Proc. Natl. Acad. Sci. USA
Vol. 74, No. 8, pp. 3471–3475, August 1977
Cell Biology

Biological properties of poliovirus encapsulated in lipid vesicles: Antibody resistance and infectivity in virus-resistant cells

(phospholipid vesicle/encapsulation/cellular incorporation)

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Communicated by Edward A. Adelberg, June 8, 1977

ABSTRACT **We present evidence that poliovirus can be encapsulated in synthetic large phospholipid vesicles. The virus associated with the vesicles is found to be (*i*) resistant to antiserum against poliovirus and (*ii*) infectious for cells that are normally resistant to virus infection because of a membrane restriction. Our interpretation of these results is that the virus is entrapped in the interior aqueous space of the vesicles and that this vesicle-associated virus is introduced directly into the cytoplasm of the cells via fusion of the vesicles with the cellular plasma membrane, bypassing the surface receptor-mediated restriction.**

Synthetic lipid vesicles [often referred to as liposomes (1, 2)] have been widely discussed as vehicles for the introduction of foreign materials into eukaryotic cells (3–5). Several groups have successfully introduced a variety of biologically active molecules into cells by this method (6–10). All of the experiments reported so far, however, have used either small unilamellar or multilamellar vesicles that restrict the size and stability characteristics of the molecules to be encapsulated. Many interesting experiments require an encapsulation process that produces vesicles with a large interior aqueous space that can accommodate large macromolecules or molecular aggregates efficiently, but avoids extremes of pH, temperature, detergents, solvents, or sonication. Furthermore, the vesicles must be capable of introducing their contents into the cytoplasm of a cell under physiological conditions. We have previously described a technique for making large unilamellar vesicles (LUV) which involves the fusion of small phosphatidylserine (PtdSer) vesicles by Ca^{2+}, and subsequent removal of Ca^{2+} by EDTA (11). Vesicles prepared from bovine PtdSer, a negatively charged lipid which is fluid at physiological temperatures, are taken up by cells primarily by fusion with the plasma membrane rather than by endocytosis (12). In this paper we present evidence that a large (8×10^6 dalton) molecular aggregate, a picornavirus, can be efficiently encapsulated in LUVs and that this vesicle-associated virus is biologically active when exposed to cells.

Poliovirus, a small nonenveloped plus-strand RNA virus, is easily grown and purified, and simple virus plaque assays provide an unambiguous, sensitive assay system. The virion contains no lipid; thus infection of cells does not initially involve membrane–membrane interactions. Poliovirus requires a primate-specific membrane receptor for normal infection, and the absence of this receptor on the surface of non-primate cells makes them virus-resistant (13, 14). This system has allowed us to test not only whether the encapsulated virus is biologically active but also whether the membrane-mediated restriction of resistant cells can be bypassed by introducing the virion into the cell via a vesicle.

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MATERIALS AND METHODS

Cells and Virus. Poliovirus Type I obtained from the laboratory of D. Baltimore of the Massachusetts Institute of Technology is used to infect monolayers of HeLa S3 cells grown in Eagle's minimum essential medium (EMEM) + 10% fetal calf serum (Grand Island Biological). Virus labeled with ^{35}S is grown as follows: virus stock at low multiplicity of passage is adsorbed to cells, multiplicity of infection = 1, in the absence of serum for 1 hr at 37°; at 5 hr after infection, the normal medium is replaced with EMEM without unlabeled methionine and with 10% dialyzed fetal calf serum and [^{35}S]methionine at 12.5 μCi/ml (specific activity: 315 Ci/mmol; New England Nuclear). Medium and cells are harvested 15–18 hr after infection and Nonidet P40 (Shell Chemical) is added to make a 1% (vol/vol) solution. This solution is clarified by centrifuging 10 min at $2000 \times g$; the supernatant is then spun at $100,000 \times g$ for 2 hr at 4°. The resulting pellets are resuspended in 1× reticulocyte standard buffer (RSB; 10 mM NaCl/10 mM Tris·HCl/1.5 mM $MgCl_2$ at pH 7.4) + 1% sodium dodecyl sulfate (Sigma) and layered onto sucrose gradients as described in the legend of Fig. 1. The peak fractions from gradients are pooled, pelleted by centrifuging at $100,000 \times g$ for 4 hr at 20°, resuspended in buffer, and pelleted by centrifuging twice to remove residual detergent. The final pellet is resuspended in buffer containing 100 mM NaCl, 2 mM histidine, 2 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes), 0.1 mM EDTA at pH 7.4.

Chinese hamster ovary cells (CHO-Kl, American Type Culture Collection) are grown as monolayers in F12 medium buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Grand Island Biological) + 10% fetal calf serum.

Vesicle Preparation. The techniques for making large unilamellar vesicles via Ca^{2+}-induced phase changes in the lipid were described by Papahadjopoulos *et al.* (11); the techniques for encapsulation are described in the *Results*. A brief summary follows. Chromatographically pure PtdSer from bovine brain (15) plus a trace amount of ^3H-labeled dipalmitoylphosphatidylcholine (9) is suspended by vortex mixing for 10 min in NaCl/His/Tes buffer at 10 μmol/ml followed by sonication for 1 hr at 25° in a closed tube placed in a bath-type ultrasonic cleaner (model T-80-80-1RS, Laboratory Supplies, Hicksville, NY). The solution is kept under nitrogen throughout this procedure to minimize oxidation of the lipid. Ca^{2+} is introduced either by direct addition of 100 mM $CaCl_2$ to the sonicated solution (final concentration 10 mM) and incubation at 37° for 1 hr, or by dialysis of the solution against buffer containing 100

Abbreviations: LUV, large unilamellar vesicles; PtdSer, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; EMEM, Eagle's minimum essential medium; RSB, reticulocyte standard buffer; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid; PFU, plaque-forming unit; CHO, Chinese hamster ovary cells.

mM NaCl, 2 mM histidine, 2 mM Tes, and 1.5 mM $CaCl_2$ at pH 7.4 overnight at room temperature. The resulting precipitate is pelleted by centrifuging at 2500 × *g* for 10 min. The pellet is resuspended at a lipid concentration of 10 μmol/ml in a concentrated virus solution or buffer (usually 5 μmol of PtdSer in 0.5 ml) by vortex mixing 10 min at room temperature. Several clean glass beads are added to the solution to facilitate emulsification of the lipid cochleate cylinders. EDTA (100 mM) is added directly to this solution, final concentration 15 mM, and 10–20 μl of 100 mM NaOH is added to adjust the pH to 7.4. The solution is vortex mixed 10 min at 37° followed by incubation for 30 min. The vesicles are then pelleted by centrifuging at 48,000 × *g* for 20 min, washed with buffer, and pelleted again. The final pellet is resuspended in 1 ml of NaCl/His/Tes buffer. All buffers and solutions are sterilized by filtration or autoclaving prior to addition to vesicle preparations.

Fractionation of Vesicles and Virus. Gradients are prepared in 1-ml steps of 50, 30, 10, and 2% Ficoll (Pharmacia) in NaCl/His/Tes buffer, overlayed with a 1-ml sample. Following centrifugation at 300,000 × *g* for 2 hr at 20° in an SW 50.1 rotor, the onput and individual steps are collected by pipette as 1-ml fractions.

Biological Assays of Fractionated Vesicle and Virus Preparation. Aliquots of Ficoll gradient fractions are slowly diluted 1:10 in medium (EMEM) with or without poliovirus-neutralizing antiserum (Flow Laboratories, Rockville, MD) at a concentration capable of inactivating 10^8 plaque-forming units (PFU). Samples containing antiserum are incubated 1 hr at 37°. A series of $\log_{10}$ dilutions of each sample is made in medium, and monolayers of HeLa cells in multiwell plates (Falcon Plastics) are inoculated with 0.1 ml of the dilutions followed by a 3 hr incubation at 37°. The inoculum is removed, the cells are washed, and fresh medium is added. Following a 2-day incubation, plates are stained with 0.1% crystal violet in 10% formalin and plaques are counted.

Fractions to be used for infectious center assays are diluted slowly 1:5 in medium. Pellets of 10^5 CHO or HeLa cells are resuspended in 0.25 ml of these diluted fractions and the suspensions are incubated 2.5 hr at 37° with agitation. The cells are then pelleted, treated with neutralizing antiserum for 30 min at 37°, repelleted, washed with buffer, pelleted again, and resuspended in EMEM + 10% fetal calf serum. Tenfold dilutions of the cells are made in EMEM + serum + 0.2% agarose and plated over HeLa monolayers. Plates are stained with neutral red at 0.2 mg/ml after 3 days.

Virus amplification experiments are performed on monolayers of CHO cells (10^5 cells per cm^2). Following a 3-hr incubation with 1:5 dilutions of Ficoll fractions at 37°, the inoculum is removed and cells are washed, first with buffer, then with 0.5 ml of F12 medium + 10% fetal calf serum. The medium wash is used as a 0 hr supernatant for titration. The cells are incubated 16 hr at 37° in F12 + 10% serum. The cell supernatants are collected, clarified by centrifuging for 10 min at 2000 × *g*, and titrated on HeLa monolayers.

Biohazard Considerations. We are aware that we have modified the host-range of a potential pathogen, if only for one cycle of infection. Therefore, all experiments in which virus is associated with lipid vesicles are performed in tightly sealed vessels or in laminar flow hoods which emit only HEPA- (Baker Co.) filtered air. Further, all associated laboratory personnel have been recently vaccinated against poliovirus.

RESULTS

Encapsulation of Poliovirus in LUV. All poliovirus used in these experiments is purified to ensure against possible contamination by cell membrane components. Clarified supernatants from infected cells are subjected to high-speed centrifugation and the resulting pellets are resuspended and layered onto sucrose gradients containing sodium dodecyl sulfate as described in the legend of Fig. 1. The peak fractions from such a gradient are pooled, pelleted, washed twice to remove any residual detergent, and resuspended in a small volume of buffer solution containing 100 mM NaCl, 2 mM histidine, 2 mM Tes, and 0.1 mM EDTA, at pH 7.4.

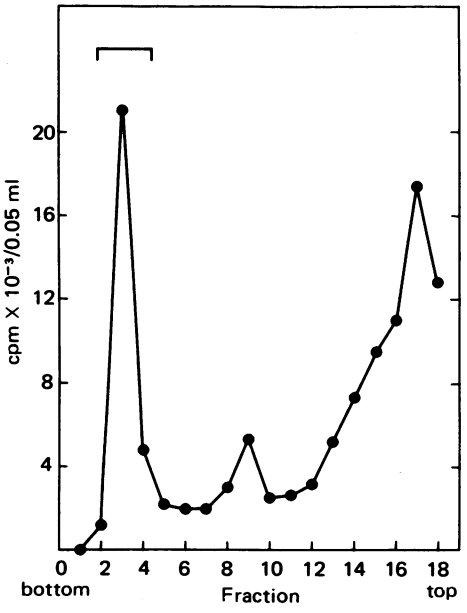

FIG. 1. Sucrose gradient purification of poliovirus. HeLa cells are infected with poliovirus type I, labeled with [^{35}S]methionine and the virus is harvested as described in *Materials and Methods*. The high-speed virus pellet is resuspended in 1× RSB containing 1% sodium dodecyl sulfate, layered onto a 32-ml gradient of 15–30% sucrose + 0.5% sodium dodecyl sulfate buffered with 1× RSB. Sedimentation is for 2 hr at 131,000 × *g*, 20°. The gradient is fractionated; 50-μl aliquots of the fractions are removed, precipitated with trichloroacetic acid, filtered, and assayed for radioactivity in a liquid scintillation counter.

The technique for preparing large unilamellar vesicles of PtdSer encompassing foreign material are essentially those previously described (11). Minor modifications were outlined in *Materials and Methods*. Purified PtdSer plus a trace amount of ^{3}H-labeled dipalmitoylphosphatidylcholine (DPPC) is suspended in buffer by sonication, resulting in dispersion of the lipid into small unilamellar vesicles 200–300 Å in diameter (11). Addition of $CaCl_2$ produces a white flocculent precipitate which, when examined by freeze-fracture electron microscopy, appears to be rolls of large lipid lamellae that we have termed cochleate cylinders (11). Following a low-speed centrifugation to pellet the cochleate cylinders, the wet pellet is resuspended in a concentrated solution of the material to be encapsulated, in this case poliovirus. Addition of EDTA induces formation of large spherical vesicles, entrapping 10–20% of the virus present and causing the flocculent suspension to become opalescent. This solution is vortex mixed and incubated for 30 min at 37°. At this point most of the lipid has been incorporated into the LUV, which vary from 0.1 to 1.0 μm in diameter. To remove the bulk of the nonencapsulated virus, the vesicles are pelleted and washed once by centrifugation at 48,000 × *g* for 20 min. The final pellet is resuspended in 1 ml of NaCl/His/Tes buffer; 50 μl is removed and the radioactivity is measured. This material in a typical experiment accounts for approximately

Table 1. Fractionation of vesicle and virus preparations

Ficoll fraction	Phospholipid (^{3}H) and virus (^{35}S) present in each fraction, cpm × 10^{-3}/ml							
	Encapsulated poliovirus		Poliovirus mixed with preformed vesicles		Vesicles alone		Poliovirus alone	
	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H	^{35}S
Onput	52.9	1.5	21.0	0.9	24.1	—	—	0.8
2%	370.9	18.9	163.9	2.7	219.7	—	—	0.4
10%	17.4	3.7	2.4	5.8	6.4	—	—	2.9
30%	5.2	7.4	3.2	88.0	3.1	—	—	80.6
50%	2.1	1.9	2.0	17.7	2.5	—	—	18.7

Samples of [^{3}H]DPPC-labeled vesicles and/or [^{35}S]methionine-labeled poliovirus are centrifuged in Ficoll velocity step gradients for 2 hr at 300,000 × *g* in an SW 50.1 rotor. One-milliliter fractions are collected; 100 μl of each is removed, precipitated with trichloroacetic acid, and filtered, and the radioactivity is measured. Counts in each channel are corrected for crossover where necessary. Poliovirus "encapsulated" in vesicles: 2×10^5 cpm poliovirus are mixed with 5 μmol of PtdSer + 5×10^5 cpm of [^{3}H]DPPC prior to formation of LUV by addition of EDTA; following LUV formation and washing, gradient onput radioactivity equals 4.5×10^5 ^{3}H cpm and 3.5×10^4 ^{35}S cpm. Poliovirus mixed with preformed vesicles: 2.5 μmol of PtdSer + 2.5×10^5 cpm of [^{3}H]DPPC are used to form LUV in buffer; after washing these LUV are mixed with poliovirus and put on a gradient. Onput radioactivity equals 2×10^5 ^{3}H cpm + 2×10^5 ^{35}S cpm. Vesicles alone: 2.5 mol of PtdSer + 2.5×10^5 cpm of [^{3}H]DPPC are used to form LUV in buffer; after washing onput radioactivity equals 2.6×10^5 ^{3}H cpm. Poliovirus alone: 1×10^5 cpm ^{35}S-labeled poliovirus is put on the gradient.

90% of the total lipid and approximately 20% of the initial virus, of which more than 50% appears to be encapsulated (Table 1). In parallel experiments where the same procedure was used in the presence of 10 mM sucrose, with or without virus, it was found that a similar percentage (10–20%) of the initial sucrose was associated with the vesicles (unpublished data). Because sucrose does not adsorb to phospholipids and is only captured within the vesicles' internal aqueous space (2), we assume that most of the vesicle-associated virus is similarly encapsulated within the vesicles.

Isolation and Characterization of Lipid-Associated Poliovirus. We further isolate the virus associated with the vesicles by fractionating the pelleted virus–LUV preparations on discontinuous Ficoll gradients consisting of 50, 30, 10, and 2% steps. Following centrifugation for 2 hr at 300,000 × *g*, individual steps are collected as fractions. A small aliquot of each fraction is removed and the radioactivity is measured. Table 1 shows the results of an experiment with [^{35}S]methionine-labeled virus and vesicles containing [^{3}H]DPPC; approximately 50% of the viral radioactivity sediments with the lipid. This represents approximately 10% of the original input virus, because the washing removes 80% of the initial ^{35}S-labeled material. When the virus is added to preformed LUV only about 2% of the virus label sediments with lipid. We conclude that the fraction of the virus with slower sedimentation properties has formed a stable association with the lipid vesicles and that this association occurs at a significantly higher rate when virus is added prior to the final stages of vesicle formation.

The assays for biological activity of the various gradient fractions are designed to answer two questions: is the vesicle-associated virus infectious? and is this virus accessible to neutralizing antibody? Each fraction is titered on permissive cells (HeLa) before and after antibody treatment; the results are shown in Table 2. The lipid-associated virus had a significantly

Table 2. Assays of plaque-forming units on HeLa cells

Ficoll fraction	Encapsulated poliovirus		Poliovirus alone		Vesicles alone
	−Ab	+Ab	−Ab	+Ab	
Onput	10^5	10^5	10^5	0	0
2%	10^7	10^6	$5 \cdot 10^3$	0	0
10%	$5 \cdot 10^6$	10^3	10^7	10^1	0
30%	10^8	10^2	10^8	$5 \cdot 10^1$	0
50%	$5 \cdot 10^6$	0	10^7	0	0
Control*			10^8	$5 \cdot 10^1$	

Fractions from virus-containing Ficoll step gradients are titered on sensitive (HeLa) cells before and after exposure to poliovirus-neutralizing antiserum (Ab); fractions from a preparation of vesicles alone are tested for overt cytotoxic effects. Virus in 10-fold dilutions is adsorbed to HeLa cells for 3 hr at 37°, the inoculum is washed away, and fresh medium is added to the monolayers. After a 2-day incubation, the plates are stained and plaques are counted. Titers are expressed in plaque-forming units/ml (PFU/ml), rounded off to the nearest factor of 5. Recovery of onput infectivity was 95–100%.
*Control: virus alone (30% Ficoll fraction) mixed with vesicles alone (2% Ficoll fraction).

higher titer than a parallel fraction (2% Ficoll) from a gradient containing virus alone, suggesting that the lipid–virus complex is infectious. Antiserum treatment of fractions containing virus but no lipid results in a 10^4-fold reduction of infectivity. Similar treatment of an encapsulated virus fraction reduces the titer by only 10-fold, indicating that a substantial fraction of this virus is not subject to the neutralizing action of the antiserum. Control experiments indicate that preformed vesicles do not inhibit antibody-mediated neutralization of the virus. Our interpretation of the antiserum resistance is that the virus is inside the vesicles, the lipid bilayer forming a barrier between encapsulated virions and external antibody molecules. A possible explanation for the disparity in the titer of the encapsulated virus fraction before and after antiserum treatment lies in the expected efficiency of infection with free versus encapsulated virus. In the absence of antibody unencapsulated virus can initiate an infection by the normal route, whereas encapsulated virus requires both interaction between the lipid vesicle and the cell and subsequent initiation of infection by the virus. Preliminary data indicate that only about 1% of the vesicle population becomes cell associated. This indicates that the infectious process with encapsulated virus may be considerably less efficient than normal infection, giving a titer biased in favor of the free virus present as a contaminant or virus released by lysis or breakage of the vesicles.

We have used Chinese hamster ovary (CHO) cells to test the ability of vesicle-associated poliovirus to bypass the receptor-mediated restriction of non-primate cells. Cells are exposed to encapsulated or naked virus fractions, washed with antibody after 3 hr, and tested for their ability to form infectious centers when overlayed on permissive cell monolayers. The data presented in Table 3 demonstrate that only encapsulated poliovirus is capable of infecting CHO cells. When a parallel sample of encapsulated poliovirus is subjected to the same treatment in the absence of CHO cells, no infectious centers are formed, indicating that virus-containing vesicles alone are not responsible for the infections. When virus and preformed vesicles are mixed with cells under the same conditions, infectious centers are produced at a very low level of efficiency. Several mechanisms could be responsible for these latter infections: virus could be trapped during a fusion event between a vesicle and a cell, entering the cytoplasm during the rearrangement of the membrane; virus could be similarly trapped during a vesicle-

Table 3. Assays of infectious centers on CHO cells

Inoculum	Infectious centers formed		
	10^4 CHO cells	10^3 CHO cells	10^2 CHO cells
Encapsulated poliovirus	Confluent lysis	12, 11, 9	4, 2, 4, 1
Poliovirus alone	0	0	0
Poliovirus mixed with vesicles	0, 2	0	0
Vesicles alone	0	0	0

Poliovirus-resistant cells are used to assay the virus and vesicles for the ability to form infectious centers. Following a 3-hr incubation with the virus and/or vesicle preparation, CHO cells are treated with poliovirus-neutralizing antibody and washed. Tenfold dilutions of these cells then are plated in agarose over virus-sensitive cell monolayers. Cultures are incubated for 3 days, stained with neutral red, and plaques are counted. Encapsulated poliovirus: 0.25 ml of a 1:5 dilution of a 2% Ficoll fraction (= 10^5 antibody-resistant PFU and 250 nmol of lipid) is mixed with 10^5 CHO cells. Poliovirus alone: 0.25 ml of a 1:5 dilution of a 30% Ficoll fraction of virus alone (= 5×10^6 antibody-sensitive PFU) is mixed with 10^5 CHO cells. Poliovirus mixed with vesicles: 0.125 ml of a 2:5 dilution of a 2% Ficoll fraction of vesicles alone (= 250 nmol of lipid) + 0.125 ml of a 2:5 dilution of a 30% fraction of virus alone (= 5×10^6 antibody-sensitive PFU) are mixed with 10^5 CHO cells. Vesicles alone: 0.25 ml of a 1:5 dilution of a 2% Ficoll fraction of vesicles alone (= 250 nmol of lipid) is mixed with 10^5 CHO cells. Infectivity of samples is determined by the method described for Table 2.

induced fusion between two cells (16); or virus could be "encapsulated" when two vesicles fuse with each other. The fact that infectious centers are formed at a significantly higher efficiency by encapsulated virus than by virus mixed with preformed vesicles indicates that these mechanisms are not responsible for the ability of the vesicle encapsulated virus to overcome the receptor restriction. The most likely mechanism for the infectious properties of this virus is fusion of the lipid vesicle with the cell and release of the encapsulated virus into the cytoplasm.

Additional data demonstrating the infection of CHO cells with encapsulated virus is provided by experiments measuring the ability of the encapsulated virus to replicate in resistant cells. CHO cells are exposed to gradient fractions containing encapsulated virus, virus alone, or virus plus preformed vesicles. After the inoculum is washed away, the cell supernatants are titered on permissive cells at 0 and 16 hr. The data in Table 4 indicate that CHO cells can amplify poliovirus only when vesicle-encapsulated virus is used; cells exposed to virus alone or virus mixed with preformed vesicles do not produce progeny virions.

Table 4. Virus amplification in CHO cells

Inoculum	Poliovirus, PFU/ml cell supernatant	
	0 hr	16 hr
Encapsulated poliovirus	$2 \cdot 10^1$	$5 \cdot 10^4$
Poliovirus alone	—	10^1
Poliovirus mixed with vesicles	—	10^1

Amplification of poliovirus in resistant cells is measured by exposing monolayers of CHO cells to virus preparations for 3 hr at 37°, washing away the inoculum, and assaying the amount of poliovirus in the cell supernatant at 0 and 16 hr after the incubation. Encapsulated poliovirus: 0.1 ml of a 1:5 dilution of a 2% Ficoll fraction (= 2×10^4 antibody-resistant PFU, and 100 nmol of lipid) is used as inoculum. Poliovirus alone; 0.1 ml of a 1:5 dilution of a 30% Ficoll fraction of virus alone (= 10^6 antibody-sensitive PFU) is used as inoculum. Poliovirus mixed with vesicles: 0.05 ml of a 2:5 dilution of a 2% Ficoll fraction of vesicles alone (= 100 nmol of lipid) and 0.05 ml of a 2:5 dilution of a 30% Ficoll fraction of virus alone (= 10^6 PFU) are used as inoculum. Infectivity of samples was determined by the method described for Table 2.

DISCUSSION

The evidence we present in this paper demonstrates that poliovirus virions can be associated with a lipid vesicle and transported into CHO cells. Most (>90%) of the vesicle-associated virus appears to be encapsulated within the interior aqueous compartments of the vesicles rather than adsorbed on the surface. This interpretation is based on the following data. (*i*) The amount of virus associated with the vesicles is consistent with predictions made on the basis of sucrose capture, a reliable measure of the trapped internal aqueous space (1, 2). (*ii*) Other macromolecules such as synthetic polynucleotides (17) and ferritin show similar efficiencies of entrapment. Freeze-fracture electron microscopy of LUV reveals spherical structures containing visible ferritin molecules within the interior aqueous space of the vesicle at approximately the same concentration as in the external bulk phase (unpublished observations). Freeze-fracture electron micrographs of virus-containing vesicle preparations show similar vesicle morphology. However, poliovirions have not been observable in these preparations, probably because of the low virus-to-vesicle ratio (about 10^{-3} virions per LUV). (*iii*) A substantial fraction of the virus sedimenting with lipid is refractory to neutralizing antiserum, suggesting that the lipid bilayer prevents access of the antibody molecules to the encapsulated virus particles. Control experiments demonstrate that lipid vesicles do not interfere with this antigen–antibody reaction when naked virus is used. The fact that we see a fraction of the virus associated with the vesicles in an antibody-sensitive form remains unexplained (see *Results*).

Several mechanisms can be envisaged for the uptake of encapsulated virus by the cells. These include fusion of the lipid vesicle with the cell membrane, endocytosis by the cell, or modification of the cell surface followed by viral entry (5). We prefer the interpretation that the virus is introduced into cells following fusion of the vesicles with the cellular plasma membrane. Previous studies have shown that similar vesicles that are "fluid" and negatively charged introduce their contents into cells predominantly by fusion rather than by an endocytotic mechanism (12). These conclusions are based on experiments which demonstrate that insertion of small molecules into cells with PtdSer vesicles is independent of cellular energy metabolism and is unaffected by treatment of cells with cytochalasin B or glutaraldehyde (12). These studies used unilamellar (sonicated) and multilamellar (shaken) vesicles, but recent experiments with LUV composed of pure PtdSer indicate that their uptake by 3T3 cells proceeds by a similar mechanism. It is possible, however, that cellular uptake of these and other vesicles by CHO and HeLa cells involves mechanisms in addition to fusion.

The fact that we can introduce into a cell a complex structure about 300 Å in diameter suggests that this type of vesicle can be used as a vehicle for the introduction of large macromolecules into cells. We need not expose the virus to significant stresses such as detergent or sonication and we do not subject the cells to any special pretreatment. Thus, the system should be flexible in terms of the structure and stability of the molecule to be encapsulated and the type of cell or tissue used as a target. Further, lipid vesicles do not seem to be cytotoxic: PtdSer at up to 500 nmol/ml (approximately 5×10^{10} LUV per ml) can be

applied to 10^5 cells without significantly affecting the plating efficiency or growth of the cells (unpublished reuslts).

Several facets of this system are now available for study. The relative efficiencies of infection of encapsulated virus versus virus alone in susceptible cells can be determined. It might also be possible to use this vesicle system to infect virus-resistant cells with purified poliovirus RNA. We feel this latter system could be very useful if we could improve upon previously reported efficiencies of RNA infection of cells (18). We have shown that synthetic double-stranded RNA molecules apparently can be introduced into cells via vesicles, but these structures represent unusually stable molecules without size or information constraints (19). The potential for extending the poliovirus and poliovirus RNA model systems to include insertion of mRNAs and other genetically active material is apparent and we hope to proceed in this direction.

The results reported here also suggest a useful model system for studying the interaction of picornaviruses with cells. It is clear that we have subverted the membrane restriction on poliovirus–hamster cell interaction, but the details of this infectious process are not understood. As discussed above, it is possible that the vesicles fuse with the cell membranes, emptying their contents into the cytoplasm. If this is the introductory mechanism, one wonders where the alterations in picornavirus structure that are part of normal infection occur. Lonborg-Holm has demonstrated (17) that upon association with cellular membranes, poliovirus assumes at least one identifiable intermediate form, designated the "A particle". It could now be possible to determine whether this intermediate can be detected upon association of virus with the lipid bilayer or whether it appears upon addition of the virus-vesicle complex to cells. The results of Lonborg-Holm using neutral lipid vesicles suggest that the structural alteration increases the lipophilic properties of the virus because only "A particles" associate with such vesicles (20).

In conclusion, phospholipid vesicles are a simple and efficient system for the introduction of biologically active macromolecules into cells; they may also represent a useful model system for the study of interactions of nonenveloped viruses with cell membranes.

We thank Dr. Karl Lonborg-Holm for valuable discussions and advice. This work was supported in part by grants from the National Institutes of Health: CA-16705 and AI-14042 (to R.T.), CA-18527 and GM-18921 (to D.P.), and the American Cancer Society: IN-54P-16 (to T.W.).

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