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Targeting Zinc Protoporphyrin Liposomes to the Spleen Using Reticuloendothelial Blockade with Blank Liposomes

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ABSTRACT. Metalloporphyrin inhibitors of heme oxygenase have been studied for use in the prevention of hyperbilirubinemia of the neonate. One report has suggested that incorporation of these drugs into liposomes can increase their localization to the spleen, dramatically reducing heme oxygenase activity in that important heme-degrading organ. We sought to further increase porphyrin delivery to the spleen by using reticuloendothelial blockade with blank liposomes 2 h before injection of 0.3 μm extruded zinc protoporphyrin liposomes (L-ZnPP). Control adult rats without hemolysis had splenic heme oxygenase activity of 1.07 ± 0.09 nmol carbon monoxide (CO)/h/mg protein. Rats treated with L-ZnPP alone had splenic heme oxygenase activity of 0.53 ± 0.16 nmol CO/h/mg protein 6 h after L-ZnPP dosing. However, rats treated with 1000 μmol of blank liposomes per kg to saturate the reticuloendothelial system 2 h before L-ZnPP administration had splenic heme oxygenase activity of 0.25 ± 0.16 nmol CO/h/mg protein at t = 6 h, which is significantly less than that of the L-ZnPP alone group ($p < 0.05$). In adult rats treated with heat-damaged red blood cells (RBC) to simulate hemolysis, treatment with 10 μmol of aqueous ZnPP per kg or 10 μmol of untargeted L-ZnPP per kg did not produce a difference from control in total body bilirubin production as estimated by CO excretion. However, RBC-treated rats given 1000 μmol of blank liposomes per kg 2 h before L-ZnPP administration produced significantly less CO than control, aqueous ZnPP-treated, and untargeted L-ZnPP-treated rats from 8 to 12 h after RBC treatment. In addition, splenic heme oxygenase activity in RBC-treated rats receiving 10 μmol of targeted L-ZnPP per kg was completely eliminated 12 h after RBC treatment. These results indicate that targeting of L-ZnPP to the spleen with reticuloendothelial blockade leads to improved *in vivo* suppression of total body bilirubin production in adult rats treated with heat-damaged RBC. More complete inhibition of splenic heme oxygenase is the likely mechanism for this improved therapeutic effect. (*Pediatr Res* 34: 1–5, 1993)

Abbreviations

ZnPP, zinc protoporphyrin
L-ZnPP, liposomal zinc protoporphyrin
SnPP, tin protoporphyrin
HO, heme oxygenase
CO, carbon monoxide
VeCO, total body carbon monoxide excretion
RES, reticuloendothelial system
RBC, red blood cell
EPC, egg phosphatidylcholine
EPG, egg phosphatidylglycerol
i.p., intraperitoneal(ly)

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Neonatal hyperbilirubinemia continues to be a common concern, with more than 6% of newborns developing significant jaundice (serum bilirubin above 220 μmol/L) nationwide (1). Bilirubin is produced from heme by a two-step pathway of which HO (EC 1.14.99.3) is the rate-limiting enzyme (2). In the neonate, bilirubin produced in the RES from senescent fetal RBC cannot be excreted by the immature conjugating mechanisms of the neonatal liver, leading to blood bilirubin levels far above those of normal adults. Severe neonatal hyperbilirubinemia is associated with kernicterus and death, although the pathophysiology of this process is still being studied and debated (3–6). Current treatments for neonatal jaundice, such as phototherapy, focus on increasing the clearance of bilirubin from the blood. An experimental treatment for neonatal jaundice is being investigated that would use analogs of heme to competitively inhibit HO (7, 8). This approach is attractive because elevated bilirubin levels can be prevented.

A variety of metalloporphyrin HO inhibitors have been studied in several animal models (7–11). The first trial of SnPP in human neonates (12), however, was clinically unremarkable (13). Perhaps this was because insufficient amounts of metalloporphyrin were available to heme-degrading tissues of the RES at the low doses of 1–2 μmol SnPP per kg used in that study. One obvious way to increase the drug localized to the RES would be to increase the dose. However, this might lead to an increase in adverse reactions as well. Liposomal incorporation may make it possible to increase the amount of porphyrin localized to the RES without dose escalation.

Liposomes are artificial lipid vesicles with one or more concentric bilayers composed mainly of phospholipid and cholesterol. They can be used to encapsulate drugs in the aqueous volume, the lipid bilayer itself, or a combination of both (14). Their usefulness for the delivery of many drugs has been limited because most liposomes are quickly scavenged by RES (15). Landaw *et al.* (16) incorporated SnPP into unsized multilamellar vesicles of EPC and demonstrated increased localization of SnPP to the spleen as compared with SnPP administered in the aqueous form, with a concomitant decrease in splenic HO activity. In addition, they showed decreased biliary bilirubin output in bile duct–cannulated rats treated with liposomal tin mesoporphyrin.

Although liposomes are cleared from the blood by the RES, they actually accumulate poorly in the spleen relative to the liver in most circumstances. In general, more than 50% of an injected liposome dose will be taken up by the liver (Kupffer's cells), with only 10% going to the spleen (17). Targeting strategies can be used to increase the liposome dose delivered to the spleen. Our goals in the present study were: *1*) to formulate and characterize a stable preparation of L-ZnPP; *2*) to assess whether L-ZnPP could be targeted effectively to the spleen using reticuloendothelial blockade; and *3*) to study in adult rats with and without hemolysis the therapeutic efficacy of targeted *versus* nontargeted L-ZnPP, as reflected by suppression of VeCO.

MATERIALS AND METHODS

Animals. Our animal use protocol was approved by the Stanford University Division of Laboratory Animal Medicine. Adult male Wistar rats (Simonsen Laboratories, Inc., Gilroy, CA) weighing 275 to 325 g were used. They were housed at 25 ± 1°C, with a 12-h light cycle starting at 0700 h, and maintained on a diet of Wayne MRH 22/5 Rodent Blox (Continental Grain Co., Chicago, IL) and unlimited water. They were fasted for 12 h before experimentation. Once experimentation was begun, they were kept in subdued light.

Liposomes. EPC and EPG were purchased from Avanti Polar Lipids, Birmingham, AL. Cholesterol was obtained from Sigma Chemical Co., St. Louis, MO. ZnPP was from Porphyrin Products, Inc., Logan, UT. All chemicals used were of reagent grade or better. Initial encapsulation studies of ZnPP were done using the ethanol injection method (18). Briefly, lipid and porphyrin powders were dissolved in ethanol to a volume of 10% of the final desired volume. This mixture was then injected slowly into vigorously stirred 100 mM potassium phosphate buffer, pH 7.4, to form liposomes. Although this method allowed us to study the bilayer binding characteristics of the porphyrin, we encountered difficulties with size stability that were likely due to inadequate solvent removal by dialysis. Thus, for animal studies, ZnPP liposomes were made by the thin film method (19). The total lipid concentration was 50 mM. EPC and EPG in a 4:1 molar ratio were dissolved in chloroform and evaporated to a thin film under vacuum on a rotary evaporator. The dry film was additionally placed at a vacuum of 5 mtorr for at least 12 h to remove residual solvent. ZnPP was made into a 3.2 mM solution by dissolving 20.0 mg of the powder into 500 μL 10% (vol/vol) ethanolamine with stirring. The volume was brought to 7.5 mL with distilled water, and 1.0 mL of 1 M potassium phosphate buffer, pH 7.4, was added. The pH was titrated to 7.4 with 1 M HCl, and the final volume was brought to 10.0 mL with distilled water. The ZnPP solution was added to the dry lipid film and the mixture placed on a wrist shaker for 30 min. The resultant liposomes were sequentially extruded under nitrogen pressure three times through two stacked 0.4-μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA) held in a commercially available extrusion cell (Lipex, Vancouver, Canada). The mean particle size was approximately 0.3 μm as measured with a Coulter submicron particle sizer (Coulter Electronics, Inc., Hialeah, FL). In the phase contrast optical microscope, the tail (diameter ≥ 0.5 μm) of the size distribution could be observed. No large liposomes or aggregates were seen. The fluorescence mode showed a uniform glow indicating homogeneous incorporation of the porphyrin molecules into the liposomes. Blank liposomes for saturation of the RES were made to a concentration of 100 mM total lipid from EPC:EPG:cholesterol (1:1:1) by the same method with hydration with normal saline. Blank liposomes were also extruded to a size of 0.3 μm. The liposomes were stored for up to 1 wk at 4–8°C. The size distribution and drug concentration did not change over this time period.

ZnPP assay. Liposomes were assayed for ZnPP concentration spectrofluorimetrically using 90% ethanol/10% 1 M HCl (vol/vol) as a solvent. The emission at 603 nm with excitation at 407 nm was read and compared with a standard curve.

Octanol/water partition coefficient. A small amount of aqueous ZnPP was diluted with water to 1.0 mL total volume. One mL of 1-octanol was added, followed by vigorous mixing for at least 1 min. The phases were separated by centrifugation at 3000 × *g* for 15 min. The amount of ZnPP in each phase was quantitated, with the partition coefficient being the amount of drug in octanol divided by the amount in water.

Encapsulation efficiency. The percentage of drug encapsulated was determined by suspending 100 μL of liposomes in 900 μL of saline in a microfuge tube and spinning at 15 000 × *g* for 15 min. The amount of ZnPP in the pellet and supernatant was determined as above with encapsulation efficiency defined as the amount of drug recovered in the pellet divided by the total drug. For liposomes used in animals, this value was uniformly above 90%.

Plasma induced leak assay. Thin film L-ZnPP preparations were assayed for leakage in human plasma. The sample was diluted 10-fold in normal saline and mixed 1:1 with pooled human plasma. The mixture was incubated for 1 h at 37°C, then separated on a 20-cm Bio-Gel A15 M column (Biorad Laboratories, Richmond, CA) eluted with degassed normal saline. Fractions of 0.5 mL were collected and assayed for ZnPP concentration. Encapsulation efficiency was defined as the ratio of the first peak (liposomes) to the total recovered drug. For these thin film preparations, 40 to 50% of the drug was found to leak out in 1 h.

Hemin solution. Hemin for simulation of hemolysis was made into a 6 mM solution similar to ZnPP. Thirty μmol hemin per kg body weight were injected by the i.p. route.

Damaged RBC. Fresh blood from two donor rats was collected by cardiac puncture into acid citrate dextrose. The RBC were isolated by centifugation at 3000 × *g* for 15 min, and incubated at 49.5 ± 0.5°C for 20 min (20). The cells were washed twice with 1.5 volumes of normal saline and resuspended in one volume of normal saline for injection of 1.5 mL via the tail vein. The total Hb was measured by the cyanmethemoglobin procedure (Bulletin 525, Sigma Chemical Co.). The amount of heme injected was approximately 30 μmol/kg body weight.

VeCO. The *in vivo* total body bilirubin production was estimated using measurements of the VeCO. This technique has been described in detail elsewhere (10, 11).

HO assay. The HO activity of supernatant fractions of homogenized liver and spleen centrifuged at 15 000 × *g* for 15 min was determined using a gas chromatographic assay described previously (10, 11, 21).

Experimental procedure. Our targeting method was first assessed in adult rats without hemolysis. An initial VeCO was determined. Control animals received either an RES saturating dose of 1000 μmol total lipid per kg i.p. as blank liposomes or an equal volume of saline i.p. followed 2 h later by an i.p. dose of blank liposomes equal to 150 μmol total lipid per kg. Experimental animals were given either an RES saturating dose of blank liposomes or an equal volume of saline, followed 2 h later by 10 μmol L-ZnPP per kg. Six h after L-ZnPP treatment, the animals were again assayed for VeCO, killed, and the livers and spleens were assayed for HO activity.

The assessment of L-ZnPP in a hemolytic model was done by two methods: first, using an i.p. dose of hemin solution; second, using an i.v. dose of heat-damaged RBC. Both sets of experiments were done using essentially the same protocol. An initial VeCO was determined at t = 0 h. A dose of 30 μmol hemin per kg (~1.5 mL i.p.) or approximately 30 μmol RBC heme per kg (1.5 mL i.v.) was administered to each animal. At this time, animals also received either an i.p. injection of saline or an i.p. dose of 1000 μmol total lipid per kg as blank liposomes to saturate the RES. At t = 2 h, the VeCO was again measured, after which the animals were given an i.p. dose of 10 μmol L-ZnPP per kg, an equal volume of blank liposomes, or 10 μmol aqueous ZnPP per

kg. Bihourly VeCO readings were then performed until CO excretion returned to near baseline levels. Animals that had been given damaged RBC were killed at t = 12 h and assayed for HO activity in the liver and spleen.

Statistics. The data are expressed as a mean ± SD; the number of determinations is given in parentheses. Data were analyzed using a one-way analysis of variance. Statistical differences between groups were determined using the Scheffe F-test.

RESULTS

Table 1 shows the encapsulation of ZnPP into liposomes by the ethanol injection method. The optimum encapsulated drug to lipid ratio was about 1:16, allowing greater than 90% of the drug to be encapsulated with no need for free drug removal. The octanol:water partition coefficient of ZnPP was found to be 3.2 ± 0.2 (not shown), which indicates that most of the encapsulated ZnPP is located in the bilayer of the liposomes (17).

The effect of targeting ZnPP liposomes to the spleen of adult rats without hemolysis is shown in Table 2. The HO activity in the spleens and livers of both L-ZnPP–treated groups was significantly ($p < 0.01$) lower than that of the controls. In addition, the splenic HO activity in the rats receiving a predose of blank liposomes to saturate the RES was significantly ($p < 0.05$) lower than that of animals who had received a predose of saline before administration of L-ZnPP. Six h after L-ZnPP administration, the VeCO of the saline-pretreated group had dropped to 79% of its initial value, and the VeCO of the liposome-pretreated group had dropped to 72% of its initial value. The VeCO of the control group did not change over this time period. The difference in VeCO between the two L-ZnPP–treated groups and the control group was significant at $p < 0.01$.

Figure 1*A* shows the results of the VeCO readings of animals that had been treated with an i.p. dose of hemin to simulate hemolysis. An initial rise in the VeCO was seen in all animals. Administration of L-ZnPP at t = 2 h blocked further rises in the VeCO over the next 10 h. L-ZnPP–treated rats, pretreated with saline, produced significantly ($p < 0.01$) less CO than liposome-treated controls at t = 6 and t = 8 h. L-ZnPP–treated rats pretreated with the blank liposomes produced significantly less CO than controls from t = 4 to t = 8 h. Aqueous ZnPP at a dose of 10 μmol/kg administered at t = 2 h produced no statistical difference from control in this model. Both groups of animals dosed with L-ZnPP showed statistical difference ($p < 0.01$) from the aqueous ZnPP-treated group, the saline-predosed group at t = 8 h, and the liposome-predosed group a t = 4, 6, and 8 h. There was no statistical difference between L-ZnPP–treated rats predosed with saline, and those predosed with an RES saturating dose of blank liposomes over the course of this experiment.

Figure 1*B* shows the results of VeCO determinations in animals treated with damaged RBC to simulate hemolysis. All rats given damaged RBC showed a rapid rise in the VeCO. Animals given a pretreatment of blank liposomes to saturate the RES 2 h before L-ZnPP showed a more rapid decline in VeCO from t = 6 to 12 h than did control, aqueous ZnPP-treated, or saline-pretreated L-ZnPP–treated animals. Lipid-pretreated animals produced significantly ($p < 0.05$) less CO at t = 8, 10, and 12 h after RBC treatment compared with all other groups.

Table 3 shows the liver and spleen HO activity of the animals from Figure 1*B* killed at t = 12 h. All ZnPP-treated groups had significantly decreased liver HO activity relative to controls. In addition, both L-ZnPP–treated groups had significantly decreased splenic HO activity relative to controls. In the lipid pretreated group, splenic HO activity was completely eliminated.

DISCUSSION

The encapsulation of porphyrins into liposomes is not a new idea. The work of Jori *et al.* with porphyrin liposomes for use in photodynamic destruction of tumors (18, 22, 23) has been extensive. This article provides the second report of the use of porphyrins and liposomes toward the goal of preventing jaundice in neonates. We have shown that ZnPP can be incorporated into liposomes of EPC and EPG with good efficiency by both the ethanol injection and thin film methods. Our thin film preparations were easily extruded to 0.3 μm without loss of drug or encapsulation efficiency. Because the negatively charged EPG was included in the bilayer, our liposomal suspension did not aggregate even after a week of storage, and our experience with other negatively charged preparations leads us to believe that this formulation could be stored much longer. Furthermore, when prepared with the proper cryoprotectant, we have found that similar preparations are easily lyophilized, increasing shelf-life dramatically. In contrast, neutral EPC preparations are in general quite unstable and would be expected to form uninjectable aggregates in a matter of days.

In adult rats without hemolysis, a dose of 10 μmol L-ZnPP per kg suppressed the VeCO and liver and spleen HO activity to a level comparable to that reached using an aqueous dose of 40 μmol ZnPP per kg (10). Our results also indicate that reticuloendothelial blockade with blank liposomes 2 h before administration of L-ZnPP increases the degree to which splenic HO is inhibited. Interestingly, it was difficult to correlate this increased splenic HO inhibition with increased therapeutic efficacy as reflected by suppression of the VeCO.

We believe that, because bilirubin production in the adult rat without hemolysis is rather limited, this model was not sensitive enough for us to discern a true difference. Yet, the results also indicate that there was essentially no benefit seen due to targeting of ZnPP to the spleen in the inhibition of the VeCO of adult rats when i.p. hemin was given to simulate hemolysis. However, when damaged RBC were injected i.v. as a hemolytic challenge, the results were strikingly different. Liposomal ZnPP produced no difference from control unless a pretreatment of blank liposomes was given to saturate the RES. This raises the question of which of these two hemolytic models is more representative of jaundice in the neonate.

Serum albumin is a well-described carrier of endogenous porphyrins. Yet, few studies of the pharmacodynamics of injected exogenous porphyrins have been reported. The photosensitizer hematoporphyrin injected as an aqueous solution associates heavily with plasma lipoproteins, especially HDL, in which it can circulate for extended periods of time (23). Other evidence indicates that hemopexin would carry a substantial portion of SnPP injected as an aqueous solution (24). The degree of porphyrin association with lipoproteins and hemopexin is related to

Table 1. *Encapsulation of ZnPP into ethanol injection liposomes of varying formulation**

Formulation	Molar ratio	[Lipid] (μmol/mL)	[ZnPP] (μmol/mL)	Encapsulation efficiency (%)	Drug:lipid (μmol/μmol)†
EPC:Ch ($n = 3$)	55:45	25	1.6	50 ± 8	1:31
EPC:EPG:Ch ($n = 3$)	45:10:45	25	1.6	56 ± 6	1:28
EPC ($n = 3$)	100	25	1.6	95 ± 4	1:16
EPC ($n = 2$)	100	25	3.2	67	1:12
EPC:EPG ($n = 3$)	80:20	25	1.6	96 ± 3	1:16

* [Lipid], lipid concentration; [ZnPP], ZnPP concentration; Ch, cholesterol.

† This reflects the ratio of encapsulated drug to total lipid. Calculation is as follows: [ZnPP] × encapsulation efficiency/[Lipid].

Table 2. *Effect of ZnPP liposomes on total body bilirubin production and tissue HO activity in adult rat**

Group	HO activity (nmol CO/h/mg protein)		VeCO (μL CO/kg/h)	
	Spleen	Liver	t = 0 h	t = 6 h
Control (n = 4)	1.07 ± 0.09	0.37 ± 0.04	19.1 ± 1.6	19.2 ± 0.5
Saline + L-ZnPP (n = 5)	0.53 ± 0.16†	0.06 ± 0.02†	18.8 ± 1.3	14.8 ± 2.0†
Liposome + L-ZnPP (n = 5)	0.25 ± 0.16†‡	0.06 ± 0.02†	19.1 ± 1.1	13.8 ± 1.1†

* An initial VeCO was determined and the animals were predosed with 1000 μmol total lipid per kg as blank liposomes or saline as indicated. Two h later, animals were given 10 μmol L-ZnPP per kg or an equal volume of blank liposomes. Six h later, the VeCO was again measured, and the animals were killed and assayed for HO in the indicated tissues.

† $p < 0.01$ relative to controls, Scheffe F-test.

‡ $p < 0.05$ relative to the saline-pretreated group, Scheffe F-test.

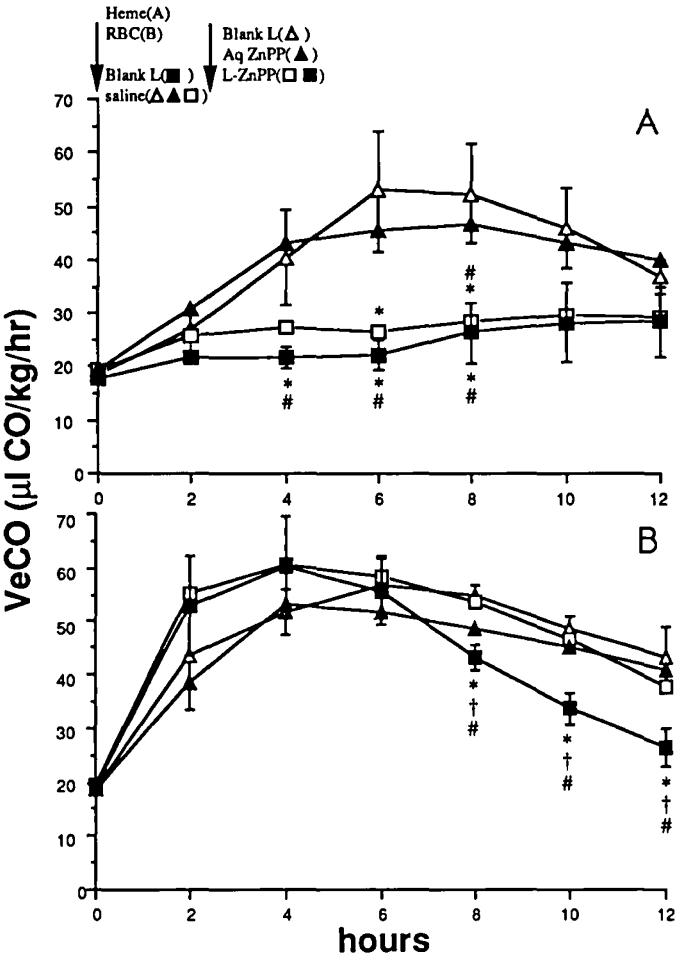

Fig. 1. CO production in adult rats with simulated hemolysis. An initial VeCO was determined at t = 0 h. All animals were then treated i.p. with either 30 μmol hemin per kg body weight (*A*) or i.v. with approximately 30 μmol heme per kg in the form of heat-damaged RBC (*B*). The *two arrows above panel A* indicate what pretreatment and treatment, respectively, each group of animals, represented by the symbols, received at the indicated time point. At t = 0, animals represented by ■ were pretreated with 1000 μmol total lipid per kg body weight as blank liposomes (*Blank L*) per kg body weight (n = 4). Animals represented by ▲ were given 10 μmol aqueous ZnPP (*Aq ZnPP*) per kg body weight (n = 4). Liposome encapsulated ZnPP (L-ZnPP) was given to both the saline-pretreated (n = 3, □) and the liposome-pretreated (n = 3; ■) animals. VeCO readings were continued until t = 12 h. (* indicates $p < 0.01$ compared with controls; † indicates $p < 0.01$ compared with saline-pretreated L-ZnPP–treated group; # indicates $p < 0.05$ compared with aqueous ZnPP group).

Table 3. *Heme oxygenase activity (nmol CO/h/mg protein) in tissues of adult rats from Figure 1B killed at t = 12 h**

Group	Spleen	Liver
Control (n = 4)	1.33 ± 0.44	0.31 ± 0.05
Aqueous ZnPP (n = 4)	0.73 ± 0.20	0.23 ± 0.03†
Saline + L-ZnPP (n = 3)	0.25 ± 0.07†	0.12 ± 0.02†
Liposomes + L-ZnPP (n = 3)	ND†‡	0.09 ± 0.02†‡

* ND, none detected.

† $p < 0.01$ compared with control, Scheffe F-test.

‡ $p < 0.01$ compared with aqueous ZnPP group, Scheffe F-test.

hydrophobicity (23, 24). Because hemin, like SnPP and hematoporphyrin, is hydrophobic, it likely would associate with plasma lipoproteins and hemopexin after injection and would be cleared from the circulation by the hepatocyte, not the RES macrophage. Thus, injection of aqueous hemin would preferentially stimulate liver, specifically hepatocyte, HO activity. In contrast, heat-damaged RBC are cleared from the circulation by the macrophages of the RES, particularly those in the spleen (20). A similar mechanism is responsible for clearing damaged RBC from the circulation of the neonate with hemolytic disease (1). Thus, injection of heat-damaged RBC into the rat is probably a better model for studying neonatal jaundice than injection of aqueous hemin. When we focus only on the results of the VeCO readings in animals treated with damaged RBC, we see a clear benefit of targeting ZnPP to the spleen.

In general, we have demonstrated more complete inhibition of liver HO activity than of splenic HO activity *in vivo* with administration of aqueous ZnPP (10, 11). As mentioned earlier, this is probably due to delivery of the majority of injected porphyrin to the hepatocyte by hemopexin and lipoproteins. However, a large portion of the heme from senescent RBC is probably converted to bilirubin in the spleen (1). In the present study, the splenic HO activity that was not eliminated in the aqueous ZnPP-treated group and the saline-predosed L-ZnPP-treated group from Figure 1*B* was evidently enough to allow those animals to keep pace with controls as far as total body CO production. Because the splenic HO activity of the liposome-pretreated rats represented in Figure 1*B* was totally eliminated, a significant decrease in total CO production was observed from t = 8 to 12 h relative to the other three groups. These results confirm the hypothesis of Landaw *et al.* (16) that delivery of adequate porphyrin to the spleen represents a major stumbling block to the optimum use of metalloporphyrin HO inhibitors as a therapy for neonatal jaundice.

We have demonstrated in a rodent model that active targeting of L-ZnPP to the spleen using reticuloendothelial blockade can increase HO inhibition there, with a corresponding decrease in VeCO when damaged RBC are injected as a hemolytic challenge. This targeting method, however, is cumbersome and might have adverse consequences, such as increased risk of infection in the high-risk neonate. Recent work using sterically stabilized liposomes with extended blood circulation times (Stealth liposomes, Liposome Technology, Inc., Menlo Park, CA) has shown that almost 50% of an injected dose of large liposomes can be

delivered to the spleen, with most of the balance being delivered to the liver and less than 10% going to other tissues (25, 26). This phenomenon depends on the size of the liposome and the use of special lipids in the bilayer (27). The use of Stealth liposomes to encapsulate relatively lipid-soluble metalloporphyrin inhibitors of HO such as ZnPP might increase the potency of these drugs by allowing maximum delivery of inhibitor to heme-degrading tissues. At the same time, porphyrin would not be delivered to tissues where it might cause adverse reactions. We would predict large increases in the therapeutic efficacy of these drugs were this proved to be the case.

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