Cellular Cytoplasmic Delivery of a Polypeptide Toxin by Reconstituted Influenza Virus Envelopes (Virosomes)†

Romke Bron,‡ Antonio Ortiz,§ and Jan Wilschut*

Department of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

Received August 26, 1993; Revised Manuscript Received December 13, 1993®

ABSTRACT: In this paper it is demonstrated that reconstituted influenza virus envelopes (virosomes) fuse efficiently with membranes of the endosomal cell compartment of cultured cells, after internalization through receptor-mediated endocytosis. As a consequence, molecules, encapsulated in the virosomal interior, are transferred to the cell cytoplasm. This process was monitored on the basis of delivery of subunit A of diphtheria toxin (DTA), initially encapsulated in the virosomal lumen. Virosome-mediated cytoplasmic delivery of DTA resulted in a virtually complete inhibition of cellular protein synthesis. DTA delivery was blocked by factors inhibiting the pH-dependent fusion activity of viral hemagglutinin, i.e., 20 mM NH_4Cl, preincubation of the virosomes at low pH, and anti-hemagglutinin antibodies. Quantitation of the extent of virosome-mediated delivery of biologically active DTA demonstrated that a lower bound of approximately 10% of the virosomes entering the cells deposited their aqueous contents into the cytosol. This is in good agreement with the final extent of virosome fusion of 40%, as determined by a fluorescence lipid mixing assay based on the dilution of pyrene-labeled phosphatidylcholine from the virosomal into the endosomal membrane. At an added concentration of 1 μM virosomal lipid per 50 000 cells, depending on the condition of the experiment, a minimum of 4000 virosomes per cell were found to be internalized. Virosomes could also be induced to fuse with the cell plasma membrane by a transient lowering of the extracellular pH, as detected by appearance of virosome-encapsulated DTA in the cytoplasm. The results demonstrate that virosome-mediated delivery of polypeptide toxin molecules can be used as a sensitive means to study the interactions of enveloped viruses with host cells. In addition, virosomes may be used as versatile vehicles for the delivery of water-soluble molecules to the cytoplasm of cultured cells.

Enveloped viruses have developed an efficient membrane fusion strategy in order to deposit their genome into the cytosol of the host cell. This fusion process can be initiated directly at the cell surface under the neutral pH conditions of the extracellular medium. This is the case for Sendai virus (Haywood & Boyer, 1982; Hoekstra & Klappe, 1986) and presumably also for HIV[1] (Stein et al., 1987; Mcclure et al., 1988). Alternatively, the fusion reaction can be activated after uptake of intact virus particles into endosomes through receptor-mediated endocytosis. In this case, fusion is activated by the mildly acidic pH in the lumen of the endosomes, and the target membrane for fusion of the viral envelope is the endosomal membrane. Influenza virus represents one of the best documented examples of an enveloped virus, requiring acidic pH for expression of its fusion activity (Matlin et al., 1981; White et al., 1981; Stegmann et al., 1986). The membrane fusion reaction of influenza virus, catalyzed by the hemagglutinin (HA) spike glycoprotein, is very fast and efficient. As a consequence the viral nucleocapsid is delivered to the cell, while the envelope components remain behind in the endosomal membrane.

Previously, we have shown that influenza virus envelopes can be reconstituted by solubilization of the viral membrane in the nonionic detergent $C_{12}E_8$, removal of the viral nucleocapsid and matrix protein by ultracentrifugation, and subsequent extraction of the detergent by hydrophobic Bio-Beads SM2 (Stegmann et al., 1987b). This procedure had been developed earlier for the vesicular stomatitis virus (VSV) (Metsikkö et al., 1986). For this virus, as well as for influenza virus, it results in full recovery of the original fusion capacity, as judged by polykaryon formation, hemolysis, or dilution of fluorescent lipid analogues into model or cellular target membranes. In addition, the physical characteristics and morphology of the virosomes, prepared according to this procedure, are similar to those of the intact viral envelopes (Metsikkö et al., 1986; Stegmann et al., 1987b; Bron et al., 1993).

An attractive feature of virosomes is that during the reconstitution process a variety of reporter molecules can be incorporated, either in the virosomal membrane or in the aqueous interior. This enables the application of a number of assays for the assessment of viral fusion that cannot be used with intact virus particles. This is quite important, since there is only a limited number of assays available for direct monitoring of the membrane fusion activity of intact virions, and we have demonstrated that, in addition, some of these may be prone to artifacts under certain conditions (Stegmann et al., 1993).

† This study was supported by the Netherlands Organization for Scientific Research NWO (fellowship to R.B. under the auspices of the Netherlands Chemical Foundation SON) and the European Molecular Biology Organization (long-term fellowship to A.O.).

* To whom correspondence should be sent.

‡ Present address: MRC Institute for Molecular Cell Biology, Membrane Biology Laboratory, University College London, Gower Street, London WC1E 6BT, England.

§ Present address: Department of Biochemistry and Molecular Biology, Veterinary Faculty, University of Murcia, E-30071 Murcia, Spain.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: BHK, baby hamster kidney; BSA, bovine serum albumin; CHEMS, cholesteryl hemisuccinate; DOPE, dioleoylphosphatidylethanolamine; DT, diphtheria toxin; DTA, diphtheria toxin A subunit; FCS, fetal calf serum; GMEM, Glasgow's modification of Eagles' medium; HA, hemagglutinin; HEPES, 4-(hydroxyethyl)-1-piperazineethanesulfonic acid; HHB, Hanks' salt solution, buffered with 10 mM HEPES to pH 7.4 and supplemented with 0.1% (w/v) BSA; HIV, human immunodeficiency virus; HNE, 150 mM NaCl and 0.1 mM EDTA, buffered with 5 mM HEPES to pH 7.4; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PK, proteinase K; Pyr-PC, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine; R_{18}, octadecylrhodamine B; SFV, Semliki Forest virus; SIV, simian immunodeficiency virus; TCA, trichloroacetic acid; VSV, vesicular stomatitis virus.

0006-2960/94/0433-9110$04.50/0

In the present paper, we demonstrate that influenza virus virosomes have the capacity to deliver encapsulated water-soluble molecules to the cytosol of cultured cells. During the reconstitution procedure, subunit A of diphtheria toxin (DTA; 21 kDa) was encapsulated in the virosomes. DTA catalytically inactivates elongation factor 2 (EF-2), involved in cellular protein synthesis, through an ADP-ribosylation reaction (Honjo et al., 1968). In the absence of the B subunit, which carries the receptor-binding activity of the toxin, DTA cannot penetrate cells. It is demonstrated that virosome-mediated cytoplasmic delivery of DTA is strictly dependent on the fusogenic activity of the reconstituted HA molecules and involves communication between the virosomal lumen and the cytosol. Thus, virosome-mediated DTA delivery can be used to monitor the fusion activity of influenza virus in cultured cell systems. In addition, on the basis of their delivery capacity, influenza virus virosomes can be applied as vehicles for the introduction of foreign water-soluble substances into cells.

MATERIALS AND METHODS

Chemicals, Toxins, and Reagents. Octa(ethylene glycol)-mono(*n*-dodecyl) ether ($C_{12}E_8$) was from Nikko Chemicals (Tokyo, Japan). Bio-Beads SM-2 were from Bio-Rad (Richmond, CA). Metrizamide was obtained from Nycomed, AS (Oslo, Norway). Nicked diphtheria toxin (DT) was purchased from Calbiochem (La Jolla, CA) or Connaught (Willowdale, Ontario, Canada). Fragment A of diphtheria toxin (DTA) was purified as described (Collins & Huang, 1987) and checked for purity by SDS–PAGE. The diphtheria toxin from Connaught contained a detectable amount of unnicked toxin that was separated from the A subunit by gel filtration on Sephadex G-100. 3-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (Pyr-PC) was purchased from Molecular Probes (Eugene, OR). A rabbit anti-HA antiserum, raised against the X-47 strain, was obtained from Dr. J. C. de Jong, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, and found to be cross-reactive with X-97 HA, as determined by sandwich ELISA. All other chemicals were of analytical grade, unless indicated otherwise.

Viruses and Cells. The recombinant influenza A strains X-97, NIB24, and NIB26 (all of the H3N2 subtype) were provided by Solvay Duphar B. V. (Weesp, The Netherlands). The viruses had been propagated in 11-day-old fertilized hen eggs and were purified and stored as described before (Stegmann et al., 1985), except that after sedimentation from the allantoic fluid the virus was further purified by equilibrium density centrifugation on a 20–60% linear sucrose gradient at 100000*g* for 16 h (Stegmann et al., 1993). Viral phospholipid was determined, after extraction of membrane lipids (Bligh & Dyer, 1959), by phosphate analysis (Böttcher et al., 1961). Protein was determined according to Peterson (1977). The fusion efficiencies and characteristics of the NIB24 and NIB26 strain were quite similar, as determined in various model systems, by application of fluorescence assays for lipid mixing. The apparent threshold of fusion of both strains was pH 6.2. The threshold of fusion of the X-97 strain was pH 5.7 (results not shown).

BHK-21 cells were grown in GMEM (Flow Laboratories Ltd., Irvine Ayrshire, Scotland), supplemented with 10% tryptose phosphate broth, 5% fetal calf serum (FCS), and 2 mM glutamine.

Reconstitution of Viral Envelopes. Virosomes were prepared essentially by the method described by Metsikkö and co-workers for the vesicular stomatitis virus (Metsikkö et al., 1986), as adapted for influenza virus (Stegmann et al., 1987b; Bron et al., 1993). Briefly, virus (1.5 μmol of phospholipid) was sedimented by ultracentrifugation and solubilized in 100 mM $C_{12}E_8$ in HNE, the nucleocapsid was removed by ultracentrifugation (85000*g*, 30 min), and the supernatant (containing the viral envelope proteins and lipids) was shaken with Bio-Beads SM-2 to remove the detergent. Subsequently, the virosome preparation was centrifuged on a 10–40% (w/v) discontinuous sucrose density gradient in HNE at 130000*g* for 90 min, and the virosomes were collected from the interface between the sucrose layers. Probes were introduced as follows. Lyophilized DTA was dissolved in the reconstitution mixture at 25 μM. After formation of the virosomes as above, the virosomes collected from the sucrose gradient were diluted in HNE, sedimented at 150000*g* for 1 h, resuspended in 0.5 mL of HNE, mixed with 1.0 mL of 52.5% (w/v) metrizamide in HNE, subjected to flotation on a 35–30–0% (w/v) metrizamide gradient in HNE (150000*g*, 2h), and collected from the top of the gradient. Pyr-PC was dried from a solution in chloroform/methanol (1:1), first under a stream of nitrogen, and then at high vacuum for 1 h. Subsequently, the reconstitution mixture was added to the dry probe film to a molar ratio of viral lipids to Pyr-PC of 9:1.

Cytoplasmic Delivery of DTA. BHK-21 cells, grown to 5 × 10^4 cells per well in 96-well trays (Costar, Cambridge, MA), were washed with 200 μL of Hanks' medium (137 mM NaCl, 5.4 mM KCl, 0.40 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$, 0.40 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 5.6 mM glucose), buffered to pH 7.4 with 10 mM HEPES and supplemented with 0.1% BSA (HHB). Virosomes were allowed to bind to the cells in a volume of 50 μL of HHB at 4 °C for 1 h. Unbound virosomes were removed by two washes with ice-cold HHB. Virosome internalization was induced by the addition of 200 μL of HHB, prewarmed to 37 °C, and incubation of the cells at 37 °C. After 1 h the buffer was replaced by GMEM + 5% FCS. Fusion at the plasma membrane was induced by incubation in fusion buffer (135 mM NaCl, 5 mM HEPES, 5 mM MES, 5 mM sodium citrate and 20 mM NH_4Cl), set to various pH values, after virosome binding in the cold. After this acid treatment, the buffer was replaced by GMEM + FCS and 20 mM NH_4Cl. After 16 h, cellular protein synthesis was assessed by determination of [^{3}H]Leu (Amersham) incorporation during a 15-min incubation of 0.5 μCi per well in 50 μL of HHB. Protein was precipitated by two washes with 100 μL of ice-cold 5% TCA per well, followed by a 10-min incubation on ice. Precipitated proteins were dissolved in 200 μL of 0.1 N NaOH per well and quantified by liquid scintillation counting. All presented data are averages of four determinations.

Fluorescence Measurement of Intracellular Fusion of Virosomes. Measurement of intracellular fusion of Pyr-PC-labeled virosomes was carried out essentially as described before (Bron et al., 1993; Stegmann et al., 1993). Briefly, virosomes were allowed to bind to trypsinized BHK cells at 4 °C for 1 h in HHB. After removal of unbound virosomes, 50 μL of the virosome–cell complexes was injected into 1.95 mL of prewarmed (37 °C) HHB in a quartz cuvette and subsequently incubated for 1 h at 37 °C under continuous stirring. The decrease of the pyrene excimer fluorescence was monitored continously at 480 nm in an Aminco Bowman Series 2 fluorometer (SLM Aminco, Urbana, IL), while exciting at 343 nm. The extent of fusion (f) was calculated as $f(t) = 100[E_0 - E(t)]/(E_0 - E_{cbg})$, where $E(t)$ represents the excimer fluorescence intensity at 480 nm at time t, and E_0 and E_{cbg} represent the intensity at time zero and the

Table 1: Cytoplasmic Delivery of DTA to BHK-21 Cells by Influenza Virosomes[a]

condition	[^{3}H]Leu incorporation (dpm) ± SEM
untreated cells	43453 ± 2311 (0%)
free DTA[b]	42998 ± 2664 (1%)
empty virosomes[c]	42152 ± 2463 (3%)
DTA in virosomes[c]	1434 ± 73 (97%)
DTA in virosomes,[c] + anti-HA antiserum[d]	41367 ± 2346 (5%)
DTA in virosomes,[c] virosomes acid-pretreated[e]	43118 ● 1824 (1%)
DTA in virosomes,[c] + 20 mM NH_4Cl	37598 ± 1767 (2%)
untreated cells, + 20 mM NH_4Cl	37982 ± 1744 (0%)

[a] Levels of [^{3}H]Leu incorporation in TCA-precipitable material by BHK-21 cells, grown in 96-well trays, determined 16 h after the incubation with DTA or virosomes. Virosomes were derived from influenza strain X-97 and prepared in the presence of 25 μM DTA. Numbers in parentheses indicate relative levels of protein synthesis inhibition. [b] 100 μg/mL, present during 3 h at 37 °C. [c] Virosomes (20 μM added phospholipid) were allowed to bind for 1 h at 4 °C, and, after removal of unbound virosomes, the cell-associated virosomes were allowed to be internalized for 1 h at 37 °C. [d] Virosomes, preincubated in HHB at 37 °C for 30 min with rabbit anti-HA antiserum (1:50 dilution). [e] Virosomes, pretreated for 5 min at pH 5.4 and 37 °C.

background fluorescence intensity of the cells at 480 nm, respectively. The fluorescence data were processed using the software provided with the Aminco Bowman Series 2 fluorometer.

In Vitro Translation. Dilutions of toxins or virosomes were prepared in 5 mM DTT, 50 μM NAD^+ and 0.5% Triton X-100. The assay mixture (for 36 data points) was prepared by mixing 280 μL of rabbit reticulocyte lysate, 8 μL of amino acid mixture (minus leucine), 8 μL of RNasin, 16 μL of Brome Mosaic virus RNA (all obtained from Promega Co., Madison, WI), and 5 μL of [^{3}H]Leu (5 μCi) and 35 μL of H_2O. Prior to use, the RNA was heated for 10 min at 70 °C. Standard dilutions of toxins in 1 μL were titrated in sterile round-bottom 96-well trays (Costar). To the toxins, 9 μL of assay mixture was added, and the samples were incubated for 30 min at 37 °C. The reaction was stopped by the addition of 100 μL of 1 N NaOH/3% (v/v) H_2O_2 to the wells. In addition, 10 μL of 0.2% (w/v) BSA was added. After 10 min at 37 °C, 125 μL of ice-cold 50% (w/v) TCA was added, and the plates were kept on ice for 30 min. Subsequently, the plates were centrifuged for 10 min at 1400 rpm. The supernatant was removed and replaced by 200 μL of ice-cold 10% (w/v) TCA. After 10 min on ice, the plates were centrifuged at 1400 rpm for 10 min. The supernatant was removed, and the precipitated proteins were dissolved in 2× 100 μL of 0.1 N NaOH and quantified by liquid scintillation counting.

RESULTS

Influenza Virus Virosomes Mediate Transfer of DTA to the Cytoplasm. In order to investigate the potential of virosomes to mediate transfer of water-soluble molecules to the cytosol of cultured cells, subunit A of diphtheria toxin (DTA) was used as a marker for the virosomal interior. DTA-containing virosomes were added to BHK-21 cells grown in 96-wells trays, and delivery of the toxin to the cytosol was monitored by inhibition of the capacity of the cells to incorporate [^{3}H]Leu into proteins. The results, shown in Table 1, indicate that free DTA (100 μg/mL) did not reach the cytosol. However, when the toxin was encapsulated in virosomes, cellular protein synthesis was almost completely inhibited, indicating that transfer of DTA to the cytoplasm had occurred. Empty virosomes had no significant effect on cellular protein synthesis. Delivery of the encapsulated toxin

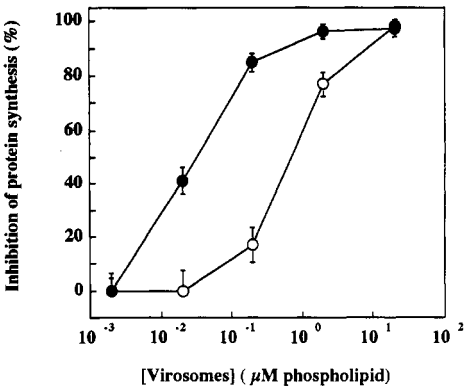

FIGURE 1: Dose dependence of virosome-mediated DTA delivery and the effect of virosome prebinding. Virosomes (derived from strain NIB26) were incubated with BHK-21 cells, grown in 96-well trays, in 50 μL of HHB at 37 °C for 3 h. Subsequently, the buffer was diluted with 200 μL of GMEM + FCS (closed circles). Alternatively, virosomes were allowed to prebind at 4 °C for 1 h. Unbound virosomes were removed, and, after a wash with ice-cold HHB, internalization was induced by the addition of prewarmed (37 °C) HHB and incubation at 37 °C. After 1 h the buffer was removed and replaced by medium (open circles). The extent of protein synthesis inhibition was determined 16 h after virosome internalization, as described under Materials and Methods, taking the levels obtained after treatment of cells with empty virosomes as the 0% level.

occurred from within endosomes in a pH-dependent fashion, as it was blocked by NH_4Cl, an inhibitor of endosomal acidification (Ohkuma & Poole, 1978). Pretreatment of the virosomes at acidic pH, which results in the abolishment of the fusion activity of HA molecules of this subtype (Puri et al., 1990), impaired the capacity of the virosomes to deliver DTA to the cytosol. Delivery was also strongly inhibited by preincubation of the virosomes with an anti-HA antiserum. Together these data indicate that transfer of the toxin molecules to the cytosol is specifically mediated by the fusion of the membrane of the virosomes with the membrane of the endosomal cell compartment and reflects the fusogenic activity of HA.

Figure 1 presents the extent of DTA-induced protein synthesis inhibition as a function of the virosome dose. Two different protocols were followed. The first protocol involved, as in Table 1, prebinding of the virosomes to the cells in the cold, followed by removal of unbound virosomes. Subsequently, internalization of cell-bound virosomes cells was induced by addition of warm (37 °C) buffer and incubation for 1 h at 37 °C. The second protocol did not involve prebinding and removal of unbound virosomes. Rather, DTA-containing virosomes at different concentrations were incubated with the cells for 3 h at 37 °C. In either case, the cells were incubated in medium for an additional period of 16 h, after which protein synthetic capacity was assessed. The results indicate that, in the first protocol, 50% inhibition of protein synthesis was induced at 1 μM added virosomal phospholipid. In the second protocol, the same effect was found at a concentration of virosomal phospholipid of only 50 nM. Since in the first protocol the unbound virosomes were removed, the difference between the dose dependencies of DTA delivery under these two conditions presumably reflects the difference in the number of virosomes taken up by the cells.

Virosome-Mediated DTA Delivery Involves Continuity between the Virosomal Lumen and the Cell Cytosol. In order to gain further insight into the precise mechanism of virosome-mediated DTA delivery to the cells, we first investigated whether the virosome-associated DTA was exclusively en-

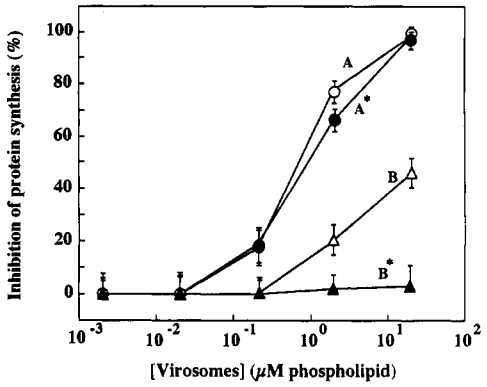

FIGURE 2: Effect of encapsulation of DTA in virosomes on the efficiency of cytoplasmic delivery to BHK-21 cells. Virosomes (derived from strain NIB24) were either prepared in the presence of 25 μM DTA (open circles; A) or mixed with 25 μM DTA after reconstitution (open triangles; B). After banding on a 10–40% (w/v) discontinuous sucrose gradient, half of each preparation was incubated with proteinase K (10 μg/mL) for 10 min at 37 °C (closed symbols: A* and B*, respectively). All virosome preparations were subsequently subjected to flotation on a 35–30–0% (w/v) metrizamide gradient at 150000*g* for 2 h. The virosomes were allowed to bind to BHK-21 cells at 4 °C, and, after removal of unbound virosomes, internalization was induced by incubation for 1 h at 37 °C. Protein synthesis inhibition was determined as in Figure 1.

capsulated within the aqueous interior of the virosomes. In the procedure used to remove the free DTA, the virosomes were routinely subjected to (i) sedimentation on a discontinuous 10–40% (w/v) sucrose gradient, (ii) dilution of the banded virosomes in HNE buffer and subsequent sedimentation by centrifugation at 150000*g* for 60 min in a SW50.1 rotor, and (iii) resuspension of the sedimented virosomes and subsequent flotation on a 35–30–0% (w/v) metrizamide gradient. Experiments with radioiodinated DTA (data not shown) indicated that after this protocol, starting with 1.5 μmol of viral lipid and 25 μM DTA, 3.5% of the initial DTA was associated with the virosomes. Only a fraction of this virosome-associated DTA, representing 0.7% of the initial DTA, was in fact encapsulated within the aqueous lumen as indicated by resistance against an exhaustive treatment with proteinase K (PK) (results not shown). This corresponds to an average of four DTA molecules per virosome.

In order to test whether DTA molecules associated to the outside of the virosomes might contribute to the observed impairment of the cellular protein synthesis, four different virosome preparations, with different localizations of DTA with respect to the virosomal membrane, were prepared and tested for their ability to deliver the toxin to the cytosol. Preparation A was prepared by reconstitution in the presence of 25 μM DTA and subjected to the separation procedure, described above. Preparation B involved empty virosomes, mixed with 25 μM DTA and subsequently subjected to the same separation procedure. After the sucrose gradient, portions of the two preparations were treated with PK, yielding preparations A* and B*. These four virosome preparations, as well as empty virosomes, treated similarly, were tested for their ability to induce inhibition of cellular protein synthesis following the internalization protocol involving prebinding of the virosomes to the cells in the cold and removal of unbound virosomes. The data, presented in Figure 2, show that, at high concentrations, the virosomes of preparation B had some effect on cellular protein synthesis. However, preparation A induced half-maximal inhibition at a concentration approximately 50-fold lower than preparation B. Thus, although a small fraction of the toxin molecules residing on the outside of the virosomes may gain access to the cytosol, those initially

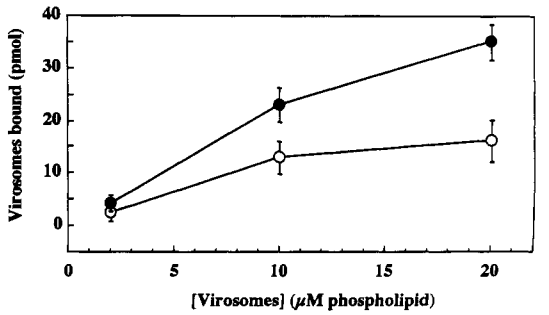

FIGURE 3: Extent of binding and internalization of virosomes by BHK-21 cells. Virosomes (derived from strain NIB24), labeled with [^{3}H]cholesteryl oleate, were allowed to bind to BHK-21 cells, grown in 96-well trays (50 000 cells per well). Unbound virosomes were removed. After a wash with ice-cold buffer, the cells were immediatedly detached by trypsinization, after which the amount of virosomes in the cell suspension was quantified by liquid scintilation counting (closed circles). Alternatively, the cells with bound virosomes were incubated in HHB for 1 h at 37 °C, washed and trypsinized, after which the amount of virosomes that remained cell-associated was determined (open circles).

trapped in the virosomal interior are transferred to the cytosol much more efficiently. This is substantiated by the observation that degradation of the external DTA hardly affected the toxicity of the virosomes with the encapsulated DTA (preparation A*), whereas the virosomes with DTA exclusively on the outside totally lost their activity after this treatment (preparation B*). From this we conclude that the virosome-mediated toxin delivery is almost exclusively due to toxin molecules initially present within the virosomal lumen and thus reflects the establishment of continuity between the virosomal interior and the cellular cytosol.

Binding and Internalization. On the basis of the above conclusion with regard to the mechanism of virosome-mediated delivery of DTA to cells, we set out to quantify the process. This involved determination of the extents of virosome binding and internalization and assessment of the amount of biologically active DTA delivered to the cells.

Using [^{3}H]cholesteryloleate as a marker, we first determined the binding and internalization of virosomes by BHK cells, under the conditions of the toxin delivery experiments. The virosomes were diluted to 2, 10, or 20 μM phospholipid in HHB and incubated in a volume of 50 μL for 1 h at 4 °C with BHK cells, grown in 96-well trays. After removal of unbound virosomes, the cells were either trypsinized directly or incubated for 1 h at 37 °C to allow internalization of the cell-associated virosomes, after which the cells were washed and trypsinized as well. In either case, the amount of cell-associated virosomes was determined by measurement of the radioactivity in the trypsinized cell suspension. The results, presented in Figure 3, indicate that, at the highest virosome concentration, 35.2 pmol of virosomal phospholipid was bound to 50 000 cells at 4 °C. This corresponds to 3.5% of the added total, or 8500 virosomes per cell. After warming to 37 °C and subsequent internalization, 16.3 pmol remained associated, corresponding to 1.6% of the added total, or 3900 virosomes per cell. The release of virosomes from the cells after warming is presumably induced by the activation of the virosomal neuraminidase [see Stegmann et al. (1987a)]. Observations by fluorescence microscopy, involving virosomes labeled with BODIPY-phosphatidylcholine, indicated that after 1 h of incubation at 37 °C all cell-associated virosomes had been internalized by the cells (results not shown).

Determination of the Number of Cytosolic DTA Molecules Based on the Rate of Protein Synthesis Inactivation. Next, we estimated the amount of DTA delivered to the cell cytosol

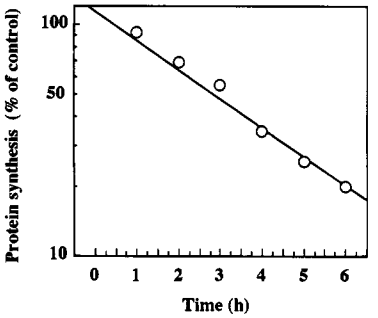

FIGURE 4: Rate of protein synthesis inhibition after internalization of DTA-containing virosomes. DTA-containing virosomes (strain NIB24; 20 μM added phospholipid) were allowed to prebind to BHK-21 cells in the cold, and internalization of cell-associated virosomes was induced as in Figures 1 and 2. At the indicated time points after the onset of internalization the protein synthesis capacity of the cells was determined as in Figure 1.

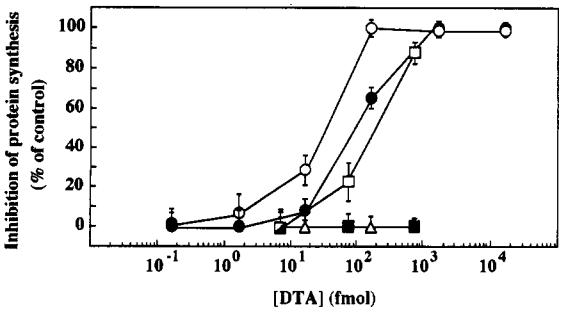

FIGURE 5: In vitro inhibition of protein synthesis by DTA. The capacity of DTA to inhibit protein synthesis after various treatments was assessed with a rabbit reticulocyte lysate as described under Material and Methods. All points are the mean of duplicate measurements: DT, reduced by 5 mM DTT (open circles); purified and lyophilized DTA (closed circles); DTA in the presence of $C_{12}E_8$-solubilized influenza virus envelopes (open squares); DTA, after the Bio-Beads treatment (closed squares); DTA, encapsulated in virosomes (open triangles).

on the basis of the biological activity of the toxin. Moynihan and Pappenheimer (1981) have determined the relationship between the DTA concentration and the rate of ADP-ribosylation of EF2 in vitro. It was found that the reciprocal half-time of ADP-ribosylation of EF2 was a linear function of the DTA concentration, the slope being 1.2 nM^{-1} min^{-1}. It was argued that the rate of ADP-ribosylation of EF2 is directly proportional to the rate of inactivation of protein synthesis, and this parameter was used to relate an in vivo observed rate of protein synthesis inhibition to the DTA concentration involved [see also Chu et al. (1990)].

In order to estimate the cytosolic DTA concentration after fusion of DTA-containing influenza virus virosomes via this approach, the levels of protein synthesis were determined by a 15-min pulse labeling with [^{3}H]Leu, with intervals of 1 h, after internalization of DTA-containing virosomes. From the data, presented in Figure 4, a half-time of inactivation of the cellular protein synthesis capacity of approximately 2 h and 15 min was determined. The DTA, introduced into the cytosol, thus catalyzed the ADP-ribosylation of 50% of the cellular EF2 within 2 h and 15 min. In vitro this would correspond to the activity of 6.7 pM DTA (Moynihan & Pappenheimer, 1981). If a cell volume of 3 pL is assumed for BHK cells (Moynihan & Pappenheimer, 1981), this would correspond to approximately 12 cytoplasmic DTA molecules per cell.

Multiplication of the number of cell-associated virosomes per cell after 1 h of endocytosis (3900; see Figure 3) with the number of encapsulated DTA molecules per virosome (approximately 4, see above) gives that under this condition 15 000–16 000 virosome-encapsulated DTA molecules are associated per cell. As we could trace the activity of only 12 DTA molecules in the cytosol, we tested whether the specific activity of the toxin was affected during the reconstitution procedure, by using an in vitro translation assay involving a rabbit reticulocyte lysate. The virosomal membranes were lysed in 0.5% Triton X-100 in order to release the encapsulated DTA molecules. The same amount of detergent was added to the standard DTA. The results in Figure 5 show that, at the highest virosome concentration that could be tested without an effect of empty virosomes, no detectable DTA activity was found in this assay, although the number of DTA molecules that was physically present should severely affect the in vitro translation process. This suggested that, surprisingly, the catalytic activity of the virosome-encapsulated DTA was reduced during the reconstitution process.

To determine at which stage of the reconstitution procedure this apparent inactivation of DTA had occurred, the inhibitory activity of purified DTA was compared to the activity of whole DT (reduced by 5 mM DTT) and purified DTA in the presence of $C_{12}E_8$-solubilized viral membranes, before and after the Bio-Beads treatment. The results (Figure 5) indicate that already some specific activity had been lost during the purification and/or lyophilization of the toxin, but the greatest loss of activity was found after the Bio-Beads treatment in the presence of 100 mM $C_{12}E_8$. Thus, during the reconstitution procedure the specific activity of the toxin is reduced at least 100-fold. Hence, the activity of 12 fully active DTA molecules corresponds to the activity of at least 1200 DTA molecules that had undergone the reconstitution procedure. Thus, of 15 000–16 000 internalized DTA molecules a lower bound of 1200 gain access to the cytosol, i.e., at least 8%.

Extent and Kinetics of Intracellular Fusion of Influenza Virosomes As Asssessed by Lipid Mixing. As a final aspect of the quantification of intracellular virosome fusion we determined the extent of lipid mixing during internalization of the virosomes by BHK cells and compared it with virosome-mediated DTA delivery. Lipid mixing of virosomes was determined on-line on the basis of dilution of virosome-incorporated Pyr-PC (Stegmann et al., 1993). The results, shown in Figure 6A, indicate that within 1 h a substantial fraction (>40%) of the incoming virosomes fused with the endosomal membrane. Thus, the fraction of virosomes that delivers its aqueous contents to the cytoplasm (a lower bound of 8%, see above) is in agreement with the fraction that merges with the membranes of the endosomal compartment. For the intact NIB26 virus we have determined a similar extent of lipid mixing after 1 h of internalization (Stegmann et al., 1993).

Figure 6A shows clearly that the onset of fusion was preceded by a lag phase of approximately 2–3 min, similar to what we have observed for the intact virus (Stegmann et al., 1993). This suggests that the initial site of fusion of the NIB26 influenza virus is the early endosomal compartment, as has been determined before for the SFV (Kielian et al., 1986; Schmid et al., 1989), which has a pH dependence of fusion (White et al., 1980; White & Helenius, 1980) quite similar to that of this particular influenza strain.

Time Course of DTA Delivery during Entry of Influenza Virosomes via the Endocytic Pathway. To determine the time course of penetration of DTA into the cytosol during virosome internalization, 20 mM NH_4Cl was added at different time intervals after the onset of the internalization process. Incubation of cells with NH_4Cl leads to a virtually instantaneous neutralization of the pH in the endo/lysosomal

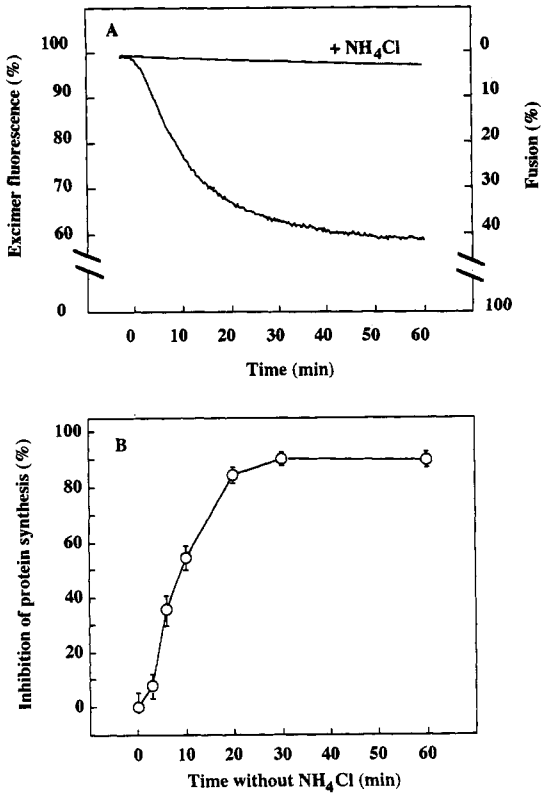

FIGURE 6: Time course of DTA penetration and lipid mixing during internalization of influenza virus virosomes by BHK-21 cells. (Panel A) Virosomes (strain NIB26; 50 μM phospholipid) containing 10 mol % Pyr-PC were allowed to bind to 20 × 10^6 trypsinized BHK cells in 1 mL of HHB at 4 °C. Unbound virosomes were removed in two centrifugation steps at 300g in 4 mL of HHB at 4 °C. Virosome–cell complexes were resuspended in 0.5 mL of ice-cold HHB. Virosome internalization was induced by the injection of aliquots of 50 μL (2 × 10^6 cells) into 1.95 mL of prewarmed (37 °C) HHB with or without NH_4Cl (20 mM), and fusion was monitored continuously as the decrease of the pyrene excimer fluorescence at 480 nm. (Panel B) DTA-containing virosomes (strain NIB26; 5 μM added phospholipid) were allowed to bind at 4 °C to BHK-21 cells. After removal of unbound virosomes, internalization was induced by incubation at 37 °C for 1 h. At the indicated time points, the buffer was replaced by HHB containing 20 mM NH_4Cl. Protein synthesis inhibition was determined as in Figure 1.

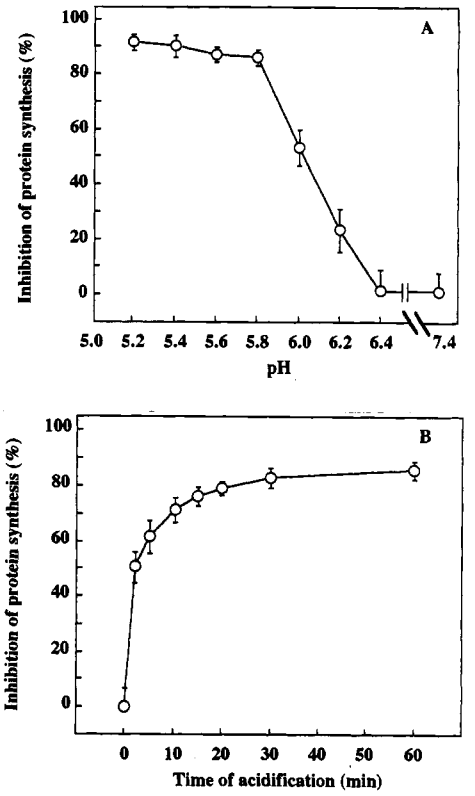

FIGURE 7: Virosome-mediated delivery of DTA via the plasma membrane. DTA-containing virosomes (5 μM added phospholipid; derived from strain NIB26) were allowed to bind to the plasma membranes of BHK-21 cells for 1 h at 4 °C. Unbound virosomes were removed by a wash with buffer. (Panel A) Fusion was induced by a 2-min incubation at 37 °C in fusion buffer, set to the indicated pH values. Protein synthesis inhibition was determined as described in the legend to Figure 1. (Panel B) Fusion was induced by incubation of the cells at pH 5.0 and 0 °C for the indicated time intervals. After completion of the incubation the fusion buffer was replaced with ice-cold medium containing 20 mM NH_4Cl. Subsequently, the cells were maintained at 37 °C, and protein synthesis inhibition was determined as in Figure 1.

compartment (Ohkuma & Poole, 1978). The virosome dose used in this experiment was chosen such that incubation of the cells for 1 h without NH_4Cl, during virosome internalization, resulted in 90% inhibition of protein synthesis. The results, shown in Figure 6, panel B, indicate that the time course of virosome-mediated toxin delivery correlated well with that of lipid mixing (panel A).

Cytoplasmic DTA Delivery Mediated by Virosome Fusion with the Plasma Membrane. Influenza virus can be induced to fuse with the plasma membrane of cultured cells by a brief exposure of cell-bound viruses to a medium with a mildly acidic pH (White et al., 1981). To investigate whether virosomes could mediate transfer of encapsulated DTA via this pathway, the virosomes (derived from strain NIB26) were allowed to bind to the plasma membrane of BHK cells for 1 h at 4 °C and induced to fuse by a 2-min incubation with fusion buffer, set to various pH values. The cells were continuously kept in the presence of 20 mM NH_4Cl, in order to prevent fusion from within endosomes. The results in Figure 7A indicate that cellular protein synthesis was impaired after exposure of the cell-bound virosomes to a pH of 6.2 or lower, the maximal effect being reached around pH 5.6–5.8. This correlates well with the pH dependence of fusion of the NIB26 strain, as determined by hemolysis, and fusion with liposomal membranes (results not shown). Importantly, the threshold pH of virosome-mediated DTA delivery via the plasma membrane is significantly higher than the threshold pH of DTA translocation (Papini et al., 1988; London, 1992).

It has been observed by several investigators that influenza HA can induce fusion at temperatures as low as 0 °C (White et al., 1982; Stegmann et al., 1990; Tsurudome et al., 1992), albeit at a low rate. If virosomes had the capacity to deliver DTA to cells at low temperatures, this would further extend the evidence for the notion that HA can express its activity at 0 °C and, in addition, substantiate that virosome-mediated DTA delivery reflects the fusogenic activity of HA. Therefore, virosomes, prebound to the plasma membranes of BHK cells were exposed at 0 °C to fusion buffers, adjusted to pH 5.0. After various time intervals the fusion buffer was replaced by ice-cold GMEM + FCS and 20 mM NH_4Cl. The virosome dose chosen for this experiment (5 μM phospholipid) was just sufficient to induce 90% inhibition of protein synthesis, after prebinding and a 1-h endocytic internalization of the virosomes as in Figure 1. The results, presented in Figure 7, panel B, show that the cellular protein synthesis was indeed inhibited after acidification of the DTA-containing virosomes at 0 °C. The degree of inhibition increased with increasing time of acidification. The incubations at low pH and low temperature did not significantly affect the levels of [^{3}H]Leu incorporation into the cells.

DISCUSSION

This paper presents evidence indicating that molecules encapsulated in the lumen of reconstituted influenza virus envelopes are deposited efficiently into the cytosol of cultured cells through low-pH-dependent fusion of the virosomal envelope with the endosomal membrane, after internalization of the virosomes by the cells via receptor-mediated endocytosis. Cytosolic delivery of virosome-encapsulated compounds can also be accomplished by induction of fusion between the virosomal membrane and the plasma membrane of the cell through a transient acidification of the extracellular medium. Since virosomes exhibit cellular entry functions similar to those of the intact virus, they provide a versatile model system for studying the membrane interactions and fusion characteristics of native viral envelopes. In addition, virosomes may be used as vehicles for delivery of foreign molecules to the cytoplasm of living cells.

The data, presented in Figure 2, demonstrate that virosome-mediated transfer of DTA molecules to the cytosol is almost exclusively due to toxin molecules, originally encapsulated in the virosomal interior. Free toxin molecules may percolate to the cytosol during virosome fusion, but this is only observed at a high virosome dose. A similar phenomenon has been observed by Metsikkö and co-workers (1986), who demonstrated that the plasma membrane of BHK cells became permeable for anti-tubulin IgG molecules after fusion of high doses of VSV virosomes. It is possible that permeabilization of the cellular target membrane is facilitated by the residual detergent left in the virosomal membranes (Stegmann et al., 1987b; Metsikkö et al., 1986). However, we emphasize that also intact influenza virus has been shown to induce leakage during fusion from within endosomes (Jakeman et al., 1991). Accordingly, we have observed cytoplasmic transfer of free DTA after co-internalization of UV-irradiated intact influenza virus (results not shown). Importantly, our present results show that the extent of this leakage phenomenon is very small relative to the extent of cytoplasmic delivery of substances initially enclosed in the virosomal or viral interior. The role of leakage during fusion is probably even less significant than the results in Figure 2 suggest, considering that the free toxin molecules, mixed with preformed empty virosomes, had not undergone the inactivating $C_{12}E_8$/Bio-Beads treatment (see Figure 5) and thus were presumably far more active than the virosome-encapsulated toxin molecules. Therefore, virosome-mediated cytoplasmic delivery of DTA can be considered as an event, analogous to nucleocapsid penetration from intact virus.

The data presented in Figures 6 and 7 illustrate that cell intoxication after virosome-mediated cytoplasmic DTA delivery provides a sensitive means to characterize virosome–cell interactions. Indeed, the pH and temperature dependence of fusion and the time course of intracellular fusion are clearly reflected by the levels of protein synthesis inhibition of the target cells after treatment with DTA-containing virosomes. Importantly, toxin delivery is fully inhibited by 20 mM NH_4Cl. Therefore, a false-positive fusion signal under this nonfusion condition, as may occur with R_{18}-dequenching (Stegmann et al., 1993), does not hamper the assessment of the intracellular fusion reaction via this approach. Furthermore, the extent of virosome-mediated DTA delivery, a lower-bound of 8% of the cell-internalized virosomes depositing their contents into the cytosol, is in good agreement with that of virosome fusion as assessed by a fluorescence lipid mixing assay, representing 40% fusion of the incoming virosomes. Finally, assessment of toxin delivery is more straightforward than the monitoring of viral infection, in the sense that it does not rely on many events following fusion, such as uncoating, correct processing of the viral genome, and synthesis and assembly of viral components. This feature may be important when the interactions of enveloped viruses with different target cells are compared.

Besides providing a model system for the investigation of viral fusion functions, virosomes may be used as vehicles for the delivery of water-soluble foreign molecules to cells. Virosomes represent a more versatile delivery system than the system described by Doxsey et al. (1985), involving fusion of loaded erythrocyte ghosts to cells carrying the influenza virus HA on their surface. Although the potential delivery capacity of the latter system is quite high, the use of this type of carrier is limited to cultured cells expressing the HA, whereas in the case of virosomes the membrane fusion function resides on the carrier. Virosomes are also more versatile than the cationic liposomes, first described by Felgner et al. (1987), that are being used extensively for cellular delivery of nucleic acids both in cultured-cell systems (Düzgünes & Felgner, 1993) and, under certain conditions, in intact animals (Zhu et al., 1993). The use of cationic liposomes is limited to delivery of primarily nucleic acids. In fact, cationic liposomes with nucleic acids do not appear to be a genuinely vesicular carrier (Gershon et al., 1993), but rather a condensed complex of the positively charged lipids with the negatively charged nucleic acid that, by an as yet unidentified mechanism, traverse the cellular membrane barrier. Virosomes, on the other hand, as demonstrated in the present study, do represent vesicular carriers in the strict sense that they fuse in a largely nonleaky fashion with cellular membranes, thereby establishing a continuum between the virosome lumen and the cell cytoplasm. Virosomes have also been demonstrated to have the capacity to deliver genes to cells (Gould-Fogerite et al., 1989).

The relative delivery efficiency of the virosomes is high. We estimated that minimally 8% of the virosomes that are internalized by the cells deliver their contents to the cytoplasm, while with the Pyr-PC lipid mixing assay a final extent of 40% fusion was observed. These values are not in contradiction; in fact there is no reason to assume that a fraction of the virosomes, fusing on the basis of the lipid mixing data, would *not* deliver their aqueous contents to the cytoplasm. In the protocol involving prebinding in the cold and removal of unbound virosomes, 40% fusion corresponds to a number of about 1500 virosomes per cell, at a virosome concentration of 20 μM. In the protocol involving continuous uptake of the virosomes this number is considerably, perhaps even 20-fold (see Figures 1 and 3), higher. Depending on the number of molecules encapsulated per virosome (in our case, this equaled on average 4), a substantial amount of foreign compound may thus be delivered to the cell cytoplasm. The delivery efficiency of influenza virus virosomes is considerably higher than that of pH-sensitive liposomes, composed of cholesteryl hemisuccinate (CHEMS) and dioleoylphosphatidylethanolamine (DOPE) (Chu et al., 1990). Relative DTA delivery by liposomes of the latter composition has been estimated to be $\geq$0.01% vs $\geq$8% for the virosomes. This difference is presumably due to the essentially different mechanisms, underlying cytoplasmic delivery, as mediated by virosomes or pH-sensitive liposomes. As demonstrated in this paper, DTA delivery by virosomes is due to the establishment of continuity between the virosomal lumen and the cell cytosol, as a result of fusion of the virosomal with the endosomal membrane (Figure 2). On the other hand, cytoplasmic delivery by pH-sensitive liposomes involves massive pH-induced

disruption of the vesicles, during which a small fraction of the molecules may leak to the cytosol as a result of occasional concomitant destabilization of the endosomal membrane (Wang & Huang, 1989).

The internal volume of virosomes prepared by the $C_{12}E_8$ method is small (Metsikkö et al., 1986; Stegmann et al., 1987b). As a consequence, the trapping efficiency is low, and, in the present study, over 99% of the DTA was not encapsulated in the vesicles. This low encapsulation efficiency is primarily due to the low lipid concentrations used in the protocol. Work aimed at improving the encapsulation efficiency of the reconstitution procedure, while retaining the fusion capacity of the vesicles, is currently in progress in our laboratory.

Our observation that treatment with Bio-Beads results in an appreciable loss of biological activity of DTA indicates that, for delivery purposes, the use of virosomes, prepared according to the present reconstitution procedure, is limited to molecules that tolerate the Bio-Beads treatment or to applications for which preservation of the native conformation of the encapsulated molecules is not critical. In this respect, we emphasize that the membrane fusion activity of the influenza HA is not affected by the Bio-Beads treatment, indicating that a loss of biological activity of proteins upon exposure to Bio-Beads is not a general phenomenon. An example of virosome-mediated delivery that does not necessarily require preservation of the native conformation of the encapsulated molecules is given by the introduction of antigens into the MHC class I presentation pathway. It is well established that class-I-restricted antigen presentation, which results in priming of CD8+ cytotoxic T lymphocytes, involves cytosolic processing of proteins to small peptides and subsequent transport of the peptides across the membrane of the endoplasmic reticulum (Monaco, 1992). Since the antigens are degraded in the cytosol of presenting cells, it is unlikely that prior preservation of their conformation during virosome encapsulation is of critical importance. Therefore, virosomes prepared by the $C_{12}E_8$/Bio-Beads reconstitution procedure should be capable of initiating an MHC-class-I-restricted CTL response through efficient introduction of encapsulated protein antigens into the cytosol of antigen-presenting cells. In this respect, reconstituted Sendai virus envelopes containing an SIV-derived antigen have been demonstrated, in Rhesus macaques, to have the capacity to prime a *bona fide* CTL response against SIV-infected cells (Miller et al., 1992).

REFERENCES

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Biophysiol. 37*, 911–913.

Bron, R., Ortiz, A., Dijkstra, J., Stegmann, T., & Wilschut, J. (1993) *Methods Enzymol. 220*, 313–331.

Böttcher, C. J. F., van Gent, C. M., & Fries, C. (1961) *Anal. Chim. Acta 24*, 203–204.

Chu, C.-J., Dijkstra, J., Lai, M.-Z., Hong, K., & Szoka, F. C. (1990) *Pharmacol. Res. 7*, 824–834.

Collins, D., & Huang, L. (1987) *Cancer Res. 47*, 735–739.

Doxsey, S. J., Sambrook, J., Helenius, A., & White, J. (1985) *J. Cell Biol. 101*, 19–27.

Düzgünes, N., & Felgner, P. L. (1993) *Methods Enzymol. 221*, 303–306.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., & Danielsen, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7413–7414.

Gershon, H., Ghirlando, R., Guttman, S. B., & Minsky, A. (1993) *Biochemistry 32*, 7143–7151.

Gould-Fogerite, S., Mazurkewiecz, J. E., Raska, K., Voelkerding, K., Lehman, J. M., & Mannino, R. J. (1989) *Gene 84*, 429–438.

Haywood, A. M., & Boyer, B. P. (1982) *Biochemistry 21*, 6041–6046.

Hoekstra, D., & Klappe, K. (1986) *J. Virol. 58*, 87–95.

Honjo, T., Nishizuka, Y., Hayaishi, O., & Kato, I. (1968) *J. Biol. Chem. 243*, 3553–3555.

Jakeman, K. J., Smith, H., & Sweet, C. (1991) *J. Gen. Virol. 72*, 111–115.

Kielian, M. C., Marsh, M., & Helenius, A. (1986) *EMBO J. 5*, 3103–3109.

London, E. (1992) *Biochim. Biophys. Acta 1113*, 25–51.

Matlin, K. S., Reggio, H., Helenius, A., & Simons, K. (1981) *J. Cell Biol. 91*, 601–613.

Mcclure, M. O., Marsh, M., & Weiss, R. A. (1988) *EMBO J. 7*, 513–518.

Metsikkö, K., van Meer, G., & Simons, K. (1986) *EMBO J. 5*, 3429–3435.

Miller, M. D., Gould-Fogerite, S., Shen, L., Woods, R. M., Koenig, S., Mannino, R. J., & Letvin, N. L. (1992) *J. Exp. Med. 176*, 1739–1744.

Monaco, J. J. (1992) *Immunol. Today 13*, 173–179.

Moynihan, M. R., & Pappenheimer, A. W. (1981) *Infect. Immun. 32*, 575–582.

Ohkuma, S., & Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3327–3331.

Papini, E., Sandoná, D., Rappuoli, R., & Montecucco, C. (1988) *EMBO J. 7*, 3353–3359.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Puri, A., Booy, F. P., Doms, R. W., White, J. M., & Blumenthal, R. (1990) *J. Virol. 64*, 3824–3832.

Schmid, S., Fuchs, R., Kielian, M., Helenius, A., & Mellman, I. (1989) *J. Cell Biol. 108*, 1291–1300.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1985) *Biochemistry 24*, 3107–3113.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1986) *J. Biol. Chem. 261*, 10966–10969.

Stegmann, T., Morselt, H. W. M., Scholma, J., & Wilschut, J. (1987a) *Biochim. Biophys. Acta 904*, 165–170.

Stegmann, T., Morselt, H. W. M., Booy, F. P., van Breemen, J. F. L., Scherphof, G., & Wilschut, J. (1987b) *EMBO J. 6*, 2651–2659.

Stegmann, T., White, J. M., & Helenius, A. (1990) *EMBO J. 9*, 4231–4241.

Stegmann, T., Schoen, P., Bron, R., Wey, J., Bartoldus, I., Ortiz, A., Nieva, J. L., & Wilschut, J. (1993) *Biochemistry 32*, 11330–11337.

Stein, B. S., Gowda, S. D., Lifson, J. D., Penhallow, R. C., Bensch, K. G., & Engleman, E. G. (1987) *Cell 49*, 659–668.

Tsurudome, M., Gluck, R., Graf, R., Falchetto, R., Schaller, U., & Brunner, J. (1992) *J. Biol. Chem. 267*, 20225–20232.

Wang, C.-Y., & Huang, L. (1989) *Biochemistry 28*, 9508–9514.

White, J., & Helenius, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3273–3277.

White, J., Kartenbeck, J., & Helenius, A. (1980) *J. Cell Biol. 87*, 264–272.

White, J., Matlin, K., & Helenius, A. (1981) *J. Cell Biol. 89*, 674–679.

White, J. M., Kartenbeck, J., & Helenius, A. (1982) *EMBO J. 1*, 217–222.

Zhu, N., Liggitt, D., Liu, Y., & Debs, R. (1993) *Science 261*, 209–211.