CHAPTER 3

JOINT TARGETING OF GLUCOCORTICOIDS WITH LONG-CIRCULATING LIPOSOMES INDUCES COMPLETE REMISSION OF EXPERIMENTAL ARTHRITIS

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Objective. To increase the therapeutic activity of glucocorticoids in experimental arthritis by encapsulation in long-circulating PEG-liposomes, which show the ability to preferentially accumulate in inflamed joints after i.v. administration.

Methods. Rats with adjuvant arthritis (AA) were i.v. treated with liposomal and free prednisolone phosphate (PLP) a few days after the first signs of the disease. The effect on paw inflammation scores during the weeks after treatment was evaluated. Liposome biodistribution and joint localization was investigated by labeling the preparation with radioactive $^{111}$In. By studying PLP encapsulated in other types of liposomes, which show a distinctive tissue distribution pattern and reduced accumulation in inflamed joints, the importance of targeted delivery to inflamed joints for the increased therapeutic effect was illustrated.

Results. Liposomal PLP proved to be highly effective in a rat adjuvant arthritis model. A single injection of 10 mg/kg resulted in complete remission of the inflammatory response for almost a week. In contrast, the same dose unencapsulated prednisolone did not reduce inflammation, while only a slight effect was observed after repeated daily injections. Evidence was found that preferential glucocorticoid delivery to the inflamed joint is the key factor explaining the observed strong therapeutic benefit obtained with the liposomal preparation, excluding other possible mechanisms like splenic accumulation or prolonged release of prednisolone in the circulation.

Conclusion. Targeted delivery using long-circulating liposomes is a promising, novel means to successfully intervene in arthritis with glucocorticoids.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, involving joint inflammation and progressive cartilage destruction (1). RA is primarily an inflammatory disease of the connective tissue characterized by spontaneous remissions and exacerbations (flare-ups).

Glucocorticoids can be highly effective in treating joint inflammation, but their systemic application is limited due to a high incidence of serious adverse effects, especially related to long-term treatment (2,3). In addition it is generally assumed that, contrary to the so-called ‘disease modifying anti-arthritic drugs’, glucocorticoids only suppress the inflammatory process, leaving the progression of disease-related joint destruction unaffected (4,5).

Two aspects are important for the efficacy-safety issues related to systemic glucocorticoid treatment. First is the unfavorable pharmacokinetic behavior of glucocorticoids upon i.v. administration, which is characterized by rapid clearance in combination with a large volume of distribution. Therefore high and frequent dosing is often necessary to achieve an effective concentration of glucocorticoid at inflamed target sites. Combined with the second aspect, namely the profound physiological activity of glucocorticoids in many different tissues, this explains the high risk of occurrence of side effects (6).

We hypothesized that a drug-targeting approach could increase the therapeutic index (7). Glucocorticoids can be incorporated in particulate carriers that show enhanced localization in the target site relative to the drug in unbound form. One of the most interesting carriers for drug-targeting in inflammatory disorders is the long-circulating liposome (LCL) system. Liposomes are small lipid bilayer vesicles (LCL are approximately 100 nm in diameter) with an aqueous core that can be used to entrap water-soluble agents. Often water-soluble polymers like poly(ethylene glycol) (PEG) are attached to the surface of LCL to reduce adhesion of opsonic plasma proteins that would otherwise induce recognition and rapid removal from the circulation by macrophages in liver and spleen (8-10). Using this approach PEG-coated LCL can remain in the circulation with a half-life as long as 50 hours (11,12). Studies with radiolabels entrapped in PEG-liposomes have indicated that PEG-liposomes can selectively extravasate in inflammatory tissues, by virtue of increased permeability of the local vascular endothelium (13,14).

To evaluate whether drug targeting can improve delivery and therefore the efficacy of glucocorticoids, we studied pharmacokinetics, tissue distribution, target localization and therapeutic activity of PLP-PEG-liposomes in a rat model of experimental arthritis. The results indicate that liposomal encapsulation can strongly increase the therapeutic efficacy of PLP. To investigate the critical role of the drug-targeting effect on the improved therapeutic activity, liposome types with short circulation times and reduced localization in the arthritic sites were investigated. The present study shows that enhanced joint accumulation of PLP realized with the long-circulating liposomal formulation explains the increased activity.
METHODS

Preparations
Liposomes were prepared by the film-extrusion method (15). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 2.0:1.0. For PEG-liposomes 7.5\% of DPPC is replaced by PEG 2000-distearoyl phosphatidylethanolamine (DSPE) conjugate (Avanti Polar Lipids Inc, Alabaster, AL), resulting in a composition containing DPPC, PEG-DSPE and cholesterol 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 PLP in sterile water. The resulting lipid dispersion was sized by repeated extrusion through a series of polycarbonate filter membranes. Unencapsulated prednisolone 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). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Small PEG and non-PEG liposomes were sized to a diameter between 90 and 100 nm while the diameter of large PEG liposomes was set between 450 and 500 nm. Phospholipid content was determined with a phosphate assay (16) in the organic phase after extraction liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content. With high performance liquid chromatography using a mobile phase of acetonitrile-water with pH of 2 and monitoring the eluens with a UV-detector, which was set at 254 nm, both prednisolone and its phosphate ester could be measured in one single run. The liposomal preparation contained around 5 mg PLP/ml and an average of 60 μmol/ml phospholipid.

Adjuvant Arthritis
The institutional Committee of Animal Experiments approved all animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (200-250 g) were obtained from Maastricht University (Maastricht, the Netherlands). To induce arthritis, 100 ml of incomplete Freund’s adjuvant containing 1 mg of heat-inactivated Mycobacterium tuberculosis H37RA (both purchased from DIFCO laboratories, Detroit, MI) was injected intracutaneously at the base of the tail. 10 days after the immunization, the first signs of joint inflammation became apparent, together with a loss of body weight due to the upcoming of the disease. 20 days post-immunization the disease reached maximal severity, after which the inflammation process gradually resolved (18).
Clinical scoring
Starting at day 10, rats were daily weighed and examined for visual signs of inflammation. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, oedema and deformation of the joints. The sum of these four grades for each animal is the clinical score.

Therapeutic efficacy
Rats were treated on day 14 or 15 post-immunization, when the average score of all rats in the experiment reached 7, which is about half the maximal scores reached in these experiments. On the day of treatment, groups of five rats were formed with equal average clinical scores. All preparations were given intravenously via the tail vein. When multiple injections of free glucocorticoid were required, each following day treatment was given at the same time of the day. Clinical score and body weights were monitored daily for up to 2 weeks post-treatment. During the last week of the experiment scoring, was performed less frequently.

Labeling procedure
Liposomes containing diethylene-triamine penta-acetic acid (DTPA) co encapsulated with PLP in the aqueous core were incubated with radioactive $^{111}$In-oxine at 60 °C for 1 hr. Efficient transportation of the label through the lipid bilayer at this temperature followed by chelation by DTPA in the liposomal interior resulted in a labeling efficiency exceeding 90% of the total radioactivity added. Non-encapsulated $^{111}$In-oxine was removed by gel filtration on a Sephadex PD-10 column. The final preparation contained 120 mCi $^{111}$In-label, 40 µmol phospholipid and 3 mg PLP per ml. Since biodistribution studies require less activity than scintigraphic imaging studies, liposomes used for biodistribution were mixed with an equal amount of unlabeled PLP liposomes, in order to keep the total PLP content of each dose constant.

Imaging studies
Whole body scintigraphy was performed with three rats at six time points after injection of 100 mCi $^{111}$In-labeled liposomes containing 2.5 mg PLP in the tail vein. Rats were anesthetized and placed prone on a single-head gamma camera equipped with a parallel-hole low energy collimator (Siemens Orbiter, Hofmann Estates, IL). Per image 300,000 counts were acquired and stored in a 256 * 256 matrix.

Biodistribution studies
At four time points (1, 4, 24 and 48 hours) after injection of a dose of liposomes containing 50 mCi $^{111}$In-label and 2.5 mg PLP, five rats were euthanized with an overdose pentobarbital. After death, samples of blood, liver, spleen, lungs, kidneys, small intestine, muscles, front and hind paws were collected and counted in a shielded well-type gamma
counter. Injection standards were counted with the tissue samples to correct for physical decay and the percentage of liposome uptake per organ as well as uptake per gram tissue were calculated.

**Prednisolone plasma concentration**

Blood samples for determination of prednisolone plasma concentrations were centrifuged to obtain plasma and stored at −80 °C. After the radioactivity in the samples had decayed (approximately 30 days), plasma was extracted according to the method reported by Derendorf et al. (17). An HPLC-method similar to that described above was used for the determination of both PLP and prednisolone in the extracts. The detection limit of the method was approximately 20 ng/ml.

**Statistical analysis**

For comparing clinical scores between groups, the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups with respect to other parameters, one-way analysis of variance was used. P values of less than 0.05 were considered significant.
RESULTS

Pharmacokinetics

Figure 1 A shows that encapsulation of PLP in PEG-liposomes resulted in a change of the pharmacokinetics of the drug. First, the plasma concentration immediately after injection of 5 mg/kg increased from approximately 1 µg/ml to over 100 µg/ml by encapsulating the drug in PEG-liposomes. Calculation of the volume of distribution revealed that PEG-liposomes reduced the distribution volume of PLP from approximately 5 l/kg to less than 50 ml/kg, indicating that PEG-liposomes limit the distribution of the drug to a volume only slightly larger than the plasma volume itself.

Furthermore, liposomal encapsulation strongly reduced the elimination rate of the drug. As unencapsulated (‘free’) drug in plasma, PLP is rapidly converted into prednisolone. Indeed, neither PLP nor prednisolone were detectable anymore in the plasma samples within 1 hour after injection of the free drug whereas i.v. injection of the liposomal drug yielded an exponential decline of PLP with a half-life of approximately 18 hours. Clearly, the liposomes protected PLP against rapid clearance, and therefore the plasma-concentration time profile of PLP was largely determined by the clearance of the liposome particles. This was confirmed by comparing the radioactivity the plasma samples with the PLP-concentration in the same plasma samples (Figure 1 B). The fact that both PLP and the liposome-associated radiolabel showed the same elimination profile indicated that the majority of PLP measured in plasma remained liposome-associated. Interestingly, despite the stability of the formulation in the circulation, low quantities of free prednisolone could be detected (Figure 1 A).

Figure 1. Pharmacokinetics. (A) Plasma concentration of PLP (closed circles) and prednisolone (open circles) after 5 mg/kg liposomal PLP, and PLP (closed squares) and prednisolone (open squares) after 5 mg/kg unencapsulated PLP. (B) % of the injected dose in plasma of liposome label (squares) and encapsulated PLP (circles). Each data point represents the mean of 5 rats ± SD.
Tissue distribution

The scintigraphic images of three rats acquired at different time points after injection of $^{111}$In-labeled PLP-PEG-liposomes are shown in Figure 2 A. High blood levels were observed, as reflected by a clearly visible region in the thorax (heart and large vessels), which is in accordance with the results presented in Figure 1. Liver and spleen were also clearly visualized, showing that they represent important organs of liposome uptake. The inflamed joints of front and hind paws became clearly and increasingly visualized at 4, 20, 24 and 48 hrs after injection. This observation points at gradual accumulation and retention of the liposomes at inflamed sites and excludes other possible explanations like increased blood flow in inflamed tissues.

In Figure 2 B quantitative biodistribution data at 48 hrs post-treatment are shown for arthritic and healthy rats. Similar percentages of the injected dose per gram were found in tissues isolated from arthritic rats and healthy rats, with the exception of the paws. Hind paws of arthritic rats had a 7-fold higher liposome uptake than hind paws of healthy rats, indicating localization of liposomes to the inflamed areas.

Figure 2. In vivo fate of labeled liposomes. (A) Whole body scintigraphic images recorded at 0 to 48 hours post injection of $^{111}$In-labeled PLP-PEG-liposomes (B) Organ distribution of $^{111}$In-PEG-liposomes in healthy rats (open bars) and arthritic rats (black bars) 48 hours post injection.

Therapeutic activity

Figure 3 A shows the anti-inflammatory activity of free and liposomal PLP at different doses in the rat model of AA. A single dose of 10 mg/kg free PLP did not result in a significant effect on paw inflammation scores. In contrast, the same dose encapsulated in PEG-
Liposomes resulted in the complete disappearance of the clinical signs of AA within two days. Resolution of the disease symptoms lasted until day 20 (6 days post-treatment) after which joint inflammation gradually reappeared, reaching the same score as the saline control group around day 28 (two weeks post-treatment). Liposomal PLP was also effective at a 10-fold lower dose (1 mg/kg), and, even at a dose of 0.1 mg/kg, significant suppression of paw inflammation was observed two days after treatment ($p < 0.05$). Only liposomal PLP significantly reversed disease-induced weight loss. Within 8 days these rats completely regained the body weight they lost due to the disease. During the same period, the groups that received saline and free PLP kept loosing weight (Figure 3 B).

Repeated dosing with daily injections of 10 mg/kg free PLP only induced slight reduction of paw inflammation (Figure 4). This dosing regimen was based on clinical experience in RA patients with glucocorticoid ‘pulse’ therapy (4,5,19) which generally involves 3 to 5 daily injections of 1 gram (methyl)prednisolone. Even though daily treatment of AA rats was continued up to 7 days, no further remission of paw inflammation was accomplished. One day after finishing the last free PLP-injection (day 21), inflammation scores already returned to the average score of the control groups. In contrast, a single injection of liposomal PLP again resulted in almost complete disappearance of joint inflammation until day 20. Figure 4 also shows that control treatment with a single injection of empty liposomes did not have a significant effect.
Importance of targeting effect

To study the importance of targeting, two different liposome types with shorter circulation times and reduced joint localization were prepared. (1.) PEG-liposomes with a relatively large mean diameter, referred to as ‘large PEG-liposomes’, which are known to mainly target to the spleen and (2.) liposomes without the PEG-coating, referred to as ‘non-PEG-liposomes’, which show enhanced uptake by liver and spleen.

Figure 4. Therapeutic activity of liposomal PLP vs multiple treatment free PLP. Therapeutic activity of a single injection of 10 mg/kg PLP-PEG-liposomes (black circles), and 7 daily injections of 10 mg/kg free PLP (black squares). Control treatments are: empty PEG-liposomes (open circles) and saline (open squares). Each point represents the mean of 5 rats ± SEM. Arrow indicates treatment.

Figure 5. Importance of targeting effect. (A) Plasma concentration-time curves of small PEG-liposomes (black squares), non-PEG-liposomes (gray circles), and large PEG-liposomes (black triangles) Error bars indicate SD. (B) Targeting of the different types of liposomes in spleen (left) and inflamed paws (right). Error bars indicate SD. (C) Therapeutic activity in AA of 10 mg/kg PLP in small PEG-liposomes (black circles), non-PEG-liposomes (black diamonds) and large PEG-liposomes (black triangles) compared to saline as control treatment (open squares). Error bars indicate ± SEM. Arrow indicates treatment. All data in (A), (B) and (C) represent the mean of 5 rats.
In Figure 5 A and B the *in vivo* behavior of both liposome types compared to the small PEG-liposome type is shown. As compared to the small PEG-liposomes, both preparations indeed showed a reduced circulatory half-life with clearly diminished accumulation in the inflamed joints. A much larger fraction of both liposomal formulations was taken up by the spleen.

The therapeutic activity of the three different PLP-liposomal formulations was compared in the AA model. The small PEG-liposomes were clearly the most effective preparation, followed by the non-PEG-liposomes whereas the large PEG-liposomes were only marginally effective (Figure 5 C). Although therapeutically much less active, large PLP-PEG-liposomes completely abolished disease-induced splenomegaly within two days. This indicates that PLP in large PEG-liposomes is potentially equally active as PLP in small PEG-liposomes but that the reduced activity in the joints is caused by reduced localization of PLP in the joints (spleen weight at 48 hrs: 0.38 ± 0.07 g after treatment with large PLP-PEG-liposomes; 0.42 ± 0.04 g after treatment with small PLP-PEG-liposomes versus 1.41 ± 0.19 g in diseased non-treated rats and 0.36 g ± 0.03 g in healthy rats).

**Systemic availability**

To address the question whether the low quantities of free prednisolone levels observed after injection of liposomal PLP (see Fig 1 A) contribute to the anti-inflammatory effect, prednisolone levels were determined after injection of the three liposome types described above. As shown in Figure 6, the plasma concentration profiles of free prednisolone were not significantly different, despite the clear differences in circulation half-lives and therapeutic activity (see Figure 5). The absence of a clear correlation between the plasma concentration profiles of free prednisolone in the circulation and the anti-inflammatory activity of the three different liposomal preparations does not indicate an essential role of these low systemic glucocorticoid levels in the therapeutic activity of PLP-PEG-liposomes.
DISCUSSION

The first part of this paper indicates that an i.v. targeted drug delivery approach using long-circulating PEG-liposomes could be a highly attractive alternative to current glucocorticoid treatment strategies, such as i.v. pulse therapy or local intra-articular injections (19-22). Long-circulating liposomes provide the opportunity to achieve high concentrations of glucocorticoid selectively at all arthritic joints by simple i.v. injection. The results indicate that in rat experimental arthritis a single i.v. injection of glucocorticoid-PEG-liposomes can already induce a strong, rapid and long-lasting therapeutic benefit. Complete remission of joint inflammation can be accomplished within two days of treatment, and the therapeutic benefit of the injection lasts for up to two weeks. Furthermore, the results show that an equivalent dose of free PLP is not effective. Limited efficacy, which is certainly not lasting, can be realized only with a treatment regimen involving daily injections of free PLP.

The second part of this paper is devoted to the question of whether the increased therapeutic activity of liposomal PLP can be attributed to drug targeting. The results in this paper indeed point to a crucial role of drug targeting. Encapsulation of PLP in PEG-liposomes strongly influenced the pharmacokinetics of i.v. injected glucocorticoid as shown in Figure 1. I.v. dosing of free PLP in rats resulted in only low and short-lasting plasma concentrations, presumably due to the rapid conversion of PLP into prednisolone. In its turn, free prednisolone was also quickly eliminated, which explains the lack of therapeutic activity of the free drug. Liposomal encapsulation markedly enhanced the concentration of PLP in the circulation. Apparently, liposomal encapsulation protects PLP against conversion and degradation. It also prevents it from the rapid and extensive tissue distribution, which occurs with free PLP. The plasma concentration-time profile of PLP largely resembled the plasma concentration-time curve of a liposome-associated radioactive marker, indicating that leakage of PLP from circulating liposomes does not play a significant role. Since the volume of distribution of PEG-liposomes is much smaller than the free drug, liposomal encapsulation may not just enhance the concentration of drug at the target site, but also lower drug concentrations at non-target tissues. The possibility of reduced toxicity may further improve the therapeutic index of glucocorticoid upon encapsulation in long-circulating PEG-liposomes.

A second confirmation of the crucial role of drug targeting in the enhanced therapeutic activity of liposomal PLP relates to the observation of selective accumulation of the liposomes at arthritic sites (Figure 2). Co-encapsulation of a radioactive marker indicated that the amount of liposomes that is taken up by the inflamed paws increased during the first 24 hrs post-injection, despite decreasing concentrations of liposomes in the circulation. This accumulation process appears to be inflammation-driven, as the degree of joint localization is much lower in healthy rats. Besides the inflamed paws, liver and spleen also appeared to be important organs of liposome uptake in rats with AA. Hepatosplenic uptake is largely responsible for the elimination of liposomes from the circulation and appears to be mediated by resident macrophages in these organs (23,24).
Interestingly, macrophages in lymphoid organs such as lymph nodes and spleen have been shown to be involved in the development of AA (25). The presumed uptake of glucocorticoid-PEG-liposomes by macrophages in the spleen may therefore (partly) explain the therapeutic activity of the preparation. Although it was found that PLP-PEG-liposomes completely reversed disease-induced splenomegaly within two days, the effect on joint inflammation did not relate to spleen targeting, as became evident from the effect obtained with the large PLP-PEG-liposomes. This liposome type, which homed to the spleen to an even higher extent without the capacity to localize in the inflamed joints, completely reversed splenomegaly as well, but only induced a partial remission of joint inflammation.

Comparison of the activities of large PLP-PEG-liposomes and small PLP-non-PEG-liposomes to the activity of small PLP-PEG-liposomes, reveals that the therapeutic activity of liposomal glucocorticoid is related to joint accumulation. From our studies it appears that the better liposomes can accumulate in the inflamed joint, the stronger the therapeutic benefit. The process of spleen homing inversely correlated with the process of joint accumulation. Increased spleen uptake may have contributed to the shorter circulation half-life of large PEG-liposomes and liposomes without PEG, resulting in less joint accumulation and consequently in less anti-inflammatory activity. These relationships stress the importance of a prolonged residence time in the circulation for the realization of sufficient joint localization to guarantee strong anti-inflammatory activity of liposomal glucocorticoids.

Besides a strong alteration of pharmacokinetics induced by liposomal encapsulation of PLP, Figure 1 also shows that sustained low levels of free prednisolone in the circulation were generated. Since little to no leakage of PLP from circulating liposomes occurred, it is hypothesized that a certain quantity of encapsulated PLP is released back into the circulation. Possibly, after hepatic and splenic uptake of liposomal PLP, phagocytes in these organs generate and release active prednisolone. The phenomenon of drug release from liposomes mediated by phagocytes in liver and spleen has previously been shown to occur with several liposomal preparations of cytotoxic drugs (26,27). The fact that large PEG-liposomes and non-PEG-liposomes also generated such low levels of free prednisolone supports the hypothesis regarding the drug release by liver and spleen phagocytes. As the therapeutic activity of the latter two liposome types is limited, it is indicated that the phenomenon of sustained levels of active prednisolone in the circulation is not responsible for the increased therapeutic activity achieved by liposomal encapsulation of PLP.

In conclusion, the results in this study indicate that a single i.v. dose of glucocorticoids encapsulated in long-circulating PEG-liposomes can lead to rapid, complete and durable resolution of joint inflammation as a result of enhanced and preferential localization of the liposomal drug in the inflamed joints. This novel approach could offer important advantages over existing therapies in arthritis, such as pulse therapy and intra-articular injections.
REFERENCES
