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Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts

Nurit Ballas [a], Nehama Zakai [a], Ilan Sela [b] and Abraham Loyter [a]

[a] *Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem and* [b] *Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot (Israel)*

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Liposomes composed of phosphatidylcholine (PC) and cholesterol, and bearing the hydroxyl form of the quaternary ammonium detergent diisobutylcresoxyethoxyethyldimethylbenzylammonium (DEBDA [OH^-]),were able to mediate functional transfer of tobacco mosaic virus RNA (TMV RNA) into tobacco and petunia protoplasts. Functional transfer of TMV RNA was revealed by the appearance of specific capsid polypeptides within 48 h after transfection. Transfer of TMV RNA with the aid of liposomes bearing the quaternary ammonium detergent was achieved using two alternative methods. TMV RNA was enclosed within large unilamellar liposomes composed of phosphatidylcholine and cholesterol, and bearing positively charged detergent. Incubation of such liposomes loaded with TMV RNA with plant protoplasts resulted in transfer of the enclosed RNA into the recipient protoplasts. In addition, functional transfer was also achieved by a complex formed between liposomes bearing the detergent and externally added (non-enclosed) TMV RNA. Maximum transfection was obtained with a complex composed of lipids, a quaternary ammonium detergent and TMV RNA, at a ratio of 1:0.5:0.035 (w/w). Using such a complex, under optimal conditions, about 30% of the recipient protoplasts were transfected with TMV RNA. Liposomes lacking the quaternary ammonium detergent practically failed to transfer TMV RNA into tobacco or petunia protoplasts.

Introduction

Resealed membrane vesicles loaded with biologically active macromolecules have been used as an efficient vehicle for the introduction of the entrapped molecules into living cells [1–4]. Vesicles made of pure phospholipids, namely, liposomes, were shown to carry proteins, RNA or DNA into cultured animal cells [1,2] and into plant protoplasts [4,5]. However, since such liposomes are unable to fuse with biological membranes, their use as a vehicle for microinjection has necessitated the addition of fusogenic agents, such as poly(ethylene glycol) or glycerol [1]. Induction of endocytosis by such reagents also stimulated the uptake of loaded liposomes into cultured cells [1,6].

Liposomes have also been used as drug delivery systems in in vivo experiments [3]. In several cases, it has been demonstrated that administration of liposomes loaded with specific drugs

Abbreviations: DEBDA[OH^-], diisobutylcresoxyethoxyethyldimethylbenzylammonium; PC, phosphatidylcholine; FITC, fluorescein isothiocyanate; REV, reverse-phase evaporated vesicles; SUV, small unilamellar vesicles; TMV, tobacco mosaic virus; cpw solution, cells and protoplasts washing solution.

Correspondence: Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel.

showed an improved therapeutic index as compared to injection of the free drug [3,7].

Recently, it has been reported that resealed vesicles can also be formed by mixing non-ionic detergents with cholesterol and phospholipid molecules [8]. The properties of such vesicles, which have been given the name niosomes, have been described, and their potential use as delivery systems has been investigated [8].

In our laboratory, we have prepared resealed vesicles which possessed certain similarities to niosomes by mixing the quaternary ammonium detergent, DEBDA[OH^-], with cholesterol and phosphatidylcholine (PC) [9]. Fusion between such positively charged liposomes could be induced, as was demonstrated by fluorescence dequenching studies, upon the addition of negatively charged polymers, such as poly(aspartic acid) [9].

In the present study, it is shown that liposomes bearing the positively charged detergent, DEBDA [OH^-], can also serve as a biological carrier, namely, to promote the transfer of TMV RNA into plant protoplasts. TMV RNA enclosed within such liposomes or added externally was functionally transferred into tobacco or petunia protoplasts, as was inferred from the appearance of specific TMV capsid polypeptides.

Materials and Methods

Chemicals. Phosphatidylcholine (PC), cholesterol, calf thymus DNA and DEBDA[OH^-] (1.0 M in methanol) were purchased from Sigma. Anti-TMV antibody raised in rabbits and chickens was a generous gift from Dr. D. Heller from the Department of Animal Science (Rehovot, Israel). FITC-labeled goat anti-rabbit antibody and goat anti-rabbit antibody conjugated to alkaline phosphatase were purchased from Miles Yeda. Cellulase R-10 and macerozyme R-10 were purchased from Yakult Pharmaceutical Industry (Nishinomiya, Japan). Pectolyase Y-23 was obtained from Seishin Pharmaceutical Co., (Chiba, Japan). All other chemicals were of analytical grade.

Isolation of tobacco and petunia protoplasts. Tobacco and petunia protoplasts were isolated from exponentially growing cells, essentially as described before [10,11].

Tobacco cells (*Nicotiana tabacum* L., 'Wisconsin-38') were washed once with cpw solution [12] containing 13% mannitol (cpw 13M) at $300 \times g$ for 5 min, and then resuspended in 3 vols. cpw 13M containing 1.5% cellulase, 0.1% macerozyme and 0.01% pectolyase. After 3 h of incubation at 37°C with shaking (100 rpm), the protoplasts were washed twice with 13% mannitol, layered on a cushion of 23% sucrose solution, and centrifuged at $600 \times g$ for 10 min. The top layer was then washed with MS-S medium [10], containing 12% mannitol (MS-S 12 M).

Petunia hybrida cells were washed with cpw solution supplemented with 10% mannitol (cpw 10 M), and resuspended in 2 vols. cpw 10 M containing 2.0% cellulase, 0.3% macerozyme and 0.01% pectolyase. The enzyme solution was purified by passing through a Sephadex G-25 column before incubation with the cells. After incubation for 15 h at 26°C, the protoplasts were washed with MS '150' medium [11]. The viability of both petunia and tobacco protoplasts was determined by phenosafranine staining [13].

Preparation of liposomes (REV and SUV) bearing DEBDA[OH^-]. Reverse-phase evaporated vesicles (REV) and small unilamellar vesicles (SUV) were prepared from PC and cholesterol using a bath sonicator (Lab Supply Co., Model 61121 SP16, 80000 cycles/s) [14,15]. Addition of DEBDA[OH^-] to the liposomes and removal of free DEBDA[OH^-] by SM-2 Bio-Beads were preformed essentially as previously described [9]. Briefly, REV or SUV (2 mg lipids, PC/cholesterol, 1 : 0.5 (w/w), in 500 μl of 150 mM sodium acetate, (pH 7.2)) were placed in a tube containing 2 mg of DEBDA[OH^-] suspended in 60 μl acetate buffer. After 20 min of incubation with gentle shaking at 37°C, free, non-liposome-associated DEBDA [OH^-] was removed by the addition of SM-2 Bio-Beads (SM-2 Bio-Beads/ DEBDA[OH^-], 20 : 1 (w/w)). Under these conditions, the ratio lipids/DEBDA[OH^-] (w/w) in the liposome suspension was 1 : 0.5. In some experiments, SM-2 Bio-Beads were not used to remove free DEBDA[OH^-] micelles from the liposome suspension (for details, see legend in the appropriate tables and Fig. 1). The PC/cholesterol ratio in all liposomes was 1 : 0.5 (w/w). DEBDA[OH^-] and phospholipid content were determined by the

methods of Bradford [16] and Stewart [17], respectively.

Encapsulation of TMV RNA in REV liposomes. RNA was extracted from TMV as described [18]. TMV RNA (160 μg in 400 μl 0.15 M acetate buffer (pH 7.2)) was added to the lipid solution (4 mg PC, 2 mg cholesterol in 1 ml diethyl ether). The suspension obtained was vigorously shaken for 1 min and sonicated for 5 s in a bath sonicator. REV were obtained after evaporation of the diethyl ether and agitation of the gel formed [14]. The liposomes thus formed were washed twice in 16 ml acetate buffer (80000 × *g*, 30 min at 4° C) and then incubated with DEBDA[OH^-] (1 : 1 (w/w)) and SM-2 Bio-Beads, as described above. With the use of radioactive RNA molecules, it was determined that about 20–25% of the added RNA molecules were trapped within the REV, giving a ratio of 32–40 μg of RNA enclosed within 6 mg of lipid vesicles (not shown).

Preparation of the liposome · DEBDA[OH^-] · TMV · RNA complex. REV and SUV were prepared and incubated with DEBDA[OH^-] to give empty liposomes bearing DEBDA[OH^-], as described above. TMV RNA, in concentrations specified in the legends to figures and tables, was then added to liposome · DEBDA[OH^-] with continuous vortexing. The addition of the TMV RNA induced an increase in the turbidity of the suspension, with the appearance of some precipitates, indicating the formation of the liposome · DEBDA[OH^-] · TMV-RNA complex. No increase in the turbidity was observed when TMV RNA was added to liposomes lacking DEBDA[OH^-].

Transfection of plant protoplasts by TMV RNA and quantitative determination of TMV capsid protein. Briefly, liposomes loaded with TMV RNA and bearing DEBDA[OH^-], or the liposome · DEBDA[OH^-] · TMV-RNA complex were added to a suspension of $2 \cdot 10^6$ plant protoplasts/ml. Following incubation with gentle shaking at 28° C, the tobacco and petunia protoplasts were washed twice with MS-S 12M or MS '150' medium, respectively, and then cultured at 28° C in the same medium at a density of $2 \cdot 10^5$ protoplasts/ml. If not otherwise stated, viral antigen (capsid protein) was estimated after 48 h of culturing by enzyme-linked immunosorbent assay [19].

Results

Transfer of TMV RNA into plant protoplasts by the combination of a quaternary ammonium detergent and phospholipid vesicles

The data presented in Fig. 1 show that liposomes, with enclosed TMV RNA and bearing DEBDA[OH^-], are able to transfer their content into plant protoplasts in a functional form. This was inferred from the appearance of intracellular TMV-specific polypeptides (Fig. 1). When loaded liposomes lacking DEBDA[OH^-] were used (Fig. 1A), the efficiency of the TMV RNA transfer into the tobacco protoplasts was very low (about 50 ng of viral antigen/10^6 protoplasts). As can be seen, the extent of transfection was dependent upon the culturing period and the amount of loaded liposomes added to the protoplast suspension (Fig. 1B). Essentially the same results were obtained with petunia protoplasts (not shown, see also Table I).

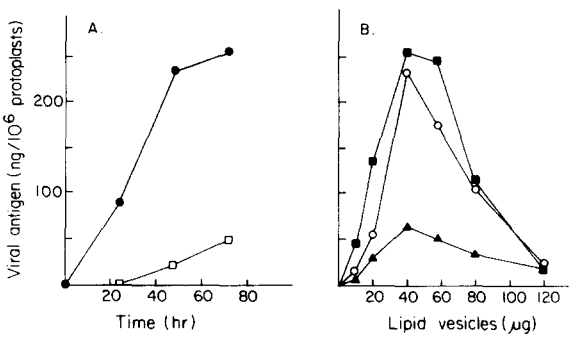

Fig. 1. The ability of loaded liposomes to transfer TMV RNA into tobacco protoplasts. Encapsulation of TMV RNA within REV, addition of DEBDA[OH^-] and removal of free DEBDA[OH^-] were as described in Materials and Methods. (A) Loaded liposomes (40 μg lipids with about 0.2 μg RNA), bearing (●) or lacking (□) DEBDA[OH^-], were incubated at 28° C with 10^6 tobacco protoplasts, in a total volume of 1 ml MS-S 12M medium. After 90 min of incubation, cells were washed and cultured at 28° C as described in Materials and Methods. Aliquots of 300 μl were removed at different times of culturing, and the formation of TMV antigens was estimated by enzyme-linked immunosorbent assay [19]. (B) Increasing concentrations of the loaded liposomes bearing DEBDA[OH^-] were incubated with tobacco protoplasts for 90 min at 28° C, as described in (A). Protoplasts were cultured in MS-S 12M medium and TMV antigens were estimated after 24 (▲), 48 (○), and 72 (■) h of culturing, as described in Materials and Methods.

TABLE I

TRANSFECTION OF TOBACCO AND PETUNIA PROTOPLASTS BY TMV RNA MOLECULES: EFFECT OF LIPOSOMES AND DEBDA[OH$^-$]

Enclosure of TMV RNA within REV, incubation of REV with DEBDA[OH$^-$] and removal of non-lipid-associated free DEBDA[OH$^-$] were as described in Materials and Methods. Tobacco protoplasts (10^6 in 1 ml of MSS 12M) were then incubated with loaded liposomes (60 μg of lipids with about 0.3 μg enclosed TMV RNA), bearing or lacking DEBDA[OH$^-$], for 90 min at 28°C. The liposome·DEBDA[OH$^-$]·TMV-RNA complex was formed as described in Materials and Methods. Briefly, to a continuously vortexed suspension of REV bearing or lacking DEBDA[OH$^-$] (15 μl of 4 mg/ml PC/cholesterol, 1:0.5 (w/w) bearing 2 mg/ml of DEBDA[OH$^-$]), a solution of TMV RNA (3.0 μl of 0.1 mg/ml) was added. The complex thus obtained was then incubated with 10^6 tobacco or petunia protoplasts as described above. The protoplasts were washed and cultured as described in Materials and Methods. Formation of TMV antigens was estimated after 48 h of culturing by the enzyme-linked immunosorbent assay [19]. Essentially, the same results were obtained in two separate experiments. However, the results given represent data obtained from one experiment. n.d., not done.

System	Viral antigen (ng/10^6 protoplasts)	
	Nicotiana tabaccum	*Petunia hybrida*
TMV RNA encapsulated in liposome-DEBDA[OH$^-$]	240	n.d.
liposome	25	n.d.
TMV RNA complexed to liposome-DEBDA[OH$^-$]	435	370
liposome	15	12

The TMV RNA, however, did not have to be enclosed within the phospholipid vesicles in order to become transfected. The results summarized in Table I show that the combination of empty liposomes carrying DEBDA[OH$^-$] and exogenous, non-trapped TMV RNA also caused transfection of either tobacco or petunia protoplasts. The appearance of TMV-directed capsid protein was slightly higher with empty liposomes complexed with external TMV RNA than with liposomes carrying encapsulated TMV RNA (see Table I). Table I also shows that only DEBDA[OH$^-$]-bearing liposomes were able to mediate the functional transfer of external TMV RNA into plant protoplasts. Very few or no TMV-specific polypeptides were detected in either tobacco or petunia protoplasts incubated with empty liposomes lacking DEBDA[OH$^-$] to which TMV RNA had been added externally. Most of the experiments described in the present work were performed with petunia protoplasts. However, essentially the same results were obtained using tobacco protoplasts.

The detergent DEBDA[OH$^-$], by itself, did not mediate any transfection of petunia protoplasts by TMV RNA, possibly because a high degree of cell death results when petunia protoplasts are incubated with the detergent in the absence of the phospholipid vesicles. As shown in Table II, transfection of plant protoplasts was caused only by the complex formed between TMV RNA and liposomes bearing DEBDA[OH$^-$]. Sequential addition of the individual complex components, i.e., incubation of petunia protoplasts first with DEBDA[OH$^-$]-bearing liposomes and subsequently with TMV RNA did not result in cell transfection.

Transfection of petunia protoplasts by the liposome·DEBDA[OH$^-$]·TMV-RNA complex

The results in Fig. 2 show that maximum efficiency of transfection was achieved by incubation of petunia protoplasts with the liposome·DEBDA[OH$^-$]·TMV-RNA complex for only 5

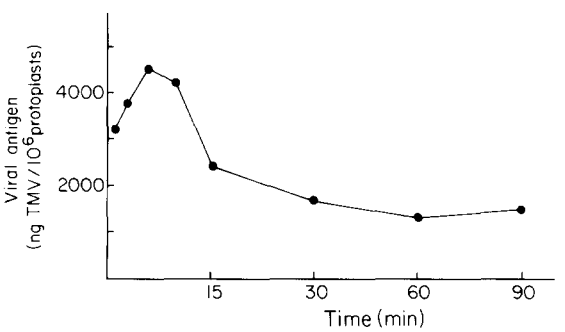

Fig. 2. Transfection as a function of the incubation period with the liposome·DEBDA[OH$^-$]·TMV-RNA complex. The liposome·DEBDA[OH$^-$]·TMV-RNA complex (1/0.5/0.035 (w/w)) was formed as described in Materials and Methods and in the legend to Table I. Samples of liposome·DEBDA[OH$^-$]·TMV RNA (84 μg of lipids, 42 μg of DEBDA[OH$^-$] and 3 μg of TMV RNA) were added in parallel to several flasks each containing $2 \cdot 10^6$ petunia protoplasts, in a final volume of 1 ml MS '150' medium. The various systems were incubated at 28°C with gentle shaking and, at the indicated times, were removed, washed and cultured for 48 h. TMV antigens were determined by enzyme-linked immunosorbent assay.

TABLE II

FUNCTIONAL TRANSFER OF TMV RNA INTO PETUNIA PROTOPLASTS: REQUIREMENT FOR THE LIPOSOME·DEBDA[OH^-]·TMV-RNA COMPLEX

Petunia protoplasts, TMV RNA and REV bearing DEBDA[OH^-] were obtained as described in Materials and Methods. In Expts. 1 and 2, TMV RNA (0.8 μg), and in Expt. 3, DEBDA[OH^-] (5.0 μg) were incubated with petunia protoplasts ($2 \cdot 10^6$) in a total vol. of 1 ml MS '150' medium for 5 min at room temperature (21° C), after which DEBDA[OH^-] (5.0 μg in Expt. 2) or TMV RNA (0.8 μg in Expt. 3) was added, and these systems were further incubated for 90 min at 28° C. In Expt. 4, a complex was formed between TMV RNA (8.0 μl of 0.1 mg/ml) and DEBDA[OH^-] (5 μl of 1 mg/ml) by addition of the RNA molecules to a continuously vortexed suspension of DEBDA[OH^-]. The complex was then added to $2 \cdot 10^6$ petunia protoplasts, and the suspension obtained was incubated for another 90 min at 28° C. In Expt. 5, petunia protoplasts ($2 \cdot 10^6$ in a final volumes of 1 ml) were incubated for 5 min with REV (PC/cholesterol, 1:0.5 (w/w)) bearing DEBDA[OH^-]), after which TMV RNA (0.8 μg) was added and the protoplasts were then incubated for an additional 90 min at 28° C. In Expt. 6, a complex was formed between liposomes bearing DEBDA[OH^-] and TMV RNA (80 μg of lipids, 40 μg of DEBDA[OH^-] and 0.8 μg of TMV RNA) as described in Materials and Methods. The complex was then incubated with petunia protoplasts ($2 \cdot 10^6$ in a final vol. of 1 ml) for 90 min at 28° C. At the end of the incubation periods, protoplasts in the various experiments were washed, cultured for 48 h, and the presence of TMV antigens was estimated as described in Materials and Methods.

Expt. no.	Addition to petunia protoplasts		TMV antigens (ng/10^6 protoplasts)
	1st	2nd	
1	TMV RNA [a]	–	0
2	TMV RNA [a]	DEBDA[OH^-]	0
3	DEBDA[OH^-] [b]	TMV RNA	0
4	DEBDA[OH^-]·TMV RNA	–	0
5	Liposome·DEBDA[OH^-]	TMV RNA	0
6	Liposome·DEBDA[OH^-]·TMV RNA	–	400

[a] Essentially the same result, i.e., no transfection, was obtained with the use of 0.3–5.0 μg of TMV RNA.
[b] The use of DEBDA[OH^-] at amounts higher than 5.0 μg was avoided because it caused a high degree of cell death.

min at 28° C. At incubation periods longer than 15 min, the extent of transfection was drastically reduced (Fig. 2). Essentially the same kinetics were obtained by the use of liposome-DEBDA[OH^-] with encapsulated TMV RNA (not shown).

More RNA can be complexed to the external surface of a vesicle that can be encapsulated within it, and therefore, the efficiency of transfection with the former was always higher than with the latter system. Indeed, the amount of TMV RNA used in the experiments summarized in Fig. 2 was about 5-fold higher than in the previous experiments (for example, see Fig. 1), hence the increase in transfection efficiency in this and subsequent experiments. It is noteworthy that in most of our experiments, about 200–300 ng of viral antigen/10^6 protoplasts were obtained using loaded liposomes as compared to 2000–6000 ng of viral antigen/10^6 protoplasts following transfection with the liposome·DEBDA[OH^-]·TMV-RNA complex.

Another factor that contributed to the higher

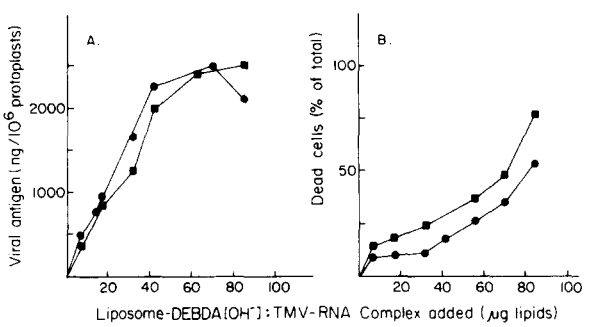

Fig. 3. Transfection of petunia protoplasts by liposome·DEBDA[OH^-]·TMV-RNA complexes formed with REV or SUV. (A) Efficiency of transfection. (B) Effect on cell viability. (A) Formation of REV (●) and SUV (■) bearing DEBDA[OH^-] and addition of TMV RNA (lipid·DEBDA[OH^-]·TMV RNA, 1:0.5:0.035) was as described in Materials and Methods. Increasing amounts of the liposome·DEBDA[OH^-]·TMV-RNA complex were incubated for 5 min at 28° C with $1 \cdot 10^6$ petunia protoplasts, in a final volume of 0.5 ml of MS '150' medium. At the end of the incubation period, protoplasts were washed, cultured for 48 h and assayed for TMV-capsid polypeptides. (B) At the end of 5-min incubation period with the liposome·DEBDA[OH^-]·TMV-RNA complex, the protoplasts were stained with phenosafranine for estimation of cell viability [13]. (●) REV, (■) SUV.

efficiency of transfection is the short incubation times of the liposomes with petunia protoplasts, which contributed another 2-fold increase in the rate of transfection. This may be due to an increase in the viability of the transfected protoplasts. Evidently, viability of the recipient protoplast was inversely proportional to the time of incubation with the liposomes.

Liposomes possessing a small internal volume and a low trapping efficiency (SUV) were shown to be very poor vehicles for transfer of macromolecules into living cells [14]. Fig. 3A, however, demonstrates that SUV were equally effective in mediating the transfer of TMV RNA into petunia protoplasts as REV. This observation further supports the view that the TMV RNA is carried by these liposomes externally. It is also demonstrated in Fig. 3A that the efficiency of transfection increases (with both SUV and REV) in parallel with the increase of the amount of the liposome · DEBDA[OH^-] · TMV-RNA complex, up to 40 μg lipid (complexed with about 1.5 μg TMV RNA), at which point cell death starts to increase more rapidly (Fig. 3B).

The effect of the lipid: DEBDA[OH^-]: RNA ratio on cell transfection and cell viability

The transfection of protoplasts with TMV RNA is mediated by a three-component vesicle in the form of a complex. The following experiments were designed in order to establish the optimal composition of the triparticle vesicle, the various parameters of transfection and also corroborate its complex nature. While, in Fig. 3, vesicles were of a certain fixed composition, Fig. 4A presents results of experiments in which two parameters (21 μg DEBDA[OH^-] and 1.5 μg TMV RNA) remained constant by amount (as well as by ratio), but the amount of lipid in the vesicles was varied. A distinct optimum in the degree of transfection was noted only with specific ratios of all the three component. Moreover, at this level of DEBDA

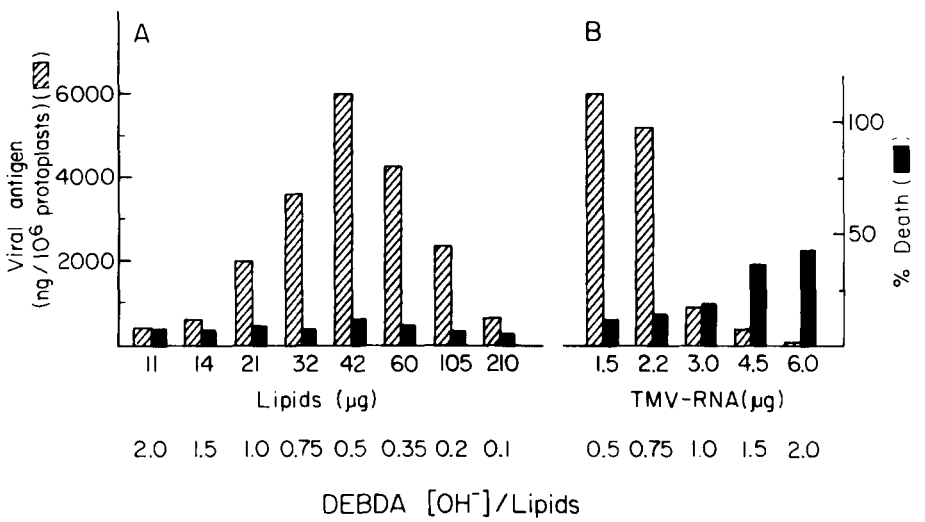

Fig. 4. Formation of liposome·DEBDA[OH^-]·TMV-RNA complex with different ratios among its components: effect on the degree of cell transfection and viability. (A) Increasing amounts of REV (11–210 μg lipids, PC/cholesterol, 1:0.5 (w/w)) were incubated with 21 μg of DEBDA[OH^-] and suspended in a final volume of 25 μl of 150 mM acetate buffer (pH 7.2). After 30 min of incubation with vigorous shaking, TMV RNA (1.5 μg) was added to give a ratio of DEBDA[OH^-]:TMV RNA of 1:0.07 (w/w). The liposome·DEBDA[OH^-]·TMV-RNA complex formed was then incubated with 10^6 petunia protoplasts, in a final volume of 0.5 ml of MS '150' medium, as described in Materials and Methods. (B) Increasing amounts of DEBDA[OH^-] (21–84 μg) were added to a constant amount of REV (42 μg of lipids), suspended in a final volume of 25 μl of 150 mM acetate buffer (pH 7.2), to give REV/DEBDA[OH^-] of various w/w ratios. After 30 min of incubation at room temperature with vigorous shaking, increasing amounts of TMV RNA (1.5–6.0 μg) were added, to give a w/w ratio of DEBDA[OH^-]/TMV-RNA of 1:0.07. The complex formed was incubated with 10^6 petunia protoplasts as described in Materials and Methods. Formation of TMV capsid protein was estimated after 48 h of culturing, as described in Materials and Methods.

[OH^-], very little cell death was observed. Under these conditions, the addition of SM-2 Bio-Beads for removal of excess DEBDA[OH^-] was not required.

When the amount of lipid in the vesicles was kept constant (42 μg lipid), and the amount of the DEBDA[OH^-] and TMV RNA (ratio 1 : 0.07) was varied (Fig. 4B), the optimum transfection was again found with a complex consisting of 42 μg lipid, 21 μg DEBDA[OH^-] and 1.5 μg TMV RNA. Any increase in the amount of DEBDA[OH^-] and TMV RNA caused a decrease in the efficiency of transfection and an increase in cell mortality (Fig. 4B). These results support a model of a three-component complex. This is further supported by the experiment summarized in Fig. 5. In this experiment, the amount of TMV RNA was varied. An increase in the amount of TMV RNA after a certain point did not result in a better transfection and even brought about some reduction of transfection. Therefore, it follows that only a proper ratio among lipid, DEBDA [OH^-] and TMV RNA (1 : 0.5 : 0.035–0.05 (w/w), respectively) makes a good transfecting vesicle.

It is noteworthy that protoplasts treated with a vesicle complex of these ratios show a high degree of cell viability.

As can be seen (Table III), most of the protoplasts (85–90%) survived the treatment with the 'transfection complex.' On the other hand, it appears that the ability of the cells to divide is affected by treatment with the liposome · DEBDA[OH^-] · TMV-RNA complex. The number of treated cells which remained alive following 7 days of culturing was about 40% of control, untreated cells. This amount could, however, be increased to about 70% of untreated cells if the protoplasts were washed with liposomes composed of PC/cholesterol (1 : 0.5 (w/w)) after treatment with the complex. It is conceivable that any free micelles of DEBDA[OH^-] which might have been

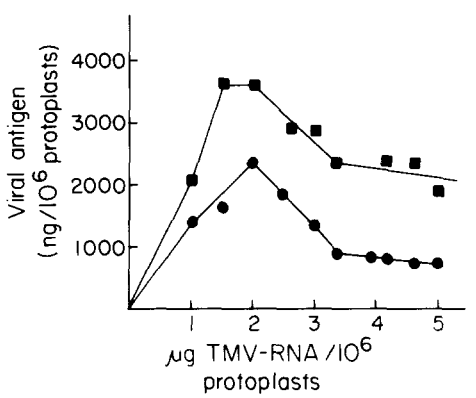

Fig. 5. Transfection of petunia protoplasts by liposome·DEBDA[OH^-]·TMV-RNA complex: effect of RNA concentration. Samples containing 42 μg and 59 μg of DEBDA[OH^-] were incubated with 84 μg lipids of REV, as described in Materials and Methods and in Fig. 4, to give a w/w ratio of lipids/DEBDA[OH^-] of 1 : 0.5 (■) and 1 : 0.75 (●), respectively. After 30 min of incubation at room temperature, increasing amounts of TMV RNA were added. The complex formed, liposome·DEBDA[OH^-]·TMV RNA, was then incubated with $2 \cdot 10^6$ petunia protoplasts. All other experimental conditions were as described in Materials and Methods.

TABLE III

THE ABILITY OF TRANSFECTED PROTOPLASTS TO SURVIVE AND UNDERGO CELL DIVISION

Petunia protoplasts ($2 \cdot 10^6$) were transfected by liposome·DEBDA[OH^-]·TMV-RNA complex (84 μg lipid, 42 μg DEBDA[OH^-] and 3 μg TMV RNA), as described in Materials and Methods and in the legend to Fig. 5. After 5 min of incubation at 28 °C, protoplasts were washed, and the pellet obtained was resuspended in 0.5 ml of MS '150' medium and incubated for 30 min at 28 °C. At the end of the incubation period, a mixture of either a suspension containing 300 μg of lipids (SUV, PC/cholesterol 1 : 0.5, of 8 mg/ml) or 10 μg calf thymus DNA was added. Following 10 min of incubation with gentle shaking at 28 °C, the protoplasts were washed once again with MS '150' medium. Samples were removed for staining with phenosafranine [13], and the protoplasts were transferred and cultured in MS '150' at a density of $2 \cdot 10^5$/ml, as described in Materials and Methods. After 48 h of culturing, samples were removed for estimating TMV-specific polypeptides, as described, and the remaining cells were allowed to grow in the same medium. Following 7 days of culturing, the packed volume of the transfected cultured cells was estimated (300 × g, 5 min). The packed volume obtained from cultures of untransfected cells was considered 100%.

Additions to wash medium	TMV antigens (ng/10^6 protoplasts) [a]	Viability (% of cells living)	Packed volume of cells (% of control) [b]
None	2700	84	40
Liposomes	2800	92	70
DNA	2600	95	70

[a] Estimated after 48 h.
[b] Estimated after 7 days.

harmful to the cells were washed and neutralized by the added liposomes. Another way to improve the ability of the cells to undergo cell division is washing with non-functional DNA molecules which supposedly are able to form complexes with the positively-charged DEBDA[OH^-].

Quantitative determination of the degree of cell transfection

The frequency of cell transfection was estimated using specific anti-TMV capsid protein antibodies and immunofluorescence techniques [20]. The results in Fig. 6 show that by employing this method, a linear correlation is observed between the percentage of transfection and the amount of TMV capsid polypeptides found intracellularly by enzyme-linked immunosorbent assay. From the results presented in the figures, it appears that 2000 ng of TMV capsid protein represents about 10% of cell transfection (10% of the cells in the population expressed TMV capsid protein). Under the conditions described in the present work, up to about 30% of the protoplasts were shown to be transfected by the liposome · DEBDA[OH^-] · TMV-RNA complex.

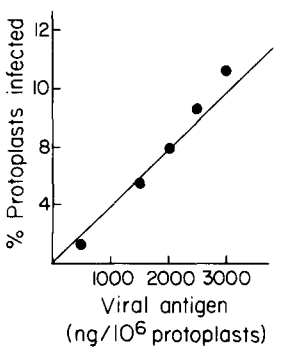

Fig. 6. The correlation between the amount of intracellular TMV-capsid polypeptides and the degree of cell transfection. Petunia protoplasts were transfected with increasing amounts of TMV RNA complexed to liposome-DEBDA[OH^-] (1:0.5) as described in Fig. 5. Duplicate samples of transfected cells were prepared and fixed on glass slides for staining with fluorescent antibody exactly as described before [23]. The fixed samples then were immersed in phosphate-buffered saline (0.15 M NaCl/50 mM phosphate buffer (pH 7.2)) containing 10% newborn calf serum. After 30 min of incubation at room temperature, the glass slides were removed and reimmersed in TBS solution (50 mM Tris-HCl (pH 8.6)/0.05% Tween 20/2% bovine serum albumin), containing rabbit anti-TMV antibody. After incubation at room temperature, the slides were washed five times with phosphate-buffered saline and then stained with fluorescently labeled goat anti-rabbit antibody.

Discussion

In the present work, we have shown that liposomes bearing a positively charged detergent are able to deliver TMV RNA into tobacco and petunia protoplasts in the absence of facilitators such as poly(ethylene glycol). In the present system, the detergent may serve a dual function. It probably mediates binding of the PC/cholesterol liposomes to the plant protoplast and, at the same time, promotes a transient increase in the permeability of the protoplast membrane. This is inferred from the observation that TMV RNA incubated with either petunia or tobacco protoplasts, in the presence of liposomes bearing the detergent DEBDA[OH^-], induces the synthesis of TMV-specific capsid polypeptides. Under optimal conditions, (10^6 protoplasts incubated with as little as 1.5 μg TMV RNA), synthesis of TMV capsid protein could be detected in up to 30% of the cells.

Our present results show that the liposome · DEBDA[OH^-] · TMV-RNA complex was much more efficient with respect to the functional transfer of TMV RNA into plant protoplasts than liposomes loaded with TMV RNA. This may result from the following. (a) External TMV RNA is more susceptible to the transformation process than RNA enclosed within the liposomes; (b) relatively higher amounts of TMV RNA can be added externally than can be enclosed within liposomes bearing DEBDA[OH^-]. The fact that, in order to be active in transfection processes, nucleic acid should not be enclosed within liposomes makes the present method simple, efficient and reproducible.

Synthesis of TMV-specific polypeptides indicates that incubation with the detergent did not induce excessive cell death. A direct estimation showed that cell viability remained high and could be further increased by washing off the detergent with either lipid vesicles or DNA molecules. Under these conditions, cell mortality was very low and practically negligible.

Transfection of plant protoplasts with TMV RNA was obtained only by the combination of DEBDA[OH^-] and lipid vesicles. DEBDA[OH^-],

by itself, was ineffective in inducing transfection of plant protoplasts by TMV RNA. Attachment between liposomes bearing DEBDA[OH^-] and plant cell plasma membranes is probably due to electrostatic interaction between the positively charged detergent and the cell-surface negative charges.

The view that negatively charged molecules can interact with liposomes bearing DEBDA[OH^-] is supported by recent experiments which show that fusion of such liposomes can be induced by the addition of poly(aspartic acid) [9]. This was inferred from studies using fluorescently labeled liposomes and the energy transfer method [9]. Like poly(aspartic acid), RNA molecules may also induce fusion between liposomes and DEBDA[OH^-]. It is conceivable, although not yet proven, that during the fusion process, some of the added RNA molecules can be enclosed within the fused liposomes (Fig. 7, model I). Such loaded liposomes may subsequently act to fuse with membranes of plant protoplasts due to the presence of liposome-associated DEBDA[OH^-] molecules, resulting in the microinjection of the entrapped RNA molecules (Fig. 7, model I). If true, such a mechanism will explain our results which showed that: (a) DEBDA[OH^-], by itself, was ineffective in inducing the uptake of TMV RNA; (b) only the simultaneous addition of TMV RNA, DEBDA[OH^-] and phospholipid vesicles caused transfection of plant protoplasts; (c) large amounts of TMV RNA caused inhibition of transfection (this may have been due to masking of the positively charged groups of DEBDA[OH^-] by excess of RNA molecules); (d) the need for liposome · DEBDA[OH^-] · TMV-RNA complexes with a certain and well-defined weight to weight ratio. Only liposomes with DEBDA[OH^-], whose amount reached about 50% of the lipid, were shown to be highly fusogenic. This model may also explain why SUV-DEBDA[OH^-] complexes were active in promoting uptake of TMV RNA.

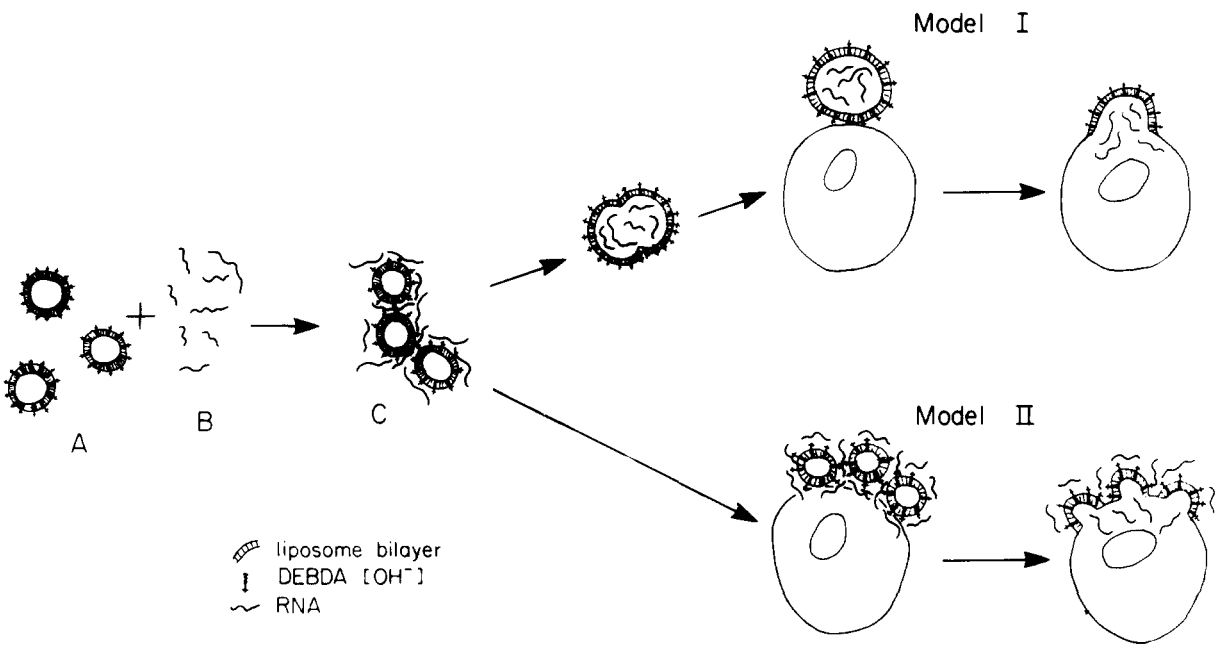

Fig. 7. Transfer of TMV-RNA into plant protoplasts by the liposome·DEBDA[OH^-]·TMV-RNA complex: a suggested scheme. (A–C) Formation of the liposome·DEBDA[OH^-]·TMV-RNA complex. Model I: Enclosure of TMV RNA within liposomes bearing DEBDA[OH^-] due to RNA-induced fusion of such liposomes. Binding and fusion of liposomes loaded with TMV RNA and bearing DEBDA[OH^-] with plant protoplasts. Model II: Binding of the liposome·DEBDA[OH^-]·TMV-RNA complex to plant protoplasts and then transfer of external RNA into plant protoplasts due to a transient increase in the protoplast membrane permeability.

Induction of fusion between SUV and DEBDA[OH^-] will significantly increase the vesicular internal volume, allowing trapping of relatively large amounts of TMV RNA.

Although the model discussed above might explain most of our results, the possibility that TMV RNA molecules, which remain externally adsorbed to the liposome-DEBDA[OH^-] bilayer, are also introduced into the plant protoplasts cannot be excluded (Fig. 7, model II). Fusion of liposome-DEBDA[OH^-], carrying externally complexed TMV RNA, with plant protoplasts may also result in transfer of the RNA into the cell cytoplasm (Fig. 7, model II).

In spite of extensive effort, in the present system it was impossible to demonstrate, using fluorescence dequenching methods, [9] fusion with liposomes bearing DEBDA[OH^-], due to a high level of intrinsic fluorescence in the plant protoplasts used. It should be mentioned, however, that preliminary studies in our laboratory by the use of electron microscopy techniques have demonstrated fusion between liposomes bearing DEBDA[OH^-] and erythrocyte membranes (not shown).

Based on the above observations and considerations, the process of endocytosis as an alternative route by which the liposome · DEBDA[OH^-] · TMV-RNA complex enters into plant protoplasts cannot be eliminated. Such a mechanism has been suggested for the uptake of lipid vesicles composed of PC and stearylamine [21]. Such positively charged liposomes have been shown to attach effectively to plant protoplasts and to be able to transfer the cauliflower mosaic virus DNA into evacuolated turnip plant protoplasts [21]. Also, uptake of phosphatidylserine/cholesterol liposomes loaded with TMV RNA has been suggested to be mediated by endocytotic-like processes [22]. It is noteworthy that experiments in our laboratory have shown that RNA and DNA molecules complexed to liposomes bearing DEBDA[OH^-] are highly protected from digestion by added RNAses and DNAses, respectively, and therefore should not be susceptible to nucleases present in the cell growth medium (not shown).

Preliminary experiments in our laboratory have shown that liposomes prepared by the present method, namely, bearing DEBDA[OH^-], can also serve as a carrier for transferring macromolecules into cultured animal cells. Incubation of such liposomes containing enclosed SV 40 DNA or ricin A with hepatoma-tissue-cultured cells resulted in fusion-mediated microinjection of the liposome content into the hepatoma cells (in preparation). These preliminary experiments as well as those described in the present work clearly show that resealed liposomes bearing positively charged detergent may offer a new and powerful way for functional transfer of macromolecules into plant protoplasts as well as into animal cultured cells.

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