Chapter 13

Fusion of Enveloped Viruses with Biological Membranes

Fluorescence Dequenching Studies

Nor Chejanovsky, Ofer Nussbaum, Abraham Loyter, and Robert Blumenthal

1. INTRODUCTION

Enveloped virions penetrate eukaryotic cells by two alternative routes (Choppin and Scheid, 1980; White *et al.*, 1983). Envelopes of viruses belonging to the paramyxovirus group fuse with cells' plasma membranes at pH 7.4 and consequently microinject their content, the viral nucleocapsid, directly

Abbreviations used in this chapter: $C_{12}E_8$, octaethyleneglycolmono(*n*-dodecyl)ether; CF, carboxyfluorescein; chol, cholesterol; CL, cardiolipin; DPA, dipicolinic acid; DQ, dequenching; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; FRET, fluorescence resonance energy transfer; gang, gangliosides; HA, hemagglutinin; HA_0, precursor of the influenza hemagglutinin glycoprotein; HEG, human erythrocyte ghosts; HSV, herpes simplex virus; HTC, hepatoma tissue cultured; N-NBD, 7-nitro-1,2,3-benzoxydiazole-4-amino; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; R18, octadecylrhodamine B-chloride; RIVE, reconstituted influenza virus envelope; ROVs, right-side-out (erythrocyte membrane) vesicles; RSVE, reconstituted Sendai virus envelope; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus.

Nor Chejanovsky, Ofer Nussbaum, and Abraham Loyter Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. **Robert Blumenthal** National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

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into the cell cytoplasm (Choppin and Scheid, 1980; Loyter and Volsky, 1982; White *et al.*, 1983). A different way of entry has been described for most other enveloped virions such as those belonging to the orthomyxovirus, toga, rhabdo, and herpes groups. Such viruses are taken into cells by endocyticlike processes. Fusion of the viral envelopes with the endosomal or lysosomal membranes is triggered by the intraorganelle low-pH environment and leads to the introduction of the viral content into the intracellular space (Chopin and Scheid, 1980; White *et al.*, 1983). Fusion of the pH-dependent virions with the plasma membrane can be triggered by lowering the pH of the medium containing the virus-associated cells.

A variety of different approaches have been applied to study the complex problem of analyzing intracellular events concomitant with virus penetration. Classically, electron microscopy has been used for such analysis, since individual virions associated with the plasma membrane or localized intracellular can be accounted for with that technique. However, according to Dales (1973), "the methodology of electron microscopy is fraught with artifacts. As a result a number of controversies have arisen regarding the true pathways for penetration." The detailed discussion offered by Dales (1973) of the difficulties and pitfalls in preparation of specimens and evaluation of electron microscopic images to analyze pathways of viral penetration is still very worthwhile reading.

In the ensuing years a number of complementary approaches have been used, and there seems to be a consensus emerging concerning entry mechanisms.

2. RECEPTORS FOR ENVELOPED VIRUSES

The infection of cells by animal viruses involves two main steps: (1) binding of the virus particles to specific cell receptors and (2) introduction of the viral nucleocapsid into the cell cytoplasm (Choppin and Scheid, 1980; Dimmock, 1982). The attachment of the viral particles to the cell surface is mediated by the viral glycoproteins that constitute the spikes in the viral envelope and specifically recognize cellular receptors for the virus on the plasma membrane of the target cells (Rott and Klenk, 1977; White *et al.*, 1983). Thus, the specificity of this recognition process depends on the molecular structure of those viral glycoproteins and their correspondent receptors. The absence of the viral receptors on a specific cell would confer it resistance to viral infection (Rott and Klenk, 1977; Choppin and Scheid, 1980). Some common characteristics can be ascribed to the process of binding of enveloped viruses to the cell surface:

1. The attachment of the viral particle to its cellular receptor is a rela-

tively slow process. For example, binding of the Sendai virus to human erythrocytes is maximal at 15 min (Wolf *et al.*, 1983) and binding of Semliki Forest virus to BHK-21 cells takes about 20–40 min (Fries and Helenius, 1983).

2. The attachment of the viral particles to their cellular receptor is almost irreversible. Elution of viruses bound to the cell surface was achieved by enzymatic digestion of the cellular receptor or, alternatively, by inactivation of the correspondent binding glycoprotein (Helenius *et al.*, 1980; Chejanovsky *et al.*, 1984).

The number of viral particles that are able to penetrate the target cell is mainly controlled by the number of viral receptors available on the cell surface (Helenius *et al.*, 1980; Chejanovsky *et al.*, 1984). The multiple copies of the viral glycoproteins present in the viral envelope contribute to the multivalent binding of the virus to the cell surface (Kielian and Helenius, 1986). The distribution of the bound virus particles on the cell surface is not always even and depends on the cell type and organization of the cell surface. For example, a high concentration of the Semliki Forest virus near the microvilli of BHK-21 cells was reported (Fries and Helenius, 1983).

The next section deals with the different cellular receptors for the different viral groups.

2.1. Myxoviruses

This viral family includes the influenza viruses of humans and lower animals. The viral envelope of viruses belonging to this group comprises three proteins. Two of them are surface glycoproteins, designated HA (hemagglutinin) and NA (neuraminidase), and are responsible for the viral binding and fusion properties and for enzymatic removal of sialic acid residues of the cell surface, respectively (Rott and Klenk, 1977; Choppin and Scheid, 1980; White *et al.*, 1983). Sialic acid residues from glycoproteins and glycolipids were found to serve as the cellular receptor for influenza viruses (Schulze, 1975; Rott and Klenk, 1977). Moreover, different human influenza isolates bearing antigenically different hemagglutinin serotypes were able to differentiate microdomains of the gangliosides such as the sialic acid species (NeuAc, NeuGc) and the sequence of sialic acid linkages (NeuAc alpha 2-3 gal, NeuAc alpha 2-6 gal) preferentially as specific viral receptors (Suzuki *et al.*, 1985). For example, the influenza strain A/PR/8/34, which is serotype H1 for hemagglutinin, recognized IV-3 NeuAc-nLc4Cer containing the NeuAc alpha 2-3 gal sequence preferentially over IV-6 NeuAc-nL c4Cer containing NeuAc alpha 2-6 gal, where the H2 serotype (A/Japan/305/57) recognized the NeuAc alpha 2-6 gal sequence preferentially over NeuAc alpha 2-3 gal (Suzuki *et al.*, 1985).

2.2. Paramyxoviruses

The most representative member of this family of viruses is the Sendai virus. Two glycoproteins are associated with the membrane of the paramyxovirus (Tozawa *et al.*, 1973), namely, the hemagglutinin neuraminidase (HN) and the fusion factor (F) (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). The HN glycoprotein mediates the binding of the viral envelope to its cellular receptor (Homma and Ohuchi, 1973). This glycoprotein specifically recognizes sialic acid containing glycoproteins and glycolipids on the target cell surface (Poste and Pasternak, 1978; Choppin and Scheid, 1980; Dimmock, 1982). Removal of membrane sialic acid residues by treatment with neuraminidase (*Vibrio cholerae* neuraminidase) results in the inhibition of the Sendai virus binding (Rott and Klenk, 1977; Dimmock, 1982). Further insertion of sialoglycolipids into the membranes of neuraminidase-treated cells allowed the subsequent infection of the host cell by Sendai virus particles (Markwell *et al.*, 1981). The neuraminidase activity residing in the HN molecule of Sendai virions seems to play a role in the spreading of the virus in the infected host (Choppin and Scheid, 1980).

2.3. Togaviruses

The knowledge acquired in the penetration of the togaviruses was due to the extensive research performed on the entry of the two alphaviruses Semliki Forest and Sindbis (Helenius *et al.*, 1980; White *et al.*, 1983). The viral nucleocapsid is enclosed in a lipid bilayer membrane in which the viral glycoproteins that constitute the spikes are embedded (Helenius *et al.*, 1980). Three glycoproteins compose the spikes E_1, E_2, and E_3. E_1 and E_2 are transmembrane proteins and E_3 is noncovalently associated with these and is external to the lipid bilayer (Helenius *et al.*, 1980). It has been shown that Semliki Forest viruses were bound preferentially to the major histocompatibility antigens HLA-A and HLA-B present on the surface of JY cells and to the H2-K and H2-L antigens present in peripheral murine lymphocytes (Helenius *et al.*, 1978). However, cells devoid of H2 antigens can be efficiently infected with Semliki Forest virus (Oldstone *et al.*, 1980).

Proteolytic digestion of host cell membranes with the appropriate proteases inhibited Sindbis virus binding to its target cell (Smith and Tignor, 1980). Neuraminidase treatment of the cells marginally affected the binding ability of the Sindbis virus (Smith and Tignor, 1980). Studies performed by chemical cross-linking of the Sindbis virus spike glycoproteins bound to the cell surface of JY and Daudi cells identified an M_r 90,000 protein (Massen and Terhost, 1981). The exact molecular nature of the alphaviruses receptor is still unclear.

2.4. Rhabdoviruses

The prototype of this group is the vesicular stomatitis virus (VSV). The viral envelope contains a single glycoprotein (G) embedded in the viral membrane (Wagner, 1975). The exact nature of the VSV cellular receptor was difficult to establish. The extremely wide host range of the VSV suggested that its cellular receptor is a component present in virtually all animal cells. Competition studies showed saturable binding sites for VSV on Vero cells (Schlegel *et al.*, 1982). Protease treatment of recipient cells did not affect VSV binding but the addition of phosphatidylserine (PS) did. Moreover, PS inhibited VSV plaque formation (Schlegel *et al.*, 1983). Reconstituted VSV envelopes bearing the VSV G-protein were able to bind and fuse at low pH with phosphatidylserine liposomes (Eidelman *et al.*, 1984). However, the specificity of PS as the VSV receptor is still questionable.

Other investigators studied the effect of incubation of VSV particles with gangliosides on the infectivity of the virus to CER cells. In addition, gangliosides from mammalian brains and from CER cells were able to inhibit the VSV attachment to susceptible CER cells (Sinibaldi *et al.*, 1985). Moreover, VSV bound very poorly to CER cells treated with glycosylases. Deglycosylated CER cells reacquired their susceptibility to infection by VSV after coating with gangliosides, suggesting the participation of the gangliosides in the receptorial structure for VSV in the CER cells system (Sinibaldi *et al.*, 1985).

2.5. Retroviruses

The extensive research performed on the biology of the human T-lymphotropic viruses, which are members of the retrovirus family, paved the way to the characterization for the cellular receptor of the human T-lymphotropic virus III (HTLV-III). The HTLV-III virus (AIDS virus) has a selective tropism to a subset of T lymphocytes, which are defined by the expression of the surface glycoprotein T_4 (CD_4 antigen) (Klatzmann *et al.*, 1984). Monoclonal antibodies directed against T_4 molecule were able to block infection of T_4^+ lymphocytes by HTLV-III (Klatzmann *et al.*, 1984). This blocking effect was shown to be specific to these antibodies; monoclonal antibodies against the histocompatibility locus antigens (HLA) class II or anti T-cell natural killer (TNK) target did not prevent HTLV-III infection (Klatzmann *et al.*, 1984). In a parallel study, pseudotypes of vesicular stomatitis virus bearing HTLV-III envelopes were tested for its ability to infect and promote formation of syncytia by mixing virus-producing and cell-bearing receptors for HIV-III (Dalgleish *et al.*, 1984). Receptors were present only on cells. It was found that anti-CD_4 antibodies inhibited syncytia formation and blocked

the pseudotypes. When the genes for T_4 were expressed on the surface of T or B lymphocytes or other human cell lines, such cells became susceptible to HTLV-III infection (Maddon *et al.*, 1985; Dewhurst, 1987). The T_4 molecule serves as the cellular receptor to HTLV-III and interacts with the viral envelope glycoprotein gp110 (McDougal *et al.*, 1986).

2.6. Herpesviruses

The envelope of the herpesviruses comprises several glycoproteins embedded in the lipid bilayer (Choppin and Scheid, 1980). The cellular receptor for the Epstein-Barr virus, a member of the herpesvirus family, was recently identified (Fingeroth *et al.*, 1984). The rank order to binding of fluoresceinated EBV to four lymphoblastoid cell lines (SB, JX, Molt-4, and Raji) was identical to the rank order of binding of monoclonal antibodies that recognized the complement receptor type 2 (CR2) as measured by analytical flow cytometry. Transfer of CR2 from SB cells to protein-bearing *Staphylococcus aureus* particles (absorbed with anti-CR2 monoclonal antibody) conferred on them the specific ability to bind ^{125}I-labeled EBV (Fingeroth *et al.*, 1984). Pretreatment of cells with anti-CR2 monoclonal antibody followed by treatment with goat $F(ab)_2$ fragments to mouse IgG blocked the binding of fluoresceinated EBV on SB cells (Fingeroth *et al.*, 1984). Based on these experiments, it was concluded that the complement receptor molecule (C3d receptor CR2) constitutes the cellular receptor for EBV. Further studies had shown that an immunotoxin made from a monoclonal antibody against the C3d receptor and the toxin gelonin was able to inhibit at very low concentrations (10^{-11} M) the ability of EBV to induce polyclonal proliferation of normal B lymphocytes (Tedder *et al.*, 1986).

2.7. Other Enveloped Viruses

The spectrum of cellular receptors for the enveloped virus families of Poxvirus, Iridovirus, Hepadnavirus, Coronavirus, Bunyaveravirus, and Arenavirus is not as yet identified.

3. INTERACTION OF ENVELOPED VIRUSES WITH RECEPTOR-DEPLETED CELLS

A different approach to study the interaction between enveloped animal viruses and target cell membranes was to study the ability of artificial receptors to mediate the viral entry to the host cell. The Sendai virus was mainly used as the model in these studies.

3.1. Use of Antimembrane Antibodies or Polypeptide Hormones to Mediate Virus Attachment

Biologically active Sendai virus envelopes can be obtained by solubilization of the viral membrane in Triton X-100 and subsequent removal of the detergent. Reconstituted Sendai virus envelopes (RSVE) constituted a closed vesicle bearing the two viral glycoproteins, hemagglutinin neuraminidase (HN) and the fusion factor (F), embedded in the lipid bilayer devoid of the nucleocapsid (Volsky and Loyter, 1978). Two alternative methods were used to promote the binding and subsequent fusion of the RSVE with viral-receptor-depleted living cells (viral receptor depletion was performed by neuraminidase digestion of the cellular sialic acid residues):

1. An antibody raised against recipient cell membranes was inserted into the viral envelope. Anti-human erythrocyte IgG was covalently bound to the hydrophobic molecule dodecanethiol and further inserted into the RSVE particles by co-reconstitution (Gitman and Loyter, 1985). The obtained RSVE bearing the anti-human erythrocytes antibody was shown to bind and subsequently fuse with the viral-receptor-depleted erythrocytes. Thus, a cell surface antigen was able to function as an efficient alternative cellular receptor for Sendai virions (Gitman and Loyter, 1984).
2. Anticell specific antibodies or alternatively polypeptide hormones were covalently coupled to the Sendai virus envelope glycoproteins after they had been solubilized with Triton X-100 (Nussbaum *et al.*, 1984). Using this method, RSVE bearing the covalently attached anti-human erythrocyte antibodies or insulin molecules was able to attach and fuse to virus-receptor-depleted human erythrocytes and rat hepatoma cells (Gitman *et al.*, 1985). Again, in this case a cellular antigen or the cell insulin receptor served as functionally active cellular receptors for Sendai virions.

3.2. Implantation of Receptors or Binding Proteins for Enveloped Virions into Recipient Cell Membranes

The ability of different gangliosides to mediate the binding and fusion of the influenza virus with chicken asialoerythrocytes was used as a model system (Suzuki *et al.*, 1985). As mentioned before, insertion of the ganglioside GM3 into the chicken asialoerythrocyte membranes restores their susceptibility to the influenza virus binding and fusion activities (Suzuki *et al.*, 1985). Similarly, insertion of sialoglycolipids into desialyzed cells allowed their infection by Sendai virus particles [see paramyxoviruses in this review and Markwell *et al.* (1981)].

Receptor negative cells to the Epstein-Barr virus were converted to receptor positive cells by fusion-mediated transfer (Volsky *et al.*, 1980). Mem-

branes rich in receptors for EBV from the human lymphoma cell line Raji were co-reconstituted with RSVE and the formed EBV-receptor-bearing RSVEs were subsequently fused with the EBV receptor negative cells. By this procedure those EBV receptor negative cells became susceptible to EBV infection (Volsky *et al.*, 1980).

The ability of anti-Sendai antibodies to mediate the binding of the virus particles to virus-receptor-depleted cells was also studied (Nussbaum *et al.*, 1984). Anti-Sendai antibodies were chemically coupled to the surface of desialyzed human erythrocytes by the use of the bifunctional cross-linking reagents *N*-succinimidyl-3-2(2-pyridyldithio) propionate or succinimidyl-4-(*p*-maleimidophenyl) butyrate. These antibodies served as attachment sites and were recognized by biologically active Sendai virus particles. Virus erythrocyte fusion was observed following attachment to anti-Sendai antibody-bearing cells (Nussbaum *et al.*, 1984).

A different system was developed by using the binding pair affinity of avidin molecule to biotin (Guyden *et al.*, 1983). Avidin was chemically coupled to surfaces of human red cells and biotin was concomitantly coupled to Sendai virus particles. The couple avidin–biotin served as an alternative recognition system between the treated Sendai virus particles and the target cell. The attachment of the virus particles to the cell surface through the HN binding activity was blocked by preincubation of the particles with an anti-HN monoclonal antibody (Fab preparation) (Guyden *et al.*, 1983).

Targeting of the Sendai virus to desialyzed human erythrocytes was also achieved by utilizing hybrid antibody molecules (Chejanovsky *et al.*, 1985). Anti-Sendai anti-human erythrocyte hybrids [$F(ab)_2$ molecules] were prepared by chemical coupling of anti-Sendai Fab fragments with anti-human erythrocyte Fab fragments. The hybrid molecules obtained were able to mediate the binding of Sendai virus particles to desialyzed human erythrocytes. Incubation of the virus with desialyzed erythrocytes at 37°C resulted in extensive cell fusion (Chejanovsky *et al.*, 1985).

In summary, it has extensively been shown that binding proteins other than membrane receptors can mediate the binding and fusion of virus particles with recipient cell membranes.

4. THEORETICAL ASPECTS OF THE USE OF FLUORESCENCE DEQUENCHING TO MEASURE VIRAL FUSION

In the past, membrane fusion events have been identified and characterized mainly by microscopic techniques (Poste and Pasternak, 1978; Duzgunes, 1985; Blumenthal, 1987). However, since those methods do not readily lend themselves to quantitation of the fusion reaction, new biophysical tech-

niques needed to be developed. In this chapter we focus on techniques using fluorescence.

The first fluorescence dequenching assay was based on self-quenching properties of the water-soluble dye carboxyfluorescein and was originally used to monitor transfer of contents of liposomes to cells (Weinstein *et al.*, 1977). The fluorophore trapped at high concentration inside a liposome emits only a few percent of the fluorescence that it would if released and diluted into the surrounding medium or into the cytoplasmic compartment of a cell. This assay indicated that the amount of liposome–cell fusion was much smaller than originally estimated by other techniques (Blumenthal *et al.*, 1977). On the other hand, the carboxyfluorescein dequenching assay has found a wide range of applications, for example, to monitor release of vesicle contents by a variety of agents such as serum components, enzymes, and cells [for a review see Weinstein *et al.* (1984)]. It has also been used to monitor leakage of vesicle contents during fusion.

Hoekstra *et al.* (1984) was the first to use fluorescence dequenching methods for studying and measuring the kinetics of fusion between enveloped virions and biological membranes. These authors made use of the self-quenching and hydrophobic properties of the fluorescent dye octadecyl rhodamine B-chloride (R18) [originally synthesized by Keller *et al.* (1977) and can be obtained from Molecular Probes, USA]. Because of the hydrophobic properties of the octadecyl chain, this probe can readily be inserted into envelopes of animal virions as well as into biological membranes or liposomes. Hoekstra *et al.* (1984) have shown that incubation of an ethanolic solution of R18 with Sendai virions results in spontaneous incorporation of the fluorescent dye into the viral envelopes. Under the experimental conditions used about 50–70% of the probe became virus-envelope associated, reaching a surface density of 2–3 mol % of the total viral phospholipids and its decrease was shown to be proportional to the fluorescence dequenching (Hoekstra *et al.*, 1984). Experiments by Hoekstra *et al.* (1984) as well as in our laboratories (Citovsky *et al.*, 1985) have shown that the incorporation of R18 into Sendai virus envelopes does not impair the viral biological activities, namely, its ability to induce hemolysis and to promote cell–cell fusion. It has been well established that hemolysis induced by Sendai virus reflects a process of virus–membrane fusion (Maeda *et al.*, 1977).

Quenching that results from collisional encounters between fluorophore and quencher is called collisional or dynamic quenching. The quencher diffuses to the fluorophore during the lifetime of the excited state; upon contact, the fluorophore returns to the ground state without emission of a photon. If the quencher is the same fluorophore it is self-quenched. In case of static quenching a complex is formed between fluorophore and quencher which is nonfluorescent.

An important aspect of collisional quenching is the distance over which fluorophore and quencher travel. The root mean square distance $[(\Delta x^2)^{1/2}]$ over which a quencher can diffuse during the lifetime of the excited state (t) is given by $\Delta x^2 = 2Dt$, where D is the free diffusion coefficient. Consider a typical case of a small, soluble fluorophore with a lifetime of 4 nsec and a diffusion coefficient of 2.5×10^{-5} cm^2/sec (both fluorophore and quencher). The distance over which the fluorophore has to diffuse to produce adequate quenching is 44 Å. To be effective, collisional quenchers must have high efficiency; that is, each collision results in loss of photon emission of the fluorophore. Generally, they are effective in the concentration range of 100 mM.

A simple calculation may determine whether the carboxyfluorescein quenching is consistent with dynamic or static quenching. The dye encapsulated into vesicles at concentrations of 10 mM or greater gives rise to about 50% fluorescence quenching (Weinstein *et al.*, 1984). Since at 10 mM the average distance between fluorophore is about 55 Å, dynamic quenching could possibly be the prevailing mechanism of self-quenching.

The measurement of fluorescent lifetimes is the most definitive method to distinguish static and dynamic quenching. In the case of static quenching no change in fluorescent lifetimes is expected, since the complexed fluorophores are nonfluorescent and the only observed fluorescence is from the uncomplexed fluorophore. Chen and Knutson (1987) found a normal lifetime (> 4 nsec) for over 95% of the fluorescence of carboxyfluorescein encapsulated in liposomes at high concentrations, indicating that the fluorescence decrease may be dominated by a static quenching mechanism. On the other hand, they observe significant dynamic quenching of free CF in the range of 10–50 mM. Depending on liposome size and encapsulation efficiency, both mechanisms may be important.

Absorption spectra of the fluorophore can also be examined to distinguish static and dynamic quenching. Collisional quenching only affects the excited states of fluorophore, and thus no change in the absorption spectra is predicted. On the other hand, ground-state complex formation will frequently result in perturbation of the absorption spectrum of the fluorophore. This is seen in spectra of carboxyfluorescein at high concentrations (Chen and Knutson, 1987).

Other methods to distinguish between static and dynamic quenching are measurements of dependence on temperature and viscosity. Dynamic quenching depends on diffusion and is expected to increase with increasing temperature and decreasing viscosity. In contrast, increased temperature is likely to result in decreased stability of complexes and thus lower the value of the static quenching constants.

In the case of R18 self-quenching, none of the experiments to determine

the mechanism of quenching has yet been reported. If we assume a lateral diffusion coefficient of 10^{-7} cm^2/sec (typical of lipid molecules in a bilayer) and a lifetime of 4 nsec, the average distance of lipid probe diffusion to produce collisional quenching is 3 Å. Hoekstra *et al.* (1984) found 50% quenching at a probe surface density of 5.5 mol % R18 in DOPC liposomes. The average distance between probes at this surface density is 33 Å, assuming a lipid head group area of 50 $Å^2$. Therefore, it is unlikely that R18 quenching is collisional. More likely, the rhodamine molecules will stack up to form nonfluorescent complexes. According to the above calculation the level of R18 quenching at 1% probe surface density indicates that it must be quite self-aggregated. The fatty acyl chain is nonsaturated and in the solid phase below 50°C. Phase separation of solid chains is not uncommon in mixtures of fluid and solid lipids. Labeling of intact Sendai virions with R18 resulted in a 2–3 mol % surface density and 70% quenching of the probe (Hoekstra *et al.*, 1984). Similar results were found with VSV (Blumenthal *et al.*, 1987). This is a higher quenching than in DOPC vesicles. Hoekstra *et al.* (1984) hypothesize that the probe is only on the outer monolayer of the labeled virions, resulting in a surface density of 4–6 mol %, which would explain the higher quenching. Moreover, the probe might be more self-aggregated in the virus membrane or the additional quenching might be due to the interaction of the probe with the more polar environment of the viral spike glycoprotein (see below).

Even if dequenching takes place upon fusion of membranes and diffusion of the probe over a larger surface area, relief of self-quenching might not be 100%. Aggregation of probe molecules even at very low surface densities might lead to some level of quenching. We have found that labeling cells directly with R18 and disrupting with Triton X-100 leads to a fluorescence increase of a factor of about 1.56 (Blumenthal *et al.*, 1987). A practical consequence of this is that measurements of total dequenching should be corrected with a factor of 1.56 (see below).

In static quenching the relation between quenched fluorescence and quencher concentration is given by (Lakowicz, 1983)

$$1 - \frac{F}{F_0} = \frac{K_s Q}{1 + K_s Q} \tag{1}$$

where F is fluorescence, F_0 fluorescence in the absence of quencher, Q concentration (or mole fraction of quencher), and K_s the (self-) association constant for the complex. According to Eq. (1), the quenching range is linear for $K_s Q << 1$. Hoekstra *et al.* (1984) found linearity up to 9 mol % R18 in DOPC liposomes, indicating that $K_s < (9 \text{ mol } \%)^{-1}$.

Part of the R18 quenching in the virion might be due to solvent effects;

that is, some of the R18 is bound to the viral spike glycoproteins. The fluorescence emission spectra of many fluorophores are sensitive to the polarity of their surrounding environment. For example, the emission peak wavelength of R18 is shifted to shorter wavelengths (blue shift) as the solvent polarity is decreased. Conversely, increasing solvent polarity generally results in shifts of the emission spectrum to longer wavelength (red shifts). Those red shifts are often accompanied by a decrease in the quantum yield of the fluorophore. The shifts result from both the interaction of the dipole moment of the fluorophore with the reactive fields induced in the surrounding solvent and from specific chemical interactions between the fluorophore with one or more solvent molecules. Localization of membrane-bound fluorophores can be inferred from their emission spectra (Lakowicz, 1983). The emission spectrum of the fluorophore inserted into the membrane is compared with its emission spectra in solvents of varying polarity.

In order to determine the environment of R18 in the virus, excitation and emission spectra of R18 in ethanol, in a lipid bilayer, in a detergent micelle, and R18 bound to protein were compared (Loyter *et al.*, 1988a). The proteins were bovine serum albumin and the extracytoplasmic portion of the VSV G-protein obtained by cathepsin D digestion (Crimmins *et al.*, 1983). R18 bound to proteins was in a much more nonpolar milieu as compared to R18 in the lipid bilayer. Figure 1 shows the emission spectrum of R18 bound to G (cath D) before and after adding detergent. The peak λ_{em} of R18 bound to G (cath D) was 600 nm, whereas its value in the detergent micelle was 586 nm, closer to the value in ethanol of 576 nm. The detergent micelle environment resulted in a fivefold increase in the fluorescence intensity in addition to the blue shift (Figure 1). In the virion the maximal value of λ_{em} was 590 nm, a value between that of the polar environment of the protein and the nonpolar environment of the lipid bilayer. On the other hand, R18-labeled Sendai virus had the same λ_{ex} and λ_{em} peaks as R18 in DOPC vesicles (Hoekstra *et al.*, 1984), indicating that in Sendai the R18 was more lipid bilayer associated.

Methods based on fluorescence resonant energy transfer (FRET) to measure membrane fusion are more sensitive, since they require less probe, which could potentially perturb the fusion system. One such assay was devised for mixing of vesicle contents (Wilschut and Papahadjopoulos, 1979) and involves the fast formation of a chelation complex between Tb^{3+}, encapsulated as a citrate complex in one population of vesicles, and dipicolinic acid in the second population. Fusion and mixing of contents result in the formation of a fluorescent Tb^{3+}–dipicolinic acid complex. Presence of Ca^{2+} and EDTA in the external medium prevents the formation of the fluorescent complex outside the vesicles. Mixing of membrane components can be measured by

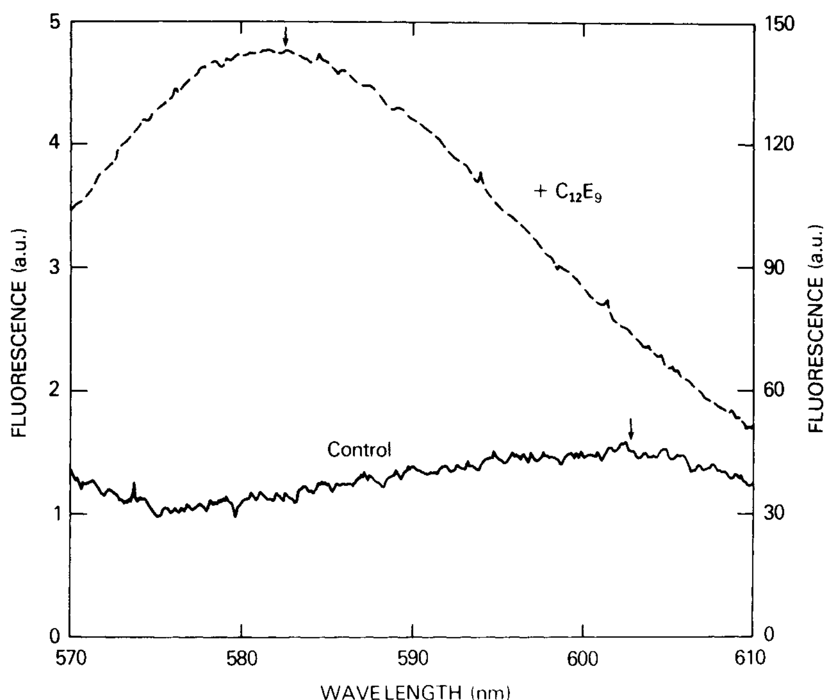

FIGURE 1. Emission spectra of R18 bound to G (cath D) before and after addition of the detergent C12E9. The data were collected using an MPF 44B spectrofluorometer (Perkin-Elmer) at an excitation wavelength of 565 nm. See text for more details.

FRET between a donor and acceptor fluorophore, both of which are attached to a phospholipid (Struck *et al.*, 1981).

Fluorescence energy transfer is the transfer of the excited-state energy from a donor to an acceptor. This transfer occurs without the appearance of a photon and is primarily a result of dipole–dipole interactions between donor and acceptor. The rate of energy transfer depends on the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles, and the distance between these molecules. It is this latter dependence on distance that has resulted in the widespread use of energy transfer as a "spectroscopic ruler." Many studies have been performed on fluorescence energy transfer between donor and acceptor pairs, which are lipid fluorophores free to diffuse in the bilayer (Fung and Stryer, 1978). The amount of energy transfer expected for fluorophores randomly distributed on the surface of a membrane has been analyzed extensively. It is a complex problem that re-

quires consideration of the geometric form of the bilayer (planar or spherical) and transfer between donors and acceptors that are on the same side of the bilayer as well as those on opposite sides.

Energy transfer results in quenching of the donor and sensitized emission of the acceptor. A variety of approaches have been used and in some instances solved numerically (Fung and Stryer, 1978; Wolber and Hudson, 1979; Snyder and Freire, 1982). In the case of lipid fluorophores randomly diffusing in the plane of the bilayer, it has been shown that quenching is only dependent on the surface density of acceptors, on the R_0 between donor and acceptor fluorophore, and on the distance of closest approach between donor and acceptor molecules. Only small amounts (<0.1 mol %) of acceptor incorporated into the membrane are needed for quenching. For the energy transfer pair N-NBD-PE and N-Rh-PE, which is widely used in liposome fusion studies, quenching is linear with acceptor concentration up to a surface density of 0.5 mol % (Struck *et al.*, 1981). At this surface density the donor was about 60% quenched. To achieve the same amount of fluorescence quenching, about 10-fold less probe needs to be used in the FRET assay as compared with the R18 dequenching assay. The ability to use less fluorescence probe, which could potentially perturb the membrane, makes the FRET assay more attractive.

It should be noted that membrane mixing assays may generate a multitude of artifactual positive results due to, for example, prefusion interactions, partial fusion, exchange of lipids, and aggregation (see Morris *et al.*, 1988). Therefore, ideally, both core mixing and membrane mixing assays should be done simultaneously or in parallel.

Regardless of what the mechanism of fluorescence quenching is, its relation to the amount of fusion needs to be established. In experiments of fusion between virions and cells, the virus is prebound to the cell surface at 4°C. Subsequently, unbound virus is removed by centrifuging the virus–cell complexes and washing. The virus–cell complexes are then warmed to 37°C and, if necessary, the pH is lowered. For that experimental protocol, the following equation has been derived expressing the relation between measured fluorescence and percentage of fusion (Loyter *et al.*, 1988a):

$$\%\ \text{fusion} = \frac{F_x - F_0}{F_t - F_0} \tag{2}$$

where F_x is fluorescence measured at a given time, F_0 is the fluorescence at time 0, and F_t is the maximal fluorescence if all the probe had transferred from virion to cell. The maximum amount of dequenching F_t is determined by disrupting the virion–cell complexes with detergent. As mentioned above, the total fluorescence of the probe in the detergent micelle may differ from

that in the cell under conditions of "infinite" dilution. For R18 in Vero cells we have determined a correction factor of 1.56 to account for this difference (Blumenthal *et al.*, 1987). The derivation of Eq. (2) assumes that the fluorophore is diluted from virion into an infinite reservoir of the cell, and that fluorophores that have already entered this reservoir do not affect those coming in.

5. FUSION OF ENVELOPED VIRUSES WITH ANIMAL CELLS AND BIOLOGICAL MEMBRANES: STUDIES WITH INTACT VIRIONS

As mentioned above, the fluorescence probe R18 can readily be inserted into envelopes of intact virus particles (Hoekstra *et al.*, 1984; Citovsky *et al.*, 1985). Incorporation of R18 and at high surface density leads to its self-quenching and its decrease was shown to be proportional to fluorescence dequenching (Hoekstra *et al.*, 1984; Citovsky *et al.*, 1985; Stegmann *et al.*, 1985). Therefore, fusion between enveloped virions and recipient membranes can be studied by determination of R18 fluorescence after the relief from its self-quenching (Hoekstra *et al.*, 1984; Citovsky *et al.*, 1985; Stegmann *et al.*, 1985; Loyter *et al.*, 1988a). The use of fluorescently labeled enveloped virions and the fluorescence dequenching method allowed studies on the various pathways of fusion (i.e., at the plasma membrane or via endocytosis), kinetics of virus–membrane fusion, effect of inhibitors, and fusion with various membrane preparations (Loyter *et al.*, 1988a).

Incubation of fluorescently labeled enveloped virions with living cultured cells resulted in fluorescence dequenching (Table I). This was demonstrated with Sendai (Hoekstra *et al.*, 1984; Citovsky *et al.*, 1985), influenza (Stegmann *et al.*, 1986; Nussbaum and Loyter, 1987), VSV (Blumenthal *et al.*, 1987), Herpes simplex 1 (HSV-1), and Semliki Forest viruses (Nussbaum and Loyter, unpublished data). The view that the fluorescence dequenching observed indeed reflects a process of virus–membrane fusion and results from the dilution of the virus-associated probe in the recipient membrane was supported by studies using inactivated unfusogenic virions (Citovsky *et al.*, 1985; Nussbaum and Loyter, 1987).

Sendai virions can be rendered unfusogenic by treatment with dithiothreitol (DTT) (Ozawa *et al.*, 1979a), trypsin (Ozawa *et al.*, 1979b), phenylmethylsulfonyl fluoride (PMSF) (Israel *et al.*, 1983), or glutaraldehyde (Chejanovsky and Loyter, 1985). No increase in the degree of fluorescence (fluorescence dequenching) was observed following incubation of treated unfusogenic Sendai virions with animal cultured cells (Citovsky *et al.*, 1985; Loyter *et al.*, 1987; Table II). A low level of fluorescence dequenching was

Table I
Interaction of Enveloped Virus with Cultured Cells and HEG: Fluorescence Dequenching Studies[a]

Virus	Recipient cells	pH of incubation	R18 EQ (%)
Sendai	HeLa	7.4	54
		5.0	38
	HEG	7.4	66
		5.0	43
Influenza	Mouse S_{49} lymphoma	7.4	48
		5.0	51
	HEG	7.4	9
		5.0	51
SFV	Vero cells	7.4	30
		5.0	55
	HEG	7.4	3
		5.0	33
HSV-1	Vero cells	7.4	65
		5.0	63
	HEG	7.4	5
		5.0	25
VSV	Vero cells	7.4	48
		5.9	56
	HEG	n.d.	n.d.

[a]Virus particles were labeled with the fluorescence probe R18 as described for labeling of Sendai or influenza virus and VSV as before (Citovsky *et al.*, 1985; Blumenthal *et al.*, 1987; Nussbaum and Loyter, 1987). Fluorescence-labeled virus particles (2 μg) were incubated with 3×10^6 cultured cells or 100–200 μg protein of erythrocyte membrane either at pH 7.4 or 5.0 (or pH 5.9 for VSV) as described before (Citovsky *et al.*, 1985; Blumenthal *et al.*, 1987; Nussbaum *et al.*, 1987). The degree of fluorescence dequenching (R18 DQ) following incubation with R18-labeled virions was determined as described elsewhere (Citovsky *et al.*, 1985; Nussbaum and Loyter, 1987; Nussbaum *et al.*, 1987).

observed also following incubation of influenza glutaraldehyde-treated virions with living cells. Infectivity as well as the fusogenic ability of influenza virions can be blocked by treatment with hydroxylamine (Schmidt and Lambrecht, 1985) or following incubation at high temperature such as 85°C (Nussbaum and Loyter, 1987) or low pH (Sato *et al.*, 1983). Indeed, no fluorescence dequenching was obtained following incubation of such treated influenza virions and cultured cells (Nussbaum and Loyter, 1987; Table II).

The correlation between the fluorescence dequenching and virus membrane fusion could also be demonstrated by the use of influenza virions bearing an uncleaved HA glycoprotein (HA_0) (Nussbaum and Loyter, 1987). HA_0 influenza virions are neither infective nor fusogenic (Klenk *et al.*, 1975). Indeed, incubation of the fluorescently labeled HA_0 influenza virions with living cells promoted a very low increase in fluorescence dequenching (Nuss-

Table II
Fusion of Enveloped Virions with Animal Cultured Cells: Effect of Inhibitors[a]

System	Virus treated with	Cell treated with	R18 DQ (%)
Sendai + HeLa cells			52
Sendai + HeLa cells	DTT	—	5
Sendai + HeLa cells	Trypsin	—	8
Sendai + HeLa cells	PMSF	—	8
Influenza + mouse S_{49} lymphoma			42
Influenza + mouse S_{49} lymphoma	Hydroxylamine	—	6
Influenza + mouse S_{49} lymphoma	Low pH	—	8
Influenza + mouse S_{49} lymphoma	—	Methylamine	18
Influenza + mouse S_{49} lymphoma	—	Ammonium chloride	21
Influenza + mouse S_{49} lymphoma	—	NaN_3	22
Influenza + mouse S_{49} lymphoma	—	EDTA	16
SFV + Vero cells	—	—	34
SFV + Vero cells	Hydroxylamine	—	6
HSV-1 + Vero cells			58
HSV-1 + Vero cells	Hydroxylamine	—	11

[a]The various virus preparations were labeled with R18 as described for Sendai and influenza before (Citovsky *et al.*, 1985; Nussbaum *et al.*, 1987). Virus particles were incubated with animal cultured cells (3×10^6 cells/system) at pH 7.4 and at the end of the incubation period, the fluorescence dequenching (R18 DQ) was determined as described elsewhere (Citovsky *et al.*, 1985; Nussbaum *et al.*, 1987). Sendai virions were treated with trypsin or PMSF as described by Israel *et al.* (1983) and with DTT as described by Loyter and Volsky (1982). Influenza, SFV, and HSV-1 were inactivated by treatment with hydroxylamine (Schmidt and Lambrecht, 1985) and pH 5.2 (Sato *et al.*, 1983) as mouse S_{49} lymphoma or Vero cells were treated with methylamine (NH_2CH_3), ammonium chloride (NH_4Cl), sodium azide (NAN_3) (50 mM each), or EDTA (5 mM) as described before (Lapidot *et al.*, 1987; Nussbaum, *et al.*, 1987). All other experimental conditions were as described elsewhere (Citovsky *et al.*, 1987; Lapidot *et al.*, 1987).

baum and Loyter, 1987). It has been well-established that mild trypsinization of the HA_0 virions causes cleavage of the HA_0 viral glycoprotein concomitantly with restoration of the viral fusogenic activity (Klenk *et al.*, 1975). Indeed, a high degree of fluorescence dequenching was observed upon incubation of trypsinized HA_0 influenza virions and living cells (Nussbaum and Loyter, 1987).

Essentially the same results were obtained when human erythrocyte membranes (human erythrocyte ghosts, HEG) were incubated with fluorescently labeled Sendai or influenza virions (Citovsky *et al.*, 1985; Stegmann *et al.*, 1986; Nussbaum and Loyter, 1987; Loyter *et al.*, 1988a; Table I). A high degree of fluorescence dequenching was observed only following incubation with active fusogenic virus particles. Using erythrocyte ghosts as recipient membranes, scientists were able to study aspects of the virus–membrane fusion process. The extent of fluorescence dequenching, which is a direct measure of the percentage of virus particles fused (Chejanovsky and Loyter, 1985; Hoekstra *et al.*, 1985), was highly dependent on the amount

of erythrocyte membrane as well as on the medium pH (Citovsky *et al.*, 1985).

Virus–erythrocyte as well as virus–cell fusion appeared to be strongly dependent on the temperature of incubation being stimulated, especially between 23 and 37°C (Hoekstra *et al.*, 1985). Fusion of Sendai virions with erythrocyte ghosts at 37°C was found to be a relatively slow process reaching maximum values within 10–15 min of incubation of 37°C and around pH 7.0–8.0 (Hoekstra *et al.*, 1984). From studies using the fluorescence dequenching method, it was inferred that about 1000 Sendai virus particles can be bound to one erythrocyte ghost as compared to 100–200 particles that actually can fuse with each erythrocyte ghost (Nir *et al.*, 1986). No increase in the degree of fluorescence was observed following incubation of treated unfusogenic Sendai virions and erythrocyte ghosts (Citovsky *et al.*, 1985; Loyter *et al.*, 1988a).

Incubation of influenza virions with human erythrocyte ghosts resulted in fluorescence dequenching only at pH values between 5.0 and 5.5 but not at pH 7.4 (Stegmann *et al.*, 1986; Nussbaum and Loyter, 1987; Table I). This is consistent with the view that the viral fusion protein, namely the HA glycoprotein, is activated only at low pH values (Huang *et al.*, 1981; Skehel *et al.*, 1982; White *et al.*, 1983). Fluorescence dequenching methods were also used to study the fusogenic properties of HSV-1 and SFV. The fusogenic properties of SFV are similar to those of influenza virions. Fusion of SFV with biological membranes was observed only at low pH values (White *et al.*, 1983). This was confirmed by recent experiments showing that incubation of fluorescently labeled SFV with erythrocyte ghosts at pH 7.4 resulted in very little (3%) fluorescence dequenching while a high degree (33%) was observed following incubation at 5.0 (Loyter *et al.*, 1988a; V. Citovsky and A. Loyter, unpublished data; Table II). Recent experiments have shown (Loyter *et al.*, 1988a) that HSV-1 also prompted fluorescence dequenching—following incubation with human erythrocyte ghosts—only at pH 5.0–5.5 and not at pH 7.4 (Loyter *et al.*, 1987; Table II).

It is noteworthy that the extent of fluorescence dequenching observed upon incubation of SFV or HSV-1 with neuraminidase–pronase-treated erythrocyte membranes was higher than that observed with nontreated membranes (Loyter *et al.*, 1988a). This is in contrast to results obtained with Sendai or influenza virions which failed to fuse—and to promote fluorescence dequenching—with such virus-receptor-depleted erythrocytes (Chejanovsky *et al.*, 1986a; Loyter *et al.*, 1988a).

The interaction of fluorescently labeled influenza virions with cultured cells showed a different pattern from that obtained with erythrocyte ghosts (Nussbaum and Loyter, 1987; Loyter *et al.*, 1988a; Table I). A high degree of fluorescence dequenching was promoted—following incubation of influ-

enza virions and culture cells—either at pH 5.0 or pH 7.4 (Nussbaum and Loyter, 1987). Very low or no fluorescence dequenching was observed at both pH values — following incubation with either inactivated unfusogenic virions or with HA_0 influenza virions (Nussbaum and Loyter, 1987; Table II). Evidently, the dequenching observed at either pH 5.0 or pH 7.4 reflects a process of virus–membrane fusion. However, only the fluorescence dequenching observed at pH 7.4, but not at pH 5.0, was inhibited by lysosomotropic reagents such as ammonium chloride and methylamine (Nussbaum and Loyter, 1987; Table II). Also, chelator of bivalent metals such as EDTA or reagents that deplete culture cells from intracellular ATP inhibited the fluorescence dequenching observed at pH 7.4 but not that obtained at pH 5.0 (Nussbaum and Loyter, 1987; Table II). Ammonium chloride and methylamine are known to increase the endosomal or lysosomal pH values (Matlin *et al.*, 1981; Svensson, 1985) while EDTA and NaN_3 inhibit endocytosis itself (Svensson, 1985). Based on these observations, it should be surmised that the fluorescence dequenching observed upon incubation with cultured cells at pH 7.4 results from fusion of influenza virus particles with membranes of intracellular organelles, while that observed at pH 5.0 is due to fusion with cell plasma membranes.

The same results were obtained following incubation of VSV, SFV, or HSV-1 with cultured cells (Blumenthal *et al.*, 1987; Loyter *et al.*, 1988a; Table I). High degrees of fluorescence dequenching were observed upon incubation at pH 5.0–5.1 and 7.4 but only that observed at pH 7.4 was inhibited by lysosomotropic reagents and EDTA.

Experiments with VSV demonstrated that fusion at the plasma membrane began immediately after lowering the pH below 6 and showed an approximately exponential time course, whereas fusion via the endocytic pathway (pH 7.4) became apparent after a time delay of about 2 min (Blumenthal *et al.*, 1987). A 10-fold excess of unlabeled virus arrested R18 VSV entry via the endocytic pathway, whereas R18 dequenching below pH 6 (fusion at the plasma membrane) was not affected by the presence of unlabeled virus. The temperature dependence for fusion at pH 7.4 (in the endosome) was much steeper than that for fusion at pH 5.9 (with the plasma membrane). Fusion via the endocytic pathway was attenuated at hyperosmotic pressures, whereas fusion at the plasma membrane was not affected by this treatment. The pH profile of Vero–VSV fusion at the plasma membrane, as measured by the dequenching method, paralleled that observed for VSV-induced cell–cell fusion. Fusion was blocked by adding neutralizing antibody to the Vero–VSV complexes (Blumenthal *et al.*, 1987). Activation of the fusion process by lowering the pH was reversible, in that the rate of fusion was arrested by raising the pH back to 7.4. The observation that pH-dependent fusion occurred at similar rates with fragments and with intact cells indicates that pH,

voltage, or osmotic gradients are not required for VSV fusion with cells (Blumenthal *et al.*, 1987).

6. USE OF FLUORESCENT DEQUENCHING METHODS TO STUDY FUSION OF ENVELOPED VIRUSES WITH BIOLOGICAL MEMBRANES LACKING VIRUS RECEPTORS

The availability of fluorescently labeled enveloped virions and fluorescence dequenching methods allowed one to study the question of whether animal viruses will interact and especially fuse other biological membranes other than cell plasma membranes. Even fusion with membranes of endocytic vesicles initiates and results from binding of virions to specific receptors on cell surfaces (White *et al.*, 1983).

In our laboratory, we studied the interaction of both Sendai and influenza virions with chromaffin granules of bovine medulla (Citovsky *et al.*, 1987a) and with prokaryotic cells, namely mycoplasmas (Citovsky *et al.*, 1987b).

Incubation of fluorescently labeled Sendai or influenza virions at pH 7.4 and pH 5.0, respectively, with chromaffin granule vesicles under isotonic conditions resulted in a very low degree of fluorescence dequenching. It is noteworthy that under physiological conditions, membranes of such organelles never encounter enveloped virions and lack receptors for these virions (Abbs and Phillips, 1980; Ekerdt *et al.*, 1981). However, when Sendai (or influenza) virions were incubated with these vesicles under hypotonic conditions, a relatively high degree (35%) of fluorescence dequenching was observed (Citovsky *et al.*, 1987a). A significantly lower degree (13%) of fluorescence dequenching was observed when treated unfusogenic (DTT, trypsin, PMSF) Sendai virions were incubated with the chromaffin granule vesicles.

Similar results were obtained when fluorescently labeled Sendai or influenza virions were incubated with right-side-out erythrocyte vesicles (ROV) from which virus receptors have been removed by treatment with neuraminidase and pronase (Citovsky and Loyter, 1985; Nussbaum *et al.*, 1987). Only incubation under hypotonic conditions resulted in fusion (fluorescence dequenching) of either Sendai or influenza virions with the virus-receptor-depleted ROVs. Increase in fluorescence dequenching was observed at pH 7.4 following incubation with Sendai virions and at pH 5.0 upon incubation with influenza virions (Citovsky and Loyter, 1985; Nussbaum *et al.*, 1987). It has been suggested that osmotic swelling of human erythrocytes promotes exposure of the masked membrane phospholipids and renders them susceptible to phospholipases or cross-linking reagents (Laster *et al.*, 1972).

The above-described observations may indicate that in order to fuse with

recipient membrane the viral envelope fusion proteins should interact directly with the membrane phospholipid bilayers. Under isotonic conditions, the interaction of enveloped virions with their membrane receptors may induce unmasking of the membrane lipid bilayer, thus making it available to the viral fusion glycoproteins (Citovsky and Loyter, 1985). These assumptions and results raise the possibility that enveloped virions will fuse with any biological membranes whose phospholipid bilayer is exposed and available to interaction with the viral glycoproteins even in the absence of appropriate virus receptors.

This was verified by recent experiments showing that Sendai or influenza virions are able to fuse with the membranes of prokaryotic cells (Citovsky *et al.*, 1987b). Incubation of fluorescently labeled Sendai influenza virions with *Mycoplasma gallisepticum* and *Mycoplasma capricolum*, but not with *Acetoplasma laidlawii*, resulted in a high degree of fluorescence dequenching. Fusion of Sendai and influenza virions with mycoplasmas was also confirmed by electron microscopic observations (Citovsky *et al.*, 1987b). The failure of Sendai and influenza virions to fuse with *A. laidlawii* may be due to the low percentage of cholesterol present in membranes of these cells (Razin and Tully, 1970).

The requirement of cholesterol for allowing virus–mycoplasma fusion was demonstrated by showing that a low degree of fusion was obtained with *M. capricolum* whose cholesterol content was decreased by modifying its growth medium (Citovsky *et al.*, 1987b). Fluorescence dequenching was not observed by incubating unfusogenic Sendai or influenza or HA_0 influenza with mycoplasmas (Citovsky *et al.*, 1987b). These results clearly demonstrated that both Sendai and influenza virions are able to fuse with mycoplasmas in spite of the fact that these prokaryotic cells lack virus receptor, namely, sialoglycolipids or sialoglycoproteins.

The above-described systems are an excellent example of the use of the fluorescence dequenching method for elucidating the molecular mechanism of virus–membrane fusion. It was possible to demonstrate using these methods that fusion of enveloped virions with biological membrane requires a high percentage of cholesterol. Such studies are almost impossible by any other methods such as observation by electron microscopy or virus-induced leakage of infected cells (Bashford *et al.*, 1985; Pasternak *et al.*, 1985).

7. ROLE OF VIRAL GLYCOPROTEINS IN THE PROCESS OF VIRUS MEMBRANE FUSION: STUDIES WITH RECONSTITUTED VIRAL ENVELOPES

The relation between the structure of viral glycoproteins and their biological function can be studied by the use of reconstituted viral envelopes.

These are membrane vesicles bearing only the viral envelope glycoproteins and devoid of the viral nucleocapsid (Loyter and Volsky, 1982; Vainstein *et al.*, 1984; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987). Studies on the biological activity of the isolated viral glycoprotein are of crucial importance for the elucidation of the as yet unknown, initial steps of virus–membrane fusion, virus penetration, and infection.

Most of the methods that have been used to reconstitute viral envelopes are based essentially on three steps: first, solubilization of intact viruses with a detergent; second, sedimentation of the internal proteins and genetic material; and third, removal of the detergent from the supernatant—a step that in most cases results in the formation of empty viral envelopes (Hosaka and Shimizu, 1972; Loyter and Volsky, 1982; Vainstein *et al.*, 1984; Stegmann *et al.*, 1987).

Two kinds of detergent have been employed for solubilization of intact enveloped virions. Detergents with high critical micelle concentration (CMC), such as octylglucoside (Helenius and Simons, 1975), which can be removed effectively by dialysis, have been used for solubilization of VSV, SFV, influenza, and Sendai virions (White *et al.*, 1983; Harmsen *et al.*, 1985; Stegmann *et al.*, 1987). On the other hand, detergents with a low CMC like Nonidet p-40 or Triton X-100 (Helenius and Simons, 1975), which cannot be removed simply by dialysis and require the addition of a hydrophobic resin such as Bio-beads SM-2, have been reported for Sendai (Loyter and Volsky, 1982; Vainstein *et al.*, 1984) or influenza (Nussbaum *et al.*, 1987). Recently, functional reconstituted influenza viral envelopes have been prepared by the use of the nonionic detergent octaethyleneglycol mono(*n*-dodecyl) ether ($C_{12}E_8$) (Stegmann *et al.*, 1987). Since this detergent possesses the same features as Triton X-100, its removal necessitated also the use of SM-2 Bio-beads (Stegmann *et al.*, 1987).

Removal of Triton X-100 by direct addition of SM-2 Bio-beads to the clear supernatant containing Sendai or influenza viral envelope phospholipids and glycoproteins results in the formation of resealed reconstituted viral envelopes (Loyter and Volsky, 1982; Vainstein *et al.*, 1984; Nussbaum *et al.*, 1987). Reconstituted Sendai virus envelopes contain only the viral hemagglutinin neuraminidase (HN) and fusion (F) glycoproteins, while those of influenza contain the viral HA (hemagglutinin) and NA (neuraminidase) glycoprotein (Vainstein *et al.*, 1984; Nussbaum *et al.*, 1987).

In Sendai virus that belongs to the paramyxovirus group, the hemagglutinin-binding activity and the neuraminidase are located on the same polypeptide, the HN glycoprotein. The virus fusion activity is located on a different polypeptide—the F glycoprotein (White *et al.*, 1983). In the influenzas that belong to the orthomyxovirus groups, the hemagglutinin and the neuraminidase are located

on two different polypeptides, the HA and the NA glycoproteins, respectively. The HA glycoprotein mediates binding to cell surface receptors and is required for virus–membrane fusion. The NA glycoprotein possesses only neuroaminidase activity (White *et al.*, 1983).

Energy transfer and fluorescence dequenching methods are used to study—on a quantitative basis—the fusion ability of reconstituted viral envelopes bearing individual viral glycoproteins or their combinations (Citovsky and Loyter, 1985; Citovsky *et al.*, 1985, 1986a; Lapidot *et al.*, 1987; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987). By using this method it was possible to compare the fusogenic properties of such envelopes to those of intact virions (Nussbaum *et al.*, 1987; Lapidot *et al.*, 1987; Stegmann *et al.*, 1987).

N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (N-NBD-PE) was incorporated into reconstituted virus envelopes during the reconstitution procedure at self-quenching concentrations (10 mol %) (Citovsky *et al.*, 1985; Citovsky and Loyter, 1985; Loyter *et al.*, 1987; Stegmann *et al.*, 1987). The R18, on the other hand, can be interred into envelopes of virus particles either by its addition during reconstitution or to already reconstituted viral envelopes (Loyter *et al.*, 1987; Nussbaum *et al.*, 1987). As opposed to R18, N-NBD-PE cannot be incorporated into envelopes of intact virions (or to already reconstituted viral envelopes), thus preventing its use for studies of fusion processes of intact virions.

Studies with fluorescently labeled reconstituted Sendai virus envelopes (RSVEs) or reconstituted influenza virus envelopes (RIVEs) have shown that envelopes obtained following solubilization of intact virions with Triton X-100, but not with octylglucoside, are as fusogenic as intact virions (Loyter *et al.*, 1987, 1988a; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987). These conclusions were based on experiments showing that the degree of fluorescence dequenching obtained following incubation of either RSVEs or RIVEs with HEG or cultured cells was close to that obtained with intact virions (Table III).

RIVEs were able to fuse with erythrocyte membrane only at pH 5.0 while with cultured cells fusion was demonstrated at pH 5.0 as well as at pH 7.4 (Nussbaum *et al.*, 1987; Table III). Only the fusion (fluorescence dequenching) observed upon incubation with cultured cells at pH 7.4 was inhibited by lysosomotropic agents (methylamine and ammonium chloride), as well as by EDTA, indicating that it is due to fusion of RIVEs taken into cells by endocytosis (Nussbaum *et al.*, 1987). The rate and the pH dependence of fusion with RSVEs and RIVEs were found to be essentially the same as those of intact virions. No increase in the fluorescence dequenching was observed when unfusogenic RSVEs or RIVEs were incubated with either HEG or cultured cells (Loyter *et al.*, 1987, 1988a; Nussbaum *et al.*, 1987; Table III). All these experiments clearly demonstrated — as had been inferred from experiments with intact virions — that

Table III
Fusion of Reconstituted Sendai or Influenza Virus Envelopes with HEG[a]

System	pH of incubation	R18 DQ
A. Sendai	7.4	56
RSVE		50
DTT–RSVE		6
HN vesicles		6
F vesicles		8
HN–F vesicles		52
B. Influenza	7.4	5
	5.0	48
RIVE	7.4	8
	5.0	42
Hydroxylamine–RIVE	5.0	8
HA vesicles	5.0	41
Hydroxylamine–HA vesicles	5.0	11

[a]Reconstituted Sendai virus envelopes (RSVEs) or membrane vesicles bearing purified hemagglutinin/neuraminidase (HN vesicles) or fusion (F vesicles) glycoproteins or both (HN–F vesicles) were prepared and fluorescently labeled as described before (Citovsky and Loyter, 1985; Citovsky *et al.*, 1986b). Reconstituted influenza virus envelopes (RIVEs) or membrane vesicles bearing only the hemagglutinin glycoprotein (HA vesicles) were prepared and fluorescently labeled as described by Nussbaum *et al.* (1987) and Lapidot *et al.* (1987).

the fluorescence dequenching observed is due to fusion between the viral envelope and the cell membranes and not to other processes such as lipid–lipid exchange.

Resealed membrane vesicles containing Sendai or influenza individual glycoproteins can be prepared following separation of the viral polypeptides on ion-exchange columns (Fukami *et al.*, 1980; Nussbaum *et al.*, 1984; Lapidot *et al.*, 1987). Membrane vesicles bearing the Sendai HN or F glycoproteins are nonfusogenic as can be inferred from experiments showing that incubation of such fluorescently labeled vesicles with biological membranes results in very little fluorescence dequenching (Citovsky and Loyter, 1985; Loyter *et al.*, 1987; 1988a; Table III). It is noteworthy that membrane vesicles bearing only the Sendai virus binding polypeptide, namely the HN glycoprotein, readily attach to cell plasma membranes and agglutinate red blood cells (Fukami *et al.*, 1980). Membrane vesicles bearing only the Sendai F glycoprotein neither agglutinate cells nor attach to their membranes (Fukami *et al.*, 1980; Nussbaum *et al.*, 1984). Recent experiments showed that even when the binding of the Sendai F vesicles to recipient membranes was mediated by a nonviral binding ligand, no increase in the degree of fluorescence was observed (Loyter *et al.*, 1987). Fluorescence dequenching, namely vesicle–membrane fusion, was observed only

with reconstituted envelopes bearing both Sendai HN and F glycoproteins within the same membrane (Citovsky and Loyter, 1985; Citovsky *et al.*, 1986a; Loyter *et al.*, 1987). These experiments raise the possibility that the Sendai virus HN glycoprotein beside being the viral binding protein also actively participates in the virus–membrane fusion step (Gitman *et al.*, 1985).

Fluorescently labeled (R18) membrane vesicles bearing only the influenza viral hemagglutinin (HA) glycoprotein were used to study its function in the fusion process (Wharton *et al.*, 1986; Lapidot *et al.*, 1987). The viral hemagglutinin glycoprotein was separated from the neuraminidase glycoprotein by agarose sulfanilic acid column and was shown to be homogeneous by gel electrophoresis and devoid of any neuraminidase activity (Lapidot *et al.*, 1987). Incubation of fluorescently labeled HA vesicles with HEG or cultured cells gave the same results as incubation of intact influenza virions or RIVEs (Lapidot *et al.*, 1987). Fluorescence dequenching was observed only with fusogenic but not with treated unfusogenic HA vesicles. Similarly, fusion of HA vesicles with living cultured cells — as opposed to fuson with HEG — was observed not only at pH 5.0 but also at pH 7.4 (Lapidot *et al.*, 1987). These results clearly showed that despite the fact that the HA vesicles are devoid of the NA glycoprotein and of neuraminidase activity they are fusogenic and behave in the same manner as intact virions (Lapidot *et al.*, 1987).

Recently, reconstituted envelopes bearing the VSV G-protein were obtained following solubilization of VSV with octylglucoside or by $C_{12}E_8$ (Metsikko *et al.*, 1986). The fusogenic activity of these vesicles was assayed for polykaryon formation (Metsikko *et al.*, 1986). Only envelopes (virosomes) obtained following the use of $C_{12}E_8$ were fusogenic, while those obtained by the use of octylglucoside did not exhibit any fusogenic activity. Membranes bearing the G-protein fuse with BHK-22 cell plasma membranes at pH 5.7–6.0 with an efficiency of fusion comparable to that of the parent virus (Metsikko *et al.*, 1986).

8. FUSION OF ENVELOPED VIRUSES WITH NEGATIVELY CHARGED AND NEUTRAL LIPOSOMES

Various methods have been employed to study and demonstrate fusion between enveloped viruses and phospholipid vesicles (Haywood, 1974; White and Helenius, 1980; Hsu *et al.*, 1983; Kawasaki *et al.*, 1983; Haywood and Boyer, 1984). Haywood (1974) was the first to demonstrate fusion between Sendai virions and lipid vesicles. Using electron microscopy techniques, Haywood showed that fusion of Sendai virions with liposomes composed of neutral lipids such as phosphatidylcholine (PC), sphingomyelin (Sph), phosphatidylethanolamine (PE), and cholesterol (chol) require the presence of virus receptors, namely, sialoglycolipids.

In more recent work fusion between influenza, SFV, or VSV and liposomes of different composition was demonstrated (White *et al.*, 1983). Virus-induced release of liposome content also has been used to follow virus–liposome fusion processes (Oku *et al.*, 1982; Kundrot *et al.*, 1983). It has been well established that fusion processes between hemolytic enveloped virions such as Sendai and influenza and living cells leads to an increase in the cell membranes' permeability to small as well as to large molecules (Bashford *et al.*, 1985; Pasternak *et al.*, 1985). Sendai virions were shown to induce hemolysis at pH values above 6.0–7.0 while the hemolytic activity of influenza virions is manifested at pH 5.2, a pH at which the viral fusion glycoprotein is activated (Huang *et al.*, 1981).

Fluorescence dequenching methods have also been used to follow — on a quantitative basis — fusion between fluorescently labeled enveloped virions and liposomes. Hoekstra *et al.* (1984) have shown that incubation of R18-labeled influenza virions with liposomes composed of negatively charged phospholipids results in fluorescence dequenching. Fluorescence dequenching was observed following incubation at pH 5.0 but not at 7.4. Recently, by the use of fluorescently labeled liposomes, it has been shown that influenza virions fuse readily with liposomes composed of negatively charged phospholipids such as phosphatidylserine (PS) or cardiolipin (CL) and very poorly with liposomes composed of neutral lipids such as sphingomyelin or sphingomyelin and cholesterol (Stegmann *et al.*, 1985). Fusion with negatively charged liposomes was pH dependent, whereas virus-induced release of calcein from these liposomes was pH independent and was observed at pH 5.0 as well as at pH 7.4 (Stegmann *et al.*, 1985; O. Nussbaum, V. Citovsky, and A. Loyter, unpublished data; Table IV).

Neither virus-induced release of liposome content nor virus–liposome fusion was dependent on the presence of virus receptor in the negatively charged liposomes (Hoekstra *et al.*, 1984; Stegmann *et al.*, 1985; Table IV). Surprisingly, a relatively high degree of fluorescence dequenching was observed following incubation of inactivated unfusogenic influenza virions with negatively charged liposomes such as those composed of PS (O. Nussbaum, V. Citovsky, and A. Loyter, unpublished data; Table IV). Furthermore, fluorescence dequenching and release of liposome content [carboxyfluoresceine (CF) dequenching] were also observed following incubation of the unfusogenic HA_0 influenza virions with PS liposomes (O. Nussbaum, V. Citovsky, and A. Loyter, unpublished data). These results raise the possibility that fusion of influenza virions with liposomes composed of negatively charged phospholipids does not reflect the biological activity of the viral glycoproteins needed for infection and penetration (Loyter and Citovsky, 1987).

Support for this view was also obtained from experiments in which fusion of Sendai virions with negatively charged liposomes was studied (Amselem *et*

Table IV
Interaction of Sendai and Influenza Virions with Phospholipid Vesicles: Virus-Induced Lysis of and Fusion with Negatively Charged and Neutral Liposomes[a]

		PC	PC/chol		PC/chol/gang		PS	
System	pH	R18 DQ (%)	R18 DQ (%)	CF release (% of total)	R18 DQ (%)	CF release (% of total)	R18 DQ (%)	CF release (% of total)
Sendai	7.4	2	43	3	48	21	68	74
DTT-treated Sendai	7.4	3	0	2	2	0	63	81
Influenza	5.0	7	31	3	42	54	50	80
	7.4	3	8	0	9	9	17	70
Hydroxylamine-treated influenza	5.0	n.d.	7	n.d.	7	2	30	68
	7.4	n.d.	5	n.d.	8	3	10	55
HA_0 influenza	5.0	n.d.	8	n.d.	9	3	35	63
	7.4	n.d.	5	n.d.	6	3	15	42

[a]Carboxyfluorescein loaded or empty liposomes composed of PC, PC/chol (1 : 0.5 molar ratio), PC/chol/gang (1 : 0.5 : 0.3 molar ratio), or PS were prepared as described by Citovsky *et al.*, (1986b). All other experimental conditions of determination of fluorescence dequenching and carboxyfluorescein release are as described by Citovsky *et al.* (1986b) and Loyter *et al.* (1988a). n.d., not done.

al., 1985, 1986; Chejanovsky *et al.*, 1986b). Incubation of fluorescently labeled Sendai virions with PS liposomes resulted in a high degree of fluorescence dequenching (Chejanovsky *et al.*, 1986b). Sendai virions also induced the release of CF from PS-loaded liposomes (Amselem *et al.*, 1985, 1986). No virus receptors, namely sialoglycolipids, were required to allow fusion with or lysis by Sendai virions of such negatively charged liposomes (Amselem *et al.*, 1985; Chejanovsky *et al.*, 1986b). Inactivated unfusogenic virions were able to fuse with liposomes composed of negatively charged phospholipids (Amselem *et al.*, 1985b; Chejanovsky *et al.*, 1986b). A high degree of fluorescence dequenching was observed following incubation of DTT- or PMSF-treated Sendai virions and PS or CL liposomes (Table IV). Fusion of Sendai virions with negatively charged lipsomes is maximal at low pH values, whereas fusion of Sendai virions with biological membranes was maximal between pH 7.0 and 9.0 (Chejanovsky and Loyter, 1985; Chejanovsky *et al.*, 1986b). Similar to intact virions, RSVEs were also able to induce lysis and to fuse with liposomes containing negatively charged phospholipids. The view that such fusion does not reflect the biological activity of the virus was further strengthened by experiments showing that membrane vesicles bearing only the Sendai-virus-binding protein, namely the HN glycoprotein, are also able to induce lysis and to fuse with liposomes composed of PS (Chejanovsky *et al.*, 1986b). Fusion was maximally expressed, at low pH values such as pH 4.0, and was not inhibited by treatment of the HN vesicles with either DTT or PMSF. Neither induction of lysis nor fusion was observed upon incubation of membrane vesicles containing the viral fusion glycoprotein (F vesicles) with the negatively charged liposomes (Chejanovsky *et al.*, 1986b).

It is noteworthy that fusion of Sendai virions or RSVEs with biological membranes is absolutely dependent on the presence of the viral fusion (F) glycoprotein, and HN vesicles neither induce lysis nor fuse with HEG or living cells (Loyter and Volsky, 1982; Citovsky *et al.*, 1986b).

Incubation of fluorescently labeled influenza or Sendai virions with liposomes composed of neutral phospholipids such as PC — as opposed to those composed of PS — resulted in very little fluorescence dequenching (Citovsky *et al.*, 1985; Loyter and Citovsky, 1987). Essentially, the same results were obtained when HA_0 or trypsinized HA_0 influenza virions were incubated with PC liposomes at either pH 5.0 or 7.4 (Loyter and Citovsky, 1987; Table IV). Incorporation of cholesterol into the PC liposomes renders them susceptible to the fusogenic activity of either Sendai or influenza virions. It has been claimed previously that fusion of SFV with phospholipid vesicles is absolutely dependent on the presence of cholesterol (White *et al.*, 1983).

Fusion of Sendai virions with PC/chol liposomes was observed at a wide range of pH values, reaching a maximal degree at pH 7.0–8.0, while that of influenza virions showed maximal values between pH 5.0 and 5.5 (Table IV).

Inactivated unfusogenic Sendai or influenza virions failed to fuse with PC/chol liposomes (Citovsky *et al.*, 1985; Loyter *et al.*, 1988b). HA_0 influenza virions also did not fuse with PC/chol liposomes while trypsinized HA_0 influenza virions readily fuse with these liposomes (Table IV). Fusion of both influenza and Sendai virions with PC/chol liposomes was found to be an unlikely process (Citovsky and Loyter, 1985; Citovsky *et al.*, 1986; Loyter *et al.*, 1988b; Table IV). Incubation of Sendai virions at pH 7.4 or influenza virions at pH 5.0 and 37°C with CF-loaded PC/chol liposomes did not induce any CF release.

Incorporation of virus receptors, namely the sialoglycolipids (gangliosides, gang), into the PC/chol liposomes renders them susceptible to the Sendai or influenza lytic activity. Thus, lysis of liposomes composed of neutral phospholipids by Sendai or influenza virions was absolutely dependent on the presence of cholesterol and virus receptors (Citovsky and Loyter, 1985; Citovsky *et al.*, 1986b; Loyter and Citovsky, 1987). Such virus-induced lysis of loaded liposomes exhibited the same features as shown by virus–membrane fusion and virus-induced lysis of living cells. It was maximally expressed at pH 7.4 when Sendai virions were used and at pH 5.0 with influenza virions (Loyter and Citovsky, 1987). Lysis of liposomes was not observed with DTT- or PMSF-treated Sendai virions or with hydroxylamine-treated influenza virions. These results support the view that in the presence of sialoglycolipids (gang), which serve as receptors for Sendai and influenza virions, lysis of loaded liposomes indeed reflects a process of virus–membrane fusion.

Essentially the same results were observed when RSVEs or RIVEs were incubated with PC/chol or PC/chol/gang liposomes. However, when membrane vesicles containing either the Sendai HN (HN vesicles) or F (F vesicles) glycoproteins were incubated with PC/chol or PC/chol/gang liposomes neither fusion nor release of the liposome content was observed (Citovsky *et al.*, 1986b). Only vesicles bearing the Sendai virus HN and F glycoproteins within the membrane were able to fuse with and to induce lysis of PC/chol/gang liposomes (Citovsky *et al.*, 1986b). From these experiments it should be inferred that even fusion with PC/chol liposomes lacking virus receptor requires the Sendai virus HN glycoprotein in addition to the F polypeptide. An active role of Sendai virus HN glycoprotein in the virus–membrane fusion step itself is thus suggested.

It appears that the following conclusions can be drawn from studies on fusion between enveloped virions and especially Sendai and influenza viruses with phospholipid vesicles: (1) Fusion with negatively charged liposomes does not reflect the biological activity of the viral envelopes. (2) Fusion with liposomes composed of neutral lipids is absolutely dependent on the presence of cholesterol and shows the same features as fusion with biological membranes. (3) Sialoglycolipids (gang), namely virus receptors, must be present in PC/chol liposomes in order to allow expression of the viral lytic activity.

9. ROLE OF CONFORMATIONAL CHANGES AND COOPERATIVITY OF VIRAL PROTEINS IN MEDIATING MEMBRANE FUSION

Significant advances have been made in recent years in elucidating the role of viral spike proteins in inducing membrane fusion. They include (1) the first high-resolution image of a membrane fusion protein, (2) the elucidation of the primary sequence of a large number of viral membrane proteins using DNA sequencing techniques, and (3) the development of genetic and chemical methods for site-specific alterations of viral protein structure.

The viral spike glycoproteins contain in their structures all the information needed for viral entry: recognition, movement to site, apposition, fusion, and dissociation. Conformational changes and subunit interactions have been studied in viral spike glycoproteins. The hemagglutinin (HA) protein of influenza virus is the best-characterized member of the family of viral spike glycoproteins that mediate membrane fusion (White *et al.*, 1983). Its structure contains the recognition site for cell surface sialic acid residues, as well as the "catalytic site" that mediates membrane fusion. The HA consists of two disulfide-linked glycopolypeptide chains, HA1 and HA2, which are derived by proteolytic cleavage from a precursor glycopolypeptide called HA0 (Klenk *et al.*, 1975). This proteolytic cleavage is absolutely required for pH-induced fusion activity of the virus. That HA is necessary and sufficient for fusion activity was shown by expressing HA in eukaryotic cells by transfection of plasmids containing cloned complementary DNAs encoding those viral proteins and monitoring pH-dependent cell–cell fusion (Gething *et al.*, 1986). Mutations in the N-terminal peptide of HA2 significantly altered fusion.

Treatment of intact influenza virus with the enzyme bromelain results in the release of nearly the entire N-terminal ectodomain of HA (95% of its mass) in a water-soluble form. The cleavage of the HA2 chain occurs close to the point where it emerges from the viral membrane (Brand and Skehel, 1972). The resulting fragment, termed BHA, has been crystallized and its three-dimensional structure determined to a resolution of 3 Å (Wilson *et al.*, 1981).

The protein is a trimeric rod-shaped molecule 135 Å in length, consisting of a stem and three globular, highly folded domains (stalks) at the top. The globular domains are composed of the HA1 chain, and they contain the binding sites for sialic acid. The head domain rests on the stem, which is composed of HA2, as well as the C-terminal and N-terminal sequences of HA1. The C terminus of HA1 is located close to the viral membrane, 22 Å from the N terminus to which it was originally linked in the HA0 precursor. The stem domain is a complex of three 76-Å-long α-helixes, which form a triple-stranded coil, stabilized by salt bridges and hydrophobic interactions. The trimer is very

stable; it does not dissociate even after treatment with SDS (Doms and Helenius, 1986). The hydrophobic HA2 N-terminal peptide implicated in fusion (fusion peptide) is tucked between the long α-helixes near the base of the molecule. Its position is stabilized by noncovalent interactions with HA1 and with residues on the same and adjoining HA2 subunits. The single HA1–HA2 interchain disulfide bond is also located in the stem region, near the base of the molecule.

Electron microscopy using negative stain reveals the HA spikes in the intact virion at neutral pH as well-ordered rectangular projections about 135 Å in length. Acid treatment results in a disordered appearance. Ruigrok *et al.* (1986) found that virus spikes become thinner and longer after acid treatment in specimens containing isolated BHA and HA, as well as in HA reconstituted in liposomes. Their interpretation is that the trimeric contacts in the head domain are broken and the HA2 stem is elongated. Doms and Helenius (1986) obtained virus particles with decreasing spike densities by partial digestion of intact virions with bromelain. Acid treatment of those particles resulted in star-shaped aggregates on the surface of the virus.

One of the most powerful methods to examine conformational transitions of HA has been the use of poly- and monoclonal antibodies that recognize known regions of the protein. The neutral form of HA has three major epitopes, termed the loop, hinge, and tip/interface, all located in the globular head domain (Webster *et al.*, 1983; Yewdell *et al.*, 1983). Following acid treatment, antibodies against the tip/interface epitope, especially those that bind close to the trimer interface, no longer interact with HA, whereas binding of antibodies to the loop and hinge epitopes is unaffected. On the other hand, monoclonal antibodies have been raised specific to the acid conformation, which bind sites in HA1 and HA2. The pH-dependent appearance of these epitopes is similar to that of fusion of the intact virion, and BHA and HA show the same changes in antigenic structure.

Other differences between neutral and acid conformations have been examined mainly using BHA. Although incapable of mediating fusion, BHA undergoes many of the conformational changes that are observed in intact HA. For instance, whereas the neutral BHA and HA are resistant to a variety of proteases, the acid forms are susceptible to digestion with trypsin, proteinase K, and other proteases (Skehel *et al.*, 1982; Doms *et al.*, 1985). The pH dependence for conversion to their protease-sensitive forms is similar to that for fusion of the intact virion from which the hemagglutinin was extracted.

pH-induced conformational changes of BHA have been monitored by circular dichroism (Skehel *et al.*, 1982), accessibility of the interchain disulfide bridge to reducing agents (Graves *et al.*, 1983), and increase in hydrophobicity as indicated by aggregation in aqueous solution, interaction with nonionic detergents, and binding to liposomes (Skehel *et al.*, 1982; Doms *et al.*, 1985).

Those irreversible changes are presumably caused by exposure of the hydrophobic fusion peptide. The uncleaved HA0 does not become hydrophobic after low-pH treatment.

In view of the subunit structure and conformational changes discussed above, it is reasonable to view the viral spike glycoproteins as allosteric proteins (Blumenthal, 1988). A model for protein function based on cooperativity through conformational changes was introduced to offer a physical interpretation for heme–heme interactions in hemoglobin (Wyman 1948). A further analysis of the control of activity of a large number of enzymes led to the conclusion that in most of them indirect interactions between distinct binding sites are responsible for the performance of their regulatory function. This led to the model of regulation of protein function by allosteric transitions (Monod *et al.*, 1963, 1965).

The model is described by the following characteristics as formulated by Monod *et al.* (1965): (1) Allosteric proteins are arranged as oligomers whose subunits are associated in such a way that they all occupy equivalent positions. This implies that the molecule possesses at least one axis of symmetry. (2) To each ligand capable of forming a stereospecific complex with the protein there corresponds one site on each protomer. In other words, the symmetry of each set of stereospecific receptors is the same as the symmetry of the molecule. (3) The conformation of each protomer is constrained by its association with the other protomers. (4) Two (at least two) states are accessible to allosteric oligomers. These states differ by the distribution and/or energy of intersubunit bonds, and therefore also by the conformational constraints imposed on the subunits. (5) As a result, the affinity of one (or several) of the stereospecific sites toward the corresponding ligand is altered when a transition occurs from one to the other state. (6) When the protein goes from one state to another state, its molecular symmetry is conserved.

A variety of models have been proposed to describe how viral proteins might interact with the target membrane to induce the lipid rearrangements required for fusion. It is reasonable to assume that the virus binds to the target membrane at many points. The nature of the binding might be electrostatic, specific site–site recognition, or penetration into the acyl chain region of the target membrane. As a result of the multiple binding and presumably following a protein conformational change, the two membranes could be physically deformed and thus brought into close contact. This latter event might precipitate fusion between the two membranes either by overcoming the repulsion forces (steric, electrostatic, hydration) or by inducing an excessive radius of curvature (Blumenthal, 1987).

On the basis of the above discussion, it appeares that the HA of influenza virus fits most of those characteristics of an allosteric protein. It therefore seems reasonable to think of the induction of fusion in terms of an allosteric model

(Blumenthal, 1988; Blumenthal *et al.*, 1988). In the proposed model, the viral spike glycoproteins are considered as regulatory proteins involved in catalysis of fusion. Initially, the model does not deal with the specific mechanism of catalysis of fusion, but rather with consequences of ligand–protein interactions on the regulation of fusion. An analogy may be drawn to allosteric enzymes discussed by Monod *et al.* (1965), where not the mechanism of catalysis but the effects of regulatory ligands, conformational changes, and/or cooperativity on the functioning of the protein are considered. The allosteric model is not dependent on mechanism, but analysis of experimental observations according to the model will provide constraints on any proposed mechanism of fusion mediated by regulatory proteins. According to the model the viral spike glycoproteins are assumed to be arranged as oligomers, consisting of a number (n) of subunits linked through quarternary interactions. In Figure 2 only two or four of the n subunits are drawn as separate entities. This oligomer is capable of undergoing a "concerted" conformational change from what is conventionally known as the *tense* or T state (inactive) to the *relaxed* or R state which is active (Monod *et al.*, 1965). Each subunit in the oligomer contains a regulatory site for a ligand (H^+) with a dissociation constant K_d. Once the protein has undergone a conformational transition from the T state to the R state, the fusion process is catalyzed, leading to the melting of the two linked membranes into the R_f state. Although all the spike proteins of a particular virion can undergo the conformational change, only those involved in the virus–target interaction are relevant. Indeed, according to the model shown in Figure 2, the transition of a single oligomer is considered sufficient to precipitate the fusion event.

In the experiments with the R18-labeled virus a single fusion event brings about the movement of the 1500 R18 molecules from the viral membrane to the target membrane where they diffuse over a larger surface area, resulting in an

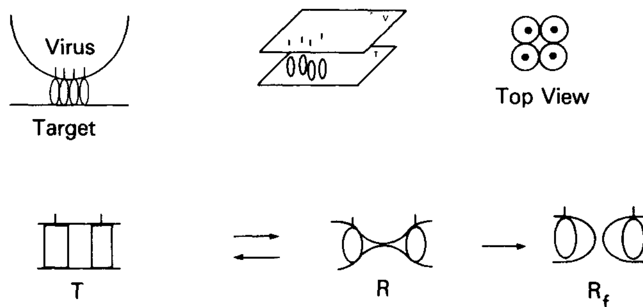

FIGURE 2. Allosteric model for membrane fusion mediated by viral spike glycoproteins. See text for further details. From Blumenthal (1988) with permission.

increased fluorescence signal. A single fusion event is induced by the transition of an oligomer in the virus–target complex from T to R to R_f (Figure 2). The total fluorescence increase is equal to the fluorescence increase per fusion event times the number of virions fused. This situation is similar to ion channel opening, which involves the conformational transition of channel protein oligomers resulting in movement of 1000 ions/msec through the channel, measured as a single channel conductance (Ehrenstein *et al.*, 1974).

By using the allosteric formalism, a simple expression for the relative rate constant for fusion can be derived (Blumenthal, 1988):

$$\text{relative rate constant} = \frac{(1 + \alpha)^n}{L + (1 + \alpha)^n} \quad (3)$$

where L is the equilibrium constant for the conformational transition between T and R states in the absence of ligand, and $\alpha = H^+/K_d$. Equation (3) is very similar to expressions derived by Monod *et al.* (1965) for the activity of allosteric enzymes.

In experiments on the interaction of VSV with Vero cells (a monkey kidney cell line), the rate of fusion was measured as a function of pH using R18 dequenching (Blumenthal *et al.*, 1987). From these data relative rate constants were calculated and plotted as a function of pH. A reasonable fit to Eq. (3) was obtained for pK_d of 6.3, $L = 1000$, and $n = 6$ (Blumenthal, 1988).

Recently, it was found that preincubation of VSV at low pH prior to binding to cells led to significant *enhancement* of fusion (Puri *et al.*, 1988). The data were analyzed in the framework of an allosteric model according to which viral spike glycoproteins undergo a pH-dependent conformational transition to an active (fusion-competent) state. Based on that analysis, it was concluded that the conformational transition to the active state is rate limiting for fusion and that the viral spike glycoproteins are fusion-competent only in their protonated form.

An alternative method to determine the size of the functional unit of the viral spike glycoprotein involved in membrane fusion is radiation inactivation analysis. Radiation inactivation of virus-induced fusion activity was measured by fluorescence dequenching of fluorescent lipid probes incorporated into liposomes (Gibson *et al.*, 1986; Bundo-Morita and Lenard, 1988), as well as by virus-induced hemolysis. With VSV it was found that the calculated functional units for both activities were similar, equivalent to about 15 viral spike glycoproteins (Bundo-Morita and Lenard, 1988). Surprisingly, however, similar studies with influenza and Sendai viruses resulted in a functional unit corresponding in size to a single protein monomer (Gibson *et al.*, 1986).

Examination of the hypotheses presented here regarding the regulatory properties of the viral spike glycoproteins in mediating membrane fusion requires

further detailed studies of the kinetics of fusion mediated by viral spike glycoproteins and analysis based on the allosteric model, in addition to further examination of the physicochemical states of those proteins in the intact virus, as well as in the isolated and reconstituted proteins using a variety of biophysical techniques.

10. CONCLUSIONS

The detailed mechanism of the process of virus–membrane fusion is still obscure. Very little is known about the molecular events that allow viral envelope glycoproteins of various groups to promote fusion with biological membranes and liposomes. Furthermore, the question of whether viral binding proteins and their membrane receptors serve only as a passive bonding pair or alternatively play an active role in the process of virus–membrane fusion is as yet unknown. From experiments showing that various binding proteins such as antibodies, polypeptide hormones and their receptors (Gitman *et al.*, 1985) or the pair avidin–biotin (Guyden *et al.*, 1983) may mediate virus binding and fusion with receptor-depleted cells should lead to the conclusion that viral binding proteins and their receptors are required only to allow attachment of viruses to recipient membranes. On the other hand, the experiments showing that hemolytic viruses such as Sendai or influenza may fuse with virus-receptor-depleted membranes only under hypotonic conditions may indicate an active role or virus receptors under physiological conditions.

This and other questions will have to be answered in future studies and answers obtained may have important clinical implications especially in developing drugs that will inhibit the initial stage of virus infection.

The availability of active fusogenic fluorescently labeled enveloped viruses and the fluorescence dequenching method (Loyter *et al.*, 1988a) made it possible to study by a simple reproducible and quantitative way the fusion of intact virions or the reconstituted envelopes with various membrane preparations.

Studies using the fluorescence dequenching method clearly showed that fusion of enveloped viruses of different groups with PC liposomes requires the presence of cholesterol (Loyter *et al.*, 1988a) and show the same feature as fusion with biological membranes. On the other hand, it appears from these studies that fusion with negatively charged liposomes does not reflect viral biological activity needed for its penetration and fusion of living cells. The interaction between enveloped viruses and liposomes of different lipid composition bearing or lacking virus receptors and the use of fluorescence dequenching methods may serve as an excellent experimental system for elucidating the yet unknown mechanism of virus–membrane fusion and the function of the viral glycoproteins and membrane receptors with this process.

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