Thrombolysis Using Liposomal-Encapsulated Streptokinase: An *In Vitro* Study (42995)

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Abstract. **The clot-lysing ability of streptokinase (SK) was examined using membrane-bound thrombi. Encapsulation of SK in large unilamellar phospholipid vesicles (liposomes) resulted in entrapping approximately 30% of its original activity. Measurements of streptokinase activity for liposomal-encapsulated streptokinase (LESK) indicated little loss of activity or leakage in Tris-buffered saline over a 24-hr period at temperatures of 4 and 23°C. However, incubation of free SK and LESK in platelet-poor plasma (PPP) at 37°C resulted in a decrease of SK activity. The retention of SK activity in LESK was considerably higher than that of unentrapped SK. Clot-dissolving time (CDT) was measured by monitoring the pressure drop during slow filtration in plasma through membrane-bound thrombi. The results indicated that both LESK and free SK were able to activate the fibrinolytic system. Without prior incubation in PPP at 37°C, the CDT of a SK and PPP mixture (SK/PPP) was 10.7 ± 1.9 min (n = 12), while that of a LESK and PPP mixture (LESK/PPP) was 12.4 ± 1.7 min (n = 12). The CDT-detected clot-lysing abilities of both SK and LESK were diminished by incubation in PPP, but to different extents. After 15- and 30-min incubations, the CDT of SK/PPP increased significantly to 15.5 ± 1.5 and 24.1 ± 2.4 min (n = 5, $P < 0.05$), respectively. In contrast, the CDT of LESK/PPP increased to 13.3 ± 0.8 min (n = 5) after 15 min of incubation and to 16.0 ± 1.1 min (n = 5, $P < 0.05$) after a 30-min incubation. These results suggest that entrapment of SK in liposomes preserves the thrombolytic potential of the plasminogen activator by limiting its exposure to the components of the plasma.** [P.S.E.B.M. 1989, Vol 192]

Streptokinase (SK), a potent plasminogen activator, has been widely used in clinical and experimental studies of humans and animals because of its thrombolytic ability. Some recent articles (1–4) have thoroughly reviewed current knowledge regarding the fibrinolytic system and the manipulation of SK. When compared with urokinase and tissue-type plasminogen activator, SK is known to reduce whole blood viscosity (5) and considered to be the least expensive (2). However, despite the low cost and clot-lysing ability of SK, its use as a thrombolytic agent has been limited because of complications associated with thrombolysis. For example, specific immunologic side effects unique to SK have been described (6, 7). Also, because of the difficulties in monitoring SK treatment, the optimal dose of SK for individuals and its timing in the treatment of thromboembolic crises are still uncertain (8). A recent study by Fitzgerald *et al.* (9) has indicated that direct intravenous administration of SK may cause platelet activation, which would further complicate the treatment. However, it is the risk of hemorrhage, characteristic of all thrombolytic agents, that underlies the concern for their therapeutic use in thrombotic disorders. In patients with myocardial infarction that have been treated with SK or urokinase, the risk of bleeding varies between 23 and 47% (1). Thus, modalities that can avoid compromising the activity of SK systemically and that promote selectivity to reduce bleeding complications should be examined. Ideally, a delivery system for plasminogen activators like SK would increase efficacy, enhance selectivity, eliminate immunologic reactions, and retard systemic deactivation to shorten reperfusion times and counter bleeding problems.

The ability of liposomes, with properties similar to those of biologic membranes (10), to encapsulate drugs, proteins, and nucleic acids has stimulated interest in the use of liposomes to transport bioactive reagents.

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Many investigations have employed liposomes as carriers for anticancer agents, antibiotic and immunologic vaccines, arthritic agents, and radiopharmaceutical markers (11–13). In addition, it has been reported that certain technetium-99m-labeled liposomes were concentrated in areas of experimental myocardial infarction in dogs following intravenous administration (14, 15). This led to the hypothesis that liposome-incorporated reagents may be targeted preferentially to the site of coronary thrombosis via this transport vehicle. If correct, this new delivery system for SK or for other plasminogen activators would be expected to improve the efficiency of dosage, and to decrease bleeding complications. Thus far, there have been few studies of the application of liposomes in thrombolytic or fibrinolytic treatments, but some investigations have shown promise in improving certain aspects of thrombolytic treatment by incorporation of urokinase or prostaglandin E_1 in liposomes (16–18).

Experiments presented here examine the ability of liposomal-encapsulated streptokinase (LESK) to activate the fibrinolytic system. Our approach also allowed us to demonstrate the protective effect of liposomes on SK activity by comparing the activity and clot-dissolving time of unentrapped and entrapped SK after incubation in plasma at 37°C.

Materials and Methods

Materials. Octylglucoside (OG) (*n*-octyl-β-D-glucopyranoside) was purchased from Calbiochem (La Jolla, CA) and dissolved in Tris-buffered saline (TBS; 20 m*M* Tris-HCl [pH 7.5], 100 m*M* NaCl). 1-Palmitoyl-2-oleoyl phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification, while radiolabeled L-α-1-palmitoyl-2-oleoyl-[oleoyl-1-^{14}C]phosphatidylcholine ([^{14}C]PC) came from New England Nuclear (Boston, MA). Triton X-100 was obtained from Research Products International (Mount Prospect, IL) and trisodium citrate was acquired from J. T. Baker Chemical (Phillipsburg, NJ). Streptokinase (SK) (Sigma Chemical, St. Louis, MO) solution was prepared fresh by dissolving 10,000 IU (1 vial) of the lyophilized powder in 1 ml of TBS. Dialysis tubing (M_r cut off 12,000–14,000) was obtained from Spectrum Medical Industries (Los Angeles, CA) and Sepharose CL-6B was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Hydrophilic polycarbonate membranes (Nuclepore Corp., Pleasanton, CA) contained 3-μm diameter cylindrical pores at a density of $2 \times 10^6/cm^2$ according to the manufacturer's literature.

Liposome Formation and Streptokinase Encapsulation. The procedures for forming large unilamellar lipid vesicles by the detergent removal method have been well documented (19, 20). Large unilamellar vesicles (LUV) were produced for the purpose of this study as follows. PC (10 mg) in 2 ml of chloroform:methanol (1:1 v/v) in a 10-ml Pyrex test tube was evaporated with a stream of nitrogen and lyophilized overnight. The thin film of dried PC coating the bottom of the tube was resuspended in 250 μl of 0.4 *M* OG in TBS by warming at 37°C for 2 hr with frequent stirring. After the PC had completely dissolved, 1.5 μg of [^{14}C] PC (0.1 μCi) were added, and this solution of lipid and detergent was then designated the PC-OG solution. The remainder of the preparation of LESK was carried out in a cold room at 4°C. For dialysis, samples contained, per ml: 50 μl of PC-OG solution, 450 μl of 0.4 *M* OG in TBS, and 5000 units of SK in 500 μl of TBS. To prepare protein-free LUV blank samples (Blank-LUV), TBS was added instead of SK solution. This mixture was then dialyzed against 1 liter of TBS for a total of 48 hr, with dialysate changes after 8 and 24 hr. The dialyzed sample (1 ml or less) was gel filtered at 1 ml/min over Sepharose CL-6B (15 cm × 1 cm i.d., preequilibrated in TBS) in order to separate LESK from nonencapsulated SK. The elution of LESK and SK from the column was detected by monitoring absorbance (and light scattering) at 280 nm (A_{280}). Vesicles eluted in the void volume, typically within two 0.5-ml fractions that were pooled and designated the LESK sample. These LUV were stored at 4°C and utilized within 48 hr. The recovery of phospholipid in the vesicles was assumed to be directly proportional to the recovery of [^{14}C]PC in the pooled vesicle fractions (typically 30%).

Vesicle Size. The Coulter Sub-Micron Particle Analyzer (model N4MD) was used to determine particle size and size distribution of the LUV by laser light scattering. The measuring principles are based on the theory of Brownian motion and photon correlation spectroscopy (21).

SK Activity and Concentration Assays. The activities and concentrations of SK before and after liposomal encapsulation were determined using available commercial kits and their procedures (activity: Helena Laboratories, Beaumont, TX; concentration: Bio-Rad, Richmond, CA). Standard SK in lyophilized powder was reconstituted with normal saline to give a total activity of 40,000 units/ml and serially diluted with TBS to create a standard activity curve ranging from 200 to 10,000 units/ml. Before an assay, the entrapped SK was released from liposomes by adding OG to a final concentration of 40 m*M*; this concentration of OG did not affect the measurement of either SK activity or concentration.

SK Adsorption to or Penetration of Liposome Membranes. To examine the association of SK with the vesicular membrane, 0.5 ml of 10,000 units/ml SK was added to 0.5 ml of Blank-LUV, incubated at 4°C for 4 hr, and then subjected to gel filtration as described above. Following the chromatography, the liposome fraction was assayed for SK activity and concentration.

Clot Formation. All blood donors (n = 16) were

healthy, nonsmoking male volunteers, who were not on medication and had fasted during the previous 12 hr. Their average age was 25 years. Venous blood was drawn from the arm of the donor into sterile disposable plastic syringes and added to trisodium citrate solution (1.1 ml 3.8% (w/v) citrate/10 ml whole blood) in siliconized test tubes. Platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood at 1000 rpm (145*g*) for 10 min in a Beckman AccuSpin centrifuge (model ACSR-IM-3; Palo Alto, CA). The upper portion (about 2.8 ml) of the supernatant was removed and designated PRP. The remainder was centrifuged at 5000 rpm (3400*g*) for another 20 min to obtain platelet-poor plasma (PPP). Platelet content in PRP and PPP was determined in duplicate with a hemacytometer using the phase contrast microscopy method as described by Brecher and Cronkite (22). The number of platelets counted in PPP (16,200 ± 3,230 platelets/mm^3) was substantially less than the number obtained for PRP (321,000 ± 24,700 platelets/mm^3) ($n = 4$, $P < 0.05$).

Hydrophilic Nuclepore membranes of the same lot number, with 3-μm diameter pore size, were employed in the pore occlusion. Their special features and preparation procedure prior to filtration have been described elsewhere (23); filtrations were performed at room temperature. To occlude pores in a membrane, 4 ml of PRP at room temperature were placed in a 30-ml plastic syringe. A total of 82.5 μl of 0.125 *M* $CaCl_2$ (Sigma Chemical Co.) were added for each ml of plasma in the syringe and the zero time was immediately recorded. The syringe was slightly stirred by hand to ensure good mixing of Ca^{2+} within the plasma. A constant volume flow rate of 0.15 ml/min through the membrane was maintained by a Sage-351 infusion pump. The pressure drop (ΔP) was monitored as a dependent variable with a Gould Statham pressure transducer (model P23ID) connected to a Gould DC amplifier/filter (model 11-4113-01) and was recorded on a chart recorder. The filtration process was stopped when the ΔP reached 120 mm Hg, and the occluded filter was then removed from the filter holder. Light suction, using a Pasteur pipette, was applied to remove the extra gel-like clot filling the filter holder before it was disassembled. The occluded membrane was then washed twice with normal saline solution (0.9% [w/v] NaCl) before being installed in a dry, clean filter holder for the next step of the experiment.

Filtrations through Occluded Membranes. SK or LESK was added to 5 ml of PPP to a final activity of 250 units/ml. Filtrations of SK/PPP or LESK/PPP through occluded membrane-bound thrombi were performed as described above. For control filtrations, occluded membranes were filtered with either PPP or Blank-LUV/PPP. After the occluded membranes were filtered with PPP, Blank-LUV/PPP, SK/PPP, or LESK/PPP, the morphology of the remnant clots and the membranes was examined under a microscope (Olympus Research model BHTU).

Incubation of SK and LESK. To study the effect of incubation with plasma proteins on SK activity, equivalent activities of unencapsulated SK and of LESK were separately injected into siliconized glass tubes containing 5 ml of the same PPP preparation. The contents of each tube were gently mixed for 1 min with a Hematology Mixer (Fisher Scientific, Pittsburgh, PA) before incubation at 37°C, and the tubes were slowly inverted three times at 5-min intervals thereafter. After either 15 or 30 min, the mixture was subjected to filtration with occluded membranes as described earlier.

Stability of LESK. The stability of LESK was examined with three simple tests. The first test involved the comparison of the activity of LESK measured immediately after being collected from gel chromatography (i.e., time zero) with that of LESK measured after 2 and 24 hr of storage at 4°C and at room temperature (23°C). Activity of each sample was measured before and after lysis with OG to determine leakage. In the second test, LESK samples (with equal activity) were incubated in PPP at 37°C for 0-, 15-, and 30-min periods as performed in the previous section. After incubation, 0.2 ml of 0.4 *M* OG was added to the LESK/PPP mixture to lyse the vesicles prior to activity measurement. For comparison, the same procedures were performed for samples of free SK. The third test required the filtration of LESK in TBS through the unoccluded and occluded membranes at flow rates of 0.15 and 1.5 ml/min. One-half-milliliter filtrate was collected after discarding the first few drops. The amount of SK released from the liposomes due to contact or viscous forces was observed with measurement of SK activity in the filtrate. Activities of LESK samples before and after filtration were measured and compared with total SK obtained by lysis with OG.

Data Analysis. All statistical comparisons were analyzed with the Student's *t* test. The statistical significance was accepted only when the probability was less than 0.05. Unless indicated otherwise, results are expressed as mean ± SD.

Results

Encapsulation Efficiency of Liposomes. The procedure developed here enabled us to encapsulate 30% of the total SK added to the sample at the beginning of the process (Table I). The radioactivity measurements show that about one third of the original amount of PC was converted to liposomes (Table I). The measurements of SK activity and concentration also showed that only a small amount of SK (~2%) was adsorbed to, or penetrated the surface of, Blank-LUV (Table I). Thus, more than 90% of the SK in the LESK preparation is encapsulated within the vesicles.

Vesicle Size and Distribution. Particle size analy-

sis shows 82 (±3)% ($n = 5$) of the vesicles in the solution have a particle diameter size of 178 (±40) nm ($n = 5$).

Pressure Drops in Filtrations. Time-dependent changes in pressure during filtration indicate the extent to which pores are being occluded during filtration with recalcified citrated-PRP or are being reopened during a filtration with SK/PPP or LESK/PPP. The ΔP's obtained after 15 min of filtration with PPP or Blank-LUV/PPP through occluded membranes were considered as the steady-state ΔP's (Fig. 1a). The steady-state ΔP's in filtrations of Blank-LUV/PPP were comparable to those obtained for PPP (i.e., 37.0 ± 7.7 mm Hg ($n = 12$) vs 35.6 ± 8.6 mm Hg ($n = 12$), respectively). In contrast, a zero or baseline pressure was observed when these solutions were filtered through the nonoccluded membranes using the same flow rate as for the case of occluded membranes. This result clearly indicated that flow resistance was due to the presence of thrombi and was not significantly affected by the LUV.

Solutions of SK/PPP and of LESK/PPP with the same streptokinase activities were filtered through the occluded membranes in order to compare their clot-dissolving capabilities. The fibrinolytic effect of unencapsulated SK and LESK was monitored by recording the ΔP as a function of time when a solution of SK/PPP or of LESK/PPP was filtered through the occluded membrane (Fig. 1b). For filtrations with these solutions, ΔP increased to a maximum, and then decreased gradually to the baseline pressure. The clot-dissolving time (CDT) was taken to be the time between the start of filtration and the time when the ΔP returned to the baseline. In the absence of preincubation with plasma, the CDT of LESK/PPP [12.4 ± 1.7 min ($n = 12$)] was slightly greater ($P < 0.05$, see Fig. 2) than that of SK/PPP [10.7 ± 1.9 min ($n = 12$)].

A reduction in the fibrinolytic activity of either unencapsulated SK or LESK would result in an increase of CDT and such a decrease in SK activity was observed when either SK or LESK was incubated with PPP (note increased CDT in Fig. 2). After 15 min of incubation, the CDT of SK/PPP increased to 15.5 ± 1.5 min ($n = 5$, $P < 0.05$), while the CDT of LESK/PPP increased less, to 13.3 ± 0.8 min ($n = 5$). After a 30-min incubation, the CDT of SK/PPP was considerably greater than that of LESK/PPP (24.1 ± 2.5 min, $n = 5$ vs 16.0 ± 1.1 min, $n = 5$; $P < 0.05$). This result indicated that the unentrapped SK was inactivated during its exposure

Table I. Recovery Efficiency of Liposomal Encapsulation

	Values before encapsulation	Values after gel filtration (mean ± SD)	Percentage (mean ± SD)	n
Phosphatidylcholine (mM)[a]	2.54	0.87 ± 0.18*	34.3 ± 7.2	9
SK Activity (units/ml)	4942 ± 42	1344 ± 240*	27.2 ± 4.8	9
SK Concentration (μM)[b]	13.1 ± 0.1	4.0 ± 0.6*	30.8 ± 4.3	9
Amount of SK adsorbed to Blank-LUV				
SK Activity (units/ml)	4945 ± 51	102 ± 6*	2.1 ± 0.1	5
SK Concentration (μM)[b]	13.1 ± 0.1	0.3 ± 0.01*	2.3 ± 0.1	5

[a] Phosphatidylcholine ($M_r = 787$).
[b] Streptokinase ($M_r = 50{,}000$).
* $P < 0.05$ compared with values before encapsulation.

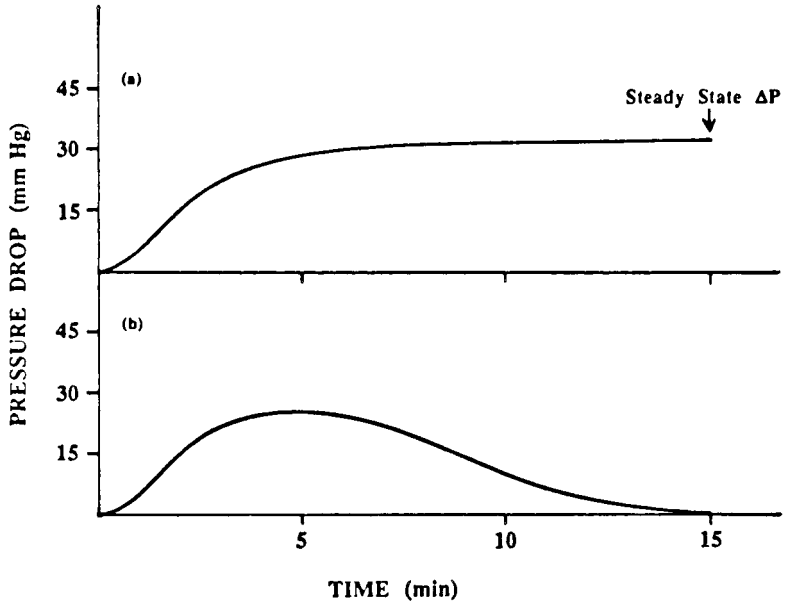

Figure 1. Time dependence of pressure drops monitored during filtrations of (a) PPP and (b) SK/PPP through the occluded membranes.Similar pressure-time histories were obtained for filtrations of Blank-LUV/PPP and LESK/PPP, respectively.

to plasma proteins, while the entrapped SK was protected by the phospholipid bilayer (see Discussion).

Stability of LESK. Both time and temperature of storage affected the stability of LESK only slightly (Fig. 3). Even for 24 hr at room temperature, the leakage of LESK is negligible (Fig. 3). However, incubation in PPP at 37°C caused a decrease of SK activity in both free SK and LESK samples (Fig. 4). For both 15- and 30-min incubation periods, the total activity of LESK samples still remained significantly higher than that of free SK samples (Fig. 4). This result corresponded reasonably with the inactivation of SK as observed in the increase of clot-dissolving time (Fig. 2). The effect of filtration forces on releasing LESK was observed with the increase of SK activity (Fig. 5) as the samples of encapsulating vesicles were filtered through the unoccluded and occluded membranes at two different volumetric flow rates.

Microscopic Study. A microscopic investigation of occluded membranes after being filtered with SK/PPP or LESK/PPP indicated that pores in the membranes were completely cleared from traces of thrombus

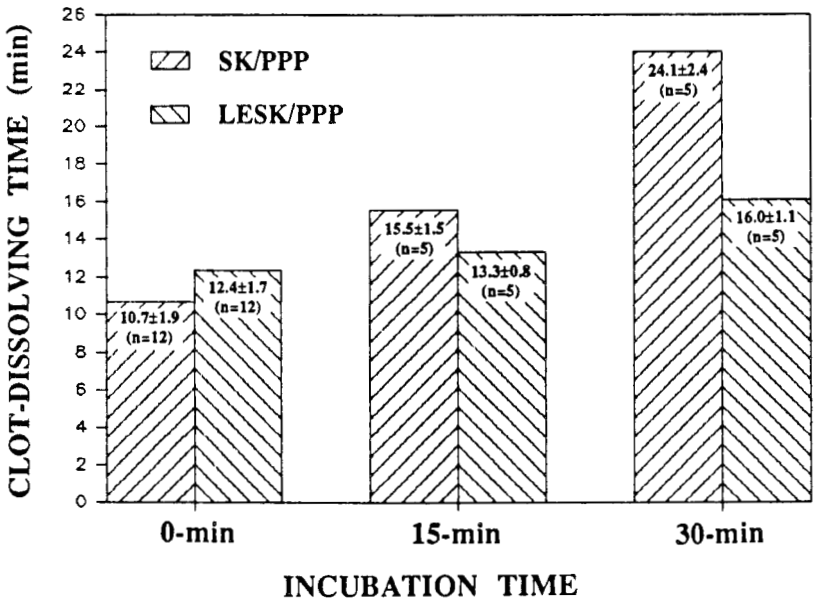

Figure 2. Incubation of SK and LESK in PPP resulted in an increase in the clot-dissolving time of membrane-bound thrombi, especially for the cases of SK/PPP.

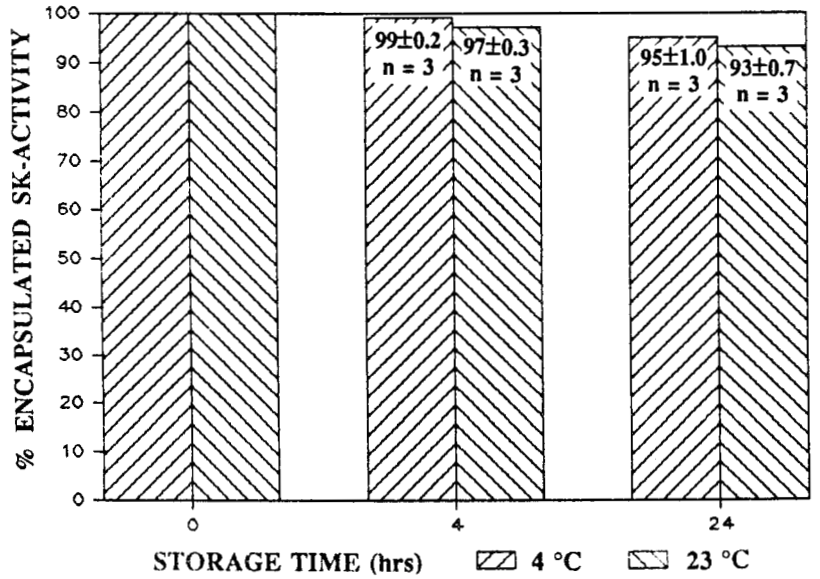

Figure 3. Encapsulated activity retained as a percentage of initial encapsulated activity indicates very little leakage or deactivation for LESK in buffered saline over 24 hr at room temperature or 4°C.

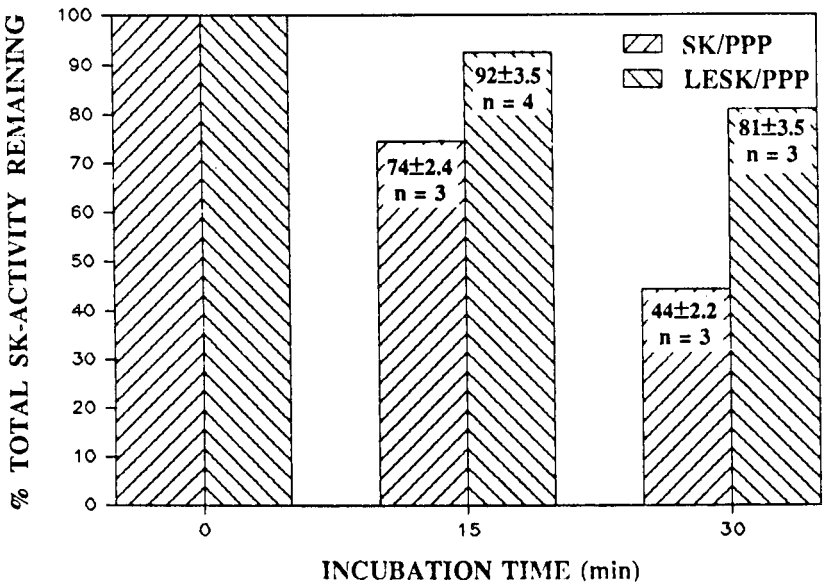

Figure 4. Incubation of free SK and LESK in PPP at 37°C resulted in a significant decrease of total SK activity, especially for the cases of SK/PPP.

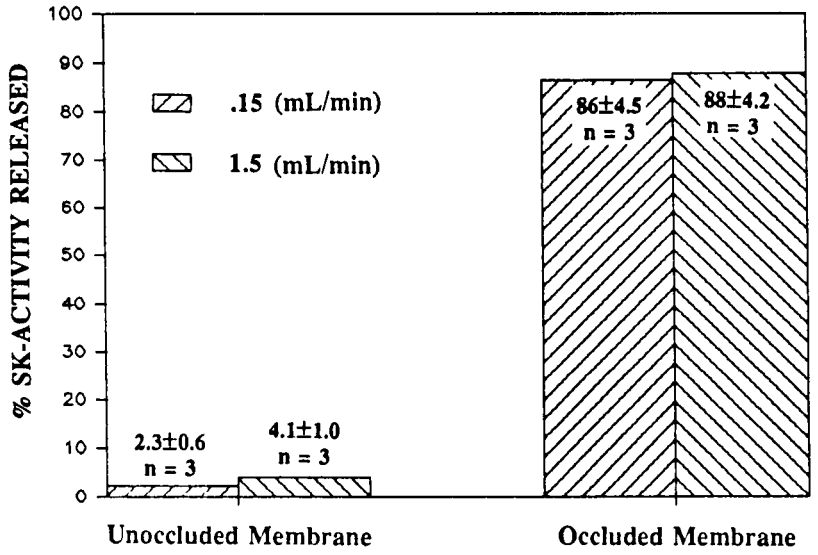

Figure 5. Most of the vesicles lysed as they were subjected to shear or contact forces while traversing the occluded membranes. In contrast, LESK remained intact for vesicles undergoing filtrations with unoccluded membranes.

formation, whereas those filtered with PPP or Blank-LUV/PPP still showed pores being occluded with large meshes of fibrin and platelet aggregates.

Discussion

Methods to enhance the application of streptokinase in thrombolytic treatments have been explored for the last two decades. The major aim of thrombolytic treatment is the dissolution of the target fibrin in an occlusive thrombus, while leaving the fluid components of the blood unaffected. SK converts the circulating proenzyme, plasminogen, to the active serine protease, plasmin, by exposing its active site (6, 24). Free circulating plasmin degrades both fibrinogen and fibrin, which is the product of the enzymatic activities that constitute the blood coagulation cascade. As a result, plasmin suppresses the coagulant phase of thrombus formation (25).

Active plasmin is rapidly neutralized in the circulation by a major physiologic plasmin inhibitor, α_2-antiplasmin (26). In the blood any plasmin generated in excess of the neutralizing effect of α_2-antiplasmin attacks not only the occlusive thrombus, but also circulating fibrinogen and other components of the hemostatic process. Damaged circulating fibrinogen and its effect on platelet aggregation have been suggested as major causes of the bleeding that occurs during infusion of SK into patients for thrombolytic treatment (27).

An alternative to direct injection of SK into blood is to encapsulate the SK in LUV prior to use. In principle, this new approach to deliver SK offers one major advantage over direct injection. The protective effects of liposomes for the SK activity suggests that liposomes would protect the entrapped SK from interaction with other blood components such as serum proteins, blood cells, and endothelial cells lining the capillary wall. This prevents premature inactivation of the SK and its descendant fibrinolytic species and may also mitigate certain bleeding complications.

In this study, membrane-bound thrombi were prepared and then used to analyze SK and LESK action in a flow-filtration study. The first step in this investigation was to ascertain that the vesicles do not interfere with the filtration test. To do this, steady-state pressure drops were measured for Blank-LUV/PPP and PPP in their filtrations through the occluded membranes. The pressure drops of the two systems were comparable, indicating that the small liposomes did not alter the resistance to flow through the thrombi, even though some of the vesicles or their lysed membranes might have attached to thrombi occluding the pores. In addition, the results also showed that residual platelets in the PPP did not contribute significantly to a further blockage of the occluded pores.

When SK was not preincubated with plasma proteins, the SK activity and the clot-dissolving time of LESK/PPP was comparable to those of SK/PPP (Figs. 4 and 2, respectively). These results indicate that the entrapped SK has a fibrinolytic ability similar to that of unentrapped SK at early times. Most important, the results showed that the liposomes were able to release SK at the thrombi while traversing the occluded pores. The mechanism by which the entrapped SK was released is not known, but several possibilities exist which are related to the chemical and physical interactions between the vesicles and their environments. In any case, data obtained for LESK in TBS indicated that physical forces alone from slow flow at two different flow rates through white thrombi are more than adequate to release streptokinase (Fig. 5).

Can the liposomes also protect the SK, as suggested above? The study of Theiss *et al.* (28) suggested that SK activity declines progressively during continuous prolonged infusion, and the reported plasma half-life of free SK in the circulation has ranged from 25 to 82 min (29, 30). In our experiments, the incubation of SK in PPP significantly decreased the thrombolytic activity of the unentrapped SK, as determined by direct measurement (Fig. 4) and, indirectly, as shown by its increasing CDT with increasing incubation time (Fig. 2). Exposed SK molecules and/or derived species therefore appear to be inactivated by proteins in the plasma. In contrast, the decrease of SK activity or the increase in the CDT was much less pronounced for LESK after an equivalent incubation period (Figs. 4 and 2, respectively). This difference indicates that liposomes were, to a degree, able to protect encapsulated SK from a deleterious interaction(s) with plasma proteins and the consequent reduction in SK activity. The fact that the CDT of LESK/PPP did increase slightly with incubation (Fig. 2) showed that the LUV used in this study did not offer complete protection for the SK, an observation confirmed by measured activity (Fig. 4). This is consistent with an earlier study in which liposomes that were stable in buffered physiologic saline were found to become leaky when incubated with serum at 37°C (31). Still, our results demonstrate that liposomes are able to retain and protect the fibrinolytic activity of SK.

Although the results reported here are promising, several aspects of this approach remain to be characterized and optimized. The leakage of SK from LESK seemed to increase with time and temperature of storage (Fig. 3). However, this leakage is considered to be negligible over a 24-hr period at a low temperature of storage, and especially when it is compared with the cases of LESK samples incubated in plasma. Earlier studies have shown that solute retention by liposomes and their half-life in the circulation can be controlled by appropriate manipulation of liposomal membrane fluidity and composition (31). In the absence of cholesterol, liposomes usually leak substantially when introduced intravenously (32). This has been attributed to interactions with plasma proteins and to lipid exchange with lipoproteins (33, 34), and can be largely inhibited by the presence of cholesterol (32, 35) or phospholipase-resistant phospholipids. Cholesterol decreases the fluidity of the nonpolar core of the phospholipid bilayer of the vesicle (31), which results in tighter packing and hence in a decrease of permeability (31, 36).

The adsorption of SK on the surface of the LUV was limited, but still measurable (Table I). Also, the leakage of entrapped SK and the number of SK molecules penetrating the bilayer lipid membrane may increase at a higher temperature. In our preparation, only about 34% of the original amount of phosphatidylcholine and 30% of the original SK were recovered in the LESK sample (Table I). Thus, further investigation of the variables in LESK preparation is necessary to obtain optimal activity of the plasminogen activator at target thrombi, an important factor in clinical applications. The variable fragility/permeability of liposomes with composition and the magnitude of physical forces at thrombus sites can be a basis for control of selectivity. Only locations with stresses above a fragility threshold will cause release of streptokinase. A target fragility threshhold is broadly defined by the level of the physical forces due to residual or emerging flow through a luminal thrombus and the magnitude of forces at a putative mural thrombi.

It is important to recognize the difference between a thrombolytic study with the membrane-bound thrombi as reported here and the situation *in vivo*. In

addition to the white thrombi formed from platelet aggregates and fibrin that are found in membrane-bound thrombi *in vitro*, *in vivo* thrombotic formation also includes red blood cells. Comparison of the geometric parameters would indicate that pathologically important occluded vessels can be orders of magnitude larger than the occluded pores. Furthermore, streptokinase in the present filtration system was not examined with whole blood, but only with platelet-poor plasma. Nevertheless, the results of our study demonstrate the potential application of liposomes, with encapsulated plasminogen activators, in thrombolytic therapy. In this study we have shown that the intermediate size of unilamellar vesicles prepared for the experiments are able to encapsulate SK and deliver it with retention of activity. More attention should be specifically given to the optimization of protocols for its preparation and to the optimal properties of the LUV, such as liposome stability or fragility, trapped volume, trapping efficiencies, liposome targeting with different surface charges, etc. The *in vitro* results of the present research indicate that further studies are warranted to examine the potential of this approach, especially for its *in vivo* application.

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