Prolonged Systemic Delivery of Peptide Drugs by Long-Circulating Liposomes: Illustration with Vasopressin in the Brattleboro Rat

Martin C. Woodle,[1,4] Gert Storm,[1,2] Mary S. Newman,[1] Jill J. Jekot,[1] Lila R. Collins,[1] Francis J. Martin,[1] and Francis C. Szoka, Jr.[3]

Received April 17, 1991; accepted August 11, 1991

The value of novel systemically long-circulating liposomes to prolong the duration of an antidiuretic hormone, arg[8]-vasopressin (VP), was investigated as a representative of low molecular weight peptides with rapid clearance. Cholesterol content was found to have a controlling effect on VP release in serum. Three types of liposomes were selected for urine production measurements in VP deficient Brattleboro rats. One contained phosphatidylserine (PS), which was rapidly cleared from the circulation. In the other two liposomes, the PS component was replaced by either phosphatidylglycerol or a novel phospholipid derivatized with polyethylene glycol (PEG); both showing prolonged circulation. Free VP (up to 8 μg/kg) gave reduced urine production for less than 24 hr. The PG formulation exhibited a dose-dependent prolonged duration of bioactivity of up to 4 days. Substitution of PEG-PE resulted in a 2-day delay followed by a prolonged duration of bioactivity for over 4 days. The duration of the prolonged bioactivity was not dose dependent but the amplitude was. This is attributed to VP release from liposomes which have distributed intact to another compartment without having been taken up by the RES. By balancing liposome circulation time, release rate, and dose, long-circulating liposomes can be applied to prolong the biological activity of a therapeutic peptide.

KEY WORDS: antidiuretic hormone; Brattleboro rat; liposomes; polyethylene glycol (PEG).

INTRODUCTION

The development of bioactive proteins and peptides as therapeutic agents is severely limited in most cases by a lack of oral bioavailability and rapid clearance from the blood (1). Liposomes have been proposed to improve their delivery by functioning as a circulating "microreservoir" for sustained release (2,3) while providing protection and reducing immunogenicity and adverse side effects. Unfortunately, liposomes have been limited by their rapid recognition and clearance from the circulation by phagocytic cells of the reticuloendothelial system (RES) (4,5).

Recently, novel liposome formulations (e.g., Stealth)[5] with reduced RES uptake and prolonged circulation times (6–8) have been developed. The formulations contained either monosialoganglioside (G_{M1}) or hydrogenated phosphatidylinositol (HPI) in "rigid" bilayers. Further achievements are attributed to steric stabilization by inclusion of polyethyleneglycol lipid derivatives (9–15).

The present studies were undertaken to investigate the utility of long circulating liposomes for sustained iv delivery of antidiuretic hormone, vasopressin (VP). VP is representative of low molecular weight peptides that are not orally active and are rapidly cleared. Bioactivity was determined by measurements of diuresis of Brattleboro rats which have a hereditary deficiency in VP production but possess functional VP receptors (16–19).

MATERIALS AND METHODS

Liposome Preparations

Large unilamellar liposomes were prepared by reverse phase evaporation (REV) (20) with the following lipids: partially hydrogenated egg phosphatidylcholine with an iodine value of 40 (Asahi Chemicals, Japan), sphingomyelin (SM) (Sigma, St. Louis, MO), cholesterol (Chol) (Croda, Fullerton, CA), phosphatidylglycerol (PG) and phosphatidylserine (PS) (Avanti Polar Lipids, Birmingham, AL), monosialoganglioside (G_{m1}) (Sigma), and a polyethylene glycol derivative of distearoyl-phosphatidylethanolamine (PEG-DSPE) (Calbiochem, San Diego, CA) as described elsewhere (13,14). The samples, prepared under aseptic conditions, contained 10 μmol phospholipid and 510 μg total arginine[8]-vasopressin (Sigma) with trace ^{3}H-labeled VP per ml in aqueous buffer (5 m*M* Tris, 100 m*M* NaCl, 0.1 m*M* EDTA) and were extruded through 0.4-μm Nuclepore filters (21). Particle size distributions (Nicomp Model 200) had a mean diameter of from 0.15 to 0.25 μm and a low polydispersity. The VP was determined by gel filtration on Bio-Gel A15 (BioRad, Fullerton, CA). Unbound VP was removed by dialysis. Plasma-induced release of VP was determined by dilution 1:5 with human serum and incubation at 37°C.

Long-circulating liposomes contained either PEG-DSPE or PG at 10% of phospholipid in SM:PC:Chol bilayers with a final mole ratio of 0.2:1:1:1, respectively (5,8). A rapidly cleared formulation contained PS as the acidic component in the same proportions. VP encapsulation of up to 22% was achieved regardless of lipid composition. Typical loading was about 10 μg/μmol total lipid, with a range of 2 to 19. Leakage during storage in saline was low, greater than 90% of the encapsulated VP retained for 2 weeks. Stability of the particle size distribution was observed; mean diameter did not change by more than 20%.

In Vivo Studies

Male adult Brattleboro rats, 220 to 400 g, congenitally deficient for VP (16), were used under standard conditions with food and water consumed ad libitum.

Circulation Time. VP blood levels were determined from radiolabeled VP in whole blood samples. A 150- to 1000-μl sample was administered via a femoral venous can-

[1] Liposome Technology Inc., 1050 Hamilton Court, Menlo Park, California 94025.
[2] Department of Pharmaceutics, University of Utrecht, Utrecht, The Netherlands.
[3] Department of Pharmacy, University of California, San Francisco, California 94143.
[4] To whom correspondence should be addressed.
[5] Stealth is a registered trademark of Liposome Technology, Inc.

0724-8741/92/0200-0260$06.50/0

nula which was then removed and the vein tied off. Blood samples of 400 μl were obtained from a chronic femoral arterial cannula. The animals were awake and unrestrained during blood drawing except for time points less than 30 min, when the anesthesia was still effective.

Urine Production Studies. The animals were acclimated to metabolic cages for at least 3 days and the baseline levels of urine flow established. Rats receiving saline were used as controls. The volume administered was adjusted to give the desired total amount of protein. For aqueous VP, dose levels of 0.2, 0.8, and 2 μg were given. With the 2-μg dose adverse side effects on breathing were observed in lighter animals which lasted up to 30 min after dosing, and higher doses were acutely lethal in some animals. For these reasons 2 μg was considered the maximum dose of aqueous VP to be given. All the liposome formulations used in this study contained at least 90% of the drug in encapsulated form. Since the unencapsulated drug should behave like aqueous VP, 10-fold higher levels of liposomal VP were given (2, 8, and 24 μg), allowing the unencapsulated fraction to be similar (0.2, 0.8, 2 μg). This gives different lipid doses but without affecting the circulation time (13,14). The highest dose of each (24 μg) was well tolerated, indicating that the liposomal VP was not immediately released.

Observations of urine production (diuresis) are expressed in two ways: cumulative diuresis as total milliliters of urine produced following dose administration and percentage of predose diuresis as milliliters per hour averaged over a 24-hr urine collection period.

RESULTS AND DISCUSSION

In Vitro Studies

In vitro studies of VP release from PEG-DSPE liposomes in the presence of 80% human serum allows identification of composition variables which influence release rates. Data for PEG-DSPE (Table I) indicate that the extent of serum-induced release is controlled by cholesterol content. Formulations composed of only SM as the neutral phospholipid showed the most rapid release, whereas a more gradual release was observed for a 1:1 mixture of PC and SM (data not shown). These results suggest that *in vivo* release rates can be regulated by the lipid composition.

In Vivo Studies

Circulation Time

Plasma pharmacokinetics in Brattleboro rats were made for VP encapsulated in three liposome formulations: one with rapid clearance containing PS and two with long circulation containing either PG or PEG-DSPE as the acidic component (Fig. 1). These results show that VP blood levels can be maintained for over 2 days following a single iv injection when encapsulated in long-circulating liposomes, compared to less than an hour with rapidly cleared liposomes (Fig. 1) or the aqueous solution (22).

Table I. Vasopressin Leakage from Stealth Liposomes Containing PEG-DSPE (10% of Phospholipid) in Human Serum

Lipid molar ratio composition (PC:SM:CHOL)	Percentage entrapment[a]		
	1 hr	24 hr	48 hr
A. 1:1:1	83	77	72
B. 1:1:0.5	79	66	60
C. 1:1:0	67	29	27

[a] Freshly prepared samples were incubated in 80% human serum, 37°C.

These measurements include a presumably biologically unavailable fraction within the liposomes. Release of this fraction to maintain plasma levels of bioactive peptide above the therapeutic threshold can be influenced and controlled by the liposome (23). Such a process was evaluated here by measurements of dual lipid and VP labels using two different lipid labels (cholesterol oleate and cholesterol oleyl ether). The results were conflicting probably because of distribution and metabolism of the lipid labels (24) arising from the relatively long time period of circulation.

Urine Production

An antidiuretic response due to VP plasma levels in the physiological range for rats, 1–8 pg/ml (25,26), was used to detect if biologically active VP is released from liposomes.

(A) Free VP. The antidiuretic response was followed with an iv dose of saline and aqueous VP at three dose levels (Fig. 2). A complete loss of diuresis for a short period during the first day was observed depending only slightly on VP dose. In most cases the recovered diuresis rate slightly exceeded that of the saline control. No further changes over the next 6 days were observed.

(B) Urine Production with Encapsulated VP. The diuresis responses obtained with the rapidly cleared liposomal VP formulation containing PS (24 μg) were similar to those of aqueous VP, despite a 10-fold difference in the dose (data not shown), indicating that only the unencapsulated VP fraction present in the formulation was bioactive. Degradation or denaturation of the VP during the liposome preparation can be ruled out since the unencapsulated VP retains its biological activity and is exposed to the same conditions. This suggests that the lack of bioactivity for the VP encapsulated in the rapidly cleared liposomes is due to RES degradation of the drug following liposome uptake.

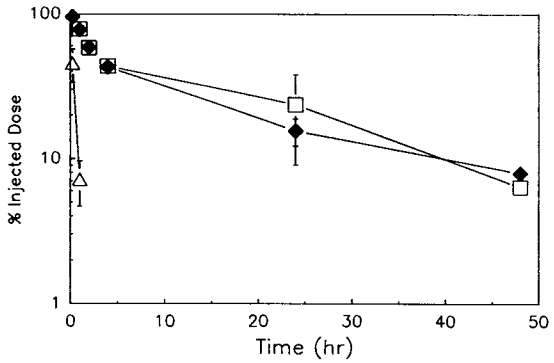

Fig. 1. Clearance of radiolabeled VP from the blood of Brattleboro rats after iv administration. Open squares, VP in PG:SM:PC:Chol liposomes; filled diamonds, VP in PEG:SM:PC:Chol liposomes; open triangles, VP in PS:SM:PC:Chol liposomes (mean ± SD, n = 3).

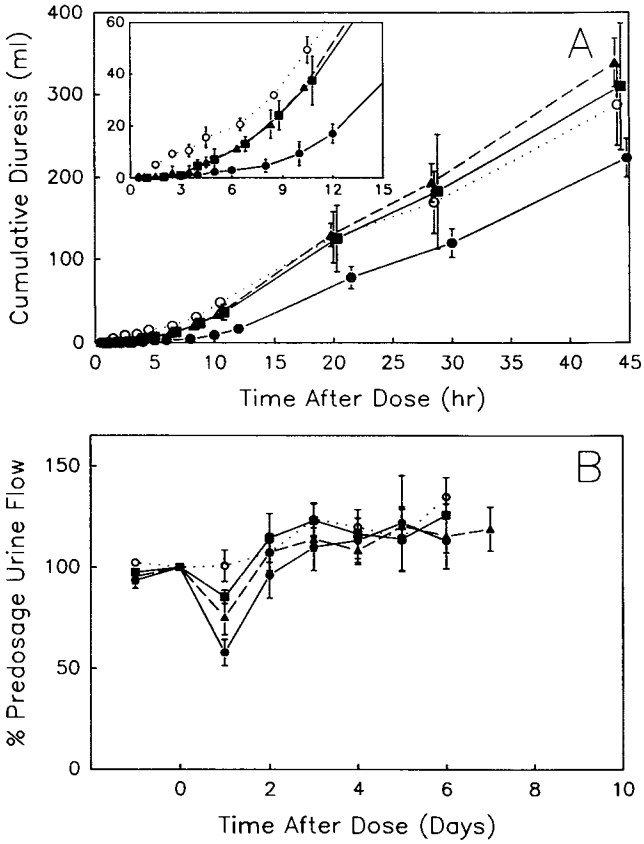

Fig. 2. (A) Cumulative diuresis and (B) diuresis as a percentage of the predosage rate, after surgery and iv dose administration of aqueous solutions of VP to Brattleboro rats. Open circles, saline control; filled squares, 0.2 μg; filled triangles, 0.8 μg; filled circles, 2 μg (mean ± SD, n = 3).

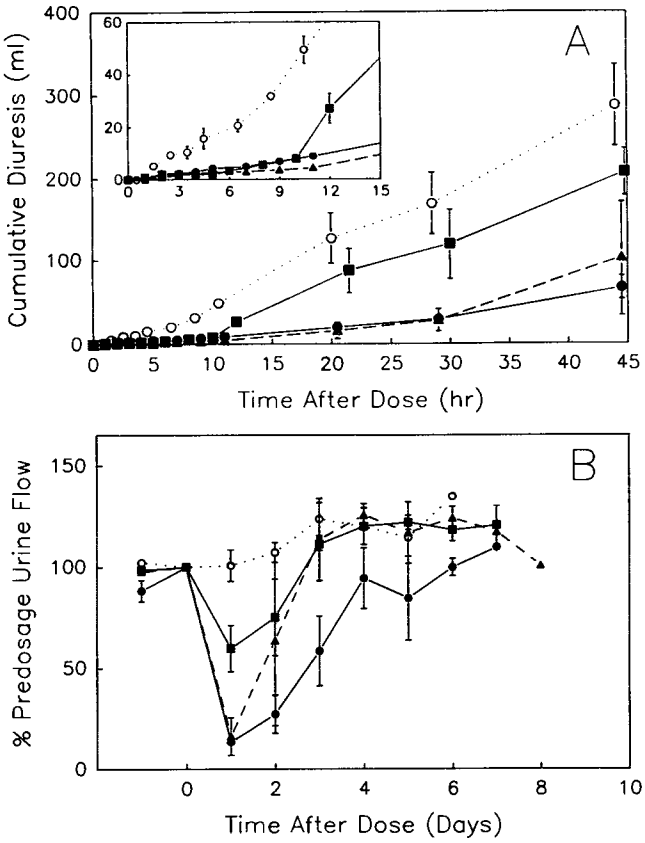

Fig. 3. (A) Cumulative diuresis and (B) diuresis as a percentage of the predosage rate of Brattleboro rats after surgery and iv dose administration of liposomal VP with a lipid composition of PG:SM:PC:Chol. Open circles, saline control; filled squares, 2 μg; filled triangles, 8 μg; filled circles, 24 μg (mean ± SD, n = 3).

When VP was administered in long-circulating liposomes containing PG, a prolonged antidiuresis was observed (Fig. 3). An immediate 10- to 12-hr delay in diuresis with only a slight dose dependence was observed, compared to a delay of only 4 to 6 hr with either free VP or the rapidly cleared PS liposome formulation. Once diuresis resumed, it did so at a rate related to the dose and remained constant for at least 30 hr. Recovery of the diuresis to the level of the saline control was dose dependent, occurring in about 2 days at the lowest dose and about 4 days at the highest dose. These results suggest that effective plasma levels are initially due to the unencapsulated VP component and are maintained for a prolonged time by release of the drug from the liposomes as they circulate.

When VP was administered in the long-circulating liposomes containing PEG-DSPE, a very different antidiuretic response pattern was observed (Fig. 4). Two apparently distinct antidiuretic responses were observed: a modest transient reduction of diuresis in the first 24 hr, followed by a second more pronounced reduction in diuresis beginning on day 2 and persisting for at least four additional days.

The reduction in diuresis on the first day after iv administration of the PEG-DSPE liposomes can be accounted for entirely by the unencapsulated VP present in the formulation. A comparison of the cumulative diuresis with that produced by aqueous VP at doses approximating the unencapsulated VP levels shows practically superimposable responses. As with the rapidly cleared liposome formulation, degradation or denaturation of the VP can be ruled out since the free component clearly retains biological activity. From these results it can be concluded that biologically active levels of VP are not released from these liposomes during the first two days post injection.

The lack of a prolonged antidiuretic effect during the first 2 days implies that little VP is released from these liposomes during circulation. Therefore, reduced levels of cholesterol, found to increase VP release *in vitro* (Table I), in the PEG-DSPE-containing formulations also were studied but at a fixed dosage of 8 μg (Fig. 5). The results agree with the *in vitro* findings showing a prolonged duration and greater amplitude of antidiuresis over the first 2 days. These data underscore the importance of balancing the circulation time of the carrier with the drug release rate during circulation.

The second, or delayed, response appears on the third day, lasts for roughly 4 days, and exhibits a very different behavior from the initial response. Its magnitude is saturated by the 8-μg dose, its duration is independent of dose, and it was observed only with PEG-DSPE-containing liposomes. Further, at a fixed dose of 8 μg, no dependence on cholesterol content was observed (Fig. 6). The possibility that this

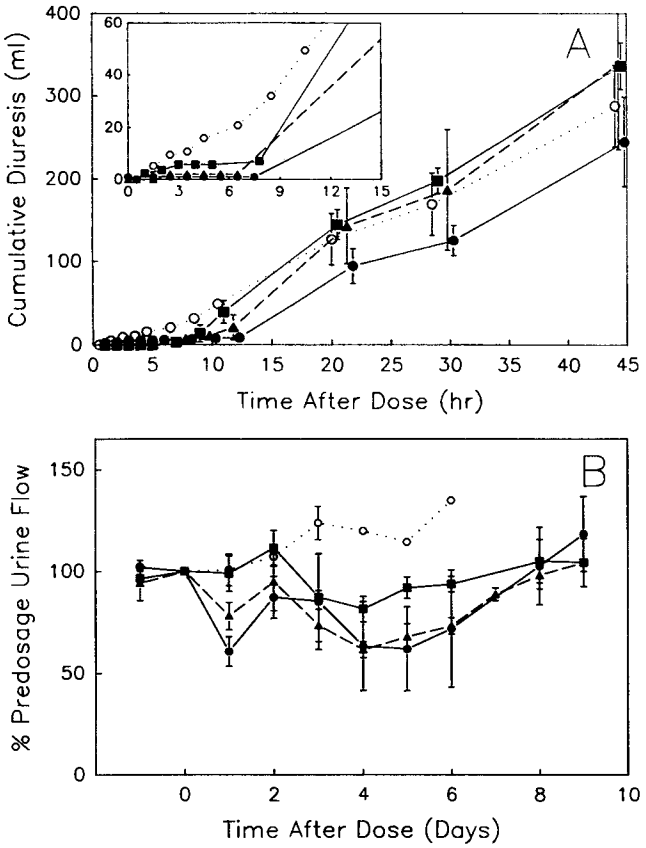

Fig. 4. (A) Cumulative diuresis and (B) diuresis as a percentage of the predosage rate of Brattleboro rats after surgery and iv dose administration of liposomal VP with a lipid composition of PEG-DSPE:SM:PC:Chol. Open circles, saline control; filled squares, 2 μg; filled triangles, 8 μg; filled circles, 24 μg (mean ± SD, n = 3).

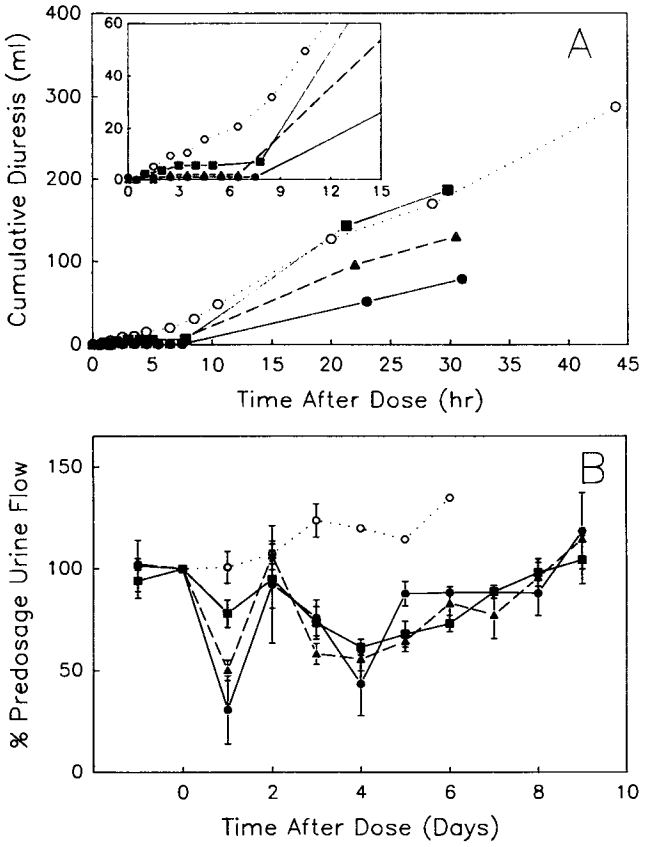

Fig. 5. (A) Cumulative diuresis and (B) diuresis as a percentage of the predosage rate of Brattleboro rats after surgery and iv dose administration of an 8-μg dose of VP encapsulated in PEG-DSPE:SM:PC liposomes with different cholesterol contents. Open circles, saline control; filled squares, 33% Chol; filled triangles, 16% Chol; filled circles, 0% Chol (mean ± range, n = 2).

second antidiuretic response is not due to release of VP from circulating liposomes but, instead, to an unexpected biological response to the PEG-DSPE formulation itself was discounted by administering the same dose of aqueous VP mixed with empty PEG-DSPE liposomes. The results of this control experiment showed no evidence of the second response (data not shown), confirming that it is due to release of biologically active VP from the PEG-DSPE-containing liposomes.

Other explanations for this behavior also have been ruled out. A transient down regulation of VP receptors (26) leading to a loss of the antidiuretic response in the presence of bioactive levels of VP during day 2 is not likely on the basis of the results obtained with the PG-containing formulation. In this case a prolongation of the diuretic response occurred which completely spans this transient period of reduced antidiuresis.

The possibility that a sudden increase in release rate from those PEG-DSPE liposomes still in circulation after 3 days accounts for the observed delayed biological effect is also unlikely. It would require that the release rate increases enough to compensate for the decline in circulating levels of liposomal VP. After 48 hr the total plasma levels of VP are less than 10% of the dose and, presumably, continue to decline thereafter. Given this clearance profile, the total VP circulating after 2 days is less than 1 μg. Previous studies with VP infusion at different rates showed a requirement for continuous infusion of 0.5 μg/day to maintain vasoconstriction (27) or 1.5 μg/day by osmotic minipump to maintain the antidiuretic response (26). Moreover, the lack of a clear-cut relationship between dose, or cholesterol content of the formulation, and the duration of the antidiuretic response (Fig. 4) also argues against this possibility.

An alternative explanation for the delayed antidiuretic response is that the PEG-DSPE-containing liposomes release little drug during circulation but a portion of the liposomes distributes intact to another anatomical compartment where a delayed release of VP occurs. To test whether plasma levels of bioactive VP are responsible for the antidiuresis, a VP antagonist was administered during the peak of the second episode of reduced diuresis to reverse the antidiuresis effect (19). Preliminary results using ip administration of one such antagonist every 3 hr over a 9-hr period on day 4 did increase the rate of diuresis (data not shown). Experiments repeating this with a longer period of antagonist administration using miniosmotic infusion pumps are currently in progress. These results, along with the dependence of the initial response on cholesterol content and the lack of a similar dependence by the delayed response, strongly sug-

gest that different mechanisms are responsible for the immediate and delayed responses.

Two possibilities for the compartment from which active VP is released can be envisioned. The rapid uptake of encapsulated VP in the PS formulation by the RES followed by apparent complete degradation of the encapsulated VP suggests that a non-RES compartment must exist in which the VP is not completely degraded but is released over several days starting on the third day. Nevertheless, it might be that a portion of the PEG-DSPE containing liposomes taken up by the RES is not degraded intracellularly (28). Subsequently, the liposomes and/or its contents could be released from these cells giving rise to the delayed response. Further studies may be helpful to distinguish among these possibilities.

CONCLUSIONS

The new long-circulating liposomes have been used to deliver cytotoxic drugs more selectively to tumors and improve targeting of ligand-bearing liposomes to cells in the vascular system (13,29,30). We applied the same approach to agents that require prolonged plasma levels but are cleared rapidly from the bloodstream. The results demonstrate the value of using certain long-circulating liposome formulations to deliver a peptide hormone. Many factors influencing liposomal drug delivery in this setting (23) have been addressed, including liposome circulation time, hormone release during circulation, blood levels of the "free" peptide required for bioactivity, clearance rate of the free drug, maximum plasma levels that can be tolerated, and the fate of encapsulated peptide cleared with the liposomes. However, a detailed characterization of many of these parameters is still lacking. In particular, many questions regarding the mechanism of the second, or delayed, biological response seen with PEG-DSPE containing long-circulating liposome formulations remain to be addressed: the sites of distribution of these liposomes, the biological interactions which lead to drug release, and the extent of cellular internalization. Nevertheless, insight has been gained into the critical factors controlling prolonged delivery of biologically active agents with this system. Long-circulating liposomes have the capacity for at least two kinds of prolonged delivery of biologically active agents which could be utilized to prolong the bioavailability of peptides from hours to as long as 1 week following a single iv injection. This approach may be most applicable to improve the delivery of agents used in the treatment of acute disorders which currently require high doses and frequent injections or infusions to maintain therapeutic blood levels.

ACKNOWLEDGMENT

Ms. Martina Tjebbes is gratefully acknowledged for expert technical assistance.

REFERENCES

1. V. H. L. Lee. Peptide and protein drug delivery: Opportunities and challenges. *Pharm. Int.* 7:208–212 (1986).
2. A. L. Weiner. Liposomes as carriers for polypeptides. *Adv. Drug Del. Rev.* 3:307–341 (1989).
3. G. Storm and D. J. A. Crommelin. Liposomes as carriers for therapeutic proteins. *Biother.* (in press).
4. J. H. Senior. Fate and behavior of liposomes in vivo: A review of controlling factors. *CRC Crit. Rev. Ther. Drug Carrier Syst.* 3:123–193 (1987).
5. U. K. Nässander, G. Storm, P. A. M. Peeters, and D. J. A. Crommelin. In M. Chasin and R. Langer (eds.), *Biodegradable Polymers as Drug Delivery Systems,* M. Dekker, New York, 1990, pp. 261–338.
6. T. M. Allen and A. Chonn. Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.* 223:42–46 (1987).
7. A. Gabizon and D. Papahadjopoulos. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA* 85:6949–6953 (1988).
8. T. M. Allen. Stealth™ liposomes: Avoiding reticuloendothelial uptake in liposomes. In G. Lopez-Berestein and I. J. Fidler (eds.), *The Therapy of Infectious Diseases and Cancer,* Alan R. Liss, New York, 1989, pp. 405–415.
9. G. Blume and G. Cevc. Liposomes for the sustained drug release in vivo. *Biochim. Biophys. Acta* 1029:91–97 (1990).
10. A. L. Klibanov, K. Maruyama, V. P. Torchillin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268:235–237 (1990).
11. M. C. Woodle, M. Newman, L. Collins, C. Redemann, and F. Martin. Improved long circulating (Stealth®) liposomes using synthetic lipids. *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 17:77–78 (1990).
12. J. Senior, C. Delgado, D. Fisher, C. Tilcock, and G. Gregoriadis. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: Studies with poly(ethylene glycol)-coated vesicles. *Biochim. Biophys. Acta* 1062:77–82 (1991).
13. D. Papahadjopoulos, T. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K.-D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* (in press) (1991).
14. M. C. Woodle, K. K. Matthay, M. S. Newman, J. E. Hidayat, L. R. Collins, C. Redemann, F. J. Martin, and D. Papahadjopoulos. Versatile liposome compositions showing prolonged circulation with steric stabilization (submitted for publication) (1991).
15. M. C. Woodle and D. D. Lasic. Sterically stabilized liposomes. (submitted for publication) (1992).
16. H. Valtin and H. A. Schroeder. Familial hypothalamic diabetes insipidus in rats (Brattleboro strain). *Am. J. Physiol.* 206:425 (1964).
17. J. Kruisbrink and G. J. Boer. Controlled long-term release of small peptide hormones using a new microporous polypropylene polymer: Its application for VP in the Brattleboro rat and potential perinatal use. *J. Pharm. Sci.* 73:1713–1718 (1984).
18. H. V. Maulding. Prolonged delivery of peptides by microcapsules. *J. Control. Release* 6:167–176 (1987).
19. W. H. Sawyer and M. Manning. Effective antagonists of the antidiuretic action of vasopressin in rats. *Ann. N.Y. Acad. Sci.* 394:464–472 (1982).
20. F. Szoka and D. Papahadjopoulos. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* 75:4194–4198 (1978).
21. F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail, and D. Papahadjopoulos. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* 557:9 (1979).
22. D. C. Klonoff and J. H. Karam. Hypothalamic and pituitary hormones. In B. G. Katzung (ed.), *Basic and Clinical Pharmacology,* 3rd ed., Appleton and Large, Los Altos, CA, 1987, pp. 423–435.

23. F. C. Szoka. Liposome drug delivery. In J. Wilschut and D. Hoekstra (eds.), *Membrane Fusion,* Marcel Dekker, New York, 1991, pp. 845–890.
24. J. T. P. Derksen, H. W. M. Morselt, and G. L. Scherphof. Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages. *Biochim. Biophys. Acta* 971:127–136 (1988).
25. B. Mohring and J. Mohring. Plasma ADH in normal and Long Evans rats and in Long Evans rats heterozygous and homozygous for hypothalamic diabetes insipidus. *Life Sci.* 17:1307–1314 (1975).
26. L. E. Cornett, S. M. Breckinridge, and T. I. Loike. Induction of V2 receptors in renal medulla of homozygous Brattleboro rats by arginine vasopressin. *Peptides* 10:985–991 (1989).
27. S. M. Gardiner, A. M. Compton, and T. Bennett. Regional haemodynamic effects of vasopressin infusion in conscious, unrestrained, Brattleboro rats. *Br. J. Pharmacol.* 97:147–152 (1989).
28. C. J. Chu. *Enhanced Cytoplasmic Delivery of Liposome Encapsulated Drugs,* Ph.D. thesis, University of California, San Francisco, 1990.
29. A. Gabizon, R. Shiota, and D. Papahadjopoulos. Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long circulation times. *J. Natl. Cancer Inst.* 81:1484–1488 (1990).
30. K. Maruyama, S. J. Kennel, and L. Huang. Liposome composition is important for highly efficient target binding and retention of immunoliposomes. *Proc. Natl. Acad. Sci. USA* 87:5744–5748 (1990).