J. Membrane Biol. 67, 137–141 (1982)

The Journal of
Membrane Biology

Threshold Effects on the Lectin-Mediated Aggregation of Liposomes: Influence of the Diameter of the Liposomes

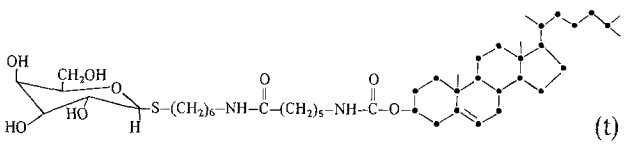
M.R. Alecio and R.R. Rando
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Summary. It had previously been found that small unilamellar liposomes of ca. 0.03 μm diameter which bear synthetic cholesterol-containing glycolipids may be aggregated by an appropriate lectin [8]. Where studied, threshold effects have been observed in that the amount of glycolipid incorporated in the liposomes must exceed a certain minimum concentration in order for aggregation to occur [3, 8, 9, 13, 14]. Threshold effects of this type may be important in mediating cell-cell and virus-cell interactions. However, before studies with small unilamellar liposomes are useful as a model for these recognition and binding phenomena, it must be shown that the observed threshold effects are not associated with the very small radius of curvature of these liposomes. This article reports that larger liposomes of average diameter 0.26 and 0.45 μm which contain the synthetic glycolipid *l* also show threshold effects when aggregated with the galactose binding lectin ricin agglutinin. Under conditions where more than 1% (mole) glycolipid is required to support the aggregation of the smallest liposomes, those of intermediate size require only 0.18% (mole) while the largest liposomes examined require between 0.095 and 0.15% (mole) depending on the method of preparation.

Key Words: threshold effect · aggregation · liposomes · ricin agglutinin · liposome diameter

Introduction

Small unilamellar vesicles of ca. 0.03 μm diameter which contain glycolipids can be aggregated by an appropriate lectin [3, 8, 9, 11, 13, 14]. Interestingly, the rates and extents of aggregation, as judged by light-scattering measurements, are not proportional to the amount of glycolipid incorporated [3, 8, 9, 11, 13, 14]. Clear threshold effects are observed in that below a certain percentage of glycolipid incorporated aggregation is not observed irrespective of the concentrations of added lectin. For example, small



(*l*)

unilamellar vesicles prepared from egg phosphatidyl choline and the synthetic glycolipid (*l*) are aggregated by the β-galactosyl binding lectin ricin agglutinin [9, 13]. Below 2% *l* in the membrane aggregation did not occur [9]. Increasing the concentration of glycolipid from 2.8 to 7% led to nearly a 100-fold increase in the apparent first-order rate constant of aggregation, and at the same time the lag period for the aggregation process decreased by approximately the same factor [9]. It was shown in this case that *l* was incorporated into the vesicles in a unimodal way at all concentrations so that the observed threshold effects could not have been an artifact of the way in which the glycolipid was incorporated into the liposomes [9]. In addition, ricin agglutinin does not itself exhibit cooperative binding of ligands [17]. Similar results were obtained when small unilamellar liposomes bearing α-mannoside containing glycolipids were treated with concanavalin A [8]. It was therefore concluded that the threshold effects are an intrinsic property of the system and not some easily rationalized artifact. If these effects were also relevant to cellular recognition phenomena, such as cell-cell adhesion and virus-cell adhesion, then large qualitative changes in recognition patterns could arise from small quantitative changes in the concentrations of some components at the cell surface. However, before the notion of threshold effects can be applied to cellular behavior it must first be determined whether these effects are associated with the very small radius of curvature found with small unilamellar vesicles (diameter ~0.03 μm). To these ends, large liposomes (mean diameters 0.25 and 0.46 μm) were prepared containing *l*. Even larger liposomes of 1–2 μm diameter did not yield reproducible results in our hands due to stability problems [16]. The ricin-mediated aggregation of these larger liposomes was compared to that of the small unilamellar vesicles. In this article we show that

0022-2631/82/0067-0137 $01.00

threshold effects are observed with the large liposomes containing *l* and that the threshold occurs at lower concentrations of synthetic glycolipid than with the small unilamellar vesicles under identical conditions.

Materials and Methods

Ricin, the β-galactoside-binding agglutinin from *Ricinus communis* was obtained from Boehringer Mannheim, Inc. Egg phosphatidyl choline was prepared and purified by the method of Litman [4]. The phospholipid was stored in chloroform solution at a concentration of 100 mM at −70 °C under an atmosphere of nitrogen. The purity of the preparation was checked routinely by thin layer chromatography (silicic acid; chloroform/methanol/acetic acid/water (70:30:2:3)). The ^{3}H-β-galactosyl glycolipid *l* was prepared by the published procedure [9] and had a specific activity of 10.5 Ci/mol. The phospholipid concentrations of liposome preparations were determined as inorganic phosphate after ashing and acid hydrolysis [1].

Liposomes

All the liposome preparations used a buffer which comprised Tris (10 mM) and 2-mercaptoethanol (3 mM) adjusted to pH 7.5.

A) Small Unilamellar Liposomes. A published method was used for the preparation of the small unilamellar vesicles (diameter ~0.03 μm) containing the ^{3}H-labeled glycolipid *l* [2]. The amount of glycolipid incorporated was determined by radioactivity counting.

B) Extrusion through Polycarbonate Membranes. The published method was used [6]. Chloroform solutions of the synthetic glycolipid *l* and egg phosphatidyl choline (12 μmol) were mixed in a vial, and the solvent was removed on a rotary evaporator to leave a thin film of lipid. Buffer was added (at least 2.5 ml), and the lipid was allowed to hydrate for 20 min before mixing vigorously for 1 min using a vortex mixer. The cloudy suspension was extruded successively through polycarbonate filters of decreasing pore size finishing with 0.4 and 0.2 μm filters for liposomes with mean diameters of approximately 0.45 and 0.26 μm, respectively. The liposomes were fractionated by chromatography on a BioGel-A 150 m column, in order to reduce the variance of the diameter of the resulting preparations. As expected, liposomes which had been extruded through a 0.2 μm filter travelled through this column more slowly than did liposomes which had been extruded only as far as the 0.4 μm filter. Fractions from the center of the peak which contained both ^{3}H and phosphate were pooled, diluted immediately to ca. 3 mM in lipid, and cooled to 0 °C.

C) Reverse Phase Evaporation Method [16]. A solution of the tritiated glycolipid in methanol was mixed with a solution of the phospholipid (ca. 10 μmol) in a small flask. The solvent was removed at 25 °C using a rotary evaporator to leave a thin film of lipid. Ether (1.5 ml) and buffer (0.5 ml) were added, and the mixture was sonicated until homogeneous (ca. 5 min). The ether was then removed cautiously using a rotary evaporator, while keeping the flask in contact with water at 10 °C. The resulting solution of liposomes was diluted with a suitable volume of buffer and traces of ether were removed under reduced pressure at 20 °C. The resulting liposomes were fractionated on a BioGel-A 150-m column and the peak fractions were collected, pooled, and cooled to 0 °C immediately.

D) Reverse Phase Evaporation Followed by Extrusion. The published procedure, which is essentially a combination of methods *B* and *C*, was followed [15]. This method is reported to yield unilamellar liposomes.

Aggregation Assays

Liposomal solutions were diluted to 0.06 mM using the buffer and brought to 10 °C in 1-ml cuvettes in the thermostatted cell compartment of a Gilford model 240 spectrophotometer. The aggregation reaction was initiated by adding ricin to a final concentration of 160 μg/ml. This concentration had previously been determined to be saturating with the small unilamellar liposomes [9]. The aggregation reaction was monitored by recording continuously the absorbance increase at 360 nm. Liposomes did not aggregate when the ricin was added in the presence of 1 mM galactose. Adding galactose (1 mM) to liposomes which had been aggregated by ricin agglutinin reduced the absorbance at 360 nm very rapidly. This indicates that the liposomes were caused to disaggregate and implies that little or no fusion of the liposomes had taken place. Glucose (1 mM) had no effect on the aggregation.

Results and Discussion

Previously light scattering at 360 nm had been shown to be an adequate quantitative assay for the aggregation of small unilamellar liposomes [9]. In Fig. 1 the adequacy of the assay, as applied to the larger liposomes used here, was investigated. In Fig. 1, 0.46 μm liposomes bearing ^{3}H-labeled *l* were treated with ricin agglutinin (160 μg/ml). At various times a portion of the reaction mixture was withdrawn and the absorbance at 360 nm was determined immediately. A second portion was centrifuged for 90 sec with a Beckman microfuge and the radioactivity remaining in the supernatant was determined. After 24 hr more than 95% of the label could be centrifuged under these conditions, showing that the reaction had gone virtually to completion. From Fig. 1 it appears that the scattering assay is sensitive only to the early steps of aggregation since large aggregates continued to be formed and could be removed by centrifugation, even after the absorbance at 360 nm had reached a stable value. Nevertheless, light scattering was conveniently used to determine whether or not aggregation had occurred. Because of the limitations of the assay, however, kinetic measurements were not attempted.

In order to compare the aggregation reaction of the large liposomes to that of the small unilamellar liposomes, aggregation studies were repeated with these latter liposomes under the low salt conditions compatible with the stability of their larger counterparts. In Fig. 2, small unilamellar vesicles containing varying amounts of ^{3}H-labeled *l* were treated with ricin (160μg/ml), and the aggregation reaction was allowed to proceed to completion. As can be seen

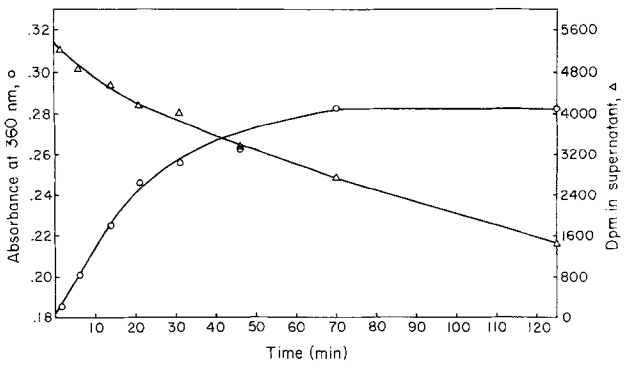


Fig. 1. Liposomes of ca. 0.46 μm diameter which had been prepared by the extrusion method (*B*) and which contained the tritium-labeled galactoside *1* (0.5% (mole)) were aggregated by adding ricin agglutinin. The reaction was monitored by recording the change in absorbance at 360 nm and by withdrawing portions (800 μl) at various times, centrifuging briefly, and then measuring the radioactivity that remained in solution. The reaction mixture contained lipid (0.06 mM), ricin (160 μg/ml), Tris (10 mM), sodium chloride (8 mM), and 2-mercaptoethanol (3 mM) and was adjusted to pH 7.6 (HCl) and maintained at 4 °C

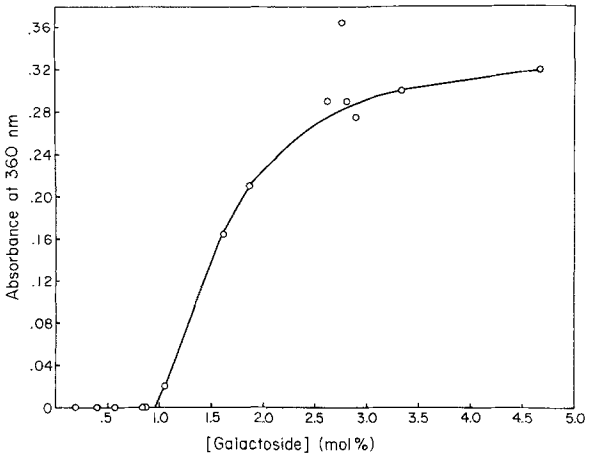


Fig. 2. Liposomes of ca. 0.03 μm diameter which had been prepared by sonication of a suspension of lipid (method *A*) and which contained the galactoside *1*, were aggregated by adding ricin agglutinin. The change in absorbance at 360 nm was measured when reaction was complete. The reaction mixture contained lipid (0.06 mM), ricin (160 μg/ml), Tris (10 mM), sodium chloride (8 mM) and 2-mercaptoethanol (3 mM) and was adjusted to pH 7.6 (HCl) and maintained at 10 °C

here, under the conditions of these experiments a threshold exists at approximately 1% (mole) galactoside such that below this concentration aggregation does not occur. Concentrations of ricin even higher than 160 μg/ml still did not support aggregation. In Fig. 3 a similar experiment was performed with the extruded liposomes of average diameter equal to 0.25 μm. Interestingly, a threshold is still observed although this time at a glycolipid concentration of 0.18% (mole). In Fig. 4*A* and *B* the same type of

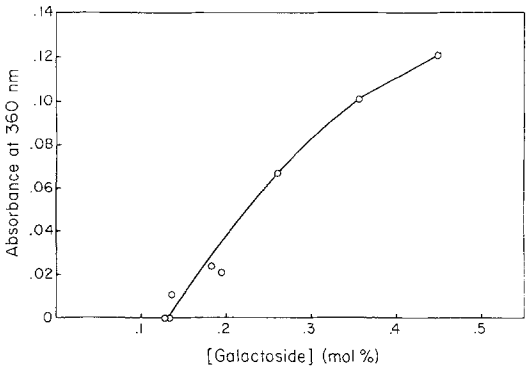


Fig. 3. Liposomes with a mean diameter of 0.25 μm which had been prepared by the extrusion method (*B*) and which contained the galactoside *1*, were aggregated by adding ricin agglutinin. The change in absorbance at 360 nm was measured when reaction was complete. The reaction conditions were as described under Fig. 2

experiment is performed with the approximately 0.45 μm diameter vesicles. In Fig. 4*A*, the threshold is measured for multilamellar vesicles prepared by the filter extrusion technique, and in Fig. 4*B* it is measured for the presumed unilamellar vesicles prepared by reverse phase evaporation followed by filter extrusion. In both cases clear thresholds are observed at 0.15% (mole) in Fig. 4*A* and at 0.095% (mole) in 4*B*. Thus in all instances threshold effects are found although they are shifted to lower glycolipid concentrations in the case of the larger liposomes.

The experiments described above show that threshold effects are not an artifact of the extreme radius of curvature of the small unilamellar vesicles but are observed in liposomes approaching the size of cells. Larger liposomes (1 μm diameter) could not be studied because of their instability. Although the threshold level of glycolipid required to support aggregation in the larger liposomes ranged from 0.18 to 0.095% (mole) this figure must be taken as an upper limit, since the actual amount of exposed glycolipid could not be determined. The determination of the exposed glycolipid in the small unilamellar liposomes was accomplished by galactose oxidase oxidation of the galactose moiety followed by titration with radio labeled acetylhydrazide [9]. This procedure was not suitable for the large liposomes because the hydrazide appeared to be bound nonspecifically to the liposomes at the low salt concentrations required to maintain their viability. Other procedures, such as periodate oxidation followed by titration of the liberated formaldehyde also were not useful because of sensitivity problems at low concentrations of glycolipid [5]. It is interesting to note, however, that the technique reported to generate unilamellar liposomes gives a lower apparent threshold level than a technique which

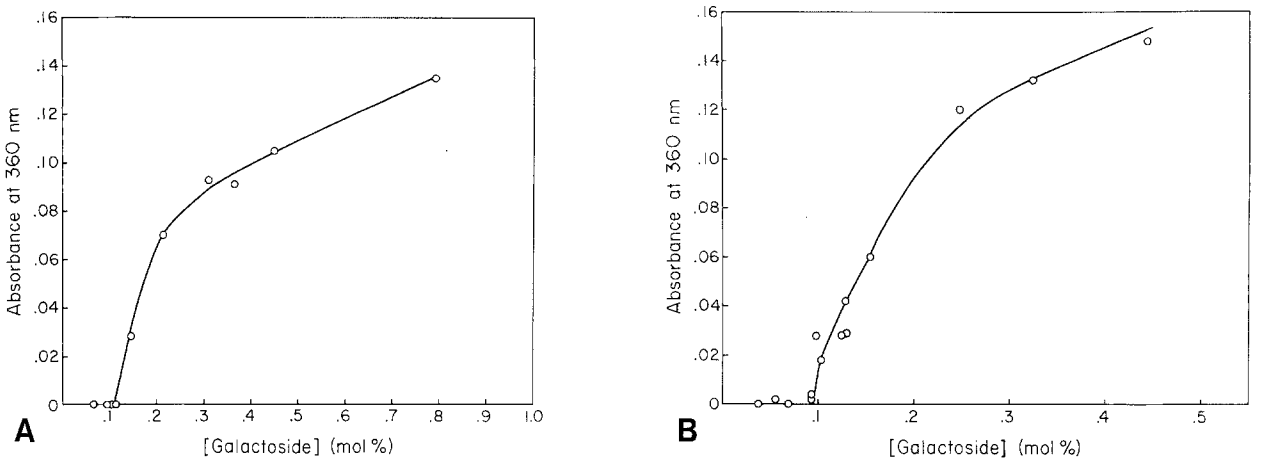


Fig. 4. Liposomes which had a mean diameter of 0.46 μm and which contained the galactoside *I* were aggregated by adding ricin agglutinin. The experiment of *A* used liposomes which were prepared by the extrusion method (Method B) while that of *B* used liposomes prepared by reverse phase evaporation followed by extrusion (method *D*). The change in absorbance at 360 nm was measured when reaction was complete. The reaction conditions were as described under Fig. 2

probably yields a mixture of multilamellar and unilamellar liposomes. Even though our threshold data on the larger liposomes must be taken as an upper limit, there can be no doubt that thresholds are observed here. The fact the threshold levels of glycolipid required to support aggregation is approximately an order of magnitude greater for the small vesicles than for the larger ones is probably due to the radius of curvature of the small liposomes. On colliding, the area of contact between small liposomes is probably much smaller than between large liposomes, since the latter are both less sharply curved and more easily deformed.

The finding of threshold effects in the aggregation of large liposomes is consistent with other results in the literature. For example, we have shown that bovine erythrocytes, which are not agglutinable by concanavalin A, can be rendered so by incorporating an α-mannoside at the cell surface [7, 10]. Sharp thresholds were observed here in that a 15% increase in the concentration of membrane-bound α-mannoside yielded cells that fully aggregated at 64 μg/ml concanavalin A from cells that did not aggregate at concentrations of concanavalin A exceeding 500 μg/ml [10]. Neuraminidase treatment of SV40-transformed hamster cells reduced the number of soy bean agglutinin binding sites by 28% and completely eliminated the lectin-mediated agglutination of these cells [18]. Finally, it has been found that liver cells bind to sugar derivatized polyacrylamide beads and that this binding is highly sensitive to the amount of sugar incorporated [12]. Relatively small changes in the sugar incorporation levels led to dramatic changes in the ability of the beads to bind the liver cells [12]. These various systems, however, do not offer the advantages of the liposomal systems for studying the mechanisms underlying the threshold effects. These advantages include the ability to incorporate a chemically defined and unimodal population of receptors and to alter other lipid components by straightforward manipulations. Studies in the mechanisms underlying the threshold effects are now in progress using the small unilamellar vesicles as a probe.

This work was supported by grant PCM 79-27214 from the National Science Foundation.

References

1. Ames, B.N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8**:115–118
2. Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E., Carlson, F.D. 1977. A simple method for the preparation of homogenous phospholipid vesicles. *Biochemistry* **16**:2806–2810
3. Curatolo, W., Yau, A.O., Small, D.M., Sears, B. 1978. Lectin-induced agglutination of phospholipid/glycolipid vesicles. *Biochemistry* **17**:5740–5744
4. Litman, B.J. 1973. Lipid model membranes. Characterization of mixed phospholipid vesicles. *Biochemistry* **12**:2545–2554
5. MacFadyen, D.A. 1945. Estimation of formaldehyde in biological mixtures. *J. Biol. Chem.* **158**:107–113
6. Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., Papahadjopoulos, D. 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* **557**:9–23
7. Orr, G.A., Rando, R.R. 1978. Synthetic concanavalin A receptors and erythrocyte agglutination. *Nature (London)* **272**:722–725
8. Orr, G.A., Rando, R.R., Bangerter, F.W. 1979. Synthetic glycolipids and the lectin-mediated aggregation of liposomes. *J. Biol. Chem.* **254**:4721–4725
9. Rando, R.R., Bangerter, F.W. 1979. Threshold effects on the lectin-mediated aggegation of synthetic glycolipid containing liposomes. *J. Supramol. Struct.* **11**:295–309

10. Rando, R.R., Orr, G.A., Bangerter, F.W. 1979. Threshold effects on the concanavalin A-mediated agglutination of modified erythrocytes. *J. Biol. Chem.* **254:**8318–8323
11. Redwood, W.R., Polefka, T.G. 1976. Interaction of wheat germ agglutinin with phosphatidyl choline liposomes containing incorporated monosialoganglioside. *Biochim. Biophys. Acta* **455:**631–643
12. Schnaar, R.L., Weigel, P.H., Kuhlenschmidt, M.S., Lee, Y.C., Roseman, S. 1978. Adhesion of chicken hepatocytes to polyacrylamide gels derivatized with N-acetylglucosamine. *J. Biol. Chem.* **253:**7940–7951
13. Slama, J.S., Rando, R.R. 1980. Lectin-mediated aggregation of liposomes containing glycolipids with variable hydrophilic spacer arms. *Biochemistry* **19:**4595–4600
14. Surolia, A., Bachhawat, B.K., Podder, S.K. 1975. Interaction between lectin from *Ricinus communis* and liposomes containing gangliosides. *Nature (London)* **257:**802–804
15. Szoka, F.C., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D. 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* **601:**559–571
16. Szoka, F.C., Papahadjopoulos, D. 1978. 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
17. Van Wauwe, J.P., Loontiens, F.G., DeBruyne, C.K. 1973. The interaction of *Ricinus communis* hemagglutinin with polysaccharides and low molecular weight carbohydrates. *Biochim. Biophys. Acta* **313:**99–105
18. Vlodavsky, I., Sachs, L. 1975. Lectin receptors on the cell surface membrane and the kinetics of lectin-induced cell agglutination. *Expl. Cell Res.* **93:**111–119

Received 16 September 1981; revised 15 December 1981