL homogenate sample.

Data Analysis

The concentration of TNF-α in the outlet perfusate was plotted against the period of perfusion of the liver for each rat. The area under the TNF-α concentration (outlet perfusate)-time curve (AUC) was estimated using the linear trapezoidal rule. Percent inhibition of TNF-α AUC as a result of MP or DMP treatment was calculated using the following equation:

$$\%\text{Inhibition} = \frac{AUC_{Positive\ Control} - AUC_{MP/DMP}}{AUC_{Positive\ Control}} \times 100$$

where $AUC_{Positive\ Control}$ and $AUC_{MP/DMP}$ refer to the TNF-α AUC in the positive control livers (LPS + no drug treatment) and livers isolated from rats treated with either MP or DMP (LPS + drug treatment), respectively. Similarly, the %inhibition of TNF-α excretion in bile by MP or DMP injection was estimated by comparing the total amounts of TNF-α excreted in bile (0 to 120 min) in each treatment and time group with the values in the positive control livers. Negative %inhibition values were considered as zero (no inhibition).

The effect of time of drug administration on the TNF-α AUC in the outlet perfusate within each drug treatment group (MP or DMP) was determined using one-factor analysis of variance (ANOVA). A two-way ANOVA was used to test the effects of time of drug treatment and the collection interval on the bile flow rates and the amounts of TNF-α excreted in bile. In the presence of a significant difference, comparison of each time point in MP or DMP treatment group with the no-treatment group (positive control) was conducted using a Dunnett's post hoc analysis. The statistical difference between the total area under the effect (%inhibition of TNF-α AUC in the perfusate or TNF-α amount in bile)-time curve for MP or DMP treatment was determined using the method of Bailer (17). All tests were performed at a significance level (α) of 0.05. Data are presented as mean ± SD.

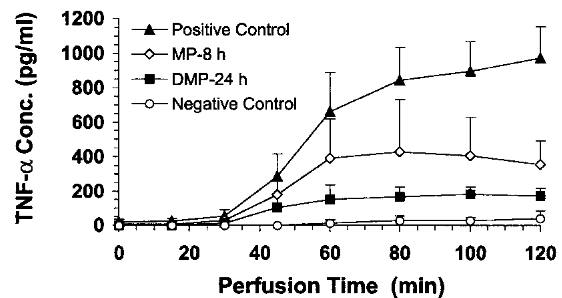

Fig. 1. The concentration-time courses of tumor necrosis factor-α in the outlet perfusate of livers isolated from untreated rats and perfused *ex vivo* in the absence (negative control) or presence (positive control) of lipopolysaccharide. Also shown are the time courses of the perfusate tumor necrosis factor-α in lipopolysaccharide-stimulated livers isolated from rats at 8 or 24 h after the intravenous (i.v.) injection of single 5 mg/kg doses (methylprednisolone equivalent) of methylprednisolone (DMP-8 h) or dextran-methylprednisolone (DMP-24 h), respectively. Symbols and bars represent average and SD values, respectively (n = 4).

RESULTS

The plots of the concentration of TNF-α in the outlet perfusate against perfusion time in negative (no drug, no LPS) and positive (no drug, LPS) control groups are presented in Fig. 1. Also included in Fig. 1 are time courses of the perfusate TNF-α concentrations at the time of maximum inhibitory effect of MP (8 h) and DMP (24 h); for brevity, the profiles for the other eight time groups after the injection of MP and DMP are not shown. The perfusate concentrations of TNF-α in the negative control livers were low (<100 pg/mL) during the entire period of perfusion. Injection of LPS into the liver (positive control) mediated a substantial increase in TNF-α concentrations (~1000 pg/mL at 120 min). The LPS-mediated increase in the concentrations of TNF-α was significantly attenuated in MP-8 h and DMP-24 h groups, with the attenuation being greater for the DMP-24 h group (Fig. 1).

The average perfusate AUC values of TNF-α at different times after the injection of MP or DMP are shown in Table I. In rats not injected with MP or DMP (time zero or positive control), the TNF-α AUC was large. When compared with the AUC values in the absence of drug treatment, both MP and DMP treatments resulted in a time-dependent decrease in the TNF-α AUC (Table I). For MP group, the TNF-α AUC values at 2, 5, and 8 h after the drug injection were significantly lower than those for the positive control livers (time zero). However, at 12 and 24 h after the drug injection, the

Table I. The Average (SD) AUC Values (pg.min/mL) of Tumor Necrosis Factor-α in the Outlet Perfusate of Livers Isolated from Rats Treated with a Single 5 mg/kg Dose (Methylprednisolone [MP] Equivalent) of MP or Dextran-Methylprednisolone (DMP) Intravenously and Stimulated *ex Vivo* with Lipopolysaccheride (n = 4 for Each Group)

	Time after drug administration (h)							
Treatment	0[a]	2	5	8	12	24	48	72
MP	6,700 (15,100)	33,900[b] (15,000)	33,500[b] (7,230)	30,400[b] (16,700)	36,300 (9,960)	52,700 (15,000)	—	—
DMP		—	39,200 (21,000)	—	29,300[b] (19,500)	14,500[b] (4,310)	28,700[b] (18,400)	48,500 (1,930)

[a] Positive control group.

[b] Significantly lower than no drug treatment based on one-way analysis of variance and Dunnett's test.

effect of MP on TNF-α AUC was not significant. For DMP group, the decrease in TNF-α AUC was significant ($p < 0.05$) at 12, 24, and 48 h after the prodrug administration. However, the apparent decreases at 5 and 72 h after the conjugate injection did not reach statistical significance (Table I).

The TNF-α AUC values in the livers of vehicle-injected rats, which were subjected to LPS infusion *ex vivo*, were 56,400 ± 6,130 and 59,300 ± 8,860 pg·min/mL for the MP and DMP dosing vehicles, respectively. These values were not significantly (one-way ANOVA) different from the TNF-α AUC value for the positive control livers (61,700 ± 15,100 pg·min/mL), which did not receive the vehicles. As expected, the TNF-α AUC for the negative control group, which did not receive LPS, was very low (1800 ± 1180 pg·min/mL).

The effects of MP and DMP expressed as %inhibition of TNF-α AUC in the perfusate are plotted against the time of drug administration in Fig. 2. The effects of MP appeared relatively early (2 h) and reached a maximum of ~50% inhibition at 8 h after the drug administration. Thereafter, the effects of MP decreased relatively rapidly, with marginal effects remaining at 24 h after the drug administration. For DMP, the effect increased relatively slowly and reached a maximum of ~80% inhibition at 24 h after the injection of the prodrug. The effect then decreased slowly, with marginal effects remaining at 72 h after the prodrug injection (Fig. 2). The total area under the effect-time curve (%inhibition·h) after DMP administration (3680 ± 406) was 4-fold greater ($p < 0.05$) than that after the administration of MP (846 ± 114).

The bile flow rates for MP- or DMP-treated rats are presented in Fig. 3. Except for the MP-2 h group, the injection of MP at different times before the liver isolation did not have any significant effect on the bile flow rates of LPS-stimulated livers; the bile flow rates in livers isolated 2 h after the injection of MP were, however, consistently lower ($p < 0.05$) than those for the positive control (no drug treatment) group at all the collection intervals (Fig. 3, top). Additionally, the collection interval did not have any significant effect on the bile flow rates ($p > 0.05$); the bile flow rates remained relatively constant during the entire 120-min perfusion period (Fig. 3, top). For DMP-injected animals, the bile flow rates of livers isolated at different times after the prodrug injection were generally similar to those for the positive control livers (no drug injection; Fig. 3, bottom). However, a statistical significance was found between the flow rates for DMP-48 h group and positive controls. This statistical difference was apparently due to lower bile flow rate values in DMP-48 h livers

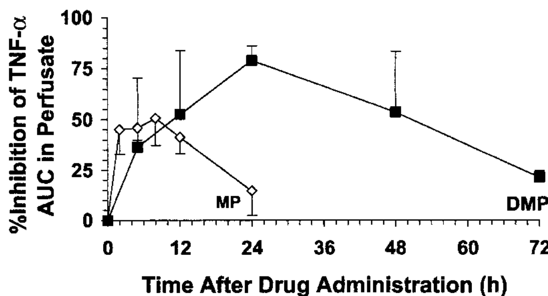

Fig. 2. Percent inhibition of tumor necrosis factor-α area under the curve in the outlet perfusate vs. lag time between drug injection and liver isolation after single i.v. doses (methylprednisolone equivalent) of 5 mg/kg methylprednisolone or dextran-methylprednisolone. Symbols and bars represent average and SD values, respectively (n = 4).

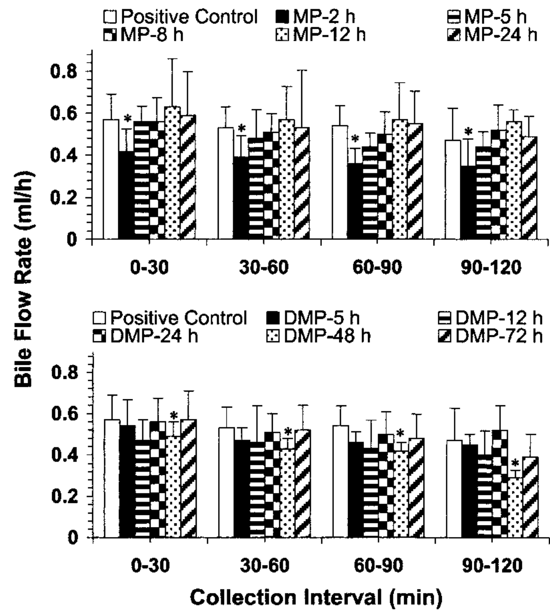

Fig. 3. Bile flow rates at different collection intervals in livers isolated from rats pretreated with single i.v. doses (methylprednisolone equivalent) of 5 mg/kg methylprednisolone (top) or dextran-methylprednisolone (bottom). *Statistically significant difference between the treatment group and positive controls (no treatment) based on two-way analysis of factor and Dunnett's test. Symbols and bars represent average and SD values, respectively (n = 4).

observed only at the 90-120 min collection interval, because the rates at the other collection intervals were not substantially different from those of the other groups (Fig. 3, bottom).

The amounts of TNF-α found in the bile of MP- or DMP-injected rats are presented in Fig. 4. For both drug-treatment

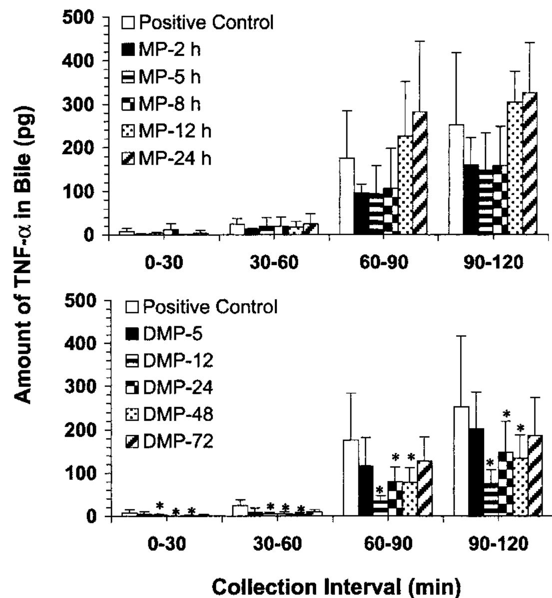

Fig. 4. Amounts of tumor necrosis factor-α secreted at different time intervals into bile after pretreatment with single i.v. doses (methylprednisolone equivalent) of 5 mg/kg methylprednisolone (top) or dextran-methylprednisolone (bottom). *Statistically significant difference between the treatment group and positive controls (no treatment) based on two-way analysis of variance and Dunnett's test. Symbols and bars represent average and SD values, respectively (n = 4).

groups, relatively small amounts of TNF-α were excreted in bile during the first 1 h of perfusion. However, the amounts of TNF-α excreted in bile during the 60-90 and 90-120 min collection intervals were relatively high (Fig. 4). Generally, significant variability was observed with regard to the LPS-induced excretion of TNF-α in the bile for all the experimental groups (Fig. 4). Further, although there was a trend toward a reduction in TNF-α biliary excretion after MP injection, the decrease did not reach statistical significance when the data for each time point was compared with the positive control values (Fig. 4, top). In contrast, significant decreases in the TNF-α biliary excretion were noticed in DMP-12 h, DMP-24 h, and DMP-48 h groups, when they were compared with data in the absence of DMP treatment (Fig. 4, bottom). The amounts of TNF-α excreted in the bile of negative control livers, which were not subjected to LPS infusion, were negligible during all the sampling collection periods (data not shown).

The effects of MP or DMP on the TNF-α amounts excreted in bile, expressed as the %inhibition of TNF-α amount in bile relative to no-drug treatment, are presented in Fig. 5 as a function of the time after each drug injection. The effect-time curves for biliary TNF-α (Fig. 5) were qualitatively similar to those for the perfusate TNF-α (Fig. 2); the inhibitory effects of MP were less intense, appeared faster, and were less sustained when compared with those of the prodrug DMP. The area under the inhibitory effect-time curve (%inhibition·h) of DMP (3390 ± 348) was significantly ($p < 0.05$) larger than that for the parent drug (521 ± 141).

The concentrations of IL-1β and IL-6 in the outlet perfusate of livers injected with LPS (positive control) were below the level of detection (data not shown). Therefore, the assays were not conducted for livers collected from drug-treated rats or in the bile samples.

The concentrations (MP equivalent) of DMP and regenerated parent drug in the liver after administration of DMP are presented in Table II. Relatively high concentrations of DMP were found in the liver (Table II). The maximum concentrations of the prodrug were observed with the first sampling time (5 h), and, thereafter, the concentrations declined with the time of injection. Seventy-two hours after the injection of DMP, relatively high concentrations of the prodrug still could be found in the liver. Additionally, free MP, regenerated from the prodrug in the liver, was detected in the liver (Table II). Similar to the concentrations of the prodrug, the highest concentrations of the regenerated MP were observed with the first sampling time (5 h). However, in contrast to the concentrations of the prodrug, the liver concentrations of MP were below the limit of quantitation of the assay at 48 and 72 h after the prodrug injection (Table II).

After the injection of an equivalent dose of the parent drug, the concentrations of MP in the liver were below the limit of quantitation of the assay (<0.1 μg/mL) at all the time points studied.

The effect-concentration relationships after DMP administration are depicted in Fig. 6. Counter-clockwise hystereses were observed when the percent inhibition in TNF-α AUC in the perfusate (Fig. 6, top) or TNF-α amount in bile (Fig. 6 bottom) was plotted against DMP concentration in the liver. The counter-clockwise hystereses could not be explained by a delay in regeneration of MP because plots of effect vs. concentration of the regenerated MP also showed a counter-clockwise relationship (Fig. 6).

Table II. The Average ± SD of Hepatic Concentrations of Dextran-Methylprednisolone (DMP) and Regenerated Methylprednisolone MP after Single 5 mg/kg Doses (MP Equivalent) of DMP Administered Intravenously at Different Times before Liver Isolation (n = 4 for Each Group)[a]

	Liver concentration (μg/g liver)	
Time (h)	DMP	Regenerated MP
5	34.9 ± 4.5	1.01 ± 0.12
12	34.7 ± 5.0	0.892 ± 0.360
24	25.1 ± 1.9	0.529 ± 0.354
48	16.9 ± 2.8	—[b]
72	13.0 ± 2.4	—[b]

[a] The concentrations were measured after 2 h of *ex vivo* perfusion.
[b] Below the limit of quantitation.

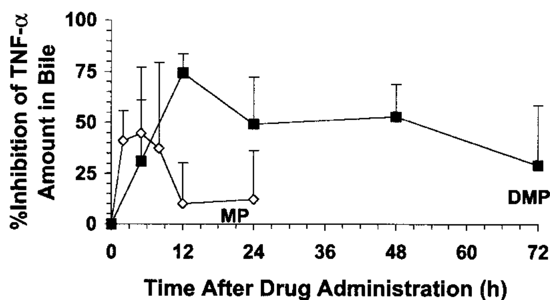

Fig. 5. Percent inhibition of tumor necrosis factor-α secretion into bile vs. lag time between drug injection and liver isolation following pretreatment with single i.v. doses (methylprednisolone equivalent) of 5 mg/kg methylprednisolone or dextran-methylprednisolone. Symbols and bars represent average and SD values, respectively (n = 4).

DISCUSSION

Based on a series of systematic studies (8,18) of dextran macromolecules with different molecular weights, we previ-

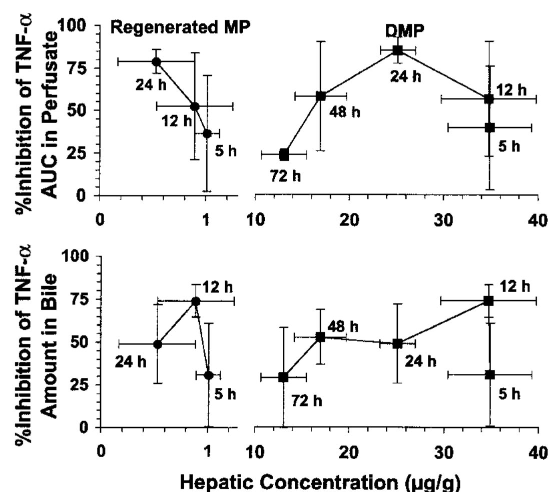

Fig. 6. Percent inhibition of tumor necrosis factor-α area under the curve in the perfusate (top) or tumor necrosis factor-α amount in bile (bottom) vs. hepatic concentration of dextran-methylprednisolone (right) or regenerated methylprednisolone (left) after pretreatment with single i.v. doses (methylprednisolone equivalent) of 5 mg/kg dextran-methylprednisolone. Symbols and bars represent average and SD values, respectively (n = 4).

ously proposed the use of relatively high molecular weight (e.g., 70 kDa) dextrans for targeted delivery of therapeutic agents to the reticuloendothelial system, including the liver. Further studies (10) using MP as a model immunosuppressant showed that dextran conjugation significantly alters the pharmacodynamics of MP in rats. After systemic administration, DMP conjugate resulted in a significant decline (80% at 24 h) in the number of splenocytes, which lasted for at least 96 h. This was substantially greater than the effects of an equimolar dose of the parent drug, which resulted in a significantly smaller (30%) and short-lived (<24 h) decline in the number of splenocytes (10). Similarly, the spleen lymphocyte proliferation assay, which is a measure of systemic immunosuppression, revealed substantial improvements in systemic immunosuppression when MP was conjugated to dextran (10). Whereas DMP injection resulted in an almost 100% inhibition of lymphocyte proliferation, the MP injection caused only 50% inhibition. Additionally, the inhibitory effects of DMP lasted longer than those after the injection of the parent drug (10). Collectively, these data indicated that dextran conjugation improves the systemic immunosuppressive effects of MP, which may be advantageous for any type of solid organ transplantation. However, because kinetic studies demonstrated substantial accumulation of DMP in the liver (9), we became also interested in determining the local immunosuppressive effects of DMP in the liver, which may be advantageous specifically in liver transplantation.

Delineation of the local immunosuppressive effects of drugs in the liver after their systemic administration is challenging because of the possible interference from the systemic immunosuppressive effects. We have recently (11) developed an *ex vivo* liver perfusion model that may be used for delineation of the local (liver) immunosuppressive effects of systemically-administered drugs without the interference of systemic factors. This model is based on LPS administration to the isolated liver, which causes nonspecific immunostimulation and activation of the Kupffer cells, resulting in the release of cytokines, such as TNF-α, in the outlet perfusate. It is believed that cytokines play significant roles in both antigen-independent and antigen-dependent inflammatory processes occurring in transplanted organs such as the liver (19). For example, antigen-independent processes during the liver preservation and subsequent reperfusion have been associated with high levels of TNF-α in the graft. Additionally, cytokines such as TNF-α and interferon-γ upregulate the expression of major histocompatibility molecules and adhesion molecule expression on endothelial cells of graft vasculature, leading to subsequent antigen-dependent inflammatory processes. With regard to T-cell function and response, cytokines increase the costimulation signals initiated by adhesion molecules such as endothelial vascular cell adhesion molecule-1. However, they cannot initiate costimulation signals by themselves. Overall, these data suggest that cytokines are important elements in a complex immune response to transplanted organs (19). Consequently, in the current study, we decided to use the levels of cytokines in the outlet perfusate as a measure of immune response.

Although both MP and DMP exhibited local immunosuppressive activity in the liver (Figs. 2 and 5), the effect was significantly more pronounced and sustained for the conjugate. This is most likely the result of the selective accumulation of DMP in the liver and its subsequent release of the parent drug, as demonstrated by relatively large concentrations of DMP and regenerated MP found in the livers of DMP-injected rats (Table II). Similarly, the lower local immunosuppressive activity of the injected MP (Figs. 2 and 5) was associated with hepatic concentrations of the parent drug, which were below the limit of quantitation of the assay. These results are consistent with a previous pharmacokinetic study of MP and DMP (9), demonstrating that conjugation of MP with dextran results in preferential accumulation of the steroid in the liver.

Other investigators have also attempted to improve the immunosuppressive profiles of drugs by means of different delivery designs such as macromolecular prodrugs (20) and liposomal formulations (21,22). For instance, Yura *et al.* (20) used a negatively charged dextran, as opposed to a neutral dextran used in our studies, for conjugation to tacrolimus. Although the conjugation resulted in significantly larger plasma AUC of the drug, the spleen and liver accumulation of the conjugate was only modestly greater than that after the injection of the parent drug. This is perhaps due to the negative charge of dextrans used by Yura *et al.* (20), which is known to reduce tissue accumulation and increase plasma concentrations of these macromolecules (23). Nevertheless, neither the systemic nor the local immunosuppressive effects of dextran-tacrolimus were examined in the above study (20).

The preferential accumulation of MP in the reticuloendothelial system, including the liver, and subsequent alterations of the pharmacodynamics of the drug have also been achieved using a liposomal formulation (21,22). Similar to dextran conjugation, the liposomal formulation of MP resulted in a superior systemic immunosuppressive effect, compared with the parent drug (22). The superiority of the liposomal MP over parent drug was further demonstrated in an experimental model of heart transplantation (24). However, the local immunosuppressive effect in the liver after systemic administration of the formulation has not been tested.

In another approach, Melgert *et al.* (25) used albumin as a carrier for targeting dexamethasone to the nonparenchymal cells of the liver. Although the overall accumulation of the conjugate in the liver was not substantially different than that after the parent drug administration, a preferential distribution of the conjugate into the nonparenchymal cells was observed (25). In our present study, the cellular localization of DMP was not determined. However, previous reports (26) suggest that dextrans preferentially accumulate in nonparenchymal cells of the liver. Because the source of liver TNF-α is mostly Kupffer cells (27), the effects of DMP on the release of TNF-α from the liver, observed here, are consistent with the preferential accumulation of the prodrug in these cells.

The anticlockwise hystereses observed in the effect-concentration relationship after DMP administration (Fig. 6) indicate a delay between the appearance of DMP in the liver and the effect. Because DMP is not active *per se* (28) and needs to regenerate MP for its effects, the hystereses could be due to the delay in regeneration of MP from DMP. However, a plot of the effect-concentration relationship for the regenerated MP also showed a similar anticlockwise relationship (Fig. 6), suggesting that the TNF-inhibitory effects of MP in the liver are indirectly related to its concentration. Indeed, other studies (11,29) have also shown that the inhibitory effects of steroids on the LPS-stimulated TNF-α production by the liver occur several hours after their iv injection. For ex-

ample, Waage (29) showed that the maximal decrease in LPS-induced serum TNF-α levels occurred at ≥5 h after the administration of dexamethasone. Further, our own recent studies (11) indicated that whereas injection of MP 5 h before the LPS-stimulation significantly reduced the release of TNF-α by the liver, the injection of the drug 1 h before LPS stimulation did not produce any significant effect. The delay in the inhibition of TNF-α release by steroids is not unexpected because these drugs reportedly inhibit TNF-α production at both the transcriptional and translational levels (30).

The DMP:MP concentration ratios in the liver tissue were relatively large (≥35-fold) at all the studied time points (Table II), suggesting a slow regeneration of MP from its prodrug. Further modifications to the design of the prodrug to result in a faster rate of regeneration may improve the effect profile of the prodrug. In future studies, we will be investigating this issue by using a lower molecular weight of dextran and/or linkers that allow a more optimum control of the regeneration rate.

In conclusion, using an *ex vivo* liver perfusion model, it was shown that systemically administered MP and DMP both have local immunosuppressive effects in the liver. The local effects of the prodrug of MP (DMP); however, appear to be more intense and sustained, compared with the equivalent doses of the parent drug. Therefore, dextran conjugation may be suitable for local delivery of immunosuppressants to the liver, which is advantageous in liver transplantation.

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