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Safety of glucocorticoids can be improved by lower yet still effective dosages of liposomal steroid formulations in murine antigen-induced arthritis: Comparison of prednisolone with budesonide

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ABSTRACT

The goal of this study was to compare the effects of liposomal and free glucocorticoid formulations on joint inflammation and activity of the hypothalamic-pituitary-adrenal (HPA) axis during experimental antigen-induced arthritis (AIA). A dose of 10 mg/kg liposomal prednisolone phosphate (PLP) gave a suppression of the HPA-axis, as measured by plasma corticosterone levels in mice with AIA and in naïve mice. In a subsequent dose-response study, we found that a single dose of 1 mg/kg liposomal prednisolone phosphate (PLP) was still equally effective in suppressing joint inflammation as 4 repeated once-daily injections of 10 mg/kg free PLP. Moreover, the 1 mg/kg liposomal PLP dose gave 22% less suppression of corticosterone levels than 10 mg/kg of liposomal PLP at day 14 of the AIA. In order to further optimize liposomal glucocorticoids, we compared liposomal PLP with liposomal budesonide phosphate (BUP) (1 mg/kg). At 1 day after treatment, liposomal BUP gave a significantly stronger suppression of joint swelling than liposomal PLP (lip. BUP 98% vs. lip. PLP 79%). Both formulations also gave a strong and lasting suppression of synovial infiltration in equal amounts. However, at day 21 after AIA, liposomal PLP still significantly suppressed corticosterone levels, whereas this suppression was not longer statistically significant for liposomal BUP. Conclusion: Liposomal delivery improves the safety of glucocorticoids by allowing for lower effective dosing. The safety of liposomal glucocorticoid may be further improved by encapsulating BUP rather than PLP.

1. Introduction

Synthetic glucocorticoids are powerful anti-inflammatory drugs that are widely applied to treat rheumatoid arthritis (RA). Recurrent and high dosing of glucocorticoids however can lead to a plethora of adverse side effects including bone demineralisation, metabolic syndrome and suppression of the hypothalamic-pituitary-adrenal (HPA) axis (McDonough et al., 2008). Therefore, modifications in glucocorticoid compounds, treatment strategy and/or delivery are aimed at improving potency and availability of glucocorticoids, while decreasing their side effects.

Delivery of glucocorticoids within long-circulating 'stealth' liposomes offers a way in which the circulation time of glucocorticoids can be improved (Allen, 1994). Liposomes sized to a diameter up to 100 nm passively extravasate in areas of increased vasodilation, e.g. due to local inflammation. In earlier studies, we and others found that a single dose of liposomal glucocorticoid is more effective than repeated doses of free glucocorticoid in several models of inflammation (Anderson et al., 2010; Metselaar et al., 2003; Crommelin et al., 1999). The targeting effect may thus allow for less frequent dosing, which was demonstrated in a murine collagen type II arthritis, where a dose of 10 mg/kg liposomal PLP was more effective than 5 repeated once-daily injections of 10 mg/kg free PLP (Metselaar et al., 2004). Furthermore, a clinical trial on long-circulating liposomal PLP in RA patients recently demonstrated their safety and efficacy (Barrera et al., 2008).

Although the pharmacokinetics and biodistribution of liposomal glucococorticoid delivery may explain their strong anti-inflammatory effect, less is known about the side effects of

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liposomal glucocorticoids. In animal models of experimental arthritis, acute side effects like weight loss and a suppression of the HPA-axis can be studied as indication for the side effects of glucocorticoids (Rauchhaus et al., 2009).

The production of endogenous glucocorticoids (corticosterone in mice) by the adrenal cortex is stimulated by adrenocorticotropic hormone (ACTH) produced and released by the pituitary gland, which in turn is regulated by corticotrophin releasing hormone (CRH) produced by the hypothalamus. The activity of the HPA-axis is regulated via a feedback mechanism of its end product, glucocorticoids, to the pituitary gland and the hypothalamus. Thus, the activity of the HPA-axis is reflected by the levels of circulating glucocorticoids in the blood (DeRijk and Sternberg, 1994).

Encapsulating different glucocorticoids into liposomes has been suggested as a way to optimize their efficacy. In most studies on liposomal glucocorticoid delivery, PLP was used, however, encapsulation of other glucocorticoids may be more effective. Of several tested liposomal glucocorticoids in a murine melanoma model, liposomal budesonide phosphate (BUP) was the most efficacious formulation in inhibition of tumor growth in these mice (Banciu et al., 2008). Budesonide is a potent glucocorticoid that is more effective at lower doses than prednisolone in the treatment of human RA (Kirwan et al., 2004). In addition, budesonide is cleared relatively rapidly from systemic circulation compared to prednisolone in their free form in humans (Ryrfeldt et al., 1982). Currently, budesonide is mostly used as treatment for asthma (Chipps, 2009), however, its characteristics make it an ideal candidate for liposomal delivery as treatment for RA as it may further reduce the required dose of liposomal glucocorticoid whereas its systemic availability is kept to a minimum due to its rapid systemic clearance.

The goal of this study was to compare the efficacy of liposomal PLP and BUP on joint inflammation during antigen-induced arthritis and investigate their effects on the HPA-axis.

2. Materials and methods

2.1. Preparation of liposomal glucocorticoids

Liposomes were prepared as described previously (Banciu et al., 2008). Briefly, a lipid formulation of dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshave, Germany), PEG 2000-distearoyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0 were dissolved in ethanol which was then evaporated from a round-bottom flask to create a lipid film. The lipid film was hydrated in water to create empty liposomes or in a solution of 100 mg/ml prednisolone disodium phosphate (PLP) (Bufa, Uitgeest, The Netherlands) in water to create liposomal PLP or the lipid film was hydrated in Budesonide Phosphate in water to create liposomal BUP. Single Unilamellar Vesicles were obtained by filtering the liposomal dispersion multiple times through polycarbonate filter membranes decreasing in pore diameter until the liposomes had a mean diameter in the range of 90–110 nm with a polydispersity of <0.2. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern ltd., Malvern, UK). Unencapsulated PLP was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Encapsulation dose of the glucocorticoid content was determined by extracting the aqueous phase from liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the glucocorticoid content. With ultra performance liquid chromatography (UPLC) (Mollmann et al., 1995; Derendorf et al., 1986), using a RP18 (5 μm) column (Merck) and a mobile phase acetonitril-water with pH of 2, connected to an UV-detector, which was set at 254 nm, both prednisolone or budesonide and its phosphate ester could be measured in one single run. The detection limit for the UPLC setup was 20 ng/ml. Liposomal preparations contained 1–10 mg/ml glucocorticoid (varying slightly between batches) and an average of 60 μmol phospholipid.

2.2. Animals

Mice (male C57Bl/6) were purchased from Elevage-Janvier (Le Genest Saint Isle, France) and were housed in filter-top cages and fed a standard diet and water provided ad libitum. All animal procedures were approved by the institutional ethics committee.

2.2.1. Antigen-induced arthritis

The antigen-induced arthritis (AIA) was performed as decribed previously (van den Berg et al., 1981). Briefly, mice at an age of 8–12 weeks were immunized with 100 μg methylated bovine serum albumin (mBSA, Sigma–Aldrich, St Louis, USA), emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, USA) which was injected into the flanks and the footpath of the forelegs. Heat-killed *Bordetella pertussis* (RIVM, Bilthoven, The Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with in total 50 μg mBSA/Freund's complete adjuvant were given in the neck region 1 week after the initial immunization. Two weeks after these injections, AIA was induced by intra-articular injection of 60 μg of mBSA in 6 μl of phosphate-buffered saline into the knee joints. Mice were weighed at the beginning and at the end of the AIA. Body weight is expressed as percentage of their body weight at the start of the AIA.

2.2.2. Treatment, sacrifice and tissue isolation

At day 3 when severe arthritis had developed mice were treated with either a single injection of liposomal glucocorticoid, repeated daily injections of 10 mg/kg free PLP or PBS. At day 14 and 21 after AIA-induction, whole blood was obtained by retro-orbital bleeding of mice anaesthetized with isoflurane (5%), into EDTA-coated Mini-Collect tubes (Greiner bio-one). Hereafter, mice were sacrificed by cervical dislocation and whole knee joints were stored in formalin for histology. After centrifugation of whole blood samples, plasma samples were stored at −80 °C.

2.3. Measurement of corticosterone

Corticosterone (B) was measured by radio-immuno assay (RIA) in blood plasma samples as described by Sweep et al. (1992). Briefly, plasma B was measured by RIA after extraction using antiserum raised in sheep against a B-21-hemisuccinate-BSA conjugate. To each plasma sample (50 μl), 100 μl 0.1 N NaOH, 100 μl B tracer ([1α,2α-N-^{3}H]B; Amersham International PLC, Amersham, Aylesbury, Buckinghamshire, United Kingdom; 10,000 dpm in 0,2% ethylene glycol (Merck)/water) and 500 μl bidistilled water were added. Extraction was carried out using 7.5 ml dichloromethane (Baker, Deventer, The Netherlands). The water phase was discarded and the dichloromethane phase was evaporated. The residue in 2 ml 0.2% EGW and mixed with 200 μl of an antiserum dilution (final dilution 1:100,000) and 100 μl B tracer (10,000 dpm/tube). Antiserum and tracer were diluted in 0.05 M borate buffer (pH 8.0) containing 0.1% human γ-globulin (Beriglobin S, Behring, Marburg, Germany). Aliquots of 300 μl eluate were taken for recovery. Amounts of 0–5000 fmol/tube B (steraloids, Inc., Wilton, NH) diluted in 0.2% EGW were used for obtaining a standard curve. After incubation overnight at 4 °C, the suspension was mixed with 150 μl dextran-coated charcoal suspension and left at 4 °C for 5 min. After centrifugation, the supernatants were decanted into counting vials,

and 4 ml Aqualuma (Lumac LSC, Olen, Belgium) was used as counting solution for radioactivity measurements. The sensitivity of the assay was 25–45 fmol/tube. The within- and between-assay coefficients of variation were 10.0% and 16.7%, respectively.

2.4. Measurement of enzymes in blood plasma

The enzymes lactate dehydrogenase (LD), alkaline phosphatase (AP), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were measured by turnover of their respective substrates by spectrophotometry on a Modular spectrophotometer (Roche Diagnostics) (Mocarelli et al., 2008).

2.5. Histology

Total knee joints of mice were isolated after sacrifice and fixed for 4 days in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Standard frontal sections of 7 μm were mounted on superfrost slides (Menzel-Gläser, Braunschweig, Germany) for histology and immunostaining. Histology was performed on sections stained with hematoxylin and eosin (HE). The severity of joint inflammation was determined as described previously (van Lent et al., 2003), by scoring the amount of cellular infiltration into the synovium using an arbitrary scale (0–3), for three representative knee joint sections for each mouse (5 mice for each treatment group). Scoring was performed in a blinded manner by two independent observers: 0, no cells; 1, mild cellularity; 2, moderate cellularity; 3, maximal cellularity.

2.6. Statistical analysis

Statistical significance of the differences between glucocorticoid-treated groups and control-treated (PBS) groups were tested by Student's *t*-test or Mann–Whitney *U*-test (non-parametric test) with the aid of Graphpad Prism 5.0 software. Data are expressed as mean ± standard error of the mean (SEM), *P*-values less than 0.05 were considered as statistically significant.

3. Results

3.1. Liposomal PLP reduces activity of the HPA axis

To study the side effects of liposomal and free PLP, we measured blood plasma levels of corticosterone, indicative for activity of the hypothalamic-pituitary-adrenal (HPA) axis. In previous studies we found that a single dose of 10 mg/kg liposomal prednisolone phosphate (PLP) strongly suppressed joint inflammation in experimental arthritis and was much more effective than 4 repeated once-daily injections of free PLP (Metselaar et al., 2004).

During AIA, plasma levels of corticosteroids strongly increase and were 240% higher at day 14 after arthritis induction when compared to naïve mice. Treatment of AIA with single dose liposomal PLP and multiple dose (4 × 10 mg/kg) free PLP suppressed mean plasma corticosterone levels by 49% (from 863 to 456 pmol/ml) and 28% (to 634 pmol/ml), respectively (Fig. 1).

Because activity of the HPA-axis is strongly related to disease activity, we also studied the side effects of glucocorticoids in naïve mice in which the arthritis is not additionally influenced by effects of the experimental arthritis. Here we found a reduction in mean plasma levels of corticosterone of 76% by single dose liposomal PLP (from 358 to 87 pmol/ml) when compared to mice injected with PBS. In contrast, multiple dose free PLP did not suppress corticosterone in naïve mice when compared to PBS injection, indicating that the 10 mg/kg dose of liposomal PLP, which is very effective in several arthritis models, causes suppression of the HPA-axis.

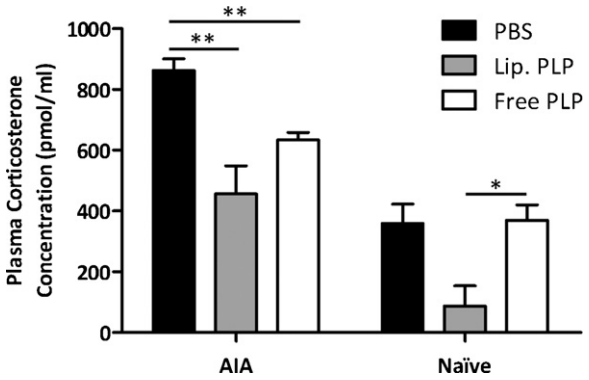

Fig. 1. Liposomal PLP suppresses corticosterone levels during AIA and in naïve mice. Mice were injected with liposomal PLP (10 mg/kg), free PLP (4 × 10 mg/kg) or PBS (control). Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney *U*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

Liposomes are largely taken up from the blood by Kuppfer cells in the liver and other cells of the mononuclear phagocytic system. Therefore, we also measured enzymes in blood plasma which are indicative for liver toxicity and cellular stress: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (AP) and lactate dehydrogenase (LD) (Fig. 2). In naïve mice, liposomal PLP had minimal effects on ALAT, ASAT, AP and LD (29%, 17%, 1% and 6%, respectively, when compared to PBS treatment).

3.2. Lower dosing of liposomal PLP maintains efficacy against antigen-induced arthritis

To see whether the single dose liposomal PLP can be lowered, without losing its efficacy compared to multiple dose free PLP, we studied the effect of different doses of liposomal PLP on joint inflammation during antigen-induced arthritis (AIA) in relation to its systemic side effects.

The different single doses of liposomal PLP (10, 5 and 1 mg/kg) all strongly reduced synovial infiltration at day 14 after AIA (11 days after treatment, Fig. 3). Suppression of joint inflammation by liposomal PLP occurred in a dose responsive manner, with an average suppression of 75%, 63% and 47% for 10, 5 and 1 mg/kg of liposomal PLP, respectively. Interestingly, a dose of 1 mg/kg liposomal PLP was still comparable in potency as 4 repeated once-daily injections of 10 mg/kg free PLP (48% suppression, Fig. 3).

As even lower doses of liposomally delivered PLP still improved its efficacy compared to the free form, we wanted to see how this would affect its systemic side effects and how this would compare against another liposomal glucocorticoid formulation.

3.3. Optimization of liposomal glucocorticoid delivery by encapsulating budesonide phosphate

To further optimize the efficacy of liposomal glucocorticoid delivery, we studied the effect of encapsulation of a novel glucocorticoid: budesonide phosphate (BUP). Budesonide is a potent glucocorticoid that has a relatively high clearance rate and may therefore have less systemic side effects than prednisolone. To compare the efficacy of both forms, mice with AIA were treated with 1 mg/kg of liposomal PLP and BUP.

To evaluate the early effects of liposomal PLP and BUP, we measured joint swelling in the knee joint already at day 1 after treatment using ^{99M}Tc-uptake. At this time point, both formulations, already significantly suppressed joint swelling when compared to control PBS-treatment (Fig. 4). Liposomal BUP proved

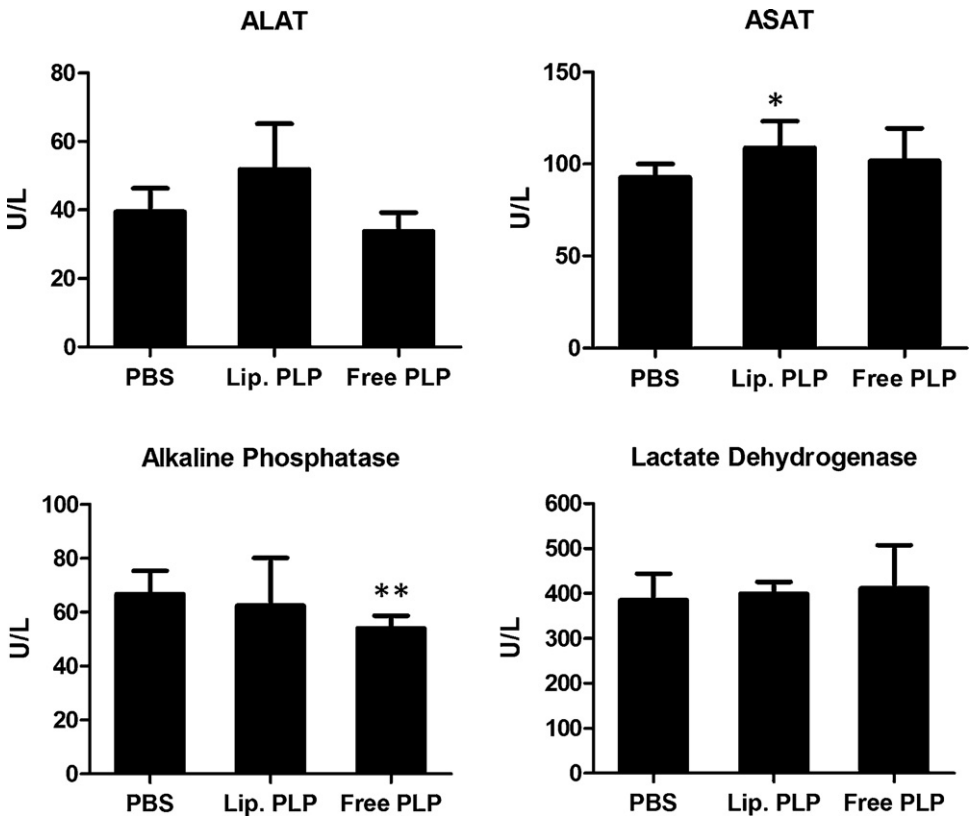

Fig. 2. Effect of Liposomal and free PLP on liver toxicity in naïve mice. Healthy mice were injected with liposomal PLP (10 mg/kg), 4× free PLP (4 × 10 mg/kg) or PBS. Plasma levels of the enzymes ALAT, ASAT, AP and LD were determined at day 5 after injection. Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney U-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

to be superior to liposomal PLP, with liposomal BUP completely suppressing joint swelling (lip. PLP 79% vs. lip. BUP 98%).

Additionally, we measured a suppression of joint inflammation by histology at day 14 and day 21 after AIA induction (Fig. 5). Both formulations were equally potent and showed a strong suppression of joint inflammation at day 14 after AIA (49% and 52% suppression for liposomal PLP and BUP, respectively). Both formulations still suppressed joint inflammation at day 21 after AIA (30% and 29% for lip. PLP and lip. BUP, respectively).

To evaluate and compare the side effects of liposomal PLP and liposomal BUP at this low dose (1 mg/kg), we again measured corticosterone levels. Firstly, we noted that corticosterone levels in control, PBS-treated mice waned by 39% from day 14 to day 21 after AIA (Fig. 6). Secondly, at day 14 of the AIA, liposomal PLP and BUP (1 mg/kg) significantly suppressed corticosterone levels by 24% and 34% from control, PBS-treated mice respectively. At day 21 after AIA, liposomal PLP still showed a low, although significant, reduction in corticosterone levels. In contrast, no significant reduction was detected anymore in the liposomal BUP treated group suggesting a faster recovery of the HPA-axis with this novel formulation.

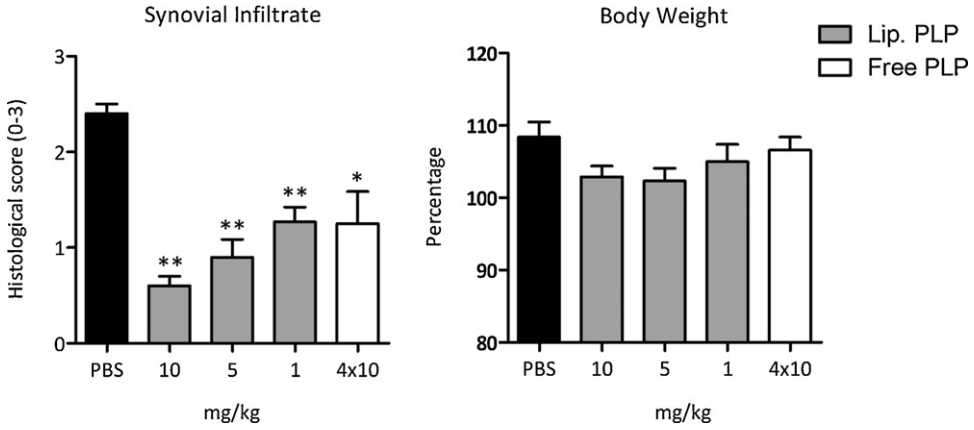

Fig. 3. Lower dosing of Liposomal PLP maintains efficacy against AIA. Mice were injected at day 3 of arthritis with 10, 5 and 1 mg/kg of liposomal PLP, free PLP (4 × 10 mg/kg) or PBS (control). Joint inflammation was determined at day 14 of the AIA. Body weight is expressed as percentage of their weight at time of sacrifice compared to their weight before treatment (day 3). Note that all PLP formulations significantly suppress joint inflammation, without significantly suppressing body weight. Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney U-test. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control (PBS) treated group.

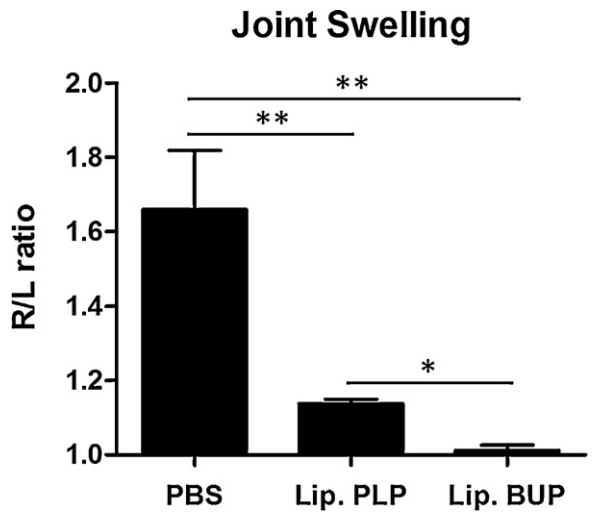

Fig. 4. Liposomal PLP and BUP suppress joint swelling during established AIA. Liposomal PLP and Liposomal BUP (1 mg/kg) were injected at day 3 after AIA. Joint swelling was determined by measurement of ^{99M}Tc-uptake at day 1 after treatment. Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney U-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

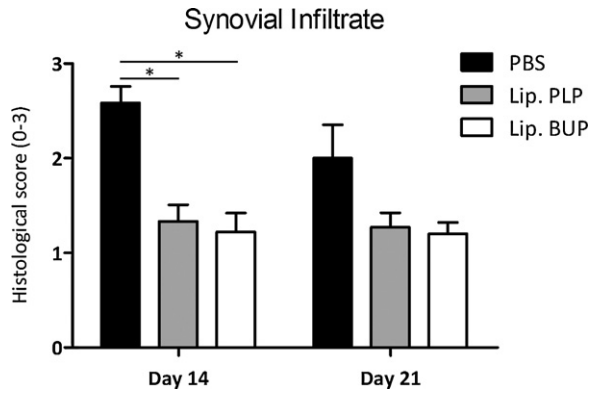

Fig. 5. Liposomal PLP and BUP give a lasting suppression of joint inflammation during AIA. Liposomal PLP and Liposomal BUP (1 mg/kg) were injected at day 3 after AIA. Joint inflammation was determined by histological scoring at day 14 and 21 after AIA. Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney U-test. $^{*}P < 0.05$.

4. Discussion

In free form, high-, pulse-dosed glucocorticoids cause unwanted side effects in the treatment of rheumatoid arthritis (Baethge et al., 1992). Liposomal delivery offers a way to decrease the side effects and increase the efficacy of glucocorticoids by increasing their circulation time and selective biodistribution towards areas of inflammation, which allows for less frequent drug dosing (Metselaar et al., 2003, 2004; Schmidt et al., 2003). In the present study we found that a single dose of 1 mg/kg of liposomal glucocorticoid was comparable in efficacy with 4 repeated once-daily injections of 10 mg/kg free PLP. Furthermore, the low dose of liposomal PLP gave a fast and durable suppression of inflammation up to day 21 of the AIA (18 days after treatment).

Liposomes sized to a diameter near 100 nm, reach the inflamed joints via passive extravasation out of dilated blood vessels, which explains their selective targeting of sites of inflammation. Long-circulating liposomes evade uptake by the mononuclear phagocytic system (MPS) due to the incorporation of poly-ethylene glycol (PEG) in their lipid membrane. However, still a large portion of the liposomes is taken up by MPS cells in spleen and liver (Metselaar et al., 2003). In the present study, we found no major effects on enzymes indicative of liver toxicity, despite their pronounced biodistribution to this organ.

Glucocorticoids are a natural product of the HPA-axis and can regulate their own production via feedback signaling to the hypothalamus. When therapeutically administered glucocorticoids enter the systemic circulation, they can regulate (suppress) the activity of the HPA-axis in a manner similar to endogenous glucocorticoids (Morand and Leech, 2001).

Studies by Metselaar et al. in rats have shown that liposomally encapsulated PLP remains available in the circulation for days, but that PLP is not released from the liposome particles while they are in the circulation (Metselaar et al., 2003). Therefore, the plasma concentration of 'free' circulating (i.e. not encapsulated in liposomes) prednisolone is very low. Nevertheless, we observed in the present study a suppression of the HPA-axis by liposomal PLP in naïve mice, which suggests that there are systemic side effects of liposomal glucocorticoids, at least for the highest dose tested. Hypothetically, low plasma levels of 'free' glucocorticoids could be induced after degradation and subsequent release by the MPS cells.

In our study, corticosterone levels in control PBS-treated mice with AIA were 2.4 times higher than in naïve mice, which demonstrates activation of the HPA-axis in this model. This is in line with earlier studies in which a high correlation between the activity of the HPA-axis and arthritis was found (Morand and Leech, 2001).

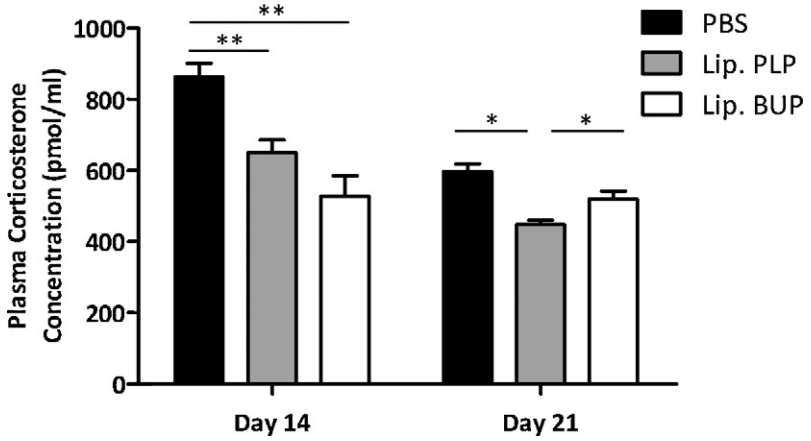

Fig. 6. Suppression of the HPA-axis by liposomal PLP and BUP (1 mg/kg). Mice were injected at day 3 after AIA. Note that liposomal BUP does not significantly suppress the HPA-axis any more at day 21 after AIA. Data represent mean ± S.E.M. ($n = 5$). Statistical significance between treatment groups was tested by Mann–Whitney U-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

The HPA-axis is stimulated by pro-inflammatory cytokines, like IL-1β, IL-6 and TNF-α, which are produced to a high extend during AIA (Bethin et al., 2000; van der Meer et al., 1996a,b; Sweep et al., 1992). The strong suppression of joint inflammation induced by liposomal glucocorticoid treatment is likely to diminish pro-inflammatory cytokine levels and thus explain the reduced activation of the HPA-axis during AIA.

Macrophages contribute for a large part to the activation of the HPA-axis as these cells are the main producers of inflammatory cytokines (van der Meer et al., 1996a,b). Stealth liposomes when given systemically, pass the endothelial layer of the blood vessels in the synovium and are directly encapsulated by the macrophages lying around the blood vessels. As these cells are of crucial importance in onset and propagation of antigen-induced arthritis (van Lent et al., 1993), direct targeting by glucocortocids may explain their rapid and strong effects on joint inflammation.

Liposomal glucocorticoid delivery may be further optimized by encapsulating novel glucocorticoids with superior characteristics. Budesonide is a more potent glucocorticoid than prednisolone (Toogood et al., 1989), therefore, liposomal BUP may still be effective at lower dosages than liposomal PLP. As budesonide has been shown to have a relatively short half life in humans and in mice, this may also result in safer use of liposomal glucocorticoid in terms of its side effects (Andersson et al., 1986). Furthermore, ACTH response studies in RA patients treated with budesonide and prednisolone showed less suppression of the HPA axis with budesonide, whereas it had an increased potency over prednisolone (Kirwan et al., 2006). Administration of slow-released oral budesonide via the ileum was previously unsuccessful in RA patients (Sheldon, 2003), however, liposomal encapsulation specifically reaches inflamed joints and may therefore be more successful. Moreover, the results in the present study demonstrate that liposomally delivered BUP is more potent than liposomal PLP in reducing joint swelling and in addition allows for a faster recovery of the HPA-axis. A high potency of liposomal BUP combined with a rapid systemic clearance thus make liposomal budesonide phosphate a good candidate for future therapy against RA.

5. Conclusions

Liposomal delivery improves the safety of glucocorticoids by allowing for lower effective dosing. The safety of liposomal glucocorticoid may be further improved by encapsulating BUP rather than PLP.

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