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INFECTION OF CELLS WITH SINDBIS VIRUS NUCLEOCAPSIDS ENTRAPPED INTO LIPOSOMES

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The nucleocapsid of Sindbis virus, a natural non-infectious complex of the viral RNA and protein molecules can be encapsulated in large, unilamellar vesicles and delivered efficiently to cells in an infectious form. It is shown that high infectivity of the vesicle entrapped nucleocapsids is partly due to the viral envelope proteins which enhance entrapment and liposome cell interaction.
We believe that the efficiency of liposome mediated gene transfer of eukaryotic cells can be increased significantly by the insertion of fusogenic viral envelope proteins into the lipid bilayer of liposomes.

INTRODUCTION

Liposome mediated transfer of macromolecules seems to be a promising new method for the genetic transformation of animal and plant cells (1, 2). Expression of new genetic information after introduction of nucleic acids (3-6), chromosomes (7), virions (8) or isolated nuclei (9) into living cells has been shown. It is suggested that at least one of the possible outcomes of cell-liposome interaction is fusion of the vesicles with the plasmamembrane (1, 2). Another type of interaction results in endocytosis, engulfment of liposomes by cells, followed by intracellular processing of the foreign material by the catabolic enzymes of the lysosomes. However, certain animal viruses (e.g. Sindbis and other alfaviruses) reportedly use this route for infection: cytoplasmic delivery of infectious viral components is achieved by the fusion of the viral envelope with the lysosomal membrane (10, 11).

In this paper we report evidence that Sindbis virus nucleocapsid, an RNase sensitive, non-infectious subviral particle (12) can be delivered into the cytoplasr

ABBREVIATIONS: NC=nucleocapsid, LUV= large unilamellar vesicles, REV=reverse phase evaporation vesicles, pfu-plaque forming unit, PBS=phosphate buffered saline, EMEM=Eagle's minimal essential medium, E_1E_2=Sindbis virus envelope proteins, HJV=Hemagglutinating Virus of Japan.

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via liposomes without being inactivated by lysosomal enzymes, and the delivery is facilitated by the presence of viral envelope proteins on the liposome surface. This fact might be exploited in liposome mediated gene transfer experiments.

MATERIALS AND METHODS

Cell and Virus

BHK 21 cells were grown in EMEM supplemented with 5% calf serum. Virus was obtained by infecting cells at low multiplicity. The medium was replaced 5-6 hrs thereafter. Envelope proteins and nucleocapsids were purified after solubilization of the viral envelope according to Helenius and Bonsdorff (13) and Kääriäinen and Söderlund (14), respectively. Nucleocapsids were ^{125}I-labelled by the chloramine-T procedure (15).

Biological Assay

Plaque assay using virus or liposome-entrapped nucleocapsids were done according to Fan and Sefton (16) on subconfluent cultures of BHK 21 cells allowing 1 hr for adsorption at 37°C in the absence of serum. Plaques were counted after 2 days of incubation. Rat anti-Sindbis serum was produced by two intravenous injections of 2×10^{11} pfu of Sindbis virus in four week intervals. Neutralization of virus or liposome-entrapped nucleocapsids with antiserum was carried out by a 1 hr incubation at 37°C followed by the plaque assay after a series of $\log_{10}$ dilutions in EMEM.

Liposomes

Brain phospholipids were purified as described by Papahadjopoulos and Miller (17). Large unilamellar vesicles and reverse phase liposomes containing radiolabelled nucleocapsids were prepared by the Ca^{++}/EDTA method (18) and according to Szoka and Papahadjopoulos (19), respectively. Liposomes were precipitated with 0.7 M NaCl and suspended in Earle's salt solution to remove unentrapped material. Infectious liposomes were produced from non-infectious nucleocapsids liberated from Sindbis virus by an ether-sonication treatment. Subsequently, the nucleocapsids were sequestered into REV (19) prepared by the addition of an acetone-insoluble, ether-soluble phospholipid fraction of ox brain in the presence of the elements of disrupted viral envelope.

Table 1 Infectivity of Sindbis virus nucleocapsids entrapped in reverse phase evaporation vesicles (REV's)

infectivity (pfu/ml)			recovery (%)[2]
original virus prep	virus, after ether and sonication[1]	nucleocapsids in rev. phase liposomes[1]	
3×10^6	0	2.7×10^4	0.90
7×10^6	0	3.5×10^3	0.05
8×10^6	0	2.2×10^4	0.28
4×10^7	6	3.0×10^3	0.08
8×10^7	10	6.1×10^5	0.76

1. Suspensions of purified Sindbis virions were mixed with ether and sonicated briefly. Infectivity was tested after this step. Phospholipids (from 35 µg to 10 mg per 10^6 pfu) were added and reverse phase evaporation vesicles were prepared. Infectivity was measured by plaque assay on BHK cell monolayers.
RNase treatment of NC loaded liposomes had no effect on infectivity.
2. Infectivity of NC loaded REV's is expressed as percentage of infectivity of the original virus stocks.

RESULTS

Delivery of infectious Sindbis RNA via liposome-entrapped non-infectious nucleocapsids was demonstrated by using unpurified nucleocapsids, freshly released from the virions by brief sonication of the virus suspension in ether. This treatment abolished virus infectivity (Table 1) even if the original titer exceeded 10^9 pfu/ml. Encapsulation of these non-infectious nucleocapsids into REV was achieved by adding acetone-insoluble phospholipids to the ether phase, then sonicating the mixture again and evaporating the organic phase according to Szoka and Papahadjopoulos (19).

Table 1 shows that liposome-entrapped nucleocapsids were highly infectious indicating entrapment of nucleocapsids in REV and delivery of intact viral nucleocapsids into the cytoplasm by fusion. The infectivity of REV-entrapped nucleocapsids occasionally approached 1% of the original virus stock. One explanation for this high infectivity can be that the original purified virus stocks contained virions that were not infectious due to membrane defects (20),

Table 2 Neutralization of Sindbis virus and nucleocapsid loaded liposomes with anti-Sindbis serum

plaque forming agent	anti-Sindbis serum	pfu/ml
Sindbis virus	none	1000
	1 µl	44
	20 µl	0
nucleocapsids in REV's*	none	1000
	1 µl	220
	20 µl	91

* An average size (0.1-0.2 µm) REV incorporated approx. ten 29 S octameric complexes (13) of Sindbis virus envelope proteins corresponding to 40 glycoprotein dimers (E_1+E_2) (26) per liposome.

The surface density of these proteins is roughly 50-100 times higher in the virions.

1 ml aliquots of virus or liposome suspensions were incubated for 60 min at 37 centigrades with 0, 1, 20 µl of anti-Sindbis virus serum. Non-neutralized infectivity was measured by plaque assay on BHK cells.

however, their nucleocapsids were capable of initiating viral replication if injected into the cytoplasm by liposomes.

Another explanation for the high efficiency of intact RNA delivery might be the presence of viral envelope proteins on the surface of the liposomes. It is known that envelope glycoproteins of Sindbis and closely related Semliki Forest virus are involved in fusion (10, 21, 22). We used an immunological approach to detect the presence of Sindbis viral envelope proteins on the outer surface of liposomes by anti-Sindbis serum. Table 2 shows the effect of antiserum on the infectivity of virus particles and REV-entrapped nucleocapsids. We found that the addition of 20 µl of antiserum resulted not only in the complete inactivation of virus particles but also in a significant decrease of the infectivity of REV-entrapped nucleocapsids indicating the presence of viral envoelope proteins on the liposome surface.

A more detailed analysis was carried out with purified [^{125}I] - nucleocapsids and envelope proteins to study the role of envelope proteins in the entrapment, binding and internalization of liposomes. [^{125}I] -nucleocapsids

Table 3 The effect of Sindbis virus envelope proteins on the entrapment of labelled nucleocapsids and on cell-liposome interaction

type of liposomes:	large unilamellar vesicles (LUV's)		reverse phase evap. vesicles (REV's)	
virus envelope proteins (E_1+E_2) per vesicle	0	10	0	10-40
entrapment[1] of nucleocapsids (%)	8.0	23.5	37.0	56.5
bound to cells[2] (%)	0.08	0.75	0.48	1.08
internalized[3] (%)	0.009	0.2o	0.053	0.152

1. Radioactivity of the used NC suspension ($1o^6$ cpm/liposome batch) is 100 %
2. Approx. $1o^4$ vesicles/cell were loaded, incubated for 1 hr at 37^o C in Eagle's salt solution, washed 3x with PBS after treatment
3. Cells were further incubated in MEM 5% serum, washed, trypsinized and pelleted through a 4% sucrose cushion

NB Labelled nucleocapsids did not bind and were not taken up by cells, neither in presence nor in absence of viral envelope proteins

were entrapped in REV or LUV both in the absence and in the presence of envelope proteins. Table 3 shows that envelope proteins enhance both entrapment and liposome-cell interactions, especially in the case of LUV. More efficient entrapment might be the consequence of interaction between envelope and capsid proteins that are able to recognize each other. This recognition may be an advantageous factor facilitating encapsulation, contrary to the entrapment of DNA or other macromolecules which is a statistical process. The presence of envelope proteins on the liposome surface may target the vesicles, enhancing their attachment to the cell surface and resulting in a more efficient binding and increased fusion, either with the plasmamembrane or with lysosomal membranes.

In similar experiments envelope proteins of HJV (Sendai virus) known to promote fusion between cells (23, 24) enhanced vesicle mediated injection of protein molecules into cells (25).

DISCUSSION

Our data demonstrate the lipsome mediated delivery of noninfectious nucleocapsids to cells. High infectivity of liposome entrapped nucleocapsids implies that RNase sensitive nucleocapsids are not degraded upon entry into the cells, indicating direct delivery of the encapsidated material into the cytoplasm.

In the presence of Sindbis viral envelope proteins more efficient internalization of vesicle entrapped nucleocapsids was observed indicating enhanced fusion of liposomes with cellular membranes due to the presence of fusogenic polypeptides on the liposome surface. Considering the natural route of infection in case of alfaviruses (10, 11) it seems more than just speculation that these liposomes are capable of delivering their content into the cells by two different ways.

They can fuse with the plasmamembrane as phophatidylserine vesicles usually do or are endocytosed and fuse with the lysosomal membrane in the low pH environment of the lysosomes, as intact virions do. Both interactions result in the liberation of the entrapped material into the cytoplasm without exposing it to the digestive enzymes of the lysosomes.

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