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Liposome Encapsulation of Retrovirus Allows Efficient Superinfection of Resistant Cell Lines

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Cell lines which are infected with retrovirus are resistant to superinfection by a related retrovirus. Packaging of whole virions within synthetic lipid vesicles allows efficient infection of such resistant cell lines. This system is more efficient in introducing encapsulated virus into infected cells than into uninfected cells.

Infection of a permissive cell line with a nondefective retrovirus yields a cell line which is a chronic producer of that virus and which is resistant to superinfection by related retroviruses (interference). Studies of avian leukosis virus and sarcoma virus infections (21, 25, 26, 29), as well as a number of mammalian retrovirus systems (11, 22, 23), have indicated that an endogenously produced viral glycoprotein binding to surface viral receptors causes the interference (7, 25, 26). Several techniques exist to overcome this block to superinfection. A different type of viral receptor on the cell surface can be employed if the virus to be superinfected is pseudotyped with a nonrelated retrovirus or other animal virus (4, 31). Alternatively, production of viral glycoprotein by the infected cell can be transiently blocked by inhibitors of glycosylation, making some of the cellular virus receptors available to bind the superinfecting virus (2, 20). Another option is use of a DNA copy of the retroviral RNA genome which can be transfected into the chronically infected cell line.

An alternative and simpler technique to those used previously would be to package virions (or virion cores) into synthetic lipid vesicles. Fusion of these vesicles with cellular membranes might then mediate an infection.

Large, unilamellar lipid vesicles can be prepared in a number of ways, including calcium-EDTA chelation (18, 27), ether injection (3, 6), removal of detergents (8, 19), and reverse-phase evaporation (28). We have found only one of these methods, reverse-phase evaporation, to be practical and efficient enough to mediate retrovirus infection. In our hands, these reverse-evaporation vesicles (REV) are without any demonstrable toxicity in all cell lines tested, including the mouse fibroblast lines NIH-3T3, BALB/c-3T3, and L-cells, a murine lymphocyte line, a mink cell line (CCL-64 [11]), and the human HeLa cell line, even at lipid concentrations as high as 66 μmol of lipid per ml and >100 μmol of lipid per 10^6 cells. Encapsulation by large, unilamellar vesicles is essentially a passive process, the result of entrapment of the aqueous phase of an emulsion as the vesicles are formed (17). The relatively large internal volumes of REV allow passive entrapment of 40 to 60% of an aqueous suspension of proteins or nucleic acids (28). To examine whether we could attain this efficiency, ^{32}P-labeled 28S RNA was used as a marker, and entrapment was measured by exclusion chromatography. RNA rather than whole virions was examined because the large size of the virions makes chromatographic separation of free virions from vesicle-encapsulated virions difficult. About 45% of the input 28S ribosomal RNA was recovered in vesicles in a typical REV preparation that incorporated both (unlabeled) retroviral virions and labeled RNA (Fig. 1). In this case, 45% is an underestimate of encapsulation because smaller vesicles would not elute in the void volume of the chromatographic column.

To examine the biological properties of an REV-encapsulated retrovirus, we used the virus produced by the G8-124 line of Moloney murine sarcoma virus (MSV)-producing cells (1). The ratio of defective sarcoma virus to the helper virus, Moloney murine leukemia virus (M-MuLV), in a typical preparation was 98:2 (15). The focus-forming unit (FFU) titer on uninfected NIH-3T3 cells for a typical 24-h harvest from G8-124 ranged from 1×10^6 to 3×10^6 FFU/ml (Table 1). Because this defective MSV was pseudotyped with the helper M-MuLV, NIH-3T3 cells which were productively infected with M-MuLV and cloned (M-NIH cells) or which were infected with a defective variant of M-MuLV so that only the M-MuLV glycoprotein was produced (clone M-13 cells) would be expected to be resistant to superinfection with MSV. Table 1 demonstrates a 10^4-fold decrease in the FFU titer of an MSV stock on M-NIH cells or M-13 cells.

The titers of different preparations of MSV-containing REV were determined on each of three cell lines (NIH-3T3, M-NIH, and M-13). The FFU titer of REV prepared from 1 ml of an MSV stock having a titer of 1×10^6 to 3×10^6 FFU/ml was approximately 2×10^3 FFU/ml on NIH cells. Although attempts were not made to separate unencapsulated MSV virions from REV-encapsulated ones, it is doubtful that any of this focus-forming activity resulted from unencapsulated, intact MSV virions because the combined treatment of an organic solvent (diethyl ether) and sonication is likely to have functionally disrupted the virions. Indeed, simple active mixing of a stock of MSV with ether, followed by evaporation of the ether, resulted in complete loss of focus-forming activity (data not shown). Preparations of liposomes made without MSV had no focus-forming activity.

When the same preparations of MSV-containing liposomes were tested for their ability to form foci on M-NIH cells, their FFU titers were substantially increased over those found on uninfected cells (Table 1). On M-13 cells, the titers of the liposome preparations were similar to those found on uninfected NIH-3T3 cells.

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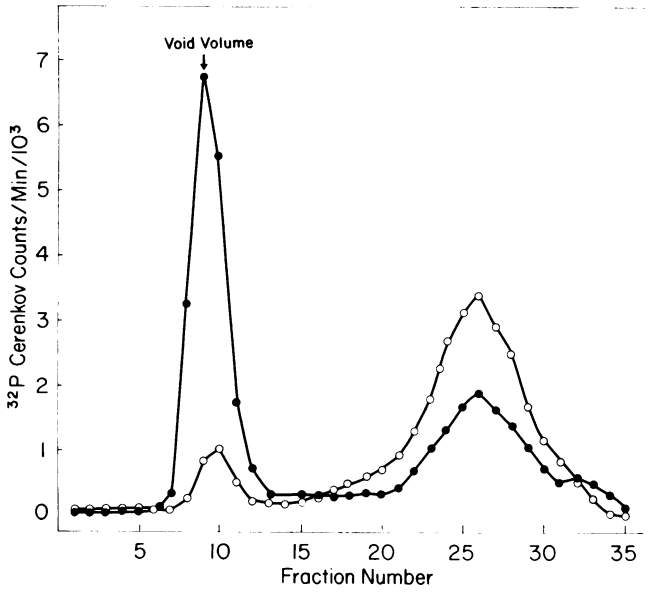

FIG. 1. Encapsulation of macromolecules into liposomes. Formation of REV is essentially as described by Szoka and Papahadjopoulos (28). We placed 33 μmol of cholesterol (Sigma Chemical Co.), 24.6 μmol of phosphatidylcholine (ex Egg, Calbiochem-Behring) and 6.6 μmol of phosphatidylglycerol (Sigma) in a flask on a rotary evaporator, and the solvent (chloroform) was removed under vacuum. Care was taken throughout the liposome preparation to keep all lipids under a nitrogen atmosphere. The lipids were redissolved in 3 ml of redistilled diethyl ether. One milliliter of phosphate-buffered saline (13.6 mM NaCl, 0.26 mM KCl, 0.8 mM $NaHPO_4$, 0.14 mM KH_2PO_4) containing 100 mg of yeast tRNA (Sigma) as carrier and MSV virions was added to the lipid-ether solution. In one preparation, this aqueous phase also contained tracer amounts of ^{32}P-labeled 28S ribosomal RNA. The two phases were sonicated in a bath-type sonicator at 0°C for 60 s, by which time a stable, milky dispersion had formed. The mixture was then placed on a rotary evaporator, and the ether was removed under vacuum at 25°C. After 5 min, an additional 2 ml of phosphate-buffered saline was occasionally added to facilitate suspension of the vesicles. Evaporation was continued for 30 min to ensure removal of all traces of the ether. In one preparation of REV, tracer amounts of ^{32}P-labeled 28S RNA were added at this point to monitor nonspecific adhesion of RNA to preformed REV. One milliliter of the vesicle preparations was then chromatographed over a 25-ml-bed-volume Bio-Gel A15 column, with phosphate-buffered saline as the buffer, to allow separation of vesicles and unencapsulated RNA. Fractions of 1 ml were collected, and the radioactivity was quantitated by Cerenkov counting. REV eluted in the void volume. All manipulations involving retroviruses were performed in closed containers or in laminar flow hoods which emit only filtered air. Symbols: ●, elution pattern of 28S RNA added before REV formation; ○, pattern of 28S RNA added to a preformed REV preparation.

Over many years, it has proved difficult to devise a method for concentrating a stock of retrovirus that preserves the biological activity of the virions. The usual concentration techniques involving centrifugation or ultrafiltration appear to damage the virions, possibly by stripping away glycoprotein from the viral membrane and rendering the virions noninfectious. Typically, after concentration procedures, no increase or even a decrease in viral titer is observed. For instance, a stock of MSV with an initial titer of 3×10^6 FFU/ml, after a 10-fold physical concentration (pelleting of virions from 10 ml of stock and resuspension in 1 ml), can produce a stock with a titer of only 3×10^3 FFU/ml (Table 1). When 1 ml of this physically concentrated virus was encapsulated into REV, however, the biological titer of the virus stock was increased up to eightfold on both infected and uninfected cells.

REV can be prepared sterilely so that the vesicles can be immediately applied to cells in culture. To remove bacteria and fungi from an REV preparation which has not been prepared sterilely, however, an REV preparation was filtered through a membrane filter (pore size, 0.8 or 0.45 μm) by using vacuum rather than pressure. Only a three- to fourfold loss of activity of an REV preparation was seen after filtration (Table 1). The combination of filter steriliza-

TABLE 1. Titers of virus and REV

Virus preparation	Titer (FFU/ml) on cell line:		
	NIH	M-NIH[a]	M-13[b]
MSV stock[c]			
No. 1	1×10^6	2×10^2	1×10^2
No. 2	3×10^6	4×10^2	1×10^2
REV preparation[d]			
No. 1	2.1×10^3	2.7×10^4	1×10^4
No. 2	1.8×10^3	3×10^4	7×10^3
Concentrated (10-fold) MSV stock[e]	3×10^3	NT[f]	NT
Concentrated (10-fold) MSV stock in REV[g]			
No. 1	8×10^3	1×10^5	2×10^4
No. 2	1.2×10^4	2×10^5	1×10^4
REV preparation after filtration[h]			
No. 1	9×10^2	6×10^3	NT
No. 2	1.5×10^3	1.2×10^4	NT

[a] Contact-inhibited clonal line of NIH-3T3 fibroblasts which have been chronically infected with M-MuLV clone 1 (9).

[b] Cloned cell line described by Shields et al. (24) which contains a defective M-MuLV provirus. It produces viral glycoprotein (gp70), but no infectious progeny virus, and does not appear to produce reverse transcriptase. It has previously been shown resistant to superinfection by MuLV and M-MuLV pseudotyped vesicular stomatitis virus.

[c] MSV stocks were titrated as follows. Cells were plated out at a density of 2×10^5 cells per 60-mm dish on the day before infection. All cell lines were carried in Dulbecco modified Eagle essential medium–10% calf serum. On day 0, 0.4 ml of serial 10-fold dilutions of MSV stocks were added to each plate in the presence of 8 μg of polybrene (Sigma) per ml of medium. The infection was continued for 1 h at 37°C with periodic rocking of the plates. The medium was then changed, and the cells were refed every 3 days until day 12, at which time foci were scored. All assays were performed in triplicate.

[d] Two representative preparations of REV prepared from MSV stocks 1 and 2, respectively. One milliliter of virus stock yielded 1 ml of liposomes. After preparation of the REV as described in the legend to Fig. 1, 1 ml of the REV suspension was added to a 60-mm dish containing 5 ml of medium and 2×10^5 cells plated out the day before. Cells were exposed to the REV suspension for 2 h at 37°C, after which the medium was changed. Cells were refed every 3 days, and foci were scored on day 12. Exposure of cells to REV prepared in the absence of MSV resulted in no foci.

[e] Virion stocks were physically concentrated by pelleting of the virus (centrifugation at 100,000 × *g* for 2 h at 4°C) and resuspension in 1/10 of their initial volumes. One milliliter of this volume was then titrated or used to make 1 ml of an REV preparation.

[f] NT, Not tested.

[g] REV prepared from the concentrated MSV stocks described above.

[h] REV preparations 1 and 2, after filtration under vacuum through an 0.8-μm-pore-size filter.

tion and the use of antibiotics in the tissue culture medium has been completely effective in preventing contamination of cell cultures after exposure to REV.

This technique for introducing retrovirus into cells is a useful method for overcoming the barrier to superinfection in cells already infected with retrovirus. Encapsulation of a picornavirus in small, unilamellar vesicles with retention of biological activity has previously been described (30). Transfection studies have shown, however, that all that is required for the initiation of picornavirus infection is entry of the RNA genome into the cell cytoplasm (13, 14). Infection by a retrovirus, on the other hand, requires at least the presence of an active reverse transcriptase in addition to an intact dimeric RNA genome and tRNA primer and perhaps other interactions between virion proteins and the viral genome. Retroviral genomic RNA alone is not infectious (12).

It would appear, then, that despite the functional disruption of the virions in the course of REV preparation, sufficient amounts of reverse transcriptase and genomic RNA (and perhaps other required proteins) remain together to allow synthesis of a provirus upon entry into a cell. In our hands, other techniques of liposome preparation, including removal of detergents (8) and calcium-EDTA chelation (18), appear too damaging to virions because, despite relatively efficient encapsulation, no biological activity would be recovered from MSV virions packaged in those ways.

Physical disruption may account for part or all of the loss of titer on uninfected cells of MSV stocks after packaging into REV. In addition to physical damage of virions, other reasons for the loss of titer can be suggested. The reverse transcriptase, for instance, may be adversely affected by the organic solvent employed in REV preparation. The effects of sonication on the integrity of the genomic RNA molecule could be implicated in the loss of biological titer, but there is reason to believe that this effect probably is not a major problem, because poliovirus RNA, which is approximately the same size as retroviral genomic RNA (9,000 bases), can be introduced into cells via REV with a specific infectivity per microgram of RNA comparable to that achieved by other transfection techniques (30; unpublished observations).

The efficiency of this system for introduction of a second retrovirus into infected cells is high (10^2 to 10^3 enhancement with concentrated virions), rivaling that reported when glycosylation inhibitors are used to allow superinfection (20), but without the toxicity found with these inhibitors. Nearly every cell (90 to 95%) which is exposed to MSV in REV can be infected, as assayed by transformation of cellular clones derived from limiting dilution after treatment with MSV in REV. Increasing the concentration of vesicles above 33 μmol of lipid per 10^5 cells does not result in higher FFU titers. This is not surprising, in that this concentration greatly exceeds the level of saturation (100 nmol per 10^6 cells) calculated by other workers (5). Treatment of cell lines with glycerol (30) or polyethylene glycol (10) after exposure to REV did not result in any consistent increase in FFU titer (data not shown).

The reason that encapsulated MSV interacts more efficiently with cells producing M-MuLV than it does with uninfected cells is not readily apparent. It is possible that the infected cells contain enough active reverse transcriptase to enhance provirus formation from introduced MSV genomic RNA. The fact that the titer of the MSV REV was not markedly elevated on M-13 cells, which do not produce an active reverse transcriptase (24), appears to support this hypothesis. However, other studies (16, 32) indicate that a large proportion of the reverse transcriptase in infected cells is in an inactive precursor form and only becomes activated upon budding of the virion.

In addition to introducing retroviruses into cells whose viral receptors are blocked by endogenous virus production, this system of REV encapsulation should prove useful in permitting entry of retrovirus into cells that lack viral receptors. Studies are now under way which explore the use of recombinant retroviral genomes packaged in virions and recombinant lambda viral genomes packaged in phage heads and then encapsulated within REV to mediate gene transfer into murine T-lymphocyte cell lines (which appear to lack retrovirus receptors) and into nonmurine cells.

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