RECONSTITUTION OF LIPID VESICLES ASSOCIATED WITH HVJ (SENDAI VIRUS) SPIKES

Purification and Some Properties of Vesicles Containing Nontoxic Fragment A of Diphtheria Toxin

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ABSTRACT

A mixture of HVJ (Sendai virus) spike proteins, the nontoxic fragment A of diphtheria toxin, lecithin, and cholesterol was solubilized in sucrose solution containing a nonionic neutral detergent. The liposomal vesicles which formed on removal of the detergent by dialysis were purified by gel filtration and centrifugation on a sucrose gradient. The resulting purified vesicles had hemagglutinating activity, hemolytic activity and, after solubilization, the enzymic activity of fragment A. The vesicles had no cell fusion activity. Electron microscopy showed that both the outside and inside of membranes of the vesicles were associated with the spikes. When the vesicles were freeze-fractured, no large aggregates of particles were seen on either face. Such fragment A-containing lipid vesicles (liposomes) with HVJ spikes bound to mammalian cell membrane and released their fragment A into the cytoplasm causing cell death. Neither fragment A-containing liposomes without spikes nor empty liposomes with spikes were toxic.

KEY WORDS lipid vesicles · HVJ spikes · fragment A of diphtheria toxin

There have been many attempts to introduce biologically active molecules into mammalian cells using lipid vesicles (5, 15, 16, 24, 29, 36). Although small amounts of a wide variety of molecules have been introduced into cells in this way, the method has not provided us with an efficient and uniform means for transporting molecules across the plasma membrane. Recently, large lipid vesicles were made from small, unilamellar vesicles (11) by treating them with Ca^{++} and then adding EDTA (26). Poliovirus, entrapped in such vesicles, could infect poliovirus-insensitive Chinese hamster ovary cells (36); however, viral growth took place in only a small proportion of the cells. Pagano and Takeichi noted that although lipid vesicles readily bound to cell surface, most of them appeared to remain intact without fusion to the membrane (23).

It is well known that HVJ (Sendai virus) promotes cell-to-cell fusion (18, 22). When the virus infects susceptible cells, the virions release their nucleoproteins into the cytoplasm after their envelopes have fused the cell membranes. We pre-

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viously showed that CRM45 (a nontoxic and enzymically active protein, serologically related to diphtheria toxin) (32, 34), entrapped within reconstituted HVJ envelopes, could be efficiently introduced into mammalian cells (35). Since it has been shown that certain proteins with hydrophobic regions, mostly isolated from membranes, can readily become associated with bilayers of artificial lipid vesicles (1, 12, 27, 30, 38), it occurred to us that, if the HVJ spike proteins (8, 28) from the viral envelopes could be similarly bound to lipid vesicles, the introduction of entrapped molecules into cells might be greatly facilitated. In our present paper, we show that liposomal vesicles with surface-associated HVJ spikes can be used to introduce substances into cells with high efficiency.

Fragment A, which contains the N-terminal 22,000 daltons (2, 3) sequence and the NAD:elongation factor 2 (EF2) ADP ribosyl (ADPR) transferase activity of diphtheria toxin (4, 9), blocks protein synthesis in eucaryotic cell extracts but is toxic neither to susceptible animals nor to cultured sensitive cells, because it cannot reach the cell cytoplasm (34). Here, we show that fragment A-containing lipid vesicles with spike proteins is highly toxic for mouse L cells, whereas fragment A-containing lipid vesicles without spikes is not toxic.

MATERIALS AND METHODS

Materials

Lecithin preparations from egg yolk (type V-E) and cholesterol (Grade I: ~99%) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Nonidet P40 (NP40) was kindly supplied by the Osaka branch of Shell Oil Chemical Co. Ltd. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG goat antibody was purchased from N. L. Cappel Laboratories Inc. (Cochranville, Pa.). [^{14}C]NAD (281 mCi/mM) and [^{14}C]lecithin (50–60 mCi/mM) were obtained from New England Nuclear (Boston, Mass.).

Isolation and NAD:EF2-ADPR Transferase Activity of Fragment A of Diphtheria Toxin

The C7(β-22) strain was isolated in the course of isolation of a nontoxinogenic β phage mutant (32). The culture supernate of C7(β-22) was concentrated in the presence of phenylmethylsulphonyl fluoride and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis without thiol. The main protein band with a mol wt ~22,000 daltons showed NAD:EF2-ADPR transferase activity. Its enzymic activity was not increased by treatment with trypsin, and no enzymic activity was found in proteins of more than 22,000 daltons. The main band with the enzymic activity is thus not a degradation product of a mutant protein of more than 22,000 daltons. A purified preparation of this mutant protein of 22,000 daltons had the same enzymic activity as fully activated diphtheria toxin on a molar basis; it was immunologically identical to fragment A of diphtheria toxin and had no toxicity to susceptible cells or animals. This mutant protein is named CRM22 and is similar to CRMs produced by C7(β-NG-2) (17), C7(β^{tox-2}) (6), and C7($\beta^{tox-112}$) (13). CRM22 purified by DE52 and Sephadex G150 chromatographies showed a single sharp band corresponding to material of 22,000 daltons on SDS-polyacrylamide gel electrophoresis. The NAD:EF2-ADPR transferase activity of each sample was assayed by a slight modification of the method of Gill and Pappenheimer (3). EF2 was purified with DE52 to remove a red colored impurity. When this purified EF2 was used in our assay system, the radioactivity of the EF2-ADPR complex was about 10 times greater than that obtained when EF2 was used before purification on DE52.

Virus and Its Envelope

HVJ, Z strain, was propagated in the chorioallantoic fluid of chick embryos. The virus was purified by differential centrifugation as described previously (20). The purified virions were resuspended in 5 mM Tris buffer (pH 7.5) and their titer measured as hemagglutinating units (HAU/ml) using chicken erythrocytes. Under our conditions, 1 HAU was equivalent to about 3×10^7 virus particles (21). Nonidet P40 was added to the virus suspension at a final concentration of 0.5% and the mixture was incubated at 20°–25°C for 30 min. The virus suspension treated with NP40 was centrifuged at 105,000 *g* for 75 min at 4°C to remove insoluble materials and nucleocapsids. The resulting supernate was dialyzed in a Neflex tube against phosphate buffered saline (PBS); the buffer was changed once a day for 3 days (10). The preparation was then centrifuged at 105,000 *g* for 60 min, and the precipitate of reassembled envelopes was resuspended in 10 mM Tris buffer, pH 7.5.

Determinations of Protein and Lipid

Protein was determined by the method of Lowry et al. (14). Lipids of reconstituted envelopes were extracted with 20 vol of mixtures of chloroform and methanol in successive ratios of 1:2, 1:1, and 2:1. The extracted lipids were weighed after evaporation of the organic solvents.

Antibody

About 10^4 HAU of reconstituted envelopes prepared

as described above were injected into rabbits subcutaneously. 3 wk later a booster injection of the same dose in complete Freund adjuvant was given. Booster injections were given at intervals until a suitable titer was obtained, and blood was taken 7–10 days after the last injection. When 2 HAU of HVJ was used as antigen, a 1:1024 dilution of antiserum inhibited hemagglutination.

Antiserum was incubated at 56°C for 30 min to inactivate complement and a globulin fraction was prepared by precipitation with 0.33 saturated ammonium sulfate followed by dialysis of the resulting precipitates against PBS (pH 7.8).

Fusion of Cells

Cell fusion was examined as described previously (22). Ehrlich ascites tumor cells harvested from mice were suspended in balanced salt solution (BSS) (140 mM NaCl, 54 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, buffered with 10 mM Tris-HCl at pH 7.6 containing 2 mM $CaCl_2$), and 0.25 ml vol of the 10% cell suspension were mixed with equal volumes of suspensions of various HAU of liposomes with spikes in BSS without Ca^{++}. The mixtures were incubated at 0°C for 5 min and then at 37°C for 30 min with shaking. After these treatments, the cells were examined for presence of fused cells in a phase-contrast microscope.

Detection of Viral Antigens on the Cell Surface

Ehrlich ascites tumor cells were washed with BSS. The washed cell suspension (10^7 cells/ml) was treated with 1,000 HAU of liposomes with spikes or with reconstituted envelopes in a manner similar to that described above for "Fusion of Cells." The cells were then washed twice with 10 ml of BSS at 0°C and suspended in one drop of BSS. Then one drop of anti-HVJ envelope serum was added to the cell suspension, and the mixture was incubated at 0°C for 20 min, washed twice with 10 ml of BSS at 0°C, and the cells were suspended in one drop of BSS. One drop of FITC-conjugated anti-rabbit IgG antibody solution diluted 1/10 with BSS was added. After incubation at 0°C for 20 min, the cells were washed at 0°C and observed under a fluorescence microscope.

Formation of Liposomes Containing Fragment A with HVJ Spikes

A mixture of 250 mg of egg yolk lecithin and 33 mg of cholesterol in chloroform and methanol (9:1) was lyophilized in the presence of nitrogen gas.

Suspensions which contained increasing amounts (between 10^4 and 10^5 HAU) of HVJ envelopes were prepared in 10 mM phosphate buffer (pH 7.2) containing 1 mM KCl, 0.3 M sucrose, and 1.8 mg fragment A per ml. 10^4 HAU were equivalent to ~0.5 mg spike proteins and 0.1 mg lipids. About 2 mg of lyophilized lecithin-cholesterol was added to 1 ml of the suspension of HVJ envelopes which were then solubilized with 0.85% NP40. This solution was dialyzed at 0°–1°C in a Neflex tube against the KCl-sucrose-phosphate buffer for 6 days; the buffer was changed each day and the dialysis tube was changed once, after dialysis for 3 days. During dialysis, the solution became turbid and finally reached an OD of 0.4 at 540 nm. To purify fragment A-containing liposomes, we applied the resulting turbid suspension to Bio-Gel A50m Bio-Rad Laboratories (Richmond, Calif.) and collected the void volume. Further purification was accomplished by centrifugation through a linear sucrose gradient (12–40% wt/vol) in 10 mM phosphate-KCl buffer (pH 7.2). The centrifugation tube contained a cushion of 60% sucrose in the same buffer. Liposomes without HVJ spikes were also prepared from 2 mg/ml lecithin-cholesterol and 0.2 mg/ml of lipids extracted from HVJ by the methods described above, except that the HVJ envelopes were omitted.

Toxicity to Mouse L Cells of Liposomes Containing Fragment A

Suspensions of 300 L cells in 2 ml of minimal essential medium (MEM) containing 10% calf serum were incubated in 30-mm plastic Petri dishes for 7.5 h at 37°C. The cells were washed once with 2 ml of chilled BSS, and 0.5 ml of a chilled suspension of fragment A-containing liposomes with spikes was added. The dishes were shaken for 20 min at 4°C, warmed to 37°C, and incubated for a further 20 min. 2 ml of Culture medium was added and incubation was continued for 1 h at 37°C. The medium was replaced by fresh medium and the cells were cultured for 7 days. The cells were finally fixed with methanol and stained with Giemsa. Experiments were performed using duplicate dishes and control dishes containing 180 and 184 colonies.

Electron Microscope Observation

Samples were stained with 3% potassium phosphotungstate (pH 6.7) for negative staining and then observed under a Hitachi 12A type electron microscope at 37 kV. For freeze-fracture, samples were fixed overnight in glutaraldehyde (2.5% solution in cacodylate buffer, pH 7.2) at 0°C, suspended with 25% glycerine in 0.1 M cacodylate buffer, pH 7.2, and incubated at 0°C for 2 h. Then they were quickly frozen in Freon 22 equilibrated with liquid nitrogen.

RESULTS

Purification of Fragment A-Containing Liposomes with HVJ Spikes

A mixture of lecithin, cholesterol, HVJ envelopes, and fragment A was solubilized in sucrose solution containing NP40, dialyzed against sucrose

solution to remove the detergent, and then applied to Bio-Gel A50m to remove free fragment A. As seen in Fig. 1, vesicles containing fragment A were eluted from the gel in the void volume as a single sharp peak. NAD:EF2-ADPR transferase activity, OD 540 nm, and hemagglutinating activity were associated with this peak. Free fragment A was eluted with a peak in fraction no. 36 and was thus well-separated from the vesicles. The fractions in the void volume were collected and applied to a sucrose density gradient to separate the liposomes with spikes from reconstituted envelopes and unbound spikes. As seen in Fig. 2 A, fractions in the void volume gave three peaks: one main peak in tube no. 16 and two small peaks in tubes nos. 3 and 12. In a separate experiment, reconstituted envelopes and nonreconstituted envelope spikes gave major peaks in tubes nos. 3 and 12, respectively. Liposomes with spikes as well as aggregated spikes were found in tube no. 12 by electron microscopy. The top tube no. 16 was found by electron microscopy to contain mainly liposomes with spikes (Fig. 5 *b*). Most of fragment A and of the hemagglutinating activity

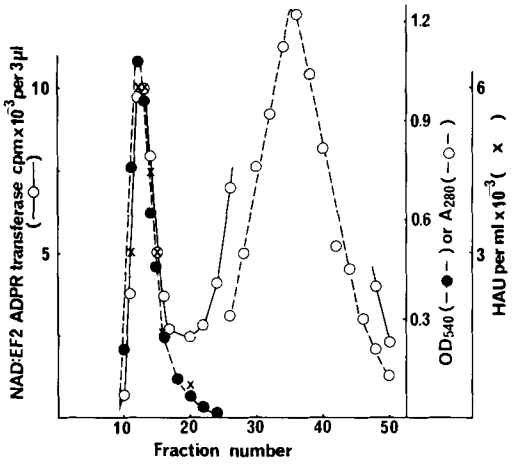


FIGURE 1 Separation of fragment A-containing liposomes with HVJ spikes from free fragment A using Bio-Gel A50m. HVJ envelopes (2.5 mg protein, 5×10^4 HAU), lecithin-cholesterol (6.5 mg), and fragment A (6.2 mg) were solubilized in 3.2 ml of 10 mM phosphate buffer containing 0.3 M sucrose, 1 mM KCl, and 0.85% NP40. The solubilized solution was dialyzed as described in Materials and Methods and was then applied to a Bio-Gel A50m column. Fractions of 20 drops were collected and the absorbance of each fraction was measured at 280 and 540 nm. NAD:EF2-ADPR transferase activity without treatment with NP40 was assayed as described in Materials and Methods. Hemagglutinating activity was also determined.

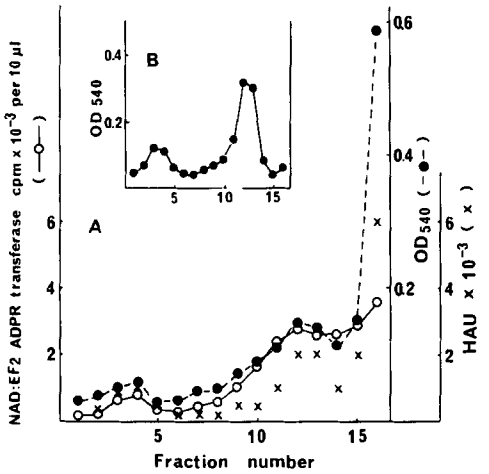


FIGURE 2 Sedimentation analysis of fragment A-containing liposomes with spikes fraction obtained in the void volume on Bio-Gel A50m filtration. Fraction nos. 11–13 of Fig. 1 were collected, applied to a sucrose gradient, and centrifuged at 25,000 rpm (Hitachi RPS 27 rotor) for 15 h at 4°C. Then fractions of 1.1 ml were collected from the bottom. The NAD:EF2-ADPR transferase activity of each fraction was estimated without resolubilization with NP40. (A) HVJ envelopes (5×10^4 HAU) were used for formation of liposomes (3.2 ml). (B) HVJ envelopes (2.5×10^5 HAU) were used for formation of liposomes (3.2 ml). The lipid content and other conditions were the same as for Fig. 2 A. Gel filtration was carried out as described for Fig. 1.

were also found in tube no. 16. Solubilization of the liposomes in tube no. 16 by treatment with NP40 resulted in about ninefold increase in enzymic activity.

On increasing the envelope protein relative to lipid in liposomes, most of the material sedimented with the peak collected in tube 12 (Fig. 2 B). There was a corresponding decrease in the pure liposome fraction at the top of the gradient. The most easily purified liposomes with spike proteins were therefore prepared using between 1 and 3×10^4 HAU of envelope spikes and 2 mg lipid solubilized in 1 ml. Under these conditions, ~0.1–0.2% of total added fragment A was recovered in the fraction of liposomes with spikes and 6–12% of the total added envelopes on the basis of hemagglutinating activity was bound to the surface of the liposomes.

Properties of Liposomes with HVJ Spikes

We tested liposomes with surface spikes for

reactivity with anti-HVJ envelope antibody. When anti-envelope antibody was added to liposomes with spikes, the turbidity of the mixture increased. The sedimentation profile on a sucrose gradient was examined before and after addition of the antibody to the liposome suspension. As seen in Fig. 3, before the addition of liposomes were found in the top fraction, but after treatment with antibody they sedimented as a single sharp peak collected in tube no. 5. More than 90% of the total enzymic activity was recovered in this tube. Solubilization of the liposomes with NP40 resulted in about 30-fold increase in enzymic activity, but the enzymic activity of the top tube was not increased by this treatment. Reconstituted envelopes treated with antibody sedimented to the bottom under these conditions. When, as a control, normal rabbit IgG was added to the liposomes with spikes, the turbidity did not increase. The liposomes with spikes treated with normal IgG were not sedimented and stayed in the top fraction.

It has been reported that concanavalin A aggregates HVJ virions (19), and we found that the purified liposomes with spikes also aggregated by this lectin.

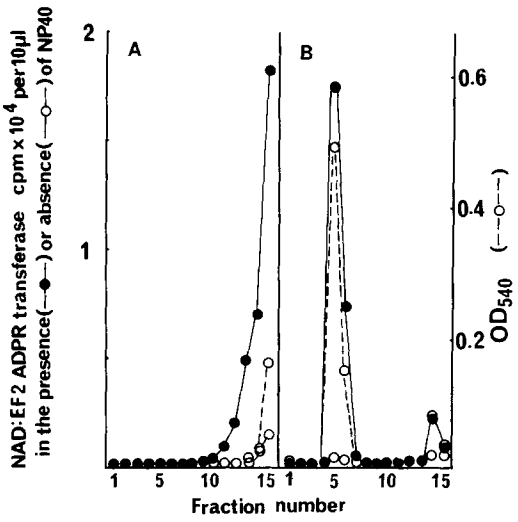


FIGURE 3 Sedimentation analysis of anti-HVJ envelope antibody-treated liposomes. (A) 0.3 ml Top fraction (purified fragment A-containing liposomes with spikes) was applied to the sucrose gradient and centrifuged as described for Fig. 2. (B) 0.3 ml Top fraction (purified fragment A-containing liposomes with spikes) treated with 1.2 ml anti-HVJ envelope antibody was applied to the sucrose gradient and centrifuged. 1-ml fractions were collected from the bottom and, after measuring their absorbance at 540 nm, the NAD:EF2-ADPR transferase activity was measured before and after treatment with NP40.

To determine whether added lecithin participated in formation of the liposomes with spikes, we reconstituted liposomes from [^{14}C]lecithin, HVJ envelopes, and fragment A and then purified them by the same method. Although >20% of the total added [^{14}C]lecithin remained on the top of the Bio-Gel column, ~68% was recovered in the purified fraction of liposomes with spikes. Anti-HVJ envelope antibody was added to the radioactive liposomes and analyzed on a sucrose density gradient under conditions similar to those described above. About 75% of the total radioactivity of the purified liposomes treated with antibody was found in the liposome-antibody complex. Lipids were extracted from the complex with chloroform and methanol, evaporated, and weighed. The weight approximately corresponded to the weight estimated from the radioactivity. These findings suggest that at least 50% of the total added lipids participates in formation of liposomes with spikes in the purified fraction.

Other viral activities of the liposomes with spikes were also examined. The hemolytic activity of the liposomes with spikes was lower than that of reconstituted envelopes and was almost the same as that of the original virus on the basis of hemagglutinating activity. Little if any cell fusion activity was observed on adding the liposomes with spikes to Ehrlich ascites tumor cells. The binding ability of liposomes with spikes to mammalian cells was compared with that of liposomes without spikes and of liposomes with HeLa cell-glycoproteins. When each [^{14}C]lecithin-labeled liposome (~6 μg lipids) was incubated with 0.5 ml Ehrlich ascites tumor cells (2×10^5) for 30 min at 37°C, ~82% of the total added radioactive liposomes without spikes was bound to the cells; however, ~35% of liposomes with spikes and 1% of liposomes with HeLa cell-glycoproteins were bound to the cells. When the binding of fragment A-containing liposomes to the cells was also examined by determination of enzymic activity of fragment A in supernate removed to cell-liposome complex, the resulting data were almost the same as described above. Although the binding amounts of liposomes with spikes were thus less than those of liposomes without spikes, significant amounts of liposomes with spikes could bind to the cells. The presence of viral antigens on cells treated with liposomes with spikes was checked by

the fluorescent antibody technique using anti-envelopes and FITC-conjugated goat antibodies (see Materials and Methods). As seen in Fig. 4, the liposomes with spikes were bound to the cell surfaces and the fluorescence intensity of cells treated with these liposomes was similar to that of cells treated with reconstituted envelopes.

Morphology of Liposomes with HVJ Spikes

The liposomes were negatively stained and observed by electron microscopy before and after purification and compared with reconstituted envelopes and native virions. As seen in Fig. 5 *a* and *b*, HVJ spikes are incorporated into both sides of liposome membrane and appear to be similar to those seen on the outer surface of reassembled envelopes (Fig. 5 *d*) and intact virions (Fig. 5 *e*). Liposomes containing lecithin alone were fragile, showed various forms, and disintegrated on storage at 4°C. Liposomes containing cholesterol in addition to lecithin were more stable, compact,

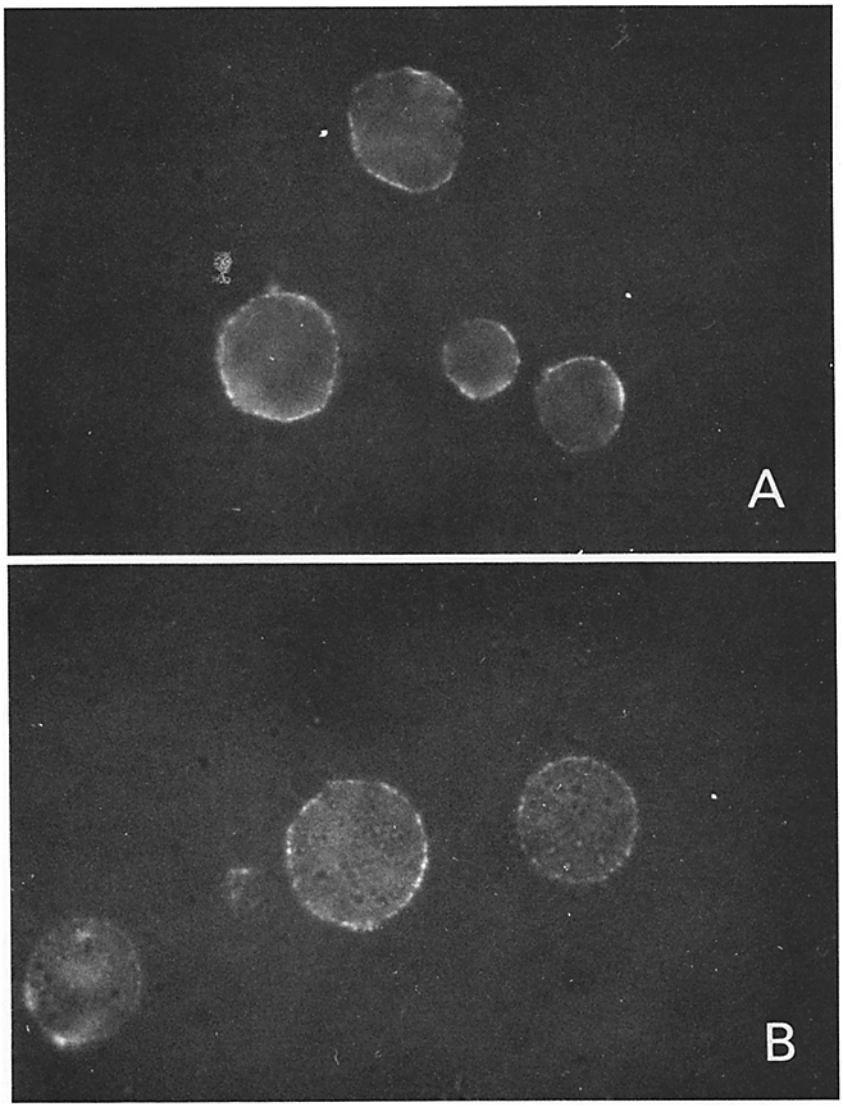
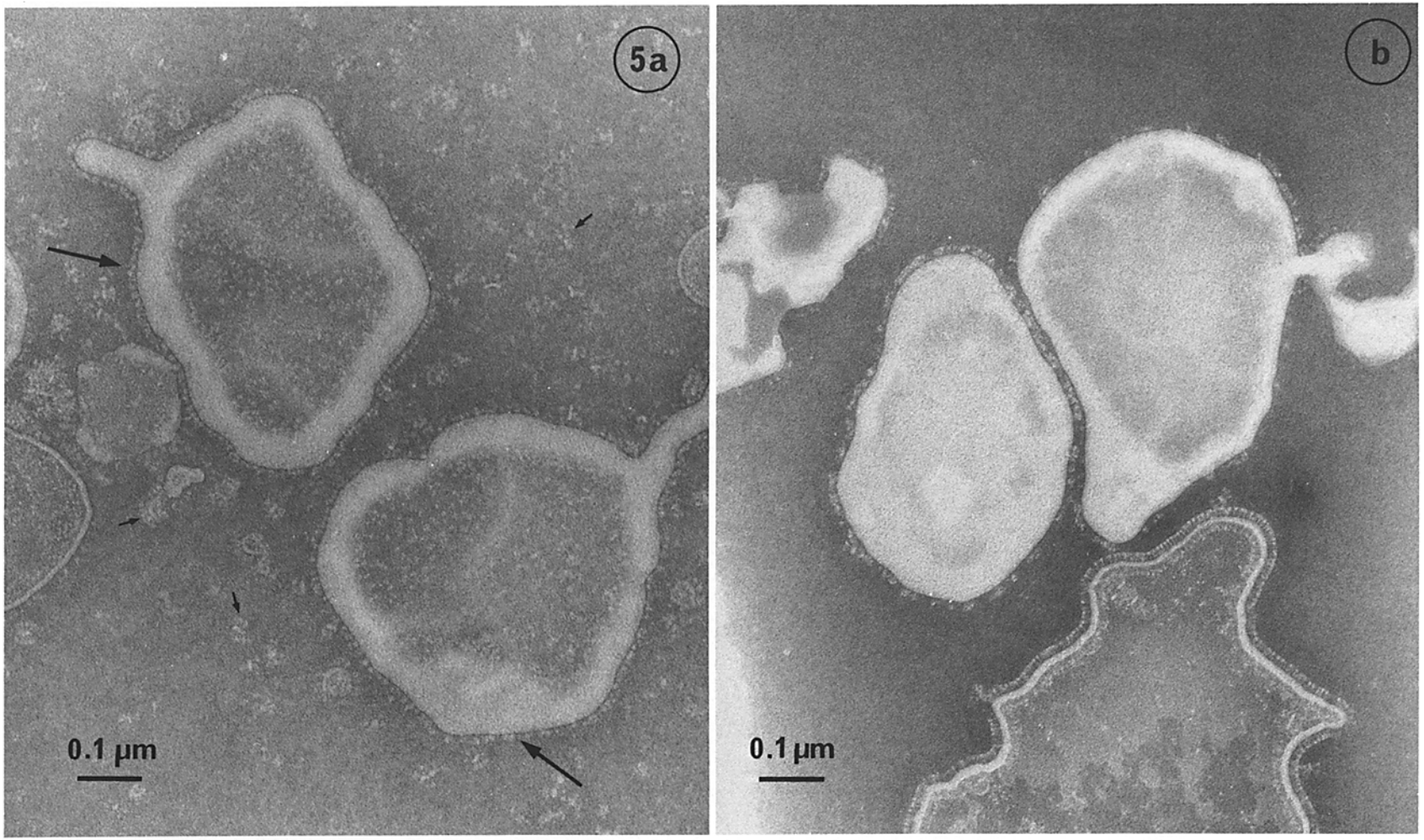


FIGURE 4 Demonstration by the fluorescent antibody technique of the presence of HVJ envelope antigens on Ehrlich ascites tumor cells treated with liposomes with spikes. (A) Cells were treated with liposomes with spikes (500 HAU). (B) Cells were treated with reconstituted envelopes (500 HAU).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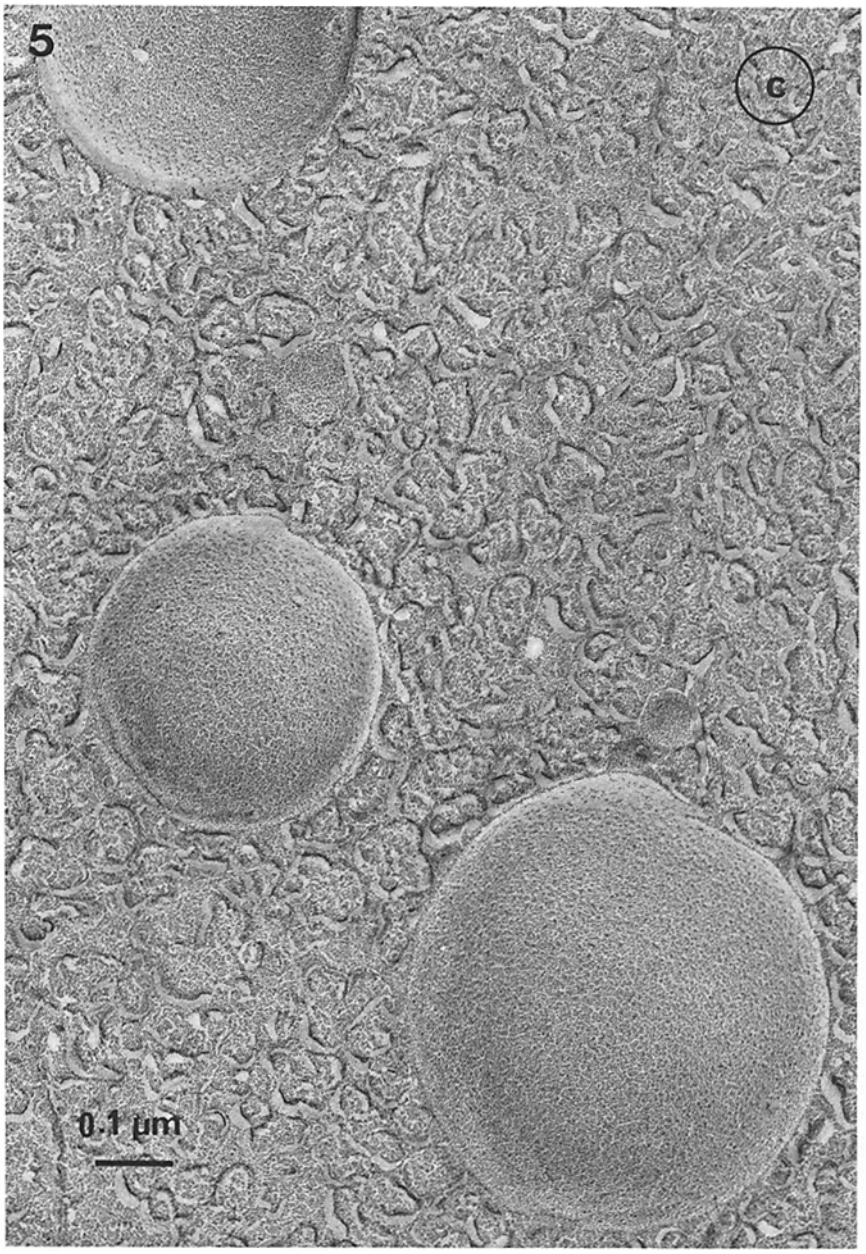
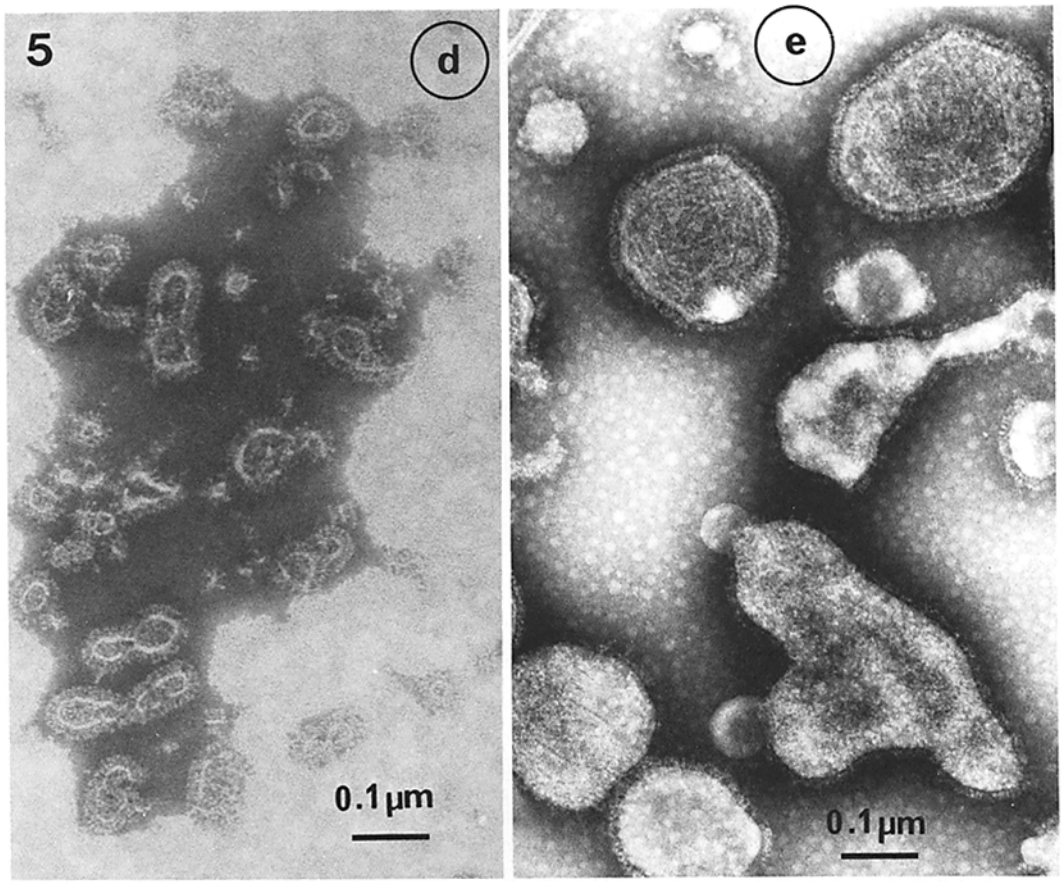


FIGURE 5 Appearance by electron microscopy of liposomes with HVJ spikes (before and after purification), reconstituted envelopes, and native HVJ virions. (*a*) Liposomes with spikes before purification, large arrows denote bound spikes and small arrows denote aggregated spikes. (*b*) Liposomes with spikes after purification. (*c*) Freeze-fracture liposomes with spikes. (*d*) Reconstituted envelopes. (*e*) Native virions. Fig. 5*a*, *b*, *d*, and *e* were negative stained. Fig. 5*c* is a replica of freeze-fracture. × 100,000.

and homogeneous. When the liposomes were freeze-fractured, no large aggregates of particles were seen on either face (Fig. 5*c*) such as those seen in mammalian cell membranes. The liposomes were much larger than those of reassembled envelopes (Fig. 5*a*, *b*, and *d*).



Toxicity of Fragment A-Containing Liposomes with Spikes

To determine the effect of HVJ spike proteins on the ability of liposomes to transport fragment A into the cell cytoplasm, we tested their toxicity for L cells by applying dilutions of fragment A-containing liposomes with and without spikes. As shown in Table I, short exposure to a liposome with spikes suspension containing only 5 ng/ml was sufficient to kill >98% of the cells. Although the binding amounts of liposomes without spikes to the cells were ~2.4 times greater than those of liposomes with spikes, cells treated with 500 ng of fragment A enclosed in liposomes without spikes killed only ~20%. In our preliminary experiments, fragment A-containing liposomes without spikes were reconstituted from lipids extracted from the virus alone, the cytotoxicity of the resulting liposomes was similar to that of liposomes without spikes shown in Table I. Empty liposomes with spikes had no measurable toxicity. These results suggest that the spike proteins associated with liposomes effectively transport fragment A trapped in the liposomes into cell cytoplasm.

DISCUSSION

Although artificial lipid vesicles enclosing a variety of hydrophylic substances within them have been prepared, such liposomal vesicles have not provided us with an effective method for introducing macromolecules into mammalian cells. As previously reported, reassembled HVJ envelopes can be used to introduce protein into cells (35). In the present paper, we have shown that artificially prepared liposomes which contain surface-bound HVJ spike proteins can be used even more effectively to introduce foreign proteins into cells.

In these experiments, we have used liposomes with spikes to introduce the fragment A of diphtheria toxin into mouse L cells. Fragment A is

TABLE I

Toxicity to L Cells of Fragment A-Containing Liposomes with HVJ Spikes

Sample	HAU/ml ng Fragment A/ml	No. of surviving colonies per dish 600 500	 180 150	 60 50	 18 15	 6 5
Fragment A-containing liposomes with HVJ spikes*		0	0	0	1	4
Fragment A-containing liposomes without HVJ spikes‡		135	162	159	182	–
Empty liposomes with HVJ spikes§		169	185	181	170	–
Fragment A		182	179	–	–	–

The effect of each sample on colony formation of L cells was determined as described in Materials and Methods. Colony counts are averages of those in duplicate plates. No entry indicates not tested.

* 0.06 OD 540 nm, 600 HAU/ml, and 500 ng fragment A/ml.

‡ 0.07 OD 540 nm, no HAU, and 500 ng fragment A/ml.

§ 0.075 OD 540 nm, 600 HAU/ml, and no fragment A.

particularly suited for such a study because, although it has no toxicity for living cells in culture medium, if a single molecule of fragment A reaches the cell cytoplasm, the cell cannot survive (37). Thus, a suspension of liposomes containing only 5 ng/ml fragment A is as toxic for L cells as a suspension of reconstituted HVJ envelopes which contain 500 ng of fragment A.

Although the reason for the superiority of artificial liposomes over reconstituted envelopes was uncertain, the following reason may be involved: First, reconstituted HVJ envelopes contain relatively little lipid and therefore soluble substances may leak out more readily than from the more stable liposomal vesicles. Second, liposomes with spikes appear to be, in the average, ~100 times greater in volume than viral envelopes as can be seen from the electron micrographs (Fig. 5).

HVJ spikes are composed of two proteins. One is a hemagglutinin with neuraminidase activity (HANA); the other, known as F protein, is presumed to be involved in fusion of the viral envelope to the cell membrane. Both of these proteins are needed for introduction of the viral genome into the infected cell (7). The requirement for HANA has been inferred from the observation that cells which have been treated with neuraminidase can no longer be infected with HVJ virions. Similarly, we have shown (in unpublished experiments) that L cells treated with neuraminidase are resistant to intoxication by fragment A-containing liposomes with spikes. Although liposomes without spikes were found to bind to cells more effectively than liposomes with spikes, fragment A-containing liposomes with spikes had much more cytotoxic activity than fragment A-containing liposomes without spikes. This suggests that F protein may cause liposome membrane to fuse with cell membrane after binding of the liposomes to cells, and that fragment A is then released into cytoplasm. It thus seems likely that both HANA and F proteins play a role in intoxication of L cells by fragment A-containing liposomes.

Papahadjopoulos et al. (25) have reported that artificial lipid vesicles can stimulate cell-to-cell fusion after prolonged incubation (24 h at 37°C). With HVJ virus, the same amount of cell fusion is observed in <30 min at 37°C. In our experiment, cells and liposomes were only incubated together for 30 min at 37°C, and no cell-to-cell fusion was observed, even by using liposomes with spikes or reassembled envelopes. This suggests that the virus may contain some other factor(s), in addition to the two spike proteins, that is required for promotion of rapid cell-to-cell fusion. It is possible that the system we have described may prove useful in helping to identify factor(s) which promote cell fusion.

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