DRUG TARGETING BY LONG-CIRCULATING LIPOSONAL GLUCOCORTICOIDS INCREASES THERAPEUTIC EFFICACY IN A MODEL OF MULTIPLE SCLEROSIS

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ABSTRACT

High-dose glucocorticoid hormones are a mainstay in the treatment of relapses in multiple sclerosis (MS). We present a way to deliver ultra high doses of glucocorticoids to inflamed sites in the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE) using a novel formulation of PEG-coated long-circulating liposomes encapsulating prednisolone (PL). \(^{3}\)H-labeled PL showed selective targeting to the inflamed CNS, where up to 4.5-fold higher radioactivity was achieved than in healthy control animals. HPLC revealed much higher and more persistent levels of prednisolone in spinal cord after PL compared to an equal dose of free prednisolone. Gold-labeled liposomes could be detected in the target tissue, mostly taken up by macrophages (M\(_{\phi}\)), microglial cells and astrocytes. Blood-brain-barrier disruption was strongly reduced by 10 mg/kg PL, which was superior to a 5-fold higher dose of free methylprednisolone (MP). PL was also superior to MP in diminishing T cell infiltration by induction of T cell apoptosis in spinal cord. M\(_{\phi}\) infiltration was clearly decreased only by PL. The rate of tumor necrosis factor-a (TNF-\(\alpha\)) positive T cells or M\(_{\phi}\) was strongly reduced by PL and by MP. No adverse effects on glial cells were detected. A single injection of PL clearly ameliorated the course of adoptive transfer EAE and EAE induced by immunization. In conclusion PL is a highly effective drug in treatment of EAE, and is superior to a five fold higher dose of free MP, possibly by means of drug targeting. These findings may have implications for future therapy of autoimmune disorders such as MS.
INTRODUCTION

Multiple sclerosis (MS) is one of the most common inflammatory disorders of the CNS. Its pathological hallmarks are demyelination and cellular infiltration of T cells (TC) and macrophages (Mφ). The most favored pathophysiological hypothesis includes a TC dominated autoimmune reaction (1).

Despite long-term immunotherapy relapses occur, which are commonly treated by repeated i.v. injections of high doses ('pulse') glucocorticoid (GC) as potent anti-inflammatory drug. The main goal is to prevent ongoing tissue destruction with loss of oligodendrocytes, axons and neurons leading to permanent functional deficits. In MS a pulse therapy with 10 mg/kg methylprednisolone (MP) for 3-5 days is the standard regimen in relapse therapy (2, 3). The optimal dosage of the pulse GC is still under debate. Recently, it was reported that treatment with ultra high dose 2 g MP i.v. per day is superior to 500 mg per day for 5 days with regard to reduction of disease activity as measured by MRI criteria (4). In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, we have previously shown, that an ultra high dose of 50 mg/kg MP i.v. is superior to eliminate inflammatory infiltrates than a ‘standard’ dose of 10 mg/kg MP i.v. and is associated with much higher tissue levels of MP (5).

The pharmacological effects of GC are based on a wide range of mechanisms of action (6). At a lower concentration GC-effects are mainly mediated by the classical GC receptor, yet at a higher concentration additional, non-genomic mechanisms may be operative, such as through membrane receptors and activation of a second messenger system (7, 8). These pathways are thought to be one possible explanation for the observed superiority of high and ultra high doses in the treatment of some autoimmune disorders (6).

The goal of this study was to investigate the effects of long-circulating prednisolone-liposomes (PL), a novel formulation for drug targeting, in treatment of EAE. These PL have been shown to exert a clear beneficial effect in an experimental rat model of arthritis (see Chapter 3). The objective of drug targeting with this formulation is to achieve ultra high tissue concentrations of GC in the inflamed target organ as compared to an equivalent dose given as free drug, and at the same time a much lower concentration systemically with a reduction of unwanted side effects.

Here we show that drug targeting by PL is highly effective in restoring the blood-brain-barrier (BBB)-integrity and in reducing cellular inflammation by induction of TC apoptosis, thereby ameliorating the disease activity of active and adoptive transfer (AT)-EAE without detectable side effects. Moreover, in contrast to free GC, Mφ infiltration was diminished after PL. The effects may be explained by ultra high tissue levels of GC, achieved by means of drug targeting. Our results may have implications for a more efficient therapy of relapses in MS and of other autoimmune disorders.
MATERIALS AND METHODS

Animals, cell culture and EAE
Female Lewis rats (Charles River, Sulzfeld, Germany) were 6-8 weeks old. All culture media and supplements were obtained from Gibco BRL (Eggenstein, Germany). Encephalitogenic TC for in vivo experiments were generated and maintained as previously described in detail (9). Briefly, primed TC (3 x 10^5/ml) were restimulated with guinea pig myelin basic protein (MBP, 20 µg/ml) in culture medium (RPMI 1640) supplemented with 1% normal rat serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, using freshly isolated and irradiated (3000 rad) thymocytes (1.5 x 10^7/ml) as antigen presenting cells.

AT-EAE was induced by tail vein injection of 10-12 x 10^6 freshly activated, MBP specific TC. Animals were inspected daily by an observer masked to the respective treatment, using a 6 grade score: 0, healthy; 1, weight loss, limp tip of tail; 2, limp tail, mild paresis; 3, moderate paraparesis, ataxia; 4, tetraparesis; 5, moribund; 6, dead (5). Disease onset in all animals was at day 2, maximum at day 5. Active EAE was induced in rats by immunization with 75 µg guinea pig MBP in 100 µg complete Freund's adjuvant (CFA) per animal by s.c. injection in the hind paws. Disease onset was at day 10 to day 12 with 100% incidence, maximum was at day 13/14.

Preparation of long-circulating PEG-liposomes
Liposomes were prepared by the film-extrusion method (10). Briefly, a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC, from Lipoid GmbH, Ludwigshafen, Germany), poly(ethylene glycol) (PEG) 2000–distearyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0. A lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone phosphate (Bupa, Uitgeest, The Netherlands) in sterile water. The resulting lipid dispersion was sized by multiple extrusion through polycarbonate filter membranes to a diameter of 90-100 nm. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Phospholipid content was determined with a phosphate assay (11) and prednisolone phosphate concentrations by reversed-phase HPLC (12). Each one ml liposomal preparation contained around 4.5 mg prednisolone phosphate and an average of 60 µmol phospholipid.

Colloidal gold-containing PEG-liposomes (13) were prepared accordingly except for the hydration step, which was performed with a freshly prepared tetrachloroaurate solution in citrate buffer. Immediately after extrusion, colloidal gold was formed by incubation of the liposomal dispersion at 37 °C.

^3H-labeled liposomes were prepared similarly except for the lipid composition: to the lipids dissolved in ethanol [^3H]-cholesteryloleylether (Amersham, Uppsala, Sweden) was added as a non-degradable liposome lipid phase marker. After rotary evaporation under reduced pressure, the lipid film was hydrated with PBS at an initial total lipid concentration
of 50 µmol/ml. Radioactivity of the liposomal dispersions was assayed in a liquid scintillation cocktail purchased from Ultima Gold (Groningen, The Netherlands) and counted in a Philips PW 4700 liquid scintillation counter. Lipid content of the liposomal dispersion was determined by assessing the loss of radioactivity of the liposomes during preparation. This mixture contained approximately 2.5 mg/ml prednisolone phosphate and 75 kBq/ml radioactivity.

**Treatment protocol and tissue sampling**

For therapeutic studies we used prednisolone PEG-liposomes (PL) and free prednisolone-phosphate or methylprednisone hemisuccinate (MP) (Urbason soluble®, Aventis, Frankfurt, Germany). Treatment regimen for AT-EAE essentially followed the protocol used in previous studies (5, 14). All experiments, except for gold-labeled liposomes (n=4), and ³H-labeled liposomes (n=4), were performed in groups of 6 animals each and reproduced at least once. 10 mg/kg body weight PL was injected i.v. in a tail vein at 2, 6, 18 and/or 42 hr before perfusion at day 5. As positive control another group received 50 mg/kg body weight MP 18 and 6 hr prior to sacrifice (5).

In a separate experiment colloidal gold-labeled liposomes were applied once at 18 hr or 42 hr. In active EAE in rats 10 mg/kg body weight PL were injected once i.v. at beginning of disease at day 12. 50 mg/kg body weight MP was administered twice i.v. at days 12 and 13. Controls received empty liposomes and/or saline i.v.

For tissue preparation anesthetized animals were perfused with HAES-steril® 6% (Fresenius, Bad Homburg, Germany), followed by paraformaldehyde 4% in 0.1 M phosphate buffer. Spinal cord was removed, postfixed, dehydrated and embedded in paraffin.

For [³H] analysis in EAE vs. healthy rats lethal CO₂-anesthesia was applied and blood was drawn by cardiac puncture and centrifuged. Subsequently, spinal cord, brain, spleen, liver, one sciatic nerve and one 5×5 mm lower back muscle as non-inflamed control tissue were taken out. All samples were stored at -80 °C until analysis. The organs of one non-treated rat were used for background radioactivity determination.

[³H]-concentrations was measured after homogenization, addition of Solvable tissue solubilizer (NEN, Dreieich, Germany) and 35% hydrogen peroxide. After overnight incubation the samples were assayed in Ultima Gold scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands). Counting time was to a statistical precision of ± 0.2% or a maximum of 5 min whichever comes first. The scintillation counter was programmed to automatically subtract background and convert counts per min to disintegrations per min. Besides tissue samples also the radioactivity of the injected dose was counted. The ratio tissue samples radioactivity over radioactivity of the injected dose yielded the rate of the injected dose value in percent.
Immunohistochemistry

Five µm cross-sections of spinal cord, spleen or liver were deparaffinized and rehydrated. Pretreatment with hydroxylamine (0.9%, from Sigma-Aldrich Chemicals, Deisenhofen, Germany) was required for albumin stain, and with protease 24 (0.4%, from Sigma-Aldrich) for Kupffer cells. As primary reagents we used: mouse mAb to a pan TC antigen (B 115-1, dilution 1:500, from HyCult biotechnology, via Sanbio, Beutelsbach, Germany) for TC; mouse mAb ED1 (diluted 1:500, from Serotec, via Biozol, Eching, Germany) for Mφ; mouse mAb ED2 (diluted 1:300, from Serotec) for Kupffer cells; rabbit polyclonal Ab to the glial fibrillary acidic protein (GFAP) from cow (diluted 1:500, from DAKO, Hamburg, Germany), incubated at 4 °C over night, for astrocytes; mouse mAb to 2', 3'-cyclic nucleotide phosphohydrolase (CNPase, diluted 1:200, from Chemicon, Hofheim, Germany) for oligodendrocytes; lectin histochemistry with a biotinylated *Griffonia simplicifolia* isolecitin B4 (GSA-I-B4, concentration 100 µg/ml, Sigma-Aldrich Chemicals), incubated 24 hr at room temperature, for microglial cells; anti-albumin Ab (diluted 1:200, from Nordic, Bochum, Germany) for detection of BBB-disruption (15). All primary reagents/antibodies were incubated for one hr at room temperature unless stated otherwise. Primary reagents/antibodies were detected using the ABC-system (DAKO), and with 3,3'-diaminobenzidine (DAB) tetrahydrochloride as chromogenic substrate. Sections were counterstained with hematoxylin for 30 sec.

Colloidal gold was visualized with a silver-enhancing solution (Sigma-Aldrich Chemicals) for 16 min in the dark. Sections were fixed by immersing in 2.5% aqueous sodium thiosulfate for 2-3 min, followed by immunohistochemistry for detection of glial or immune cells. Double labeling of apoptotic TC was performed by TUNEL as described before with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as chromogenic substrate (16). Double staining for TNF-α positive TC or Mφ was performed as previously established (13). In brief, TNF-α was detected by a rabbit polyclonal Ab (diluted 1:100, from Serotec), visualized with Vector Red (Vector) as chromogenic substrate, followed by detection of TC or Mφ, using DAB-nickel (black, from Vector) as chromogenic substrate. All sections were dehydrated and mounted in Vitro-clud® (R. Langenbrinck, Emmendingen, Germany). Analysis of inflammatory infiltrates was performed by an observer masked to the respective treatment rating 1.6-3.2 mm² of lumbar spinal cord at 250 × magnification. Apoptosis was assessed by morphological criteria (17) or TUNEL. BBB-disruption was quantified by a computer-aided grey scale measuring (Scion Image software, Scioncorp., Maryland, USA) with an Axiovert 100 microscope (Zeiss, Göttingen, Germany) and a CCD DXC 950P camera (Sony, Köln, Germany). We measured the maximal signal intensity of half a spinal cord cross section at 100 × enlargement.

Statistical analysis

Statistical analysis of the data was performed by the Student t test (Excel, Microsoft, Germany), considering *P < 0.05, **P < 0.01, ***P < 0.001 as significant P values.
RESULTS

$^3$H-labeled PL accumulate in CNS.

Selective accumulation of PL in the inflamed CNS was measured by $^3$H-labeled PL, which were injected at time points indicated in Figure 1 A. The serum level was highest 2 hr after injection and then gradually decreased about 50%, but even 42 hr after injection 3%/g organ of the injected dose was found to be circulating in AT-EAE rats as well as in healthy control animals. In the livers of the rats 1.5%/g organ were achieved after 6 hr, which remained basically unchanged until 42 hr after injection and was similar to levels of a healthy control animal. In spleen 2.7%/g organ were obtained 2 hr after the injection. Furthermore, $[^3]$H]-PL accumulated in spleen, reaching 20.3%/g organ 42 hr after injection, which was similar to healthy animals (Figure 1 A). These levels of $^3$H-labeled liposomes were in accordance with observations in experimental rat arthritis reported by Metselaar et al. (Chapter 3).

Figure 1. $^3$H-labeled PL at indicated time points measured in serum, liver and spleen (A) or spinal cord, brain and control tissues nerve and muscle (B). Radioactivity is given as percentage of the injected dose per gram organ of $^3$H-labeled PL in AT-EAE vs. healthy control rats (n= 4 per group).

The $[^3]$H-values in spinal cord, brain, peripheral nerve and muscle were lower compared to serum, spleen and liver. However, compared to the 42 hr-value in control animals without inflammation, there was a 3-fold higher $[^3]$H]-PL accumulation in CNS, in spinal cord with the highest number of inflammatory lesions and BBB-damage (18) even up to 4.5-fold (Figure 1 B). Also, in contrast to nerve and muscle from EAE-rats, only in the inflamed CNS we could observe a gradual increase of the rate of $[^3]$H]-PL, indicating an accumulation of PL. In the control nerve and muscle tissue we found no clear difference between EAE-rats and healthy control animals, except for the early time points in the nerve, where a decreasing curve similar to serum was observed. This may be best explained by contamination of the small tissue sample with blood (Figure 1 B).
Single injection of PL is effective in AT-EAE.

First we investigated the optimal timing of the PL injections. We applied a single dose of 10 mg/kg PL i.v. at 6 hr, 18 hr, or 42 hr or empty liposomes at 42 hr before perfusion at day 5 in AT-EAE. 42 hr after PL the rate of TC apoptosis was increased compared to all other groups (**P < 0.001 vs. control or 6 hr; **P < 0.01 vs. 18 hr) (Table 1) and TC infiltration was clearly reduced (**P < 0.01 vs. all groups). PL given at 18 hr before perfusion increased TC apoptosis (**P < 0.01 vs. 6 hr or control), with only slight effect on TC infiltration (Table 1). Only with treatment at 42 hr the disease course was ameliorated, which was repeated once (*P < 0.05 compared to all other groups) (data not shown).

### Table 1. Treatment of AT-EAE with a single i.v. injection of 10 mg/kg PL at indicated time points compared to empty liposomes and perfusion on day 5. Immunohistochemical staining for TC in spinal cord: rate of apoptotic TC (%) rated by morphological criteria and double labeling with TUNEL and number of infiltrating TC (per mm²) (n=5 per group, data are mean ± SD). P-values explained in text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptosis (%)</th>
<th>Infiltration (TC/mm²)</th>
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<tbody>
<tr>
<td>Control</td>
<td>12.9 ±4.5</td>
<td>177 ±50</td>
</tr>
<tr>
<td>PL 6 hr</td>
<td>15.8 ±2.5</td>
<td>180 ±53</td>
</tr>
<tr>
<td>PL 18 hr</td>
<td>22.2 ±3.0**</td>
<td>132 ±24</td>
</tr>
<tr>
<td>PL 42 hr</td>
<td>33.2 ±4.2**</td>
<td>42 ± 4**</td>
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PL is superior to free GC in AT-EAE.

We then chose the two effective treatments with 10 mg/kg PL at 42 hr and at 18 hr as therapeutic regimen with two injections. This was compared to our previous regimen of two injections of 50 mg/kg MP at 18 hr and 6 hr (5). We investigated the BBB-integrity by an immunohistochemical staining for albumin, followed by a computerized grey-scale analysis. PL (density: 114 ± 1.5) was superior to MP (density: 121 ± 1.9, ***P < 0.001 PL vs. MP); MP also restored the BBB-function as compared to controls (density: 127 ± 2.3, **P < 0.01 MP vs. controls) (2 d-f). PL clearly increased TC apoptosis (**P < 0.001 vs. control, P = 0.07 vs. MP) and reduced TC infiltration (*P < 0.05 vs. all groups) (Figures 3 A,B; 2 a-c). MP augmented the rate of TC apoptosis (**P < 0.01 vs. control), but only marginally reduced TC infiltration. In one experiment we added another group, receiving two injections of 10 mg/kg free prednisolone phosphate. Free prednisolone was less effective than 50 mg/kg MP with regard to TC apoptosis and infiltration, which is well in accord with previous results (5) (data not shown). Since liposomes are mainly taken up by Mϕ as revealed in situ by detection of gold-labeled liposomes, we also characterized Mϕ infiltration. The number of infiltrating Mϕ in spinal cord was strongly reduced by PL (*P < 0.05 vs. control, ***P < 0.001 vs. MP), whereas MP had no effect. (Figures 3 C; 2 g-i). There was no increase in the rate of apoptotic Mϕ, which was in the range of 0 – 5.5% (data not shown). Then we investigated the TNF-α production by TC and Mϕ in situ by immunohistochemical double labeling. The rate of TNF-α positive TC in
Figure 2. Immunohistochemical detection of TC infiltration, BBB-disruption, Mϕ infiltration, and gold-localization in astrocytes and Mϕ in 5 µm paraffin sections of spinal cord from AT-EAE rats at day 5. (a-c) Staining of TC (DAB, dark spots) with the mAb B115-1 and hematoxylin counterstaining (gray), in control (a), PL 10 mg/kg (b), or MP 50 mg/kg treatment (c). (d-f) Detection of BBB-disruption with anti-albumin Ab (DAB, dark) and hematoxylin counterstain, in control (d), PL 10 mg/kg (e), or MP 50 mg/kg treatment (f). (g-i) Staining of Mϕ (Vector Red, dark spots) with the mAb ED1 and hematoxylin counterstaining (gray) in control (g), PL 10 mg/kg (h), or MP 50 mg/kg treatment (i). (j, k) Detection of gold liposomes by silver-enhancing technique (black dots, indicated by arrows), in combination with staining for Mϕ (j, ED1-mAb, Vector Red), or astrocytes (k, anti-GFAP, Vector Red), and hematoxylin. Scale bar=100 µm (a-c, g-i), 200 µm (d-f), 10 µm (j, k).
spinal cord was strongly diminished by PL (32.1 ± 7.8% vs. 55.6 ±8.0% in controls, ***P < 0.001), and was equally effective as MP (34.4 ± 5.9%, ***P < 0.001 vs. controls). Also, the rate of TNF-α positive Mϕ was clearly reduced by PL (31.1 ± 7.4% vs. 52.3 ± 4.2 % in controls, ***P < 0.001), and showed the same efficacy as MP (31.1 ± 13.1%, **P < 0.01 vs. controls).

![Figure 3.](image)

Even though our study was designed to look for short-term mechanisms in situ, we could also observe a clinical benefit from PL (**P < 0.01 vs. all groups at day 5) (Figure 4 A). However, there was no beneficial effect by MP treatment, which can be explained by the start of treatment at day 4 instead of day 3 in PL. All experiments were reproduced at least once with similar results.

**PL ameliorates active EAE.**

Since PL appeared to be superior to MP in the different mechanisms investigated and beneficial clinical effects were observed in AT-EAE even with a single injection, we studied if PL could ameliorate the disease activity in an active EAE model. MBP immunized Lewis rats received one injection of i.v. 10 mg/kg PL at beginning of disease on day 12. This was compared to treatment with two injections of i.v. 50 mg/kg MP at days 12 and 13. In this experiment a single PL injection (*P < 0.05 vs. control at day 14) appeared to be equally effective as twice MP at a five fold higher dosage (*P < 0.05 vs. control at day 16) (Figure 4 B).
drug targeting by long-circulating liposomal glucocorticoids in a model of multiple sclerosis

Figure 4. Therapeutic effect on clinical scores (A) Treatment of AT-EAE with a two i.v. injections of 10 mg/kg PL 42 hr and 18 hr, or 50 mg/kg MP 18 hr and 6 hr prior to perfusion on day 5 compared to controls. (B) Treatment of active EAE with one i.v. injection of 10 mg/kg PL at day 12, or two i.v. injections of 50 mg/kg MP at days 12 and 13 compared to controls. Clinical course of disease indicated as mean +/- SD (n=6 per group).

Apoptosis in spleen or liver
We qualitatively investigated apoptosis of phagocytes and TC in spleen and liver by immunohistochemistry. We observed an induction of TC apoptosis in spleen in all PL treated groups, which was stronger as compared to free prednisolone. The frequency of apoptotic TC after injection of free prednisolone compared to controls was elevated after 2 hr and 6 hr, but not after 18 hr and 42 hr. There was no induction of apoptosis or notable change in number of Mϕ in spleen and Kupffer cells in liver in any of the treatment groups compared to controls (data not shown).

Gold-labeled liposomes are detected in spinal cord.
To detect the cellular localization of extravasated liposomes, we injected gold-labeled liposomes in AT-EAE rats once 18 hr or 42 hr before perfusion at day 5 compared to unlabeled empty liposomes. As revealed by silver-enhancing technique, gold-labeled liposomes were mostly located within phagocytic cells. Most of the cells were Mϕ in the inflamed spinal cord tissue, especially around the blood vessels (Figure 2 j). However, gold label could also be detected in astrocytes and microglia, speaking for a penetration of liposomes through disrupted BBB without the help of Mϕ (Figure 2 k). No gold particles were observed in oligodendrocytes. Also, a high amount of gold-labeled liposomes could be detected in Mϕ in spleen and in Kupffer cells in liver, which is in accord with other recent findings (Chapter 4). In EAE rats we observed no uptake of gold-liposomes in TC in spinal cord or spleen. There was no silver-enhanced signal in rats treated with unlabeled empty liposomes.
No detectable side effects on glial cells.

Since glial cells can undergo apoptosis, we wanted to examine possible side effects by the high tissue levels achieved by PL. This was especially of note for astrocytes and microglial cells, which appeared to take up liposomes, as revealed by the experiments with gold-labeled liposomes. We characterized astrocytes (GFAP), oligodendrocytes (CNPase), and microglia (GSA-I-B4) immunohistochemically. There was no induction of apoptosis in any of these glial cells and their total number per mm² remained basically unchanged after treatment with PL or MP compared to controls (data not shown).
DISCUSSION

Our experiments presented here demonstrate the beneficial effects by drug targeting of prednisolone with a new therapeutic liposomal formulation in the treatment of EAE as a model for MS. Radioactive labeling showed the accumulation of liposomes in the inflamed target organ. By a gold-labeling technique liposomes could clearly be located at the site of inflammation. These results also had functional implications: a dose of i.v. 10 mg/kg PL was superior to a five fold higher dose of i.v. free MP with regard to improvement of BBB-disruption, induction of TC apoptosis, and amelioration of cellular infiltration. Only with PL a clear reduction of inflammatory $M_\phi$ in the lesion could be achieved. In addition we observed a reduced rate of TNF-$\alpha$ expressing TC and $M_\phi$ in situ after PL treatment. As a consequence of the reduced inflammation the disease course of AT-EAE and of active EAE were ameliorated. There were no detectable side effects on glial cells in situ.

The therapeutic goal in treatment of MS-relapses is to reduce cellular inflammation as efficient as possible to prevent ongoing tissue destruction and axonal loss. The dosing of GC as mainstay of therapy in MS relapses is still a matter of debate. With regard to our previous findings in EAE (5) one of the major issues of dosing of steroids is to reach very high tissue levels, exerting multiple pathways of steroid actions according to a new model of steroid mechanisms (6). In the present study higher tissue levels of prednisolone were achieved by encapsulation of the steroid in long-circulating liposomes, which delivered the drug to the site of inflammation without a high serum concentration of the free drug.

Conventional liposomes with a relatively short circulation half-life containing hydrocortisone have been developed in the late 70ies for i.a. treatment of arthritis in experimental models and patients (19, 20). In the early 90ies the principle of selective targeting inflamed tissue was reported with cholesterol/lecithin liposomes, which showed an improved stability in the circulation. These liposomes reached the joints after i.v. injection in rats with experimental arthritis (21). Recently it could be demonstrated that a similar formulation could penetrate the BBB in EAE (22). I.v. injected liposomes encapsulating dichloromethylene diphosphonate, which suppresses $M_\phi$ activity, were beneficial in EAE and experimental autoimmune neuritis (EAN) (23, 24). For significant accumulation of the encapsulated drug in inflamed extravascular tissue it appears to be crucial that liposomes exhibit a long-circulating behavior, and it seems likely that the effects reported by de Silva et al. and Dingle at al. in the late 70ies were either mediated by a change of the pharmacokinetics of the encapsulated drug or by an indirect effect via monocytes/macrophages in liver, spleen or blood.

Prolonged circulation behavior can be accomplished with small-sized liposomes (<150 nm) composed of neutral, saturated phospholipids and cholesterol. Often water-soluble polymers like PEG are attached to the surface of long-circulating liposomes to reduce adhesion of opsonic plasma proteins that would otherwise induce recognition and rapid removal from the circulation by the mononuclear phagocyte system in liver and spleen (25-27). Using this approach PEG-coated long-circulating liposomes can remain in the
circulation with a half-life as long as 50 hrs in humans (28). The improved pharmacokinetics and target localization has led to several successful applications of this formulation in antitumor therapy (28, 29). Studies with liposome-associated radiolabels have indicated that PEG-liposomes can also successfully be employed to selectively target pathological sites in inflammatory disorders (30, 31). For our study here we encapsulated prednisolone-phosphate as active drug into long-circulating liposomes, since methylprednisolone-succinate, which we used in previous studies (5, 13), did not yield a stable formulation when encapsulated in PEG-liposomes.

To investigate whether target localization of liposomes is a direct process or a result of uptake by monocytes in blood or spleen followed by infiltration of such monocytes at the target site, we employed gold-labeled liposomes, which could be detected in spinal cord within vascular endothelium and perivascular areas non-phagocytosed as well as in inflammatory Mφ. Additionally, there was direct uptake of liposomes by resident astrocytes and microglia. Also, our radioactive data support a rapid penetration of liposomes into the CNS, where an accumulation of the injected liposomes was seen, reaching values of up to 4.5-fold higher than in healthy control animals. In contrast, non-inflamed control tissue showed similar amounts of radioactive liposomes in healthy animals and EAE-rats. Taken together these data support the hypothesis of selective targeting of PL to inflamed sites.

The theoretical prednisolone concentrations calculated based on concentrations of radioactive PL found in the spinal chord are in the range of non-genomic effects according to a new model of steroid mechanisms of action (6). These expected high levels of prednisolone after PL may have been responsible for the direct effects at the site of inflammation, such as the superior induction of TC apoptosis and the superior improvement of the BBB function. The TNF-α expression of TC and Mφ was reduced comparably to a 5-fold higher dose of free MP. However, besides clear direct effects, the reduced infiltration of TC and Mφ in the spinal cord may additionally be due to effects of PL in peripheral immune organs. We could not detect augmentation of apoptosis of Mφ in spleen. Despite high organ concentrations of PL we could not detect apoptosis in resident cells like Kupffer cells in liver or astrocytes, oligodendrocytes, or microglia in spinal cord, which rules out some important unwanted side effects. One could even speculate that the immune cells playing an active role in the disease are more susceptible to a high-dose steroid treatment than resident or resting immune cells. Also recent observations in exp. arthritis did not reveal unwanted side effects of PL.

Taken together we show that long-circulating PL given at 10 mg/kg accumulate in the inflamed organ of EAE rats. Augmentation of TC apoptosis in situ occurs rapidly and the BBB function is improved. The reduced infiltration of TC and Mφ ultimately leads to an ameliorated disease activity of active and AT-EAE. Especially the reduced Mφ infiltration, which was only seen after PL, might help to prevent ongoing tissue destruction. Thus, PL could be a therapeutic alternative to free MP, which even at a 5-fold higher dose remains less effective. Finally, employing the principle of drug targeting may reduce systemic side effects. These findings may have implications for the treatment of inflammatory disorder of the CNS such as multiple sclerosis and other autoimmune diseases.
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