Subcutaneous Administration of Superoxide Dismutase Entrapped in Long Circulating Liposomes: *In Vivo* Fate and Therapeutic Activity in an Inflammation Model

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Purpose. We are exploring liposomal delivery with the aim to change the pharmacokinetics and biodistribution of SOD to increase its therapeutic activity. From a practical point of view, a convenient route of administration would be the subcutaneous (s.c.) route. Liposomal size has been shown to be the most important factor influencing the rate and extent of drainage of liposomes from the s.c. injection site.

Methods. To monitor the *in vivo* fate of the subcutaneous administered SOD-containing liposomes in rats with a chronic arthritis inflammation, the liposomes were labeled by the co-encapsulation of the ^{111}In-DTPA complex in the internal water space.

Results. Over the initial 10h-observation period post-injection, the small-sized poly(ethyleneglycol)-liposomes (mean size about 110 nm) left the site of injection to a 2-fold higher extent (45% of the injected dose) as compared to large-sized poly(ethyleneglycol)-liposomes (mean size about 450 nm). Small-sized liposomes gave a 17-fold higher uptake in the inflamed foot than the large-sized liposomes. Comparing the localization in the inflamed foot with the non-inflamed foot, uptake was more than 15-fold higher for the small-sized liposomes as compared to the large-sized liposomes. After s.c. administration, small-sized SOD-liposomes showed substantial higher activity than large-sized SOD-liposomes. S.C. administration of small-sized SOD-liposomes is equally effective as i.v. administration of the same liposomes. I.V. administration of the large-sized SOD-liposomes yielded a significantly higher activity as compared to s.c. administration.

Conclusions. These results indicate that small-sized poly(ethyleneglycol)-liposomes can be used for the targeting of SOD to arthritic sites after subcutaneous administration.

KEY WORDS: superoxide dismutase; long circulating liposomes; subcutaneous administration; therapeutic activity; biodistribution.

INTRODUCTION

Rheumatoid arthritis is an inflammatory disorder of unknown etiology, which results in erosion, deformity, and progressive destruction of synovial joints (1) and results in the United States in more than 9 million physician visits and more than 250 000 hospitalizations per year (2). Rheumatoid arthritis treatment is still unsatisfactory and therefore any new efficacious drug will be a valuable addition to the current arsenal of antirheumatic agents. Tissue injury in the inflamed areas is essentially due to overproduction of reactive oxygen species (ROS) (3). To prevent ROS-related toxicity, the scavenger enzyme superoxide dismutase (SOD) is of potential value as a therapeutic agent (4,5). At present, there is a resurgence of interest in the therapeutic application of SOD for the treatment of rheumatoid arthritis (6). SOD is usually administered intravenously (i.v.). However, the short half-life (6 min) observed after i.v. administration strongly limits its therapeutic effect (5,7). Therefore, we are exploring liposomal delivery with the aim to change the pharmacokinetics and biodistribution of SOD to increase its therapeutic activity. In fact, our previous work has demonstrated both enhanced accumulation at body sites of arthritis and enhanced therapeutic efficacy in a rat adjuvant arthritis model, after i.v. administration of liposome-associated SOD (8,9).

From a practical point of view, a more convenient route of administration would be the subcutaneous (s.c.) route, as then administration by health professionals is not strictly required. The fate of liposomes after s.c. administration is dependent on their size. S.c. injected particulates are drained from the injected site into the circulation via the lymphatics. Depending on size and composition, a proportion of the particles will enter the lymphatic system and will eventually reach the general circulation, where they behave as if administered by the i.v. route. Liposomal size has been shown to be the most important factor influencing the rate and extent of drainage of liposomes from the s.c. injection site (10,11). Small-sized liposomes ($<$120–150 nm in diameter) can move to a large extent ($>$60% of the injected dose) from the site of injection into the bloodstream, via passage through lymphatic capillaries and regional lymph nodes. Larger liposomes have more difficulty to migrate from the s.c. interstitium into the lymphatic capillaries and will therefore remain at the site of injection to a large, almost complete extent (10–12).

Drug delivery with liposomes is based upon several mechanistic concepts, of which targeting and sustained release are the two major ones. Targeting increases the efficacy of a drug by delivering it to its site of action. Sustained release is a mechanism for increasing the efficacy and/or duration of action of a drug by maintaining therapeutic drug levels for prolonged periods of time. Clearly, if sustained release is the major objective, the use of large-sized SOD-liposomes would be preferred over small-sized SOD-liposomes as the former remain present at the s.c. injection site for a long period of time. However, we

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ABBREVIATIONS: Chol, cholesterol; DSPE-PEG, Distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000; DTPA, diethylenetriaminepenta-acetic acid; E-PC, Egg phosphatidylcholine; foot+, inflamed right foot; foot−, non-inflamed left foot; ID, injected dose; ID/g, injected dose per g of tissue; i.v., intravenous(ly); Lip, total lipid; PEG, poly(ethyleneglycol); P.I., polydispersity index; Prot, protein; (Prot/Lip), protein-to-lipid ratio; ROS, reactive oxygen species; SA, stearylamine; SD, standard deviation; s.c., subcutaneous(ly); SOD, superoxide dismutases, [EC 1.15.1.1].

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do not expect that sustained release of SOD out of a s.c. liposome depot alone would be beneficial, as SOD is very rapidly cleared upon entering the blood. Therefore, the present work was designed to study whether small-sized liposomes can be used to carry SOD after s.c. administration in liposomal form from the s.c. site to the circulation and subsequently to the arthritic site. We have previously shown that i.v. administration of SOD encapsulated in small-sized long-circulating polyethyleneglycol (PEG)-coated liposomes has significant advantages over SOD in short-circulating non-PEG liposomes in terms of localization at arthritic sites (13) and antiarthritic efficacy (9). Accordingly, the present investigations were performed with small-sized PEG-liposomes. Large-sized PEG-SOD-liposomes were also studied to check our hypothesis that sustained release at the injection site indeed is a less attractive therapeutic option.

MATERIALS AND METHODS

Chemicals

Egg-phosphatidylcholine (E-PC) was obtained from Lipoid GmbH, Ludwigshafen, FRG. Distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000 (DSPE-PEG) was obtained from Avanti Polar Lipids Inc., Alabaster, Al, USA. Cholesterol (Chol), stearylamine (SA), diethylenetriamine-penta-acetic acid (DTPA) and bovine erythrocytes Cu,Zn-superoxide dismutase (SOD) were purchased from Sigma Chemical Co. St. Louis, MO, USA. ^{111}In-8-hydroxyquinoline (^{111}In-oxine) was obtained from Mallinchrodt, Petten, The Netherlands. All other chemicals were of reagent grade.

Animals

Male Wistar rats aging more than 3 months and weighing 400–500 g were obtained from Instituto Gulbenkian de Ciência, Oeiras, Portugal. Animals were fed with standard laboratory food and water *ad libitum*.

Liposome Preparation

Large-Sized SOD-Liposomes (Non-Extruded)

Multilamellar liposomes were prepared by the dehydration-rehydration method as previously described (8). Briefly, mixtures of the appropriate amounts of lipids were dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed (32 μmol lipid per ml hydration medium) in a solution of SOD (0.5 mg/ml) in water, frozen in liquid nitrogen and lyophilized overnight. Then, buffer (0.28 M mannitol/10 mM citrate buffer pH 5.6) was added to the lyophilized powder in a volume amounting to 1/10 of the volume of the original dispersion. This hydration step lasted 30 min, and, subsequently, 0.145 M NaCl/10 mM citrate buffer pH 5.6 was added to reach the starting volume. After 30 min, nonencapsulated protein was separated from the liposome dispersion by ultracentrifugation, at 300,000 g for 20 min at 4°C in a Beckman LM-80 ultracentrifuge. Finally, large-sized SOD-liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer, pH 5.6.

Small-Sized SOD-Liposomes (Extruded)

Large-sized SOD-liposomes were subjected to a sizing procedure. The preparation procedure was exactly the same as for the large-sized SOD-liposomes except that the hydration medium contained 5 mg SOD/ml instead of 0.5 mg SOD/ml. Thirty minutes after hydration, the large-sized liposomes were extruded sequentially through polycarbonate filters ranging from 0.6 to 0.05 μm in pore size. Nonencapsulated protein was separated from the liposome dispersion by ultracentrifugation at 300,000 g for 120 min at 4° C in a Beckman LM-80 ultracentrifuge. Finally, the small-sized SOD-liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6.

^{111}In-DTPA-SOD Liposomes

Liposomes were prepared as described above with some modifications: the lipid film was dispersed in a solution of SOD in 6 mM DTPA (0.5 mg/ml for large-sized SOD-liposomes and 5 mg/ml for the small-sized SOD-liposomes). To remove the non-encapsulated DTPA, liposomes were passed through a Econo-PAC 10DG Column (Bio-Rad).

^{111}In labeling: preformed SOD-liposomes containing DTPA were labeled with ^{111}In, which was transported through the bilayer in the form of ^{111}In-oxine and trapped irreversibly in the internal aqueous phase, due to the chelation by intraliposomal DTPA. Briefly, 0.3 mCi (1.4 ml) of ^{111}In-oxine in 0.05 M Tris-HCl, pH 7.0 was added to 2.0 ml of each liposomal preparation (20 μmol of total lipid/ml) and incubated for 1 h. The nonencapsulated ^{111}In-oxine was removed by gel filtration on a Econo-PAC 10DG Column. The encapsulation efficiency of the remote labeling procedure was always higher than 85%.

Liposome Characterization

Mean liposome size was determined by dynamic light scattering with a Malvern 4700 system. As measure of particle size distribution the polydispersity index (P.I.) was used. P.I. can range from 0 (monodisperse) to 1.0 (polydisperse).

Phospholipid concentrations were determined according to Fiske and Subbarow (14) as modified by King (15).

Protein was determined according to a modified Lowry method (16) with prior disruption of liposomes with Triton X100 and sodium dodecylsulphate (17); "final protein" represents total protein associated with liposomes after removal of the non-encapsulated protein.

The SOD enzymatic activity assay was based on the ability of the enzyme to inhibit autoxidation of epinephrine to adrenochrome at pH 10.2. In case of determining liposome encapsulated enzyme, the enzyme was first released from the liposomes by the addition of 20% Triton X100 resulting in a 10% Triton X100 solution. All activity measurements were performed after dilution of the enzyme to a final concentration of 6 μg/ml, resulting in a 0.3% Triton X100 solution. Control experiments showed that this procedure did not affect SOD activity (8).

Animal Experiments

Induction of the Inflammation

Wistar rats were injected with a single intradermal injection of 0.10–0.15 ml of a suspension of *Mycobacterium butiricum* killed and dried (Difco) in incomplete Freund's Adjuvant at 10 mg/ml, into the subplantar area of the right hind paw (8). The parameter of interest of adjuvant-induced arthritis is the

swelling of the paw, which typically is established 7 days after the induction of the inflammation.

Imaging Protocol

Seven days after the induction of the inflammation, the different liposomal preparations were injected s.c. in the left fore foot, three rats per group (divided randomly). Rats were anaesthesized (halothane/nitrous oxide/oxygen) and were placed prone on a single-head gamma camera equipped with a parallel-hole medium-energy collimator (Orbiter, Siemens Inc., Hoffmann Estates, IL). The groups of rats were imaged at selected time points after injection. The scintigraphic results were analyzed by drawing regions of interest (ROI) over the s.c. site of injection and over the whole animal.

Biodistribution Protocol

To study the biodistribution of the radiolabeled liposomes, groups of 5 rats each were injected s.c. in the left non-inflamed fore foot, with a single dose of the ^{111}In-DTPA-SOD-liposomes, 7 days after the induction of the inflammation. 168 h post-injection, rats were killed with intraperitoneally injected 30 mg phenobarbital. Blood was obtained by cardiac puncture. Following cervical dislocation, different tissues were dissected, namely inflamed right foot (foot+), non-inflamed left foot (foot−), liver, spleen, lung, kidney and muscle. Dissected tissues were weighed and assayed for radioactivity in a shield well-type gamma counter.

Assessment of Therapeutic Activity

The s.c. treatment protocols involved one s.c. or i.v. injection on days 7, 11 and 15 after the induction of the inflammation (3 injections in total). Both the large-sized and the small-sized SOD-liposomes were tested at a dose of 66 μg SOD/kg body weight. Each treatment group consisted of 6 rats. For the screening of potential anti-inflammatory drugs, measuring the physical swelling of the hindpaws offers a simple and reproducible method (18). Therefore, the paw oedema was assessed by measurement of paw circumference. SOD therapeutic activity was expressed as 'percentage of oedema regression' calculated using the formula:

$$\% \text{ oedema regression} = (\text{Cat-Cbt})/(\text{Cbi-Cbt}) \times 100\ (\%)$$

where:

Cat = ankle circumference after 11 days of treatment period (day 7–18)
Cbt = ankle circumference before treatment and 7 days after induction (day 7)
Cbi = ankle circumference before the induction (day 0)

Statistical Analysis

All mean values are given ± standard deviation (SD). Statistical analysis was performed using the ANOVA test.

RESULTS AND DISCUSSION

Liposomes coated with PEG (often referred to as Stealth® or "sterically stabilized" liposomes) were chosen for this study because they have shown to be able to preferentially localize at sites of inflammation (19) after i.v. administration. Prolonged blood circulation and reduced hepatosplenic uptake (as compared to non PEG-conventional liposomes) are primary *in vivo* characteristics of PEG-liposomes. In addition, they show the valuable property of lipid dose-independent kinetics of blood clearance (20). Consequently, if s.c. administered PEG-liposomes enter the bloodstream, they will tend to accumulate at arthritic sites (13). These properties are particularly important when using SOD because the free enzyme is cleared very rapidly from the blood and local injection sites via the kidneys (half-life in blood is about 6 min in rats and 30 min in humans) (21).

After s.c. administration, large-sized and small-sized PEG-liposomes are expected to display distinct differences in *in vivo* behavior. Large-sized (non-extruded) liposomes have shown to remain almost completely localized at the s.c. injection site for long periods of time. In contrast, a major fraction of small-sized liposomes (<150 nm) (extruded) are transported via the lymphatics to the blood circulation (11). We hypothesized that any anti-arthritic activity of large-sized SOD-liposomes in the inflammation model used herein would then largely be the result of SOD released from the liposomes at the injection site. In the case of small-sized SOD-liposomes, it was reasoned that entry of small PEG-liposomes into the bloodstream and subsequently into the inflammatory focus is the main delivery route responsible for the anti-arthritic activity.

Characteristics of the two PEG-liposome formulations containing SOD used in this study, referred to as large-sized liposomes and small-sized liposomes, are presented in Table I. Both liposome formulations show the same protein-to-lipid ratio, which is important to enable comparative *in vivo* studies at the same liposomal lipid dose.

Stability Characteristics *In Vitro*

Limited stability tests for the large-sized liposomes and small-sized liposomes were performed at two pH values (pH 5.6 and 7.4). Liposomes were monitored at 37°C for 96 h and at 4°C for 1 month. Other conditions taken into account were incubation of the liposomes in 1% bovine serum albumin, pH 7.4, 37°C, for 96 h. The parameters studied were protein and lipid retention in the liposomal form, size and polydispersity, and enzymatic activity. For all formulations and under all condition schedules SOD retention remained over 95% ± 5% for the protein and lipid in liposomal form. Moreover, there was over 90% retention of enzymatic activity. Less than 5% change in particle size and no increase in polydispersity were observed.

Imaging Studies

To study the *in vivo* fate of the SOD-containing PEG-liposomes in rats with a chronic arthritic inflammation, the liposomes were labeled by co-encapsulating the ^{111}In-DTPA complex in the internal water space. This complex is a high affinity complex at physiological pH (22) and has a very short half-life due to efficient renal excretion when present as free complex in the blood circulation (half-life of 3–5 min). These characteristics make the ^{111}In-DTPA complex an appropriate label to monitor the *in vivo* fate of liposomes. Gamma-camera imaging of rats was used to monitor the fate of liposomes after s.c. administration. Two regions of interest (site of injection

Table I. Characteristics of the SOD Liposomes Studied

	Lipid composition	Molar ratio	Mean size[a] (μm)	P.I.[b]	Prot/Lip[c] (μg/μmol)
Large-sized liposomes	E-PC:Chol:DSPE-PEG	1.85:1:0.15	0.45 ± 0.10	<0.4	12–15
Small-sized liposomes	E-PC:Chol:DSPE-PEG	1.85:1:0.15	0.11 ± 0.01	<0.1	12–14

[a] Mean ± SD of 4 dispersions.
[b] P.I., polydispersity index.
[c] Prot/Lip, protein-to-lipid ratio.

and whole body) were selected. The fate of ^{111}In-DTPA-SOD-liposomes was compared with the fate of the free ^{111}In-DTPA complex after s.c. administration.

The quantitative analysis of the images obtained for the amount of radioactivity at the site of injection is shown in Fig. 1. The free ^{111}In-DTPA complex was rapidly removed (within 4 hours after injection) from the site of injection. For the large-sized SOD-liposomes, a relatively rapid initial removal (over the first 10 h post-injection) from the site of injection of about 25% of the injected dose (%ID) was observed, followed by a slower removal process. For the small-sized SOD-liposomes, a similar profile was observed, except that the degree of removal from the site of injection in the initial stage was higher, i.e., about 45% over the same 10 h-time period. Thus, over the initial 10 h-observation period, the label administered via the small-sized SOD-liposomes left the site of injection to a 2-fold higher extent as compared to the label administered via the large-sized SOD-liposomes. The disappearance of ^{111}In-label entrapped in the large-sized SOD-liposomes can be explained either by disappearance of label leaked from the liposomes at the injection site or by loss from the injection site via lymphatic drainage within a sub-population of smaller ^{111}In-liposomes.

The whole-body activity profiles derived from the quantitative analysis of the images, i.e., the amount of radioactivity remaining in the whole animal body, are shown in Fig. 2. For the free ^{111}In-DTPA complex, an excretion of about 85% ID was observed already at 4h post-injection. Thus, the rapid clearance of the free label from the injection site (Fig. 1) is followed by a rapid clearance from the body. For the large-sized SOD-liposomes, a label excretion of 10% ID was observed over the initial 10 h-time period post-injection. As during this period of time approximately 30% ID of injected label has left the injection site (Fig. 1), we deduce that about 20% ID is leaving the injection site in liposome-encapsulated form and about 10% ID had leaked from the liposomes at the injection site over the initial time frame of 10 hours. The similar slopes of the profiles shown in Fig. 1 and 2 after the initial 10 hour-period strongly suggest that sustained release of label from liposomes remaining at the injection site is the main mechanism behind the continuous decline in radioactivity at the injection site observed after the initial 10 hour-time period. In case of the small-sized SOD-liposomes, no significant whole-body clearance was observed during the first 10 h post-injection. Taking into account the observation that about 45% ID left the injection site during this time period (Fig. 1), we conclude that the small-sized liposomes leave the injection site in intact form. Image analysis revealed the presence of radioactivity in blood and tissues other than the kidneys, leading further support to the hypothesis that small PEG-liposomes are able to reach the blood circulation after s.c. administration.

Biodistribution Studies

The biodistribution profiles of the small-sized and large-sized SOD-liposomes at 7 days post-injection in rats with adjuvant arthritis are shown in Table II. The data indicate that the spleen is the main organ of accumulation for both formulations studied. The small-sized SOD-liposomes showed a splenic uptake of about 2% of the injected dose/g of tissue (%ID/g) while for the large-sized SOD-liposomes the splenic uptake was less than 0.5% ID/g. The liver uptake was less extensive and higher ($p < 0.005$) for the small-sized SOD liposomes:

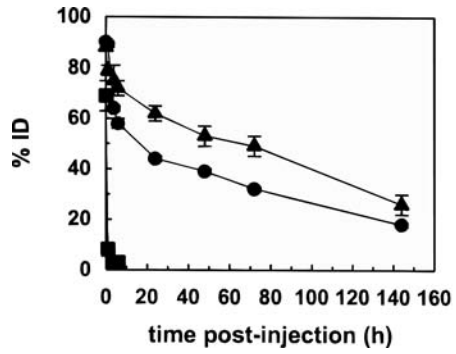

Fig. 1. Quantitative analysis of the scintigraphic images for the injection site in rats with adjuvant arthritis (three per group), s.c. injected with (●) ^{111}In-DTPA-small-sized SOD-liposomes, (▲)^{111}In-DTPA-large-sized SOD-liposomes and (■) free ^{111}In-DTPA. The injection site activity at 5 min post-injection was set at 100%. Values are the mean ±. SD (% ID = % of the injected dose).

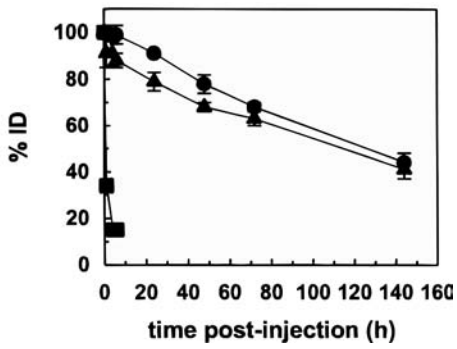

Fig. 2. Quantitative analysis of the scintigraphic images for the whole-body activity in rats with adjuvant arthritis (three per group), s.c. injected with (●) ^{111}In-DTPA-small-sized SOD-liposomes, (▲)^{111}In-DTPA-large-sized SOD-liposomes and (■) free ^{111}In-DTPA complex. The whole-body activity at 5 min post-injection was set at 100%. Values are the mean ± SD (% ID = % of the injected dose).

Table II. Biodistribution of ^{111}In-DTPA Labeled SOD Liposomes After s.c. Administration in Rats with Adjuvant Arthritis, 168 h Post-Injection

	Small-sized liposomes	Large-sized liposomes
Spleen	1.944 ± 1.900	0.315 ± 0.300
Liver	0.152 ± 0.037	0.014 ± 0.001
Kidney	0.079 ± 0.031	0.041 ± 0.011
Lung	0.027 ± 0.010	0.002 ± 0.002
Muscle	0.009 ± 0.003	0.001 ± 0.001
Blood	0.001 ± 0.001	0.001 ± 0.001
Foot+	0.121 ± 0.034	0.006 ± 0.002
Foot−	0.007 ± 0.001	0.003 ± 0.001

Note: Mean ± SD of 5 rats per group; %ID/g - % of the injected dose/g of tissue; foot+ - inflamed foot; foot− - non-inflamed foot.

0.15% ID/g versus 0.01% ID/g in case of the large-sized SOD-liposomes. Uptake by other organs was very low, with renal uptake probably related to localization of free label.

The most important result considering the aim of this study is the accumulation in the inflamed foot (foot+). S.c. administration of small-sized SOD-liposomes gave a much higher uptake in the inflamed focus (0.12% ID/g) as compared to the s.c. administration of the large-sized SOD-liposomes (0.007% ID/g). These values are significantly higher ($p < 0.01$) when compared to the values for localization in the non-inflamed foot (foot−). Inflamed foot-to-non-inflamed foot ratios are shown in Fig. 3. The mean ratio obtained with the small-sized SOD-liposomes is 10-fold higher than the mean ratio obtained with the large-sized SOD-liposomes ($p < 0.01$). One of the requirements to achieve localization of SOD-liposomes in tissues after s.c. administration is that the liposomes have to be drained from the s.c. injection site into the regional lymphatics and to reach the bloodstream in significant quantities (10,11). In fact, the substantially higher tissue levels achieved with the small-sized SOD-liposomes reflect that these liposomes reached the circulation to a higher extent than the large-sized SOD-liposomes. When the distribution resulting from the s.c route of administration is compared with that resulting from the i.v. route of administration (13), we can observe that they are similar in terms of their relative distribution characteristics: splenic uptake > liver uptake > kidney uptake. Apparently, once SOD-PEG-liposomes have reached the bloodstream after s.c. administration, they behave as if administered via the i.v. route, which indicates that SOD-liposomes have reached the circulation in an intact form. Because of the presence of PEG on the outer surface of the SOD-liposomes, it is assumed that the SOD-liposomes which reach the circulation are long-circulating and therefore able to localize preferentially at arthritic sites. In fact, the degree of localization in the inflamed foot (foot+) of the small-sized SOD-liposomes is more than 15-fold higher as compared to the localization in the non-inflamed foot (foot−). For the large-sized SOD-liposomes, the "increase factor" was much lower (less than 3-fold). The lower extent of localization of the large-sized SOD-liposomes at the inflamed site is directly related to the much smaller fraction of the injected dose reaching the bloodstream as compared to the small-sized SOD-liposomes.

Therapeutic Activity

To evaluate the anti-arthritic activity of s.c. administered SOD-liposomes in the rat adjuvant arthritis model, we have selected a dose regimen consisting of 3 injections of 33 μg SOD per rat on days 7, 11 and 15 after induction of the inflammation. This dose regimen has previously been demonstrated to result in this model in significant anti-arthritic activity when small-sized PEGylated SOD-liposomes are administered i.v. (9). I.v. injected free (i.e., nonencapsulated) SOD is not active at this dose regimen.

Figure 4 shows the anti-arthritic activity of SOD encapsulated in one of the liposome types under investigation after s.c. or i.v. administration. In all cases, liposome-encapsulated SOD displayed significant anti-arthritic activity ($p < 0.01$). After s.c. administration, small-sized SOD-liposomes showed significantly higher activity (25% ± 16%) than the large-sized SOD-liposomes (−16% ± 13%) ($p < 0.01$), which probably relates to the much larger fraction of injected liposomes reaching the blood circulation in case of the small-sized SOD-liposomes. The observation that s.c. administration of small-sized SOD-liposomes is equally effective as i.v. administration is in good agreement with the earlier observation (Fig. 1) that s.c. administered small-sized SOD-liposomes enter the bloodstream to a larger extent. Remarkably, also the large-sized SOD-liposomes showed anti-arthritic activity after s.c. administration, though much less pronounced. As free SOD is not active at this dose regimen, it is excluded that the observed activity is induced by SOD release from large-sized liposomes particles residing at the s.c. injection site. Rather, the activity seen in case of the large-sized SOD-liposomes is caused by the entry of a subpopulation of smaller particles into the bloodstream. This explanation seems in the line with the imaging results showing that a fraction

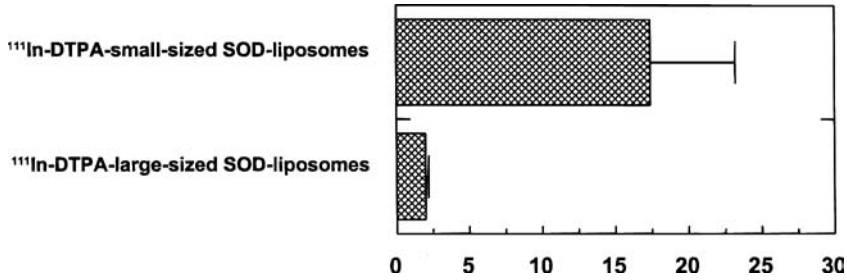

Fig. 3. Inflamed foot-to-non-inflamed foot ratio obtained with small-sized SOD-liposomes and large-sized SOD-liposomes, 168h after s.c. injection. Values represent ± SD, five rats per group.

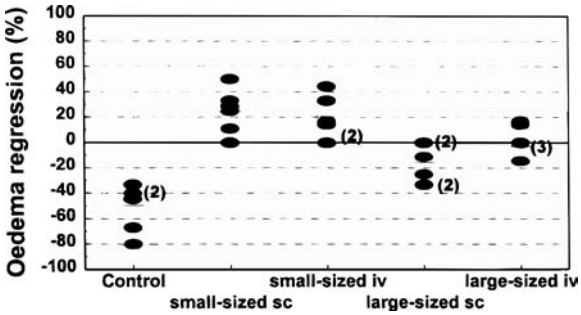

Fig. 4. Anti-arthritic activity of two liposomal SOD formulations in a rat adjuvant arthritis model: effect of route of administration. SOD-formulations were given via either the i.v. or s.c. route of administration, at a dose of 33 μg SOD per rat of SOD in small-sized liposomes and SOD in large-sized liposomes: one injection on days 7, 11 and 15 (3 injections in total). Control animals did not receive any treatment. The number of animals per group was 6; dots represent the change in swelling of the inflamed paw induced by the treatment (expressed as % oedema regression), assessed at day 18. (—: mean value; (x): number animals with the same oedema regression). Negative values point to swelling increase during the observation period starting on the first day of treatment (day 7) and ending at the day of assessment (day 18). Positive values indicate swelling decrease during the same period of time. (control = non-treated animals; small-sized sc = small-sized SOD-liposomes injected s.c.; small-sized iv = small-sized SOD-liposomes injected i.v.; large-sized sc = large-sized SOD-liposomes injected s.c.; large-sized iv = large-sized SOD-liposomes injected i.v.).

of about 20% ID out of the s.c. administered large-sized SOD-liposomes dispersion is reaching the bloodstream during the initial 10 hour-time period (Figs. 1 and 2).

Interestingly, i.v. administration of the large-sized SOD-liposomes yields a significantly higher activity (−3% ± 10%) as compared to s.c. administration ($p < 0.03$). Apparently, the number of circulating particles with a long-circulating property is higher in case of i.v. administration of large-sized SOD-liposomes. However, when compared to i.v. administration of small-sized SOD-liposomes (21% ± 10%) i.v. administered large-sized SOD-liposomes are less effective ($p < 0.05$). This finding is understandable if one takes into account that PEGylation of large particles (larger than about 250 nm) is less effective in conferring steric stabilization and consequently in prolonging circulation time (23). In addition, it should be considered that extravasation into inflamed areas decreases with increasing particle size (24).

Due to its charge, the superoxide radical ($O_2^{\bullet -}$) crosses membranes very slowly and to a small extent, unless there is an anionic channel to allow its passage through the membrane, as happens in the erythrocyte membrane (25,26). The superoxide radical can be protonated to form the hydroperoxyl radical ($HO_2^{\bullet}$). This protonated form can cross cellular membranes, in this case the liposomal membrane, as it is less polar than $O_2^{\bullet -}$. The pKa of $HO_2^{\bullet}$ is 4.7–4.8 (27) and so at physiological pH (pH = 7.4), only about 0.5% of $O_2^{\bullet -}$ exists as hydroperoxyl radical. Considering the exposed, we think that the enzyme can only be effective after being released from the liposomes as only in this situation it can react with considerable amounts of superoxide radical present in inflamed areas. Therefore, we hypothesize that the release of the protein from the liposomes is a crucial step to obtain therapeutic activity.

CONCLUSIONS

In conclusion, the present results demonstrate that PEG-liposomes can be used for the targeting of SOD to arthritic sites in a rat adjuvant arthritis model after s.c. administration. An important requirement is that the PEG-liposomes are small-sized to allow efficient drainage by regional lymphatics to the blood circulation. If these findings can be confirmed in the clinical setting, then an important hurdle to the introduction of SOD-liposomes in the routine treatment of rheumatoid arthritis is taken. The s.c. route of administration is a highly preferred over the i.v. route, because it allows patient self-administration and may avoid the need for a health professional service.

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