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Use of Forssman antigen in the study of phosphatidylcholine exchange between liposomes

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SUMMARY

An immunological technique has been developed to study phospholipid exchange between sensitized and nonsensitized liposomes. Sensitized liposomes were prepared from phosphatidylcholine, cholesterol, dicetylphosphate and Forssman antigen, whereas nonsensitized liposomes contained only phosphatidylcholine and a trace amount of inert marker. Liposomes were separated quantitatively by precipitating the sensitized liposomes with rabbit γ-globulin obtained from antiserum against red cell Forssman antigen. The technique has been used to show that little or no phospholipid exchange takes place between sensitized and nonsensitized liposomes. The addition of a partially purified phospholipid exchange protein fraction promotes exchange between the two liposome preparations.

Exchange of phospholipids has been shown to occur between cellular membranes[1–4] and between natural membranes and artificially prepared phospholipid aggregates[5]. This exchange is stimulated by a soluble protein factor, phospholipid exchange protein, from rat liver or beef heart[2,6]. For phospholipids in a bilayer structure, such as sonicated phospholipids, two kinds of movement are possible: phospholipid molecules can pass from one side of the bilayer to the other (flip-flop) or they can move about within each monolayer (lateral diffusion). Kornberg and McConnell[7,8] have determined the rates of these movements using spin-labeled phospholipids and have shown that the lateral movement is fast, more than 0.05 μm/s, while the flip-flop movement is slow; the asymmetry in the distribution of paramagnetic molecules between the two monolayers decays with a half-time of 6.5 h at 30 °C. They also showed that intervesicle exchange and vesicular fusion are very slow events.

In order to study the effect of the phospholipid exchange protein on the phospholipid exchange between liposomes, two separate populations of liposomes are necessary. This has been achieved in the present study by incorporating Forssman antigen into one of the liposome populations. The antigen-sensitized liposomes can then be separated from nonsensitized liposomes by precipitation with antisera. Alving and Kinsky[9] have shown that sensitized liposomes can be agglutinated with antiserum.

Preparation of sensitized and nonsensitized liposomes

The Forssman antigen was isolated from sheep erythrocytes as described by Yamakawa *et al.*[10] and further purified by thin-layer chromatography on Silica gel H (chloroform–methanol–water; 65:25:4, v/v). The sensitized liposomes were made from phosphatidylcholine, cholesterol, dicetylphosphate and Forssman antigen in molar ratios[11] of 1.0:0.75:0.11:0.01. Butylated hydroxytoluene (0.35 mole per mole of phosphatidylcholine) was added as antioxidant. Forssman antigen and the other lipids were combined in chloroform–methanol (2:1, v/v) and dried down under nitrogen. After addition of phosphate buffer (0.15 M, pH 7.4), the mixture was agitated briefly on a vortex mixer. After 1 h incubation at 50 °C the suspension was sonicated at 50 °C in a Branson HD-50 waterbath sonicator for 15 min and then centrifuged at 800 × *g* for 10 min, to remove easily precipitable phospholipid material.

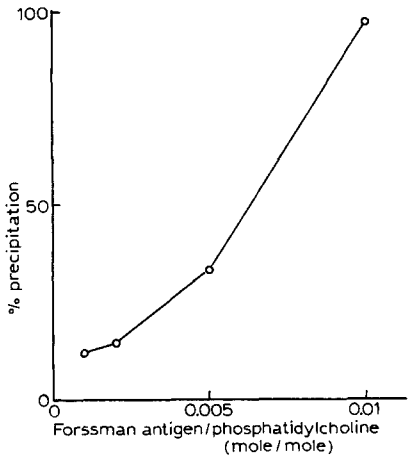
The nonsensitized liposomes were made of phosphatidylcholine and a trace amount of [^{14}C] triolein with the same technique at room temperature.

Precipitation of sensitized liposomes

The anti-sheep erythrocyte serum was prepared by immunizing rabbits subcutaneously with sheep erythrocyte ghosts[12], 1 mg protein per injection, mixed with complete Freund's adjuvant. Injections were performed once a week for 3 weeks and blood was obtained 2 weeks after the last injection. The γ-globulin fraction was prepared according to the method of Fleischman[13]. We used this fraction for the precipitation of sensitized liposomes rather than whole antiserum, because the latter contained phospholipid which could participate in the phospholipid exchange reaction.

The precipitations were performed in siliconized, disposable glass tubes, 7.5 cm × 1 cm, at room temperature. If not otherwise stated, 10 μl of sensitized liposomes (0.01 μmole phosphatidylcholine) were incubated with 50 μl of antiserum γ-globulin and 100 μl buffer. The buffer, 0.15 M phosphate buffer, pH 7.4, contained 0.1% bovine serum albumin which minimized loss of radioactivity by adsorption on the vessel walls. After incubation the tubes were centrifuged at 800 × *g* for 10 min to precipitate liposomes sensitized with Forssman antigen. Under these conditions nonsensitized liposomes did not sediment.

The effect of the amount of antigen incorporated into liposomes on completeness of precipitation is shown in Fig. 1. As can be seen, 0.01 μmole (16 μg) of antigen per μmole of phosphatidylcholine was required to obtain good precipitation. This antigen to phosphatidylcholine ratio was used for preparing liposomes in further experiments.



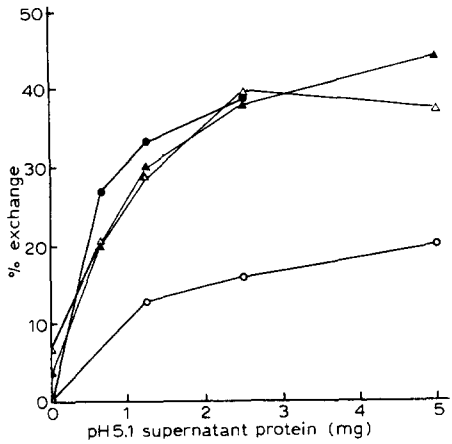


Fig. 1. Effect of Forssman antigen concentration on the precipitation of sensitized liposomes. The liposomes were made with varying ratios of Forssman antigen to phosphatidylcholine as indicated on the abscissa. The % precipitated is the % loss of radioactivity from the supernatant as compared to the total radioactivity present in the incubation. The incubation time was 3 h. The amount of antiserum γ-globulin was 320 μg per incubation. This gave maximal precipitation in each instance. When buffer was used instead of antiserum γ-globulin the precipitation was 7%.

Fig. 2. Effect of different amounts of pH 5.1 supernatant on the exchange of phospholipid between liposomes. In experiments 1 and 2, 50 μl sensitized liposomes (0.05 μmole phosphatidylcholine) were incubated with 10 μl [^{14}C,^{32}P]phosphatidylcholine (0.016 μmole phosphatidylcholine) liposomes for 60 min at 37 °C. Expt 1, exchange determined from the supernatant (○—○). Expt 2, exchange calculated from the supernatant (△—△); exchange calculated from washed pellet (▲—▲). The values shown are corrected for 100% precipitation. Expt 3, 25 μl sensitized radioactive liposomes (^{14}C/^{32}P) (0.025 μmole phosphatidylcholine) were incubated with 25 μl nonradioactive phosphatidylcholine liposomes (0.077 μmole phosphatidylcholine). ●—●, exchange calculated from the washed pellet.

In order to determine the optimal conditions for liposome precipitation similar incubations were performed for 3 h with serial dilutions of antiserum γ-globulin. With 320 μg antiserum γ-globulin 98% of the liposomes were precipitated; 160 μg gave 92%, and 80 μg 45% precipitation. When normal rabbit serum γ-globulin was used instead of antiserum γ-globulin the precipitation was 2%.

We studied the time dependency of the precipitation reaction and found that after 1 h of incubation at room temperature the precipitation was almost complete, 94%. After 2, 4 and 18 h the precipitation was 96%, 98% and 98%, respectively. When larger incubation volumes were used the incubation time had to be prolonged in order to get maximal precipitation.

Stability of antigen and phosphatidylcholine in sensitized liposomes

In order to determine whether the antigen exchanges between liposomes, doubly labeled liposomes containing [^{32}P] phosphatidylcholine and [^{14}C] triolein were used.

Nonradioactive sensitized liposomes (0.05 μmole phosphatidylcholine) in 50 μl were incubated for 3 and 12 h with 25 μl radioactive phosphatidylcholine liposomes (0.04 μmole phosphatidylcholine) containing [^{32}P] phosphatidylcholine and [^{14}C] triolein. After the incubation, the sensitized liposomes were precipitated with 500 μl antiserum γ-globulin and the radioactivity in the supernatant was determined after centrifugation for 10 min at 800 × *g*.

Table I shows that 5–8% of both ^{14}C and ^{32}P is lost from the supernatant. This is probably due to nonspecific precipitation of the radioactive liposomes or adherence to the glass, inasmuch as the same amount of ^{32}P and ^{14}C disappeared after incubation with normal rabbit serum γ-globulin or in the absence of sensitized liposomes (Table I). The experiment shows that there is little or no transfer of the antigen from the nonradioactive to the radioactive liposomes. If such transfer had occurred, the precipitation of ^{32}P and ^{14}C should have been greater in the presence of antiserum γ-globulin than with γ-globulin from normal rabbit serum.

In Table I the precent loss of ^{32}P from the supernatant fraction is the same as that for ^{14}C. This shows that there was no exchange of [^{32}P] phospholipid between sensitized and nonsensitized (^{32}P/^{14}C) liposomes. This is in agreement with the results of Kornberg and McConnell[8] obtained with spin-labeled liposomes.

TABLE I

STABILITY OF FORSSMAN ANTIGEN AND PHOSPHATIDYLCHOLINE IN SENSITIZED LIPOSOMES

The amounts of radioactivity in the ^{32}P/^{14}C-labeled liposomes used in each incubation were 4955 cpm of ^{32}P and 5745 cpm of ^{14}C. The antiserum γ-globulin used in this experiment was tested for potency in a separate experiment and was found to give 98% precipitation of sensitized liposomes.

Incubation time (h)	*Sensitized liposomes*	*γ-Globulin derived from*	*Decrease of radioactivity in supernatant (%)*	
			^{32}P	^{14}C
3	+	Antiserum	5.2	6.6
12	+	Antiserum	8.6	8.3
3	+	Normal rabbit serum	5.1	5.9
12	+	Normal rabbit serum	5.9	8.2
3	–	Antiserum	5.4	5.8
12	–	Antiserum	6.4	7.5

Influence of phospholipid exchange protein on phosphatidylcholine exchange

In order to study whether phospholipid exchange protein is active in this system, 50 μl nonradioactive sensitized liposomes were incubated with 10 μl radioactive liposomes in the presence and absence of 250 μl partially purified exchange protein. The partially purified phospholipid exchange protein was separated from the pH 5.1 supernatant[14] of beef heart by carboxymethyl cellulose chromatography, Sephadex G-75 gel filtration and isoelectric focusing (L. Johnson and D.B. Zilversmit, to be published). To the

[^{32}P] phosphatidylcholine liposomes a trace amount of [^{14}C] triolein was added in order to be able to observe the extent of coprecipitation of the radioactive with the sensitized liposomes. After 1 h of incubation at 37 °C, antiserum γ-globulin was added and after 3 h the tubes were centrifuged. The radioactivity in the supernatant was counted and from the change in ratio of ^{32}P to ^{14}C the phosphatidylcholine exchange was calculated. Table II gives the result of a representative experiment.

TABLE II

INFLUENCE OF PHOSPHOLIPID EXCHANGE PROTEIN ON PHOSPHATIDYLCHOLINE EXCHANGE

A control incubation with radioactive sensitized liposomes showed a 94% precipitation of liposomes. The exchange values are corrected for 100% precipitation. A mitochondrial incubation with the same amount of purified phospholipid exchange protein gave 17.1% exchange.

Phospholipid phosphorus		*[^{32}P] Phospholipid exchanged (% of total ^{32}P)*	
Nonsensitized (μmoles)	*Sensitized (μmoles)*	*Phospholipid exchange protein* +	–
0.25	1.51	13.7	2.1
0.5	1.51	15.2	5.0

The data show that the exchange of phosphatidylcholine between liposomes* is greatly stimulated by the presence of phospholipid exchange protein. This stimulation was also achieved by using crude pH 5.1 supernatant of beef heart homogenate. Fig. 2 shows that increasing amounts of pH 5.1 supernatant increase phospholipid exchange. Several reports have indicated that liposomes in many respects behave as cellular membranes. Thus it is of interest that the phospholipid exchange protein dependent phospholipid exchange, previously shown to occur between cellular membranes[1–5] and between liposomes and cellular membranes, also takes place between liposomes. Because sensitized liposomes can be made without protein, one has a system in which intramembranous lipid protein interaction plays no part. Another advantage, as compared to biological systems, is that the composition of the phospholipid donor and acceptor can be easily varied. A drawback of the immunoprecipitation technique is the relatively long time required for the separation of sensitized from nonsensitized liposomes. Although this point requires further study, the immunoprecipitation method appears to be a useful procedure for the study of liposome–liposome interactions.

*The possibility exists that radioactive phospholipid could have been transferred to phospholipid exchange protein and that this might have been precipitated by the antiserum γ-globulin. This would also account for loss of radioactivity from the supernatant and retention in the pellet. However, when the sensitized liposomes were omitted from the incubation mixture, which contained phospholipid exchange protein and radioactive liposomes, no radioactivity was lost from the supernatant after the addition of antiserum γ-globulin.

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