DOI: 10.1093/brain/awg176 Advanced Access publication June 4, 2003 *Brain* (2003), **126,** 1895–1904

Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis

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Summary

High-dose glucocorticosteroid hormones are a mainstay in the treatment of relapses in multiple sclerosis. We searched for a way to deliver ultra high doses of glucocorticosteroids to the CNS of rats with experimental autoimmune encephalomyelitis (EAE) using a novel formulation of polyethylene glycol (PEG)-coated long-circulating liposomes encapsulating prednisolone (prednisolone liposomes, PL). ^{3}H-labelled 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 with an equal dose of free prednisolone. Gold-labelled liposomes could be detected in the target tissue, mostly taken up by macrophages (Mϕ), microglial cells and astrocytes. Blood–brain barrier disruption was greatly 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. Mϕ infiltration was clearly decreased only by PL. The percentage of tumour necrosis factor-α (TNF-α)-positive T cells or Mϕ was greatly 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 5-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 multiple sclerosis.

Keywords: autoimmunity; neuroinflammatory diseases; long-circulating steroid liposomes; glucocorticosteroid pulse therapy; experimental autoimmune encephalomyelitis

Abbreviations: (AT)-EAE = (adoptive transfer)-experimental autoimmune encephalomyelitis; BBB = blood–brain barrier; CNPase = 2′,3′-cyclic nucleotide phosphohydrolase; GFAP = glial fibrillary acidic protein; GS = glucocorticosteroids; GSAI-B_4 = *Griffonia simplicifolia* isolectin B_4; Mϕ = macrophages; mAb = monoclonal antibody; MBP = myelin basic protein; MP = methylprednisolone; PEG = polyethylene glycol; PL = prednisolone liposomes; TNF-α = tumour necrosis factor-α; TUNEL = terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling

Introduction

Multiple sclerosis is one of the most common inflammatory disorders of the CNS. Its pathological hallmarks are demyelination and cellular infiltration of T cells and macrophages (Mϕ). The most favoured pathophysiological hypothesis includes a T cell-dominated autoimmune reaction (Noseworthy *et al*., 2000).

Despite long-term immunotherapy, relapses occur, which are commonly treated by repeated i.v. injections of high dose (pulse) glucocorticosteroids (GS) as a 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 multiple sclerosis, a high dose i.v. pulse therapy with 10 mg/kg methylprednisolone (MP) for 3–5 days is the standard regimen in relapse therapy (Milligan *et al*., 1987; reviewed in Brusaferri and Candelise, 2000). The optimal dosage of the GS pulse is still under debate. Recently, it was reported that treatment with ultra high dose 2000 mg 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 (Oliveri *et al*., 1998). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, we previously have shown that an ultra high dose of 50 mg/kg MP i.v. is superior to a 'standard' dose of 10 mg/kg MP i.v. with respect to elimination of inflammatory infiltrates and is associated with much higher tissue levels of MP (Schmidt *et al*., 2000).

The pharmacological effects of GS are based on a wide range of mechanisms of action (reviewed in Gold *et al*., 2001). At a lower concentration, GS effects are mediated mainly by the classical GS 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 (Brann *et al*., 1995; Buttgereit *et al*., 1998). 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 (Gold *et al*., 2001).

The aim 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 (Metselaar *et al*., 2003). The objective of drug targeting with this formulation is to achieve ultra high tissue concentrations of GS in the inflamed target organ as compared with 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 T-cell apoptosis, thereby ameliorating the disease activity of active and adoptive transfer (AT)-EAE without detectable side effects. Moreover, in contrast to free GS, Mϕ infiltration was diminished after PL. Ultra high tissue levels of GS, achieved by means of drug targeting, may explain the effects. Our results may have implications for a more efficient therapy of relapses in multiple sclerosis and of other autoimmune disorders.

Material 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 T cells for *in vivo* experiments were generated and maintained as previously described in detail (Gold *et al*., 1995). Briefly, primed T cells (3×10^5/ml) were restimulated with guinea pig myelin basic protein (MBP, 20 μg/ml) in 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 (1.5×10^7/ml) as antigen-presenting cells. AT-EAE was induced by tail vein injection of 10–12 $\times$ 10^6 freshly activated, MBP-specific T cells. 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 (Schmidt *et al*., 2000). Disease onset in all animals was at day 2, maximum at day 5. Active EAE was induced in rats by immunization with 75 μg of guinea pig MBP in 100 μg of complete Freund's adjuvant per animal by subcutaneous 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 (Amselem *et al*., 1993). Briefly, a 100 μmol/l lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC, from Lipoid GmbH, Ludwigshafen, Germany), polyethylene 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 (Bufa, 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 (Rouser *et al*., 1970) and prednisolone phosphate concentration by reversed-phase HPLC. Each 1 ml liposomal preparation contained ~4.5 mg of prednisolone phosphate and an average of 60 μmol phospholipid. The 4.5% prednisolone in PL proved a stable encapsulation, and no detectable leakage could be observed *in vitro* or in the circulation. Colloidal gold-containing PEG–liposomes (Huang *et al*., 1991) 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. ^{3}H-labelled

liposomes were prepared similarly except for the lipid composition: to the lipids dissolved in ethanol, [^{3}H]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 (phosphate-buffered saline) 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. The lipid content of the liposomal dispersion was determined by assessing the loss of radioactivity of the liposomes during preparation. We mixed ^{3}H-labelled, empty liposomes with non-labelled PL. This mixture contained ~2.5 mg/ml prednisolone phosphate and 75 kBq/ml radioactivity.

Treatment protocol and tissue sampling

For therapeutic studies, we used PL, free prednisolone phosphate (Bufa) or methylprednisolone-21-hemisuccinate (MP, Urbason solubile®, Aventis, Frankfurt, Germany). The treatment regimen for AT-EAE essentially followed the protocol used in previous studies (Schmidt *et al*., 2000, 2001). All experiments, except for HPLC analysis (n = 3), gold-labelled liposomes (n =4) and ^{3}H-labelled liposomes (n = 4), were performed in groups of six animals each and reproduced at least once. Prednisolone phosphate (10 mg/kg body weight), encapsulated in liposomes (PL), was injected i.v. in a tail vein at 2, 6, 18 and/or 42 h before perfusion at day 5. As positive control, another group of rats received 50 mg/kg body weight MP 18 and 6 h prior to sacrifice (Schmidt *et al*., 2000). For HPLC analysis, free prednisolone phosphate served as another treatment arm and was given once at the same time points as PL. In a separate experiment, colloidal gold-labelled liposomes were applied once at 18 or 42 h. In active EAE in rats, 10 mg/kg body weight PL was injected once i.v. at disease onset on day 12. MP (50 mg/kg body weight) was administered twice i.v. at days 12 and 13 as positive controls. Negative controls received empty liposomes and/or saline i.v. All injections were applied in a volume of 1 ml. For tissue preparation, anaesthetized animals were perfused with 6% HAES-steril® (Fresenius, Bad Homburg, Germany), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord was removed, post-fixed, dehydrated and embedded in paraffin. For HPLC and [^{3}H]PL analysis, rats received a lethal CO_2 anaesthesia. Blood was drawn by cardiac puncture and centrifuged. In HPLC experiments, spleen and liver were removed and post-fixed in 4% paraformaldehyde as above, followed by removal of the spinal cord. For analysis of ^{3}H distribution in EAE versus healthy rats, spinal cord, brain, spleen, liver, one sciatic nerve and one 5 × 5 mm piece of a lower back muscle were taken out as uninflamed control tissue. All samples were stored at –80°C until analysis. HPLC analysis of prednisolone concentration was performed by FOCUS Clinical Drug Development GmbH, Neuss, Germany, as reported previously (Möllmann *et al*., 1995; Schmidt *et al*., 2000) using coded samples. ^{3}H radioactivity was measured after homogenization, and addition of Solvable tissue solubilizer (NEN, Dreieich, Germany) and 35% hydrogen peroxide. After overnight incubation, the samples were assayed in an Ultima Gold scintillation cocktail (Packard BioScience BV, 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 subtract background automatically and convert counts per minute to disintegrations per minute. Besides tissue samples, the radioactivity of the injected dose was also counted. The ratio of tissue sample radioactivity over radioactivity of the injected dose yields the rate of the injected dose value as a percentage.

Immunohistochemistry

Spinal cord, spleen or liver cross-sections (5 μm) were deparaffinized and rehydrated. Pre-treatment 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 monoclonal antibody (mAb) to a pan T-cell antigen (B 115-1, dilution 1 : 500, from HyCult Biotechnology, via Sanbio, Beutelsbach, Germany) for T cells; 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 antibody to the bovine glial fibrillary acidic protein (GFAP) (diluted 1 : 500, from DAKO, Hamburg, Germany), incubated at 4°C overnight, 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* isolectin B_4 (GSA-I-B_4, concentration 100μg/ml, Sigma-Aldrich Chemicals), incubated 24 h at room temperature, for microglial cells; and anti-albumin antibody (diluted 1 : 200, from Nordic, Bochum, Germany) for detection of BBB disruption (Morrissey *et al*., 1996). All primary reagents/antibodies were incubated for 1 h 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 haematoxylin for 30 s. 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 labelling of apoptotic T cells was performed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) as described before with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as chromogenic substrate (Gold *et al*., 1994). Double staining

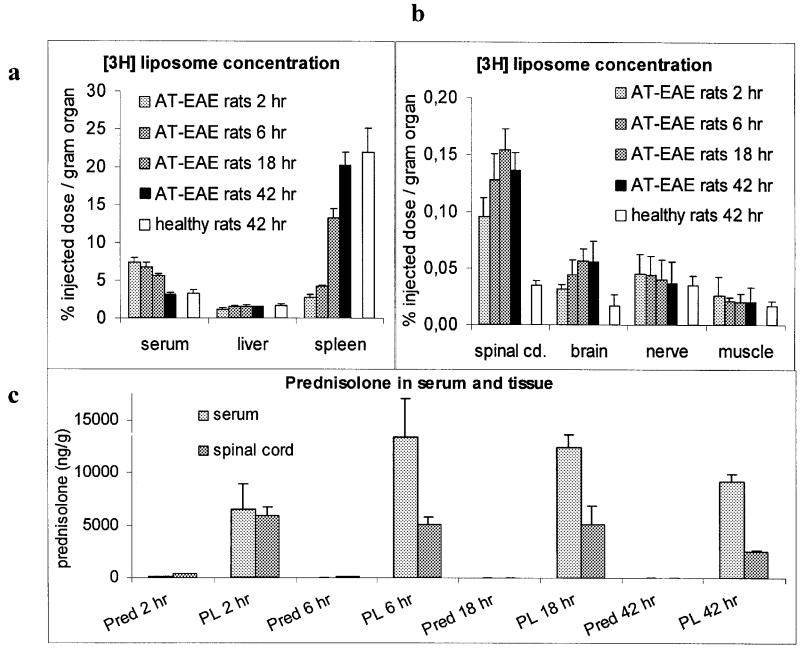

Fig. 1 ^{3}H-labelled PL measured at the indicated time points in serum, liver and spleen (**a**) or spinal cord and brain, and the control tissues nerve and muscle (**b**). Radioactivity is given as a percentage of the injected dose per gram of organ of ^{3}H-labelled PL in AT-EAE versus healthy control rats (n = 4 per group). (**c**) Prednisolone concentration as measured by HPLC in serum and spinal cord after a single i.v. injection of 10 mg/kg PL compared with 10 mg/kg free prednisolone at the indicated time points in AT-EAE rats (n = 3 per group). Data are mean ± SD.

for tumour necrosis factor-α (TNF-α)-positive T cells or Mϕ was performed as previously established (Schmidt *et al*., 2001). In brief, TNF-α was detected by a rabbit polyclonal antibody (diluted 1 : 100, from Serotec), visualized with Vector red (Vector) as chromogenic substrate, followed by detection of T cells or Mϕ, using DAB–nickel (black, from Vector) as chromogenic substrate. All sections were dehydrated and mounted in Vitro-clud® (R. Langenbrinck, Emmendingen, Germany). Examination of inflammatory infiltrates was performed by an observer blinded to the respective treatment, analysing 10 standardized visual fields, covering equal areas of grey and white matter, in two standardized lumbar (intumescentia lumbalis) spinal cord sections at 250× magnification with a total area of 3.2 mm^2 per animal. TNF-α-positive immune cells and apoptotic glial cells were analysed in 10 visual fields of one lumbar spinal cord section (1.6 mm^2). Apoptosis was assessed by morphological criteria (Wyllie, 1980) or TUNEL. Data for apoptotic or TNF-α-positive cells were indicated as the ratio of double-labelled cells and the total number of cells expressed as a percentage. BBB disruption was quantified by a computer-aided grey scale measuring (Scion Image Software, Scioncorp., MD) 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. We semi-quantitatively evaluated the frequency of apoptotic T cells and Mϕ in spleen and Kupffer cells in liver by screening two sections per animal at 250× enlargement, using a 4-grade score for (i) no; (ii) weak; (iii) medium; or (iv) strong occurrence of apoptosis.

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-labelled PL accumulate in CNS

Selective accumulation of PL in the inflamed CNS was measured by ^{3}H-labelled PL, which were injected at the time points indicated in Fig. 1a. The serum level was highest 2 h after injection and then gradually decreased ~50%, but, even 42 h 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 liver from EAE rats, 1.5%/g organ were achieved after 6 h, which remained basically unchanged until 42 h after injection and was similar to levels of a healthy control animal. In spleen, 2.7%/g organ were obtained 2 h after the injection. Furthermore, [^{3}H]PL accumulated in spleen, reaching 20.3 %/g organ 42 h after injection, which was similar to healthy animals (Fig. 1a). These levels of

Table 1 *Treatment of AT-EAE with a single i.v. injection of 10 mg/kg PL at the indicated time points before perfusion on day 5, compared with empty liposomes*

	Apoptosis (%)	Infiltration (T cells/mm^2)
Control	12.9 ± 4.5	177 ± 50
PL 6 h	15.8 ± 2.5	180 ± 53
PL 18 h	22.2 ± 3.0**	132 ± 24
PL 42 h	33.2 ± 4.2**	42 ± 4**

Immunohistochemical staining for T cells in spinal cord: percentage of apoptotic T cells analysed by morphological criteria and double labelling with TUNEL, and number of infiltrating T cells (per mm^2) (n = 5 per group, data are mean ± SD). P values are explained in the text.

^{3}H-labelled liposomes were in accordance with observations in experimental rat arthritis reported by Metselaar *et al*. (2003). The ^{3}H values in spinal cord, brain, peripheral nerve and muscle were lower compared with serum, spleen and liver. However, compared with the 42 h value in control animals without inflammation, there was a ~3-fold higher [^{3}H]PL accumulation in CNS, in the spinal cord having the highest number of inflammatory lesions and BBB damage (Lassmann, 1983) even up to 4.5-fold (Fig. 1b). Also, in contrast to nerve and muscle from EAE rats, only in the inflamed CNS could we observe a gradual increase in 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 that of serum was observed. This may be best explained by contamination of the small tissue sample with blood (Fig. 1b).

Ultra high prednisolone levels in CNS

Tissue levels of prednisolone after injection of PL versus free prednisolone were measured by HPLC. Two hours after i.v. 10 mg/kg free prednisolone, a serum level of 112 ± 40 ng/ml, and a spinal cord level of 341 ± 54 ng/g was achieved, which was in the range of previous findings with free MP (Schmidt *et al*., 2000). After 6, 18, and more so after 42 h, free prednisolone in the serum had declined to almost undetectable levels. In spinal cord, 94 ± 68 ng/g were reached after 6 h, whereas later on no prednisolone was detectable (Fig. 1c). In contrast to this, we found much higher and prolonged levels of prednisolone after treatment with i.v. 10 mg/kg PL. Two hours after injection, the concentration was 5895 ± 837 ng/ml in the spinal cord and about the same in serum. After 18 h, the serum level declined moderately, and was 9203 ± 690 ng/ml until 42 h after the injection. Only after 42 h was the prednisolone concentration in spinal cord found to decrease to 2503 ± 161 ng/mg (Fig. 1c). Prednisolone tissue levels after PL were equivalent to concentrations of >10^{-5} mol/l for at least 18 h, which is well in the range of non-genomic actions, occurring at concentrations of >10^{-9} mol/l (Buttgereit *et al*., 1998). In contrast, the maximum tissue concentration after free prednisolone reached up to 10^{-6} mol/l for only 2 h.

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, 18 or 42 h or empty liposomes at 42 h before perfusion at day 5 in AT-EAE. At 42 h after PL, the rate of T-cell apoptosis was increased compared with all other groups (***P < 0.001 versus control or 6 h; **P < 0.01 versus 18 h) (Table 1), and T-cell infiltration was clearly reduced (**P < 0.01 versus all groups). PL given at 18 h before perfusion increased T-cell apoptosis (**P < 0.01 versus 6 h or control), with only a slight effect on T-cell infiltration (Table 1). Only with treatment at 42 h was the disease course ameliorated, which was repeated once (*P < 0.05 compared with all other groups) (data not shown).

PL is superior to free GS in AT-EAE

We then chose the two effective treatments with 10 mg/kg PL at 42 and at 18 h as the therapeutic regimen with two injections. This was compared with our previous regimen of two injections of 50 mg/kg MP at 18 and 6 h (Schmidt *et al*., 2000). 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 versus MP); MP also restored the BBB function as compared with controls (density: 127± 2.3, **P < 0.01 MP versus controls) (Fig. 2D–F). PL clearly increased T-cell apoptosis (***P < 0.001 versus control, P = 0.07 versus MP) and reduced T-cell infiltration (*P < 0.05 versus all groups) (Figs 2a–c, and 3a and b). MP augmented the rate of T-cell apoptosis (**P < 0.01 versus control), but only marginally reduced T-cell 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 T-cell apoptosis and infiltration, which is well in accord with previous results (Schmidt *et al*., 2000) (data not shown). Since liposomes are taken up mainly by Mϕ as revealed *in situ* by detection of gold-labelled liposomes (see above), we also characterized Mϕ infiltration. The number of infiltrating Mϕ in spinal cord was strongly reduced by PL (*P < 0.05 versus control, ***P < 0.001 versus MP), whereas MP had no effect. (Figs 2g–i and 3c). There was no increase in the percentage of apoptotic Mϕ, which was in the range of 0–5.5% (data not shown). Then we investigated the TNF-α production by T cells and Mϕ *in situ* by immunohistochemical double labelling. The percentage of TNF-α-positive T cells in spinal cord was strongly diminished by PL (32.1 ± 7.8 % versus 55.6 ±8.0 % in controls, ***P < 0.001), and was as equally effective as MP (34.4 ± 5.9%, ***P < 0.001 versus controls).

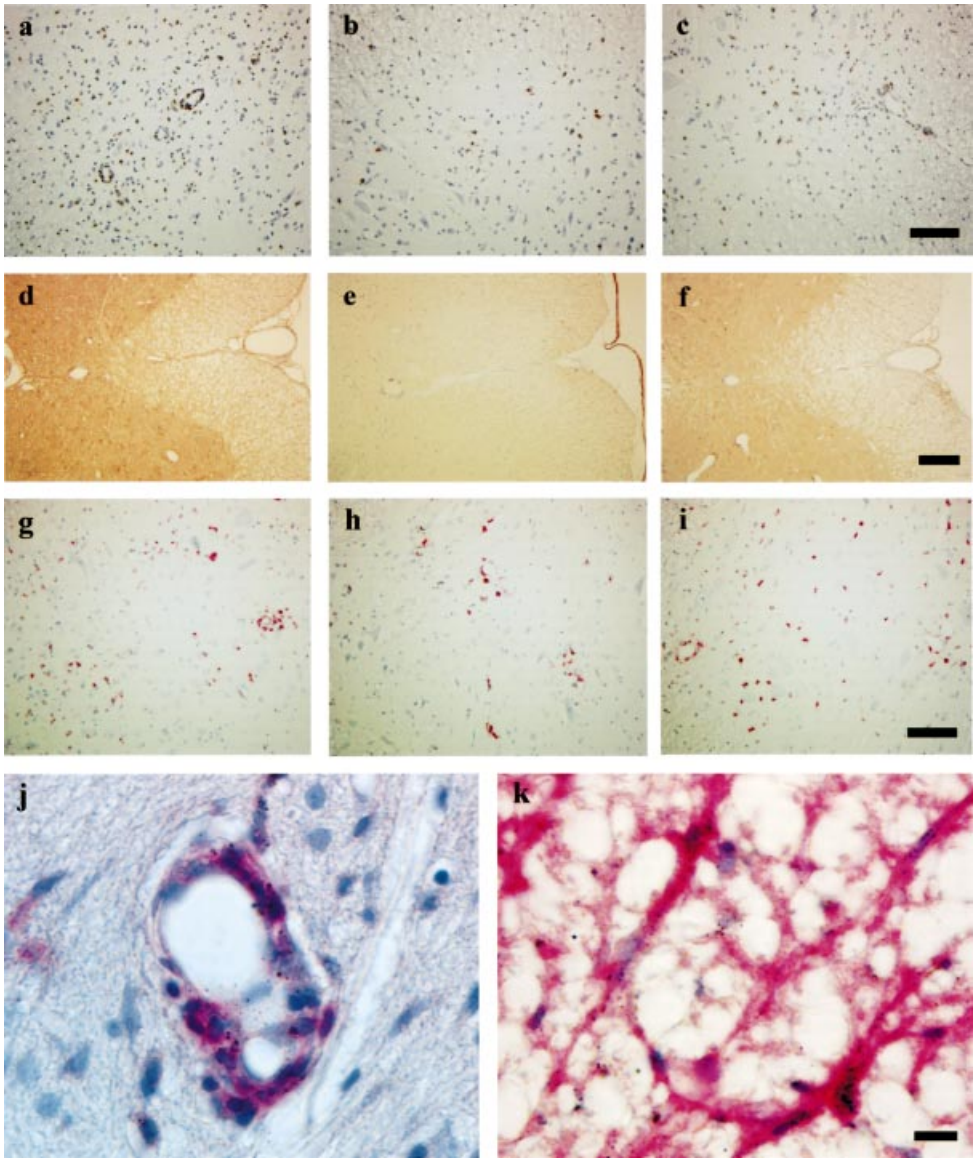

Fig. 2 Immunohistochemical detection of T-cell infiltration, BBB disruption, Mϕ infiltration and gold localization in astrocytes and Mϕ in 5 μm paraffin sections of spinal cord from AT-EAE rats at day 5. (**a**–**c**) Staining of T cells (DAB, brown) with the mAb B115-1 and haematoxylin counterstaining (blue) in control (**a**), PL 10 mg/kg (**b**) or MP 50 mg/kg treatment (**c**). (**d**–**f**) Detection of BBB disruption with anti-albumin antibody (DAB) and haematoxylin counterstain in control (**d**), PL 10 mg/kg (**e**) or MP 50 mg/kg treatment (**f**). (**g**–**i**) Staining of Mϕ (Vector red) with the mAb ED1 and haematoxylin counterstaining in control (**g**), PL 10 mg/kg (**h**) or MP 50 mg/kg treatment (**i**). (**j** and **k**) Detection of gold-labelled liposomes by the histochemical silver-enhancing technique (black), in combination with immunohistochemical staining for Mϕ (**j**, ED1 mAb, Vector red) or astrocytes (**k**, anti-GFAP, Vector red), and haematoxylin counterstain. Scale bar = 100 μm (**a**–**c**, **g**–**i**), 200 μm (**d**–**f**), 10 μm (**j**, **k**).

Also, the percentage of TNF-α-positive Mϕ was clearly reduced by PL (31.1 ± 7.4% versus 52.3 ± 4.2% in controls, $***P < 0.001$), and showed the same efficacy as MP (31.1 ± 13.1%, $**P < 0.01$ versus controls).

Even though our study was designed to look for short-term mechanisms *in situ*, we also observed a clinical benefit from PL ($**P < 0.01$ versus all groups at day 5) (Fig. 4a). 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 with 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

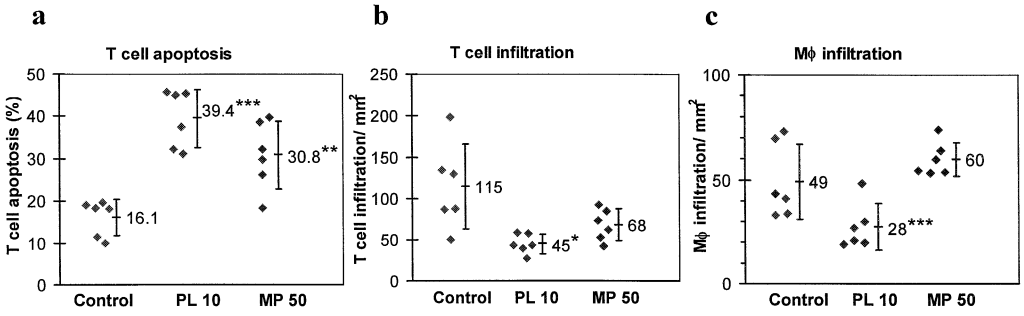

Fig. 3 Treatment of AT-EAE with two i.v. injections of 10 mg/kg PL 42 and 18 h, or with 50 mg/kg MP 18 and 6 h prior to perfusion on day 5 as compared with controls. (**a** and **b**) Immunohistochemical staining for T cells or Mϕ in spinal cord. (**a**) Percentage of apoptotic T cells rated by morphological criteria and double labelling with TUNEL. (**b**) Number of infiltrating T cells per mm^2. (**c**) Number of infiltrating Mϕ per mm^2. Each symbol represents one animal (n =6 per group); numbers and bars indicate mean ± SD. P values are explained in the text. This figure is available in colour as supplementary material at *Brain* Online.

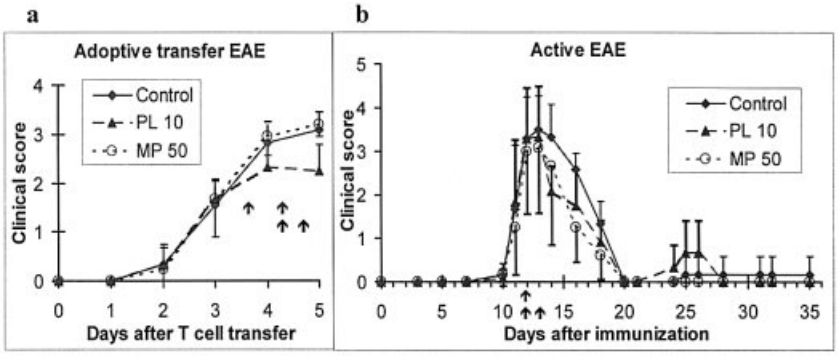

Fig. 4 (**a**) Treatment of AT-EAE with two i.v. injections of 10 mg/kg PL 42 and 18 h, or 50 mg/kg MP 18 and 6 h prior to perfusion on day 5 compared with controls. (**b**) Treatment of active EAE with one i.v. injection of 10 mg/kg PL at disease onset on day 12, or two i.v. injections of 50 mg/kg MP at days 12 and 13 compared with controls. The clinical course of the disease is indicated as mean ± SD (n = 6 per group). This figure is available in colour as supplementary material at *Brain* Online.

observed in AT-EAE even with a single injection, we investigated the possibility that 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 disease onset on day 12. This was compared with 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 versus control at day 14) was shown to be similarly effective as two injections of MP at a 5-fold higher dosage (*P < 0.05 versus control at day 16) (Fig. 4b).

Liposomes visualized in CNS tissue by gold labelling

To detect the cellular localization of extravasated liposomes, we injected gold-labelled liposomes in AT-EAE rats once 18 or 42 h before perfusion at day 5 and compared them with unlabelled empty liposomes. As revealed by the silver-enhancing technique, gold-labelled liposomes were located mostly within phagocytic cells. Most of the cells were Mϕ in the inflamed spinal cord, typically around blood vessels (Fig. 2j). However, gold label could also be detected in astrocytes and microglia, indicating a direct penetration of liposomes through the disrupted BBB without the help of Mϕ uptake (Fig. 2k). No gold particles were observed in oligodendrocytes. Gold-labelled liposomes could also be detected in Mϕ in the spleen and in Kupffer cells in the liver, which is in accord with other recent findings (Metselaar *et al.*, 2003). We observed no uptake of gold-labelled liposomes in T cells in spinal cord or spleen of EAE rats. There was no silver-enhanced signal in control rats treated with unlabelled empty liposomes.

No 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 took up liposomes as demonstrated by gold-labelled liposomes (see above). We characterized astrocytes (GFAP), oligodendrocytes (CNPase) and microglia (GSA-I-B_4) immunohistochemically. In the control group, we observed 260 ± 60/mm^2 (mean ± SD) oligodendrocytes, with 6.5 ± 1.9% apoptotic, 75 ± 12/mm^2 astrocytes, with 4.0 ± 1.5% apoptotic, and 40± 18/mm^2 microglial cells without microglial apoptosis. There was no induction of apoptosis in any of these glial cells, and their total number per mm^2 remained basically unchanged after treatment with PL or MP compared with controls (data not shown).

Enhanced T-cell apoptosis in spleen

We qualitatively investigated apoptosis of phagocytes and T cells in spleen and liver by immunohistochemistry. We observed an induction of T-cell apoptosis in spleen in all PL-treated groups, which was stronger compared with free prednisolone. The frequency of apoptotic T cells after injection of free prednisolone compared with controls was elevated after 2 and 6 h, but not after 18 and 42 h. There was no induction of apoptosis or notable change in number of Mϕ in spleen nor in Kupffer cells in liver in any of the treatment groups compared with controls (data not shown).

Discussion

Our experiments presented here demonstrate the principle of drug targeting of prednisolone with a new therapeutic liposomal formulation in the treatment of EAE as a model for multiple sclerosis. Radioactive labelling showed the accumulation of liposomes in the inflamed target organ. By a gold labelling technique, liposomes could be localized at the site of inflammation, where much higher and prolonged tissue concentrations of prednisolone as the active drug were measured by HPLC. This also had functional implications: a dose of i.v. 10 mg/kg PL was superior to a 5-fold higher dose of i.v. free MP with regard to improvement of BBB disruption, induction of T-cell apoptosis and amelioration of cellular infiltration. Only with PL could a clear reduction of inflammatory Mϕ in the lesion be achieved. In addition, we observed a reduced rate of TNF-α-expressing T cells and Mϕ *in situ* after PL treatment. As a consequence of the reduced inflammation, the disease course of AT-EAE and of active EAE was ameliorated. There were no detectable side effects on glial cells *in situ*.

The therapeutic goal in treatment of multiple sclerosis relapses is to reduce cellular inflammation as efficiently as possible in order to prevent ongoing tissue destruction and axonal loss. The dosing of GS as the mainstay of therapy in multiple sclerosis relapses is still a matter of debate. With regard to our previous findings in EAE (Schmidt *et al*., 2000), one of the major issues of dosing the steroid is to reach very high tissue levels, exerting multiple pathways of steroid actions according to a new model of steroid mechanisms (Gold *et al*., 2001). 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 were developed in the late 1970s for intra-articular treatment of arthritis in experimental models and in patients (Dingle *et al*., 1978; de Silva *et al*., 1979). In the early 1990s, the principle of selective targeting of inflamed tissue was reported with cholesterol/lecithin liposomes, which showed an improved stability in the circulation. These liposomes reached the inflamed joints after i.v. injection in rats with experimental arthritis (Love *et al*., 1990). Recently, it could be demonstrated that a similar formulation could penetrate the BBB in EAE (Rousseau *et al*., 1999). I.v.-injected liposomes encapsulating dichloromethylene diphosphonate, which suppresses Mϕ activity, were beneficial in EAE and experimental autoimmune neuritis (EAN) (Huitinga *et al*., 1990; Jung *et al*., 1993). For significant accumulation of the encapsulated drug in inflamed extravascular tissue, it appears to be crucial that liposomes exhibit a long-circulating behaviour, and it seems likely that the effects reported by de Silva *et al*. (1979) and Dingle *et al*. (1978) were mediated either by a change of the pharmacokinetics of the encapsulated drug or by an indirect effect via monocytes/macrophages in liver, spleen or blood.

Extended circulation of PL can be accomplished with small-sized liposomes (<100 nm) composed of neutral, saturated phospholipids and cholesterol. Often, water-soluble polymers such as 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 (Woodle and Lasic, 1992; Oku and Namba, 1994; Woodle *et al*., 1994). Using this approach, PEG-coated long-circulating liposomes can remain in the circulation with a half-life as long as 50 h in humans (Gabizon *et al*., 1994). The improved pharmacokinetics and target localization have led to several successful applications of this formulation in antitumour therapy (Gabizon *et al*., 1994; Hong and Tseng, 2001). Studies with liposome-associated radiolabels have indicated that PEG–liposomes can also be employed successfully to target pathological sites selectively in inflammatory disorders (Laverman *et al*., 1999; Dams *et al*., 2000).

For this study, we encapsulated prednisolone phosphate as the active drug into long-circulating liposomes, since methylprednisolone succinate, which we used in previous studies (Schmidt *et al*., 2000, 2001), 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-labelled liposomes, which could be detected in spinal cord within vascular endothelium, in

inflammatory Mϕ and yet unphagocytosed in perivascular areas. Additionally, there was direct uptake of liposomes by resident astrocytes and microglia. Another argument showing that liposomes can enter the CNS as target organ directly without the help of phagocytosis is the rapid increase of the prednisolone concentration in the tissue 2 h after injection, which is much faster than would be expected based on cellular diapedesis. Also, our radioactive studies 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, uninflamed 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 ultra high tissue HPLC levels of prednisolone after PL were in the range of non-genomic effects according to a new model of steroid mechanisms of action (Gold *et al*., 2001). These non-genomic actions are mediated by membrane-bound steroid receptors and physicochemical membrane interactions, which may occur after degradation of liposomes, possibly by extracellular proteases and also after uptake and lysosomal degradation in Mϕ. The prednisolone tissue concentration after the free steroid remained in a much lower range, which was well in accord with previous findings (Schmidt *et al*., 2000). The ultra high levels of prednisolone after PL exerted direct effects at the site of inflammation, such as the superior induction of T-cell apoptosis and the higher degree of restoration of BBB function. The TNF-α expression of T cells and Mϕ was reduced comparably with a 5-fold higher dose of free MP. However, besides clear direct effects, the reduced infiltration of T cells 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 uptake of PL, we could not detect apoptosis in resident cells such as 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 experimental arthritis did not unreveal unwanted side effects of PL (Metselaar *et al*., 2003).

Taken together, we show that long-circulating PL given at 10 mg/kg accumulate in the inflamed CNS of EAE rats, leading to ultra high tissue levels of the GS. Augmentation of T-cell apoptosis *in situ* occurs rapidly and the BBB integrity is improved. The reduced infiltration of T cells and Mϕ ultimately leads to a reduced disease activity of active and AT-EAE. The reduced Mϕ infiltration, which was only seen after PL, might help especially 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, there are indications that employing the principle of drug targeting may reduce systemic side effects. These findings might have implications for the treatment of multiple sclerosis, but also other inflammatory disorders of the CNS.

Acknowledgements

We wish to thank Professor Rudolf Martini for stimulating discussions, Gabriele Köllner, Helga Brünner and Verena Wörtmann for excellent technical assistance, and Louis van Bloois for his help with preparing the liposomes. This study was supported by funds from the state of Bavaria, Germany.

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Received January 17, 2003.
Revised March 25, 2003. Accepted March 28, 2003