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 (EC₅₀ = 7.9 nM) than do human peripheral blood lymphocytes (EC₅₀ = 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 ³H-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-lecithin (phosphatidylycholine) (PC) and L-α-phosphatidylglycerol (egg sodium salt) (PG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Methylprednisolone was obtained from Sigma (St. Louis, MO) and ³H-thymidine (6.7 Ci/mmole) 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 M₅ 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 (5-10⁴ 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% CO₂ humidified atmosphere. ³H-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 ³H-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.

Splenic 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, 5-10⁴ 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 ³H-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:

\[ E = E_{50} - \frac{E_{\max} \cdot C^r}{C^r + EC_{50}^\gamma} \]

where \( E \) is the effect (log(cpm)), \( E_{\max} \) is maximal effect of cell proliferation with PHA, \( E_{50} \) is the effect at baseline, \( C \) is MPL concentration (nM), \( EC_{50} \) is the MPL concentration which produces 50% of maximum inhibition, and \( \gamma \) 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:

\[ \frac{dC_E}{dt} = k_d \cdot (C_p - C_E) \]

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:

\[ \frac{k_{on}}{C_E + R} \rightarrow C_E R \]

\[ k_{off} \]

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

\[ \frac{d(R)}{dt} = -k_{on} \cdot (R) + k_{off} \cdot (C_E R) \]

\[ \frac{d(C_E R)}{dt} = k_{on} \cdot (C_E) \cdot (R) - k_{off} \cdot (C_E R) \]

where \( C_p R \) 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 polynomial equations:

\[ C_p = \sum C_i e^{-\lambda_i t} \]

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-