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Selective virus-mediated intracellular delivery of membrane-impermeant compounds by means of plasma membrane vesicles

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Abstract

The impermeability of the cell plasma membrane is a major obstacle to intracellular delivery of large hydrophilic molecules, such as many kinds of drugs. This contribution describes a general-purpose delivery system that employs the membrane fusion capacity of enveloped viruses to circumvent cell impermeability. Vesicles were generated from the plasma membrane of HEp-2 cells, a human cell line host for the Newcastle disease virus (NDV). They could be loaded with a fluorescent, high molecular weight dye (FITC/dextran, MW 70 KDa) or with the enzyme ribonuclease A (MW 14 KDa). These vesicles were found to fuse and deliver their lumen contents to cultured HEp-2 cells in the presence of NDV virions. When ribonuclease was employed as the encapsulated solute, viral replication was inhibited and death of the infected cells was accelerated. Implications and possible applications of this technique in antiviral therapy are discussed.

Keywords: Newcastle disease virus (NDV); HEp-2; Liposomes; Ribonuclease; Drug delivery

1. Introduction

Selective intracellular delivery of membrane-impermeable molecules has long been a goal that would improve the therapeutic potential of many drugs. Many aqueous solutes such as peptides, proteins and oligonucleotides cross-cellular membranes only with great difficulty, if at all. This limits their use both in medical therapy and in laboratory investigation. Among many methods that address this problem, a promising approach employs liposomes or similar lipidic bilayers (Dass-Crispin et al., 1997; Jaaskelainen et al., 1998) entrapping a small enclosed volume and having surface properties designed to facilitate approach, selective targeting and delivery of the encapsulated contents to specific kinds of cells.

An elaboration of this approach is to insert into the lipidic bilayer proteins derived from the envel-

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ope of a virus that is known to fuse with particular cells, thus achieving delivery of the entrapped molecules (Hug and Sleight, 1994; Ramani et al., 1998) by means of the fusogenic properties of the viral envelope proteins. The utility of these vehicles in vivo has been limited by several problems, including the immune system reaction to the viral proteins, a property which has been exploited to develop a novel category of antiviral vaccines (Mengiardi et al., 1995; Holzer et al., 1996).

In the approach described here, lipidic vesicles were generated directly from the plasma membranes of the cell type to which the drug was to be delivered. These plasma membrane vesicles (denoted PMVs) can be generated easily from cultured (Maeda et al., 1983) or isolated cells, and have been used successfully as model systems for the cell membrane, as they were shown to retain most of the parent membrane constituents intact and in the original orientation on the membrane (Donowitz et al., 1987). Moreover, vesicles of this kind have also been shown to fuse with viral envelope proteins (Puri et al., 1992), which indicates that they retain and display functional viral receptors.

It is well known that many enveloped viruses may promote, under appropriate conditions, simultaneous fusion between two cell membranes resulting in the formation of multinucleated polykaryocytes or syncytia (Seya et al., 1997; Barbeau et al., 1998). Our procedure consisted in loading PMVs with an aqueous, membrane impermeable solute, then add them to virus and intact cells in culture. Simultaneous fusion of the virus with two plasma membranes, that of the cell and that of the PMV, was in this case expected to merge the two aqueous compartments and deliver the entrapped solutes to the cytosol.

The reason for the choice of host cell-derived plasma membrane vesicles as a novel drug carrier lies in the potential advantages envisioned in this variation of the liposome system. First of all, as PMVs are derived from a tissue of the host species, or even of the host itself, it is imaginable that they would elicit a weaker response of the host immune system resulting in prolonged retention in the body. Besides, given the tissue-specificity of most virus-receptor interactions it is plausible to imagine a strong selectivity of the PMVs for delivery to the correct tissues. This approach should moreover be straightforwardly extendable to a large number of virus/cell systems, as the only requirement is that the targeted virus should be able to fuse its envelope with the cell membrane and this is the typical situation. Finally, large amounts of membrane surface area are readily obtainable from tissue cultures and PMVs can be easily generated from cells by simple procedures. The present work employed the established human cell line HEp-2 and the Newcastle disease virus (NDV), a member of the Paramyxovirus family able to infects these cells by interaction with the sialic acid of cell membrane glycoproteins.

2. Materials and methods

2.1. Cells

Human HEp-2 cells were obtained from ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagles Medium (Sigma) with added 10% fetal bovine serum (FBS, Sigma).

Newcastle disease virus (Texas GB strain) was kindly donated by Prof. K. Lam (UC Davis), propagated in fertilized chicken eggs obtained from SPAFAS (Storrs, CT) and stored in 1.5 ml aliquots of egg serum in liquid nitrogen (180 μg protein ml^{-1}).

2.2. Virus solutions

A concentrated virus solution (denoted 1:1) was prepared by ultracentrifuging 1.5 ml of egg serum virus suspension (35 K rpm, 60 min) and resuspending the pellet in 1.5 ml PBS. The resulting protein concentration was 180 μg ml^{-1}. A 1:100 dilution of this suspension was prepared in PBS. Both solutions could be stored at 4°C for up to 2 weeks without loss of infectivity.

2.3. Other materials and procedures

Octadecyl rhodamine B (R_{18}) and fluorescein isothiocyanate dextran conjugate (FITC/dextran)

were obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma. Low speed centrifugations were carried out in Sorvall RC-5B centrifuges; ultracentrifugations in a Beckman L8-60M. Fluorescence spectroscopy was carried out on a Perkin Elmer LS-50B fluorimeter. Fluorescence microscopy was performed on a Nikon Labophot microscope. Plate readings were done on a V-Max plate reader (Molecular Devices).

2.4. PMV generation

PMVs were generated according to a previously reported procedure (Maeda et al., 1983). Briefly, cells were scraped in the cold (4°C) from 3–5 175 cm^2 flasks and pelleted at low speed (5 K rpm, 10 min). They were resuspended in a three-fold volume of homogenizing buffer (10 mM Na_2HPO_4, 1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiotreitol, 0.005 mM PMSF, 0.02% NaN_3, pH 6.0) and disrupted in a sterile hood by extrusion through a sterile 10 ml syringe. The suspension was then layered over a 41% w/w sucrose cushion and ultracentrifuged (35 K rpm, 40 min). The PMVs were collected, washed twice with homogenizing buffer, resuspended in PBS, and snap frozen in liquid nitrogen for storage until use.

2.5. PMV characterization

PMVs were quantitated by the BCA protein assay and their dimensions (average diameter 1.0 μm) were obtained by the quasi-elastic light scattering (QELS) technique.

2.6. Macromolecular solute incorporation

PMVs stored in liquid nitrogen were thawed at room temperature. They were then centrifuged (15 K rpm, 15 min) and the supernatant discarded. The pellet was resuspended by fine needle homogenization in a solution of the macromolecular solute (1–2 mM) in sterile PBS. The suspension was snap frozen in liquid nitrogen for 5 min, then immediately transferred to an ice-water bath where it was left to thaw for about 1 h. The thawed suspension was centrifuged and the pellet washed with sterile PBS until the supernatant was devoid of optical absorbance signal (FITC case) or showed no detectable SDS-PAGE band (RNase case). Three washing cycles were found to be sufficient for the purpose. The PMVs were stored at 4°C until use.

2.7. Three-body PMV fusion assay

PMVs (4 ml, 80 μg ml^{-1} protein) were labeled with octadecyl rhodamine by adding 16 μl of a 1 mg ml^{-1} solution in ethanol. After 60 min incubation at 25°C, the PMVs were washed by centrifugation and resuspension in 4 ml PBS. Two samples were then prepared in the cold:

sample M: 2 ml rhodamine labeled PMVs plus 2 ml unlabeled PMVs;

sample N: 2 ml labeled PMVs plus 2 ml PBS.

Fluorescence spectra of these samples were taken at 40°C ($\lambda_{ex} = 520$ nm; $\lambda_{em} = 585$ nm). Subsequently NDV (40 μl of the 1:100 suspension) was added to each sample (afterwards labeled M_v and N_v, respectively) and the mixtures were incubated 60 min in the cold. Fluorescence spectra were again taken under the same conditions as above.

2.8. Verification of incorporation FITC/dextran

Encapsulation of FITC/dextran was verified using a solution of the fluorescein quencher Co^{2+} (10 mM in isotonic Tris buffer), which quenches fluorescence of dye physically adsorbed on the outside of the vesicles but cannot readily gain access to the intravesicular compartment. The control consisted of PMVs simply exposed to the fluor after the freeze-thaw step.

2.9. Verification of incorporation: RNase A

Encapsulation of RNase A was confirmed by SDS-PAGE gels of PMVs which had been either freeze-thawed in RNase (3 mg ml^{-1} PBS) solution or suspended in it after the freeze-thaw step.

2.10. Intracellular delivery of FITC-dextran

PMVs were prepared as described and loaded

with a 3 mM solution of FITC-dextran conjugate (MW 70 000) in PBS. The control consisted of PMVs incubated in to the loading solution but not freeze-thawed.

HEp-2 cells were grown in two well chamber slides (Labtek) to 80% confluency. They were then incubated in the cold (4°C) for 5 min. The medium was discarded and replaced with an equal volume of the viral suspension in PBS (sample F) or PBS alone (sample C). The slides were incubated for 40 min in the cold to allow virus adsorption, after which the overlaid liquids were removed and a suspension of FITC/dextran loaded PMVs was added to both wells. The slides were incubated for a further 30 min at 4°C.

The samples were then transferred to a 37°C incubator for 45 min to allow viral fusion. The wells were then rinsed thoroughly with PBS and observed and photographed using a Nikon fluorescence microscope.

2.11. NDV inhibition by intracellular delivery of ribonuclease A

PMV suspensions (250 μg protein each) in PBS (C) or PBS plus 2 mg ml^{-1} ribonuclease A (R) were freeze-thawed, washed three times in PBS and finally resuspended in 500 μl of DMEM 10% FBS. To each sample was added NDV (20 μl of 1:100 suspension) and the samples were incubated in the cold for 4 h. HEp-2 cells were cultured in 96-well plates for cytotoxicity assays. After removal of culture media, aliquots of the PMV-NDV suspension (80 μl) were added to each of two wells (R and C). Additional control wells were inoculated with virus alone (4 μl of the 1:100 suspension) or with virus plus free RNase (final concentration: 2 mg ml^{-1}). After 48 h at 37°C, 20 μl of the supernatants were taken from the primary infection wells and serially diluted two-fold along 12 well rows of 96-well plates. After 72 h the wells were checked for cytopathic effects. These secondary infected wells were also treated with 5 μl $well^{-1}$ of MTT (5 mg ml^{-1} PBS). Samples were incubated for 3 h at 37°C, fixed with glutaraldehyde (0.1% in PBS) and examined by light microscopy and plate readings ($\lambda = 570$ nm).

2.12. Cell viability assay

The viability of cells in virus- and PMV-treated samples was assessed using MTT (3[4,5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide), a yellow compound that forms a black insoluble formazan dye when metabolized (Slater et al., 1963).

2.13. RNase-induced cell death

Cells in 96-well plates were treated as above with RNase-loaded (R) or control (C) PMV-NDV mixtures, or with virus alone. Twenty-four hours after inoculation, MTT (5 μl $well^{-1}$, 5 mg ml^{-1} PBS) was added to each well. After 3 h further incubation at 37°C, cells were fixed with glutaraldehyde (0.1% in PBS) and examined by light microscopy and, after MTT solubilization in isopropanol (80 μl^{-1}), optical density plate readings ($\lambda = 570$ nm).

2.14. RNase-loaded PMVs selectivity assay

RNase A loaded PMVs, prepared and resuspended as described above, were added (80 μl) to uninfected cell wells in the absence of virus. The controls consisted of untreated cells (N) or cells treated with unloaded PMVs (C). After 24 h the wells were treated with MTT, incubated and fixed as described above.

3. Results

3.1. Efficiency of PMV loading procedures

The efficiency of the freeze-thaw PMV loading procedure is demonstrated in Fig. 1 and Fig. 2. PMVs subjected to one freeze-thaw cycle in a FITC/dextran solution (Fig. 1A) retained most of their fluorescence in the presence of the fluorescein quencher Co^{2+} (Fig. 1C). In vesicle samples simply incubated with FITC/dextran (Fig. 1B), fluorescence was almost completely quenched by Co^{2+} (Fig. 1D). When samples freeze-thawed or simply incubated with RNase solution were analyzed by SDS-PAGE, only the freeze-thawed sam-

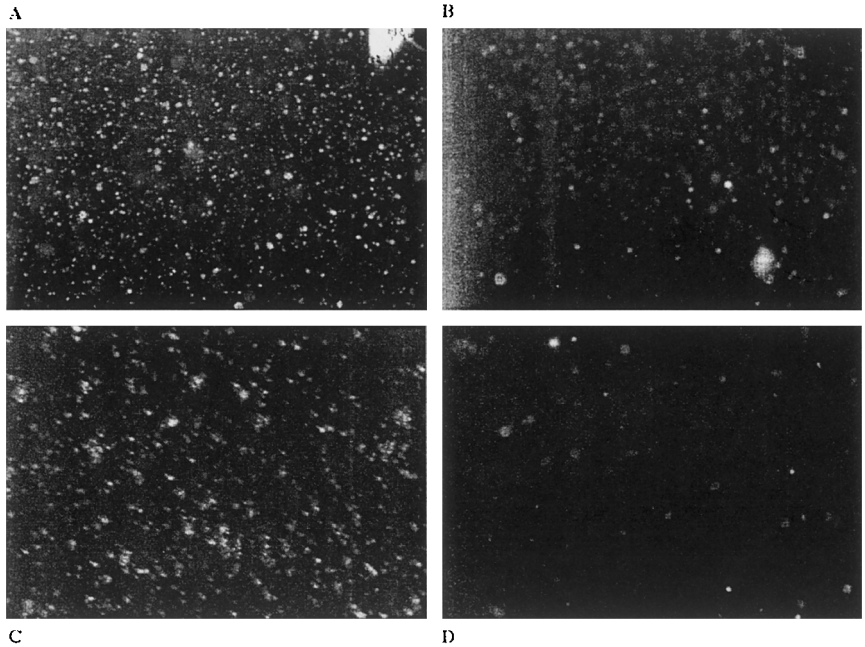

Fig. 1. Effects of Co^{2+} on PMVs loaded or exposed to FITC/Dextran. A, B, PMVs freeze-thawed or exposed to FITC/Dextran; C, D, the same samples after addition of Co^{2+} (10 mM).

ple showed a detectable protein band at 14 KDa (Fig. 2).

3.2. Three-body fusion assay

The capacity of ND virions to fuse with multiple HEp-2 PMV membranes was tested. A previously reported assay for viral fusion (Puri et al., 1993) described the use of the fluorescent membrane phospholipid octadecyl rhodamine (R_{18}) to label virion envelopes at a self-quenching concentration. Fusion between labeled virions and unlabeled cells diluted the fluor, and fluor dequenching was observed as a signal increase. This assay is commonly employed in studies of viral (Sinclair et al., 1997; Tomasi et al., 1998) and cellular (Arienti et al., 1997; Hirano et al., 1997; Edwardson, 1998) fusion events. In the present work, this assay was adapted to detect double (i.e. PMV-virus-PMV) instead of simple (PMV-virus) fusion. To achieve this, PMVs were labeled rather than virions, then fluorescence of a mixed sample of labeled (quenched) and unlabeled PMVs was monitored in the presence or

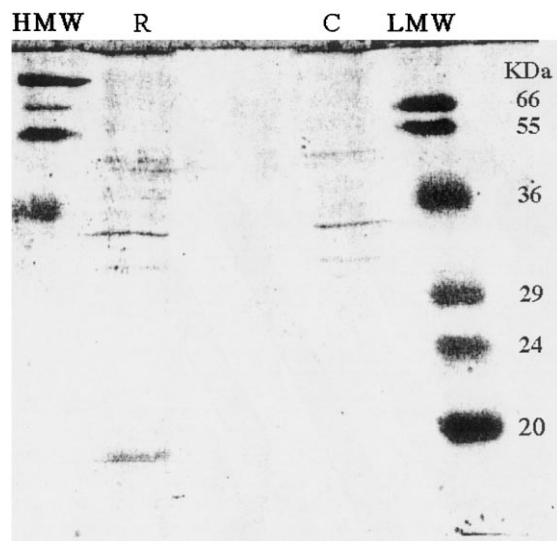

Fig. 2. SDS-PAGE gel of PMVs freeze-thawed (R) or exposed (C) to a 20 mg ml^{-1} RNase A solution. Gel stained with Coomassie dye. The molecular weights of the LMW protein standards are reported on the right.

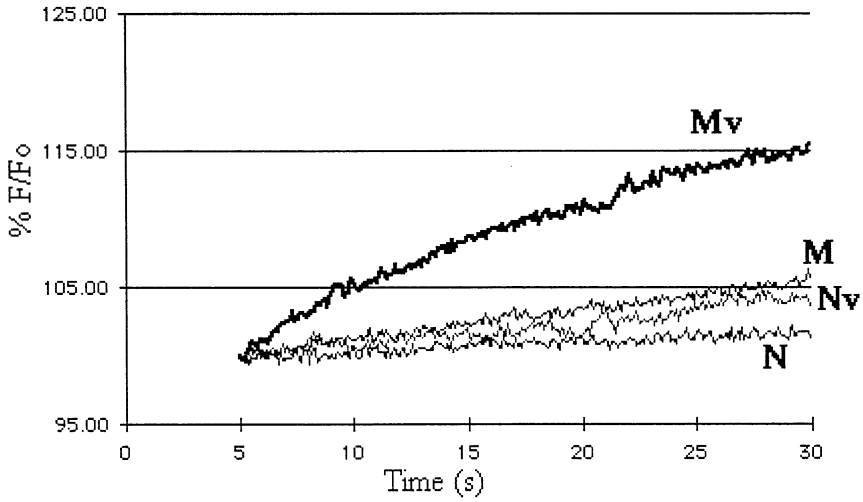

Fig. 3. Octadecyl rhodamine B dequenching experiments to monitor virus-induced membrane fusion. N, labeled PMVs; N_v, labeled PMVs plus NDV; M, labeled and unlabeled PMVs; M_v, labeled and unlabeled PMVs plus NDV.

absence of virions. Fusion of labeled PMVs with virions should have resulted in minimal dye dilution and hence dequenching, but a three-body fusion (PMV-virion-PMV), given the larger size of the PMVs, would have produced significantly greater dequenching. This would permit distinction of the two fusion events. Controls were also provided in the form of pure labeled virions and the same with labeled PMVs. Fig. 3 shows fluorescence dequenching in the mixed PMV sample (M), pure labeled PMVs (N) and both after addition of NDV (M_v, N_v). The quenching of the N sample was stable, as expected since no unlabeled membranes were present. The N_v sample showed a slight dequenching, likely reflecting dispersion of the fluor onto viral envelopes on fusion (Frumkin, 1996). The M sample also showed a slight increase in the signal, probably due to slow spontaneous fluor monomer transfer from labeled to unlabeled PMVs. The M_v sample, in contrast, showed marked dequenching, following the first-order behavior reported previously (Puri et al., 1993).

3.3. Intracellular delivery of FITC-dextran conjugate

The incubation of FITC/dextran-loaded PMVs with NDV virions and HEp-2 cells led to the appearance of diffuse fluorescence from the areas of the wells corresponding to cell volume (Fig. 4). This diffuse fluorescence was not visible in controls containing loaded PMVs but no NDV. Rather, fluorescence in the control samples appeared as smaller, bright points consistent with the volume of intact vesicles. This result is consistent with simultaneous fusion of virions with a cell and PMV, merging the internal aqueous compartments of both and allowing the highly impermeable dextran-conjugated dye to diffuse through the cell cytoplasm. In virus-free controls, the PMVs instead adsorbed to the cells or were endocytozed, the dye remaining confined in the vesicular lumen.

3.4. Intracellular delivery of ribonuclease A

Virus-induced PMV-cell fusion has the potential to deliver membrane-impermeable compounds that interfere with viral replication. One such agent is the enzyme ribonuclease A, which was encapsulated in PMVs as described above. Cells were exposed to RNase-loaded and empty PMVs, plus or minus NDV; or to NDV alone, plus or minus free RNase. RNase activity in the cytosol was monitored in two ways: direct assessment of

primary cell death, and quantitation of viral yield by the end-point serial dilution of the supernatant in a secondary cell infection. Delivered ribonucleolytic enzyme was expected to bring about cell death well in advance of virus-caused lysis. Additionally, delivered enzyme was expected to reduce viral yield directly by interfering with the RNA-based viral genome replication. Fig. 5 shows cell viability in samples treated with RNase (R) and control (C) PMVs both plus NDV at 12 h post infection. Control samples showed more extensive viability, reflected by the conversion of MTT to its dark formazan metabolite. Less extensive conversion in the RNase sample indicated substantial cell death.

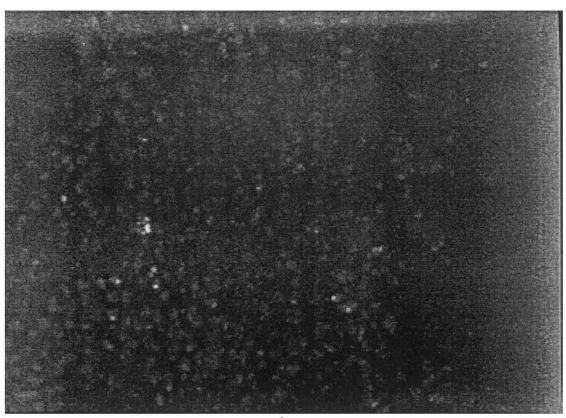

Fig. 4. Fluorescence micrographs of HEp-2 cells incubated with FITC/Dextran PMVs without (A) or with (B) NDV virions.

The viral yield of the same primary infection cultures was assessed by serial dilution of the supernatants into fresh culture samples and assessment of cell viability and end-point dilution after an average of 72 h post secondary infection. In these samples, a lower cell viability reflects greater viral yield, hence less inhibited viral replication in the primary culture. Viability was assessed both by direct fluorescence microscopy (Fig. 6 shows a typical result) and MTT assay followed by plate scans (Fig. 7). These show that incubation with RNase-loaded PMVs (Fig. 6R) reduced the viral yield in the primary infection wells by factors of 16–32-fold, as compared to samples treated with PMVs simply exposed to the RNase solution (Fig. 6C).

In a separate experiment, cultures were treated with A) NDV, plus or minus free RNase, or B) RNase or control PMVs in the absence of NDV. NDV induced identical cytotoxicity and viral yield in the presence or absence of free RNase (data not shown). In the absence of NDV, RNase loaded and control PMVs had no effect on cell viability (Fig. 8).

4. Discussion

The goal of this work was to show that vesicles obtained from the plasma membrane of a cell line may be employed to deliver large, membrane-impermeable molecules to the same cells by means of an enveloped virus capable of infecting that cell line. Our experimental system consisted of plasma membrane vesicles generated from human HEp-2 cells by a reported procedure involving hypotonic lysis. When subjected to a freeze-thaw cycle (Liu and Yonetani, 1994; Bally et al., 1988) these PMVs can encapsulate and retain hydrophilic molecules of diverse size, namely a high molecular weight polymer labeled with a fluorescent dye (FITC/dextran conjugate, average MW 70 KDa) and an enzyme (ribonuclease A, MW about 14 KDa). Thus molecular size (at least up to 70 KDa) and hydrophilicity do not pose obstacles to encapsulation in this kind of PMVs.

The next objective was to verify that these PMVs could mimic their parent cells in undergo-

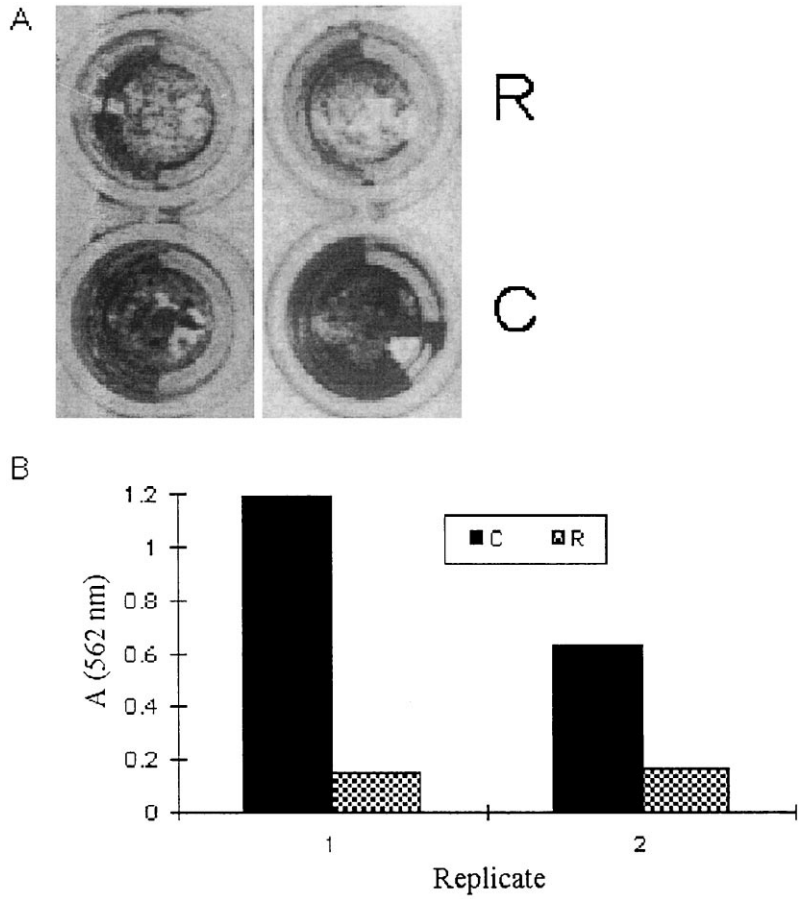

Fig. 5. (A) MTT cell viability assays 24 h after treatment with NDV plus RNase loaded PMVs (R) or empty PMVs (C). (B) plate readings of the isopropanol solubilized MTT from the same wells.

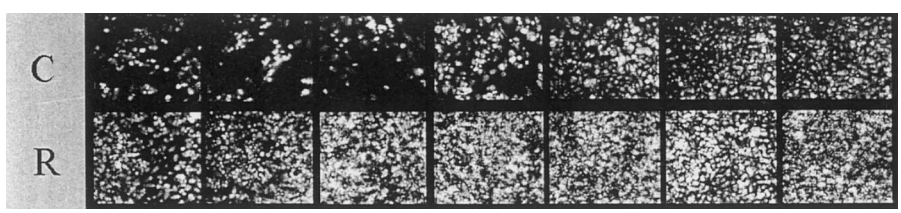

Fig. 6. Autofluorescence micrographs of HEp-2 culture wells treated with the supernatants of the primary NDV infection carried out in the presence of RNase loaded PMVs (R) or empty PMVs (C). In each row, wells were inoculated with increasing dilutions of the supernatants (1:32 to 1:2048).Micrographs were taken after 72 h infection.

ing membrane fusion with the envelope of a virus (NDV) which is capable of infecting HEp-2 cells. Early reports (Kohn, 1965) have shown that infection of NDV, as for many enveloped viruses, proceeds through fusion of the viral envelope and the plasma membrane of viable cells. It has also been reported that PMVs derived from cells expressing the CD4 receptor are capable of interaction and possibly fusion with HIV (Puri et al., 1992). Since the viral proteins which mediate fu-

sion are distributed evenly on the virion envelope, if a virion binds two host cell membranes simultaneous fusion can occur leading to coalescence of the three bodies. In the case of whole cells, this leads to the formation of polykariotic syncytia, which are routinely observed in NDV and many other kinds of viral infections (Kohn, 1965; Seya et al., 1997; Barbeau et al., 1998). Simultaneous fusion of a cell and a loaded PMV should instead result in delivery of the PMV contents to the cytosol. FITC/dextran was employed as a marker of intravesicular compartments. Fluorescence microscopy revealed that PMV-encapsulated FITC/dextran does not diffuse into the cytosol unless NDV is present. In that case, PMV treated cells display enhanced, diffuse fluorescence over the whole cell body. This result is consistent with double fusion events that have resulted in the delivery of the dye to the cell. In contrast, residual PMVs added in the absence of virions retain the punctuate, concentrated fluorescence of pure PMVs. We also noticed a sharp decrease in the number of polynucleated cells (syncytia) induced by the virus when PMVs were present. This is also expectable because, given the greater number of vesicles over cells, the former are statistically more likely to replace cells in double-fusion events.

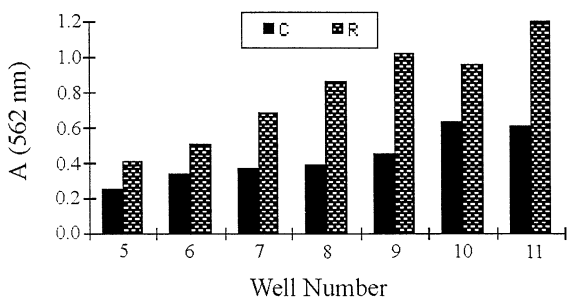

Fig. 7. Plate absorbance readings for the MTT assay performed on the same wells as in Fig. 6. Well MTT dissolved in 70 μl isopropanol. Absorbance measured at 562 nm.

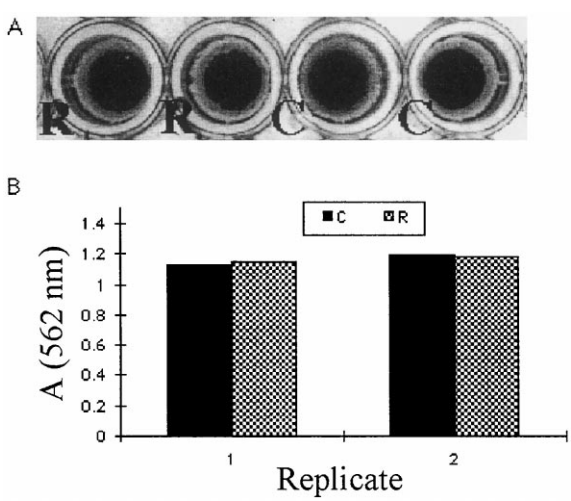

Fig. 8. MTT assay performed on HEp-2 wells treated with RNase containing PMVs in the absence of NDV (R) or with unloaded PMVs (C). B, plate readings for the MTT solubilized from the same wells are shown below.

Confirmation of the three body fusion event comes from the octadecyl rhodamine B fluorescence dequenching assay. The variation of the procedure employed could distinguish PMV-virus-PMV fusion (or 'syncytial' fusion) from simple PMV-virus fusion by the extent of dequenching. The results are consistent with PMV-PMV fusion mediated by the virus.

To corroborate the FITC evidence, and also to investigate a possible practical application of the delivery system, the same techniques were employed to achieve delivery of a membrane-impermeable compound that would inhibit viral replication, thus reducing the viral yield of NDV-infected cells. The agent examined was the enzyme ribonuclease A (RNase A), a hydrolyzing agent for RNA and hence a potential inhibitor of viral RNA replication whose impermeability to phospholipid membranes makes it ineffective if administered in solution.

Incorporation of this molecule in PMVs and addition to virions at the time of cell infection caused extensive and rapid death of the infected cells (Fig. 5) and a concomitant significant decrease in the amount of virus produced (Figs. 6 and 7) over the control (in which cell death is likely to be caused by virus infection and syncytia generation alone). These two effects are consistent with effective cytoplasmic delivery of the enzyme. When RNase-loaded PMVs were administered in the absence of virus, no cytotoxic effect was detected (Fig. 8). Since the enzyme quickly degrades all kinds of RNA and is presumably cytotoxic, it

is reasonable to assume that it does not gain access to the cell cytoplasm in that case. Besides confirming the proposed mechanism of solute delivery, this result also shows that delivery should be selective for those cells which are being infected by enveloped viruses, leaving healthy or unrelated cells unaffected. This could allow introduction of compounds able to interfere with the early stages of viral replication, such as enzyme substrate analogues or antisense oligonucleotides. In addition, since in many cases (including NDV) viral fusogenic proteins appear on the surface of infected cells at late stages of infection, inducing virion-independent syncytia in what is termed 'Fusion From Within' or FFWI (Deng et al., 1994), it is conceivable that PMVs could also fuse with late stage-infected cells and deliver specific agents (such as antibodies) which could interfere with virion release. Furthermore, it has been shown that plasma vesicles bearing a viral receptor effectively reduce viral infectivity (Puri et al., 1992), supposedly by direct fusion and scavenging of virions.

This general method is applicable to a large number of virus/cell systems, because it requires no knowledge of the specific molecular fusion apparatus involved. The only essential requirement for its applicability is the presence of a viral membrane fusion machinery consisting of viral docking/fusion proteins and a corresponding cellular receptor on distinct membranes. Membrane fusion, whether taking place directly upon virus/cell contact or subsequently to endocytosis, is believed to be the main entry route for all enveloped viruses (Kielian and Jungerwirth, 1990). These features also make it a useful research tool whenever tissue-selective intracellular delivery of membrane-impermeable compounds is required. Moreover, since techniques to inactivate viral genome and abolish infectivity while preserving fusogenic potential, such as UV-irradiation, are well known (Qiu et al., 1984), it is imaginable to employ the PMVs in delivery experiments not targeted at viral inhibition. Additionally, since these vesicles are derived from whole plasma membranes and have been shown to retain a large number of host membrane determinants besides viral receptors, they may be useful in applications where a realistic set of normal membrane functions is required.

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