Animal Viruses Are Able to Fuse with Prokaryotic Cells

FUSION BETWEEN SENDAI OR INFLUENZA VIRIONS AND MYCOPLASMA*  

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Sendai and influenza virions are able to fuse with mycoplasmata. Virus-Mycoplasma fusion was demonstrated by the use of fluorescently labeled intact virions and fluorescence dequenching, as well as by electron microscopy. A high degree of fusion was observed upon incubation of both virions with *Mycoplasma gallisepticum* or *Mycoplasma capricolum*. Significantly less virus-cell fusion was observed with *Acholeplasma laidlawii*, whose membrane contains relatively low amounts of cholesterol. The requirement of cholesterol for allowing virus-Mycoplasma fusion was also demonstrated by showing that a low degree of fusion was obtained with *M. capricolum*, whose cholesterol content was decreased by modifying its growth medium. Fluorescence dequenching was not observed by incubating unfusogenic virions with mycoplasmata. Sendai virions were rendered nonfusogenic by treatment with trypsin, phenylmethylsulfonyl fluoride, or dithiothreitol, whereas influenza virions were made nonfusogenic by treatment with glutaraldehyde, ammonium hydroxide, high temperatures, or incubation at low pH. Practically no fusion was observed using influenza virions bearing uncleaved hemagglutinin. Trypsinization of influenza virions bearing uncleaved hemagglutinin greatly stimulated their ability to fuse with Mycoplasma cells. Similarly to intact virus particles, also reconstituted virus envelopes, bearing the two viral glycoproteins, fused with *M. capricolum*. However, membrane vesicles, bearing only the viral binding (HN) or fusion (F) glycoproteins, failed to fuse with mycoplasmata. Fusion between animal enveloped virions and prokaryotic cells was thus demonstrated.  

A long-standing and unresolved problem in cell biology is the initial stages of virus-cell interaction (Rott and Klenk, 1977; Choppin and Scheid, 1980; White et al., 1983). In spite of extensive and continuous research, the detailed mechanism by which animal enveloped virions fuse with membranes of eukaryotic cells is still obscure (Rott and Klenk, 1977; White et al., 1983). Enveloped viruses belonging to the *Paramyxovirus* group, such as Sendai virus, fuse with the cell plasma membranes at neutral or basic pH values (Rott and Klenk, 1977; Choppin and Scheid, 1980; White et al., 1983). Influenza virions, on the other hand, are also able to fuse with the cell plasma membrane, but this occurs only when the pH of the incubation medium is lowered to between 5.0 and 5.2 (White et al., 1983).  

The interaction and fusion of Sendai and influenza virions with animal cells require the presence of sialic acid-containing receptors such as sialglycolipids and sialoglycoproteins (Gottschalk, 1957; Markwell and Paulson, 1980). Recently, however, we have shown that Sendai virions are able to fuse with erythrocyte membranes lacking virus receptors provided that the membrane phospholipids are exposed to the viral glycoproteins by osmotic stress (Citovsky and Loyter, 1985) or with lipid vesicles composed only of phosphatidylcholine and cholesterol (Citovsky and Loyter, 1985; Citovsky et al., 1985). Similar observations have been made following the interaction between influenza virions and phospholipid vesicles (Maeda et al., 1981; Doms et al., 1986). Based on these observations, it is tempting to speculate that viral envelopes are able to fuse with biological membranes whose lipid molecules are exposed to the viral glycoproteins, without the need of any specific virus receptors. A most attractive system to study this hypothesis is the interaction between animal enveloped virions and prokaryotic cells, especially mycoplasma. Mycoplasmata are deprived of a rigid cell wall and are known to lack any sialic acid-containing components (Razin, 1975). In addition, the composition of their membrane phospholipids can be manipulated, thus reflecting, to a certain degree, the lipid composition of the growth medium (Razin and Tully, 1970; Rottem, 1980).  

The recent use of fluorescently labeled virions (Roekstra et al., 1984; Citovsky and Loyter, 1985; Citovsky et al., 1985) and energy transfer or fluorescence dequenching methods to follow virus-membrane fusion processes allows the study of fusion between animal virions and membranes of cells other than eukaryotic cells. In this work, we have demonstrated, for the first time, fusion between Sendai or influenza virions and *Mycoplasma* cells. A high degree of fusion was observed with the Mycoplasma requiring *Mycoplasma gallisepticum* and *Mycoplasma capricolum* cells, whereas a low degree of fusion was observed with *Acholeplasma laidlawii* cells, whose membranes contain low amounts of cholesterol.

**MATERIALS AND METHODS**

**Chemicals**—PSMF, trypsin (Type III), DTT, and the ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and purchased from local suppliers.  

*The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CCCP, carbonyl cyanide m-chlorophenylhydrazone; R鰈, octadecylrhodamine B chloride; RSVE, reconstituted Sendai virus envelopes; HA, uncleaved hemagglutinin.*

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from Sigma. Octadecyl rhodamine B chloride (R<sub>18</sub>) was purchased from Molecular Probes.

**Viruses**—Sendai and influenza (A/PR/8/34) strains, H<sub>N</sub>N<sub>1</sub>, PR8 viruses were isolated from the allantoic fluid of fertilized chicken eggs (Peretz et al., 1974; Klenk et al., 1975). Influenza A, possessing HA<sub>2</sub> (virus N(H<sub>N</sub>10N7)), was grown in Madin-Darby canine kidney cells and isolated as previously described for chick embryo cells (Klenk et al., 1975). The viral hemagglutinating units and hemolytic activities were determined as described previously (Peretz et al., 1974; Huang et al., 1981). If not otherwise stated, egg grown viruses possessing cleaved hemagglutinin were used.

**Cells**—M. capricolum, M. gallisepticum, and A. laidlawii were grown in a modified Edward's medium (Razin and Rottem, 1976). The cells were harvested at the midexponential phase of growth (A<sub>450</sub> = 0.20–0.25), washed (12,000 x g for 10 min at 4 °C) in Solution Na (250 mM NaCl, 10 mM Tris-HCl, pH 7.4), and resuspended in the same buffer. For fusion experiments, the cells were used immediately after harvesting.

**Preparation of Fluorescent Virus Particles**—Sendai and influenza virions were labeled with R<sub>18</sub> as described elsewhere (Citovsky et al., 1985). Briefly, 5–7 μl of a 6 mg/ml ethanolic solution of R<sub>18</sub> was rapidly injected into 700 μl of Solution Na containing 1.7 mg of either Sendai or influenza virions. After 15 min of incubation at room temperature in the dark, the virions were washed in 20 volumes of Solution Na (100,000 x g for 30 min at 4 °C) and resuspended in the same buffer to give a protein concentration of 0.2–0.3 mg/ml. Under such conditions, R<sub>18</sub> was inserted into the viral membranes at self-quenching surface density (about 3 mol % of total viral phospholipids), and its decrease was shown to be proportional to the fluorescence dequenching (Hoekstra et al., 1985; Citovsky et al., 1985).

**Incubation of Sendai and Influenza Virions with Mycoplasmata**

**Fluorescence Measurements**—Fluorescent Sendai or influenza virions (2.0 μg of protein of Sendai virions and 6 μg of protein of influenza virions) were incubated with Mycoplasmata cells in a final volume of 400 and 200 μl, respectively, under the conditions described for each experiment. In a weight ratio of 1 μg of viral protein/100 μg of Mycoplasmata proteins, the virus:cell ratio was about 1:2. After incubation, 1 ml of Solution Na was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was estimated before and after solubilization with 0.1% Triton X-100. The fluorescence degree obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe (Hoekstra et al., 1984; Citovsky et al., 1985). All fluorescence measurements were carried out with a Perkin-Elmer MPF-4 spectrofluorometer with a 520-nm high pass filter and 590-nm emission slits to reduce light scattering.

**Protein Determination**—Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

**RESULTS**

**Fusion of Sendai Virions and Mycoplasmata**—The results in Fig. 1A show that incubation of fluorescently labeled (bearing R<sub>18</sub>) Sendai virions with cells of either M. capricolum or M. gallisepticum resulted in fluorescence dequenching, the degree of which was highly dependent on the amount of cells present in the system. Practically no fluorescence dequenching was observed following incubation of fluorescently labeled virions with A. laidlawii. It is noteworthy that whereas M. capricolum and M. gallisepticum require cholesterol for growth, A. laidlawii does not (Razin and Tully, 1970). The cholesterol is incorporated unchanged into the cell membrane of the former two species, reaching levels of up to 50 mol % (Rottem, 1980).

**Fig. 1. Fusion of Sendai virions with Mycoplasmata cells as monitored by fluorescence dequenching.** A, fusion of Sendai virions with various Mycoplasmata strains. R<sub>18</sub>-labeled Sendai virions (2.0 μg of viral protein) were incubated for 45 min at 37 °C with Mycoplasmata cell suspensions (0–400 μg of cell protein) in a final volume of 400 μl of Solution Na. After incubation, the degree of fluorescence dequenching (R<sub>18</sub>–DQ) was estimated. B, fusion of Sendai virions with M. capricolum cells; effect of temperature. The fluorescence dequenching measurements were performed at pH 7.5 as described for B, except that incubation temperatures were varied between 5 and 50 °C.

**t °C**

**5 10 15 20 25 30 35 40 45 50**

**R<sub>18</sub>–DQ (%)**

**0 20 40 60 80 100**

**Cells (μg protein)**

**0 200 400**

**Mycoplasma**

**A. laidlawii**

**M. capricolum**

**M. gallisepticum**

**Fusion of Sendai Virions and Mycoplasmata: Fluorescence Measurements—**Fluorescent Sendai or influenza virions (2.0 μg of protein of Sendai virions and 6 μg of protein of influenza virions) were incubated with Mycoplasmata cells in a final volume of 400 and 200 μl, respectively, under the conditions described for each experiment. In a weight ratio of 1 μg of viral protein/100 μg of Mycoplasmata proteins, the virus:cell ratio was about 1:2. After incubation, 1 ml of Solution Na was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was estimated before and after solubilization with 0.1% Triton X-100. The fluorescence degree obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe (Hoekstra et al., 1984; Citovsky et al., 1985). All fluorescence measurements were carried out with a Perkin-Elmer MPF-4 spectrofluorometer with a 520-nm high pass filter and 590-nm emission slits to reduce light scattering.

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**The view that the fluorescence dequenching observed indeed reflects fusion between the viral envelope and the Mycoplasmata membrane was further supported by the results presented in Table I. As can be seen in Table I, fluorescence dequenching was observed only when hemolytic Sendai virions were incubated with either one of the two cholesterol-containing Mycoplasmata species. It has been well established that virus-induced hemolysis reflects a process of virus-membrane fusion (Maeda et al., 1977b). On the other hand, incubation of nonhemolytic (i.e., DTT-, PMSF-, or trypsin-treated) Sendai virions with Mycoplasmata resulted in very little fluorescence dequenching (Table I). It is well established that the above treatments cause inactivation of the viral fusogenic activity (Ozawa et al., 1979; Israel et al., 1983).

**Similarly, very little fluorescence dequenching was observed when active fusogenic Sendai virions were incubated with mycoplasmata that were pretreated with 0.1% glutaraldehyde (Table I). Thus, glutaraldehyde-fixed cells should not be susceptible to fusion with Sendai virions but should allow lipid-lipid exchange between the viral envelope and the biological membranes (Maeda et al., 1977a).**

**Additional direct support for the claim that the fluorescence dequenching observed is indeed due to a fusion process be-
Fusion of Animal Viruses with Mycoplasma Cells

TABLE I

Requirement for hemolytic Sendai virions to allow fusion with Mycoplasma cells

| Exp I | M. capricolum | M. gallisepticum |
|-------|---------------|-----------------|
| %     |               |                 |
| Sendai virus treated with: |   |                 |
| Nothing     | 84 | 45* | 29 |
| DTT         | 6  | 7   | 2  |
| PMSF        | 8  | 8   | 6  |
| Trypspin    | 4  | 7   | 7  |

Exp II

Prior to incubation with Sendai virions, cells treated with:

|          | ND  | 48  | 33  |
|-----------|-----|-----|-----|
| Nothing   |     |     |     |
| Glutaraldehyde (0.1%) | ND | 8   | 4   |

*No detectable increase in the degree of fluorescence (R<sub>n</sub> dequenching) was observed under the same conditions following incubation of virus particles in the presence of cells.

ND, not determined.

TABLE II

Requirement for both Sendai virus envelope glycoproteins for fusion with Mycoplasma cells

| M. capricolum cells incubated with: | R<sub>n</sub> dequenching % |
|------------------------------------|-----------------------------|
| Sendai virions                     | 48                          |
| RSVE                               | 45                          |
| RSVE<sub>PMSF</sub>               | 7                           |
| F-vesicles                        | 5                           |
| NH-vesicles                       | 7                           |
| F-HN-vesicles<sub>PMSF</sub>      | 8                           |

Tabelle the viral envelope and the Mycoplasma membranes were obtained from the experiments described in Table II. As can be seen, incubation of fluorescently labeled F- or HN-virions with M. capricolum resulted in a low degree of dequenching. It is noteworthy that due to the presence of the viral binding protein, the HN-virions are able to attach to cell surfaces in the same manner as intact virions (Fukami et al., 1980). Fluorescence dequenching was observed only when fluorescent vesicles possessing the two viral envelope glycoproteins, i.e., the F-HN-virions, were incubated with the Mycoplasma cells (Table II). The results in Table II show that the degree of dequenching obtained following incubation with the coreconstituted F-HN-vesicles was very close to that obtained with intact virions or with reconstituted Sendai virus envelopes (RSVE). As expected, only a low degree of dequenching was obtained following incubation with nonfusogenic RSVE or F-HN-vesicles, namely with reconstituted viral vesicles treated with PMSF (Table II).

The results in Fig. 1B show that maximum fluorescence dequenching (45%) was observed between pH 6.0 and 9.0. At pH values below 6 or above 9, a relatively low degree of fluorescence dequenching was observed (Fig. 1B). Other workers have demonstrated that the Sendai virus fusion factor is maximally activated at pH 6.0–9.0 (White et al., 1983; Chejanovsky and Loyter, 1985).

Fusion of Sendai virions with mycoplasmata is extremely dependent on the incubation temperature, as demonstrated in Fig. 1C for M. capricolum. Maximum fusion (fluorescence dequenching) was observed at 37 °C, whereas very little fusion occurred at 17 °C and below (Fig. 1C). In addition, a decrease in the extent of fusion was observed at temperatures above 42 °C. This is probably due to thermal inactivation of the viral fusion protein (Citovsky et al., 1985; Chejanovsky and Loyter, 1985).

Electron Microscopic Observations of Fusion between Sendai Virions and M. capricolum—Fusion between intact Sendai virions and mycoplasmata was also demonstrated by electron microscopy using thin-sectioned (Fig. 2) preparations. The electron micrographs depicted in Fig. 2 show that Sendai virions (V) as well as Mycoplasma cells (MP) can clearly be identified in thin sections. The viral ribonucleoproteins can be distinguished in these preparations as long threads within the virus vesicle. No such structures are seen in the Mycoplasma cells whose intracellular space is filled with the darkly stained floccular material, as was reported before (Razin, 1975). Fused virions were prepared and stained with uranyl acetate as described elsewhere (Marti and Webster, 1986). a, cells incubated with PMSF-treated Sendai virions (prepared as described for Fig. 1). Free virus particles (V) and Mycoplasma cells (MP) can clearly be identified. b–d, virus particles whose envelopes are in the process of fusion (b and c) or are fused (d) are seen. Arrows indicate fusion areas (b and c) or fused membranes (d). Magnification × 26,300.
resulted in a much lower degree of fluorescence dequenching (10-15%) (Fig. 3A). Surprisingly, almost the same degree of fluorescence dequenching was observed following incubation at either low (pH 5.2) or neutral pH values (Fig. 3A). A low pH is required for activation of the influenza virus' infectivity as well as its ability to fuse with biological membranes (Huang et al., 1981; White et al., 1983).

The view that the fluorescence dequenching observed indeed reflects a process of virus-membrane fusion is strongly supported by the results in Fig. 3B, showing that a relatively low degree of fluorescence dequenching was obtained by incubating influenza virions, bearing HA$_{av}$, with *M. capricolum*. Activation of the nonfusogenic HA$_{av}$-containing influenza virions can be achieved by trypsin digestion which specifically cleaves the HA$_{av}$ glycoprotein (Klenk et al., 1975). The results in Fig. 3B show a marked increase in the degree of fluorescence dequenching following trypsinization of the HA$_{av}$-containing influenza virions. As can be seen (Fig. 3B), the extent of fluorescence dequenching observed upon incubation of trypsinized HA$_{av}$ influenza virions with *M. capricolum* was very close to that obtained with the egg-grown influenza virions.

The assumption that the fluorescence dequenching observed at both pH 5.2 and 7.4 is a quantitative reflection of a virus-membrane fusion process is further strengthened by the results summarized in Table III. Incubation of active, fusogenic influenza virions with human erythrocyte ghosts at pH 5.2, but not at pH 7.4, resulted in fluorescence dequenching; whereas with *M. capricolum*, an increase in fluorescence was observed at both pH values. On the other hand, a low degree

![FIG. 3. Interaction of influenza virions with Mycoplasma cells. A, fusion with various Mycoplasma strains. R$_s$-labeled influenza virions (6 $\mu$g of protein) were incubated with *M. capricolum*, *M. gallisepticum*, and *A. laidlawii* (300 $\mu$g of protein each in 200 $\mu$l of Solution Na) for 10 min at 4°C. At the end of the incubation period, the pH of the suspension was either left at pH 7.4 (filled bars) following the addition of 0.5 $\mu$l of Tris-Cl, pH 7.4, or adjusted to pH 5.2 (open bars) by the addition of 0.5 $\mu$l sodium acetate, pH 5.2. The suspensions obtained were incubated for 30 min at 37°C, at the end of which the degree of fluorescence was estimated as described under "Materials and Methods." B, interaction with HA$_{av}$-containing influenza virions. HA$_{av}$-bearing influenza virions were obtained following infection of Madin-Darby canine kidney cells with (Nh$_{H}$-N$_{v}$)) influenza virus A, as described before (Klenk et al., 1976). Trypsination of the HA$_{av}$ virions was performed by incubating 200 $\mu$l of virus with 3 $\mu$l of trypsin (bovine pancreas, Type III, Sigma) in a final volume of 200 $\mu$l of PBS (10 mM sodium phosphate and 150 mM NaCl), pH 7.4, for 20 min at 37°C essentially as described before (Klenk et al., 1976). Influenza virions (6 $\mu$g of PR8, N-HA$_{av}$, and trypsinized (Tryp) N-HA$_{av}$) were incubated with 300 $\mu$g of *M. capricolum* at pH 5.2 (open bars) or 7.4 (closed bars) as described under "Materials and Methods." At the end of the incubation period, the degree of fluorescence dequenching was estimated as described under "Materials and Methods" and for A above.

Fusion between the virus envelopes and the *Mycoplasma* cells was not observed in preparations containing nonfusogenic (PMSF-treated) Sendai virions (Fig. 2a). The same picture was obtained from preparations made of fusogenic viruses which were incubated with the *Mycoplasma* cells in the cold (not shown). A preliminary step in the fusion process between Sendai virions and *M. capricolum* appears in the thin section shown in Fig. 2b. A virus particle and a *Mycoplasma* cell are seen in tight contact. At the point of attachment (arrow), no clear viral or *Mycoplasma* membranes can be identified. Similar observations have also been found when the fusion areas between viral envelopes and plasma membranes of eukaryotic cells were examined (Toister and Loyter, 1973). Viral particles whose envelopes had completely fused with the *Mycoplasma* membranes are seen in Fig. 2 (c and d). This is particularly clear in the electron micrograph presented in Fig. 2d. The viral envelope and the *Mycoplasma* membrane appear to be connected (Fig. 2d, arrow), forming one continuous bilayer.

Fusion of Influenza Virions and Mycoplasma—Incubation of fluorescently labeled influenza virions, similar to incubation of Sendai virions, with *M. gallisepticum* and *M. capricolum* resulted in a relatively high degree (40-55%) of fluorescence dequenching, whereas incubation with *A. laidlawii*...
showing that the cholesterol content of *M. capricolum* is relatively low amounts of cholesterol (7.0 pg/mg of cells), whereas results in Fig. 5 confirm previous observations (Rottem, 1980) giving (Fig. 5, A, bar a) was much lower than that obtained after incubation with cells containing a relatively high amount of cholesterol (Fig. 5, A, bars b and c, and B, bars b and c). A clear correlation between the cholesterol content of *M. capricolum* and its susceptibility to fusion with both Sendai and influenza virions is thus evident. From the results in Fig. 5B, it appears that fusion of influenza virions with *M. capricolum*, both at pH 5.2 and 7.4, was dependent on the cholesterol content of the cells. No virus-*Mycoplasma* fusion (fluorescence dequenching) was observed with nonfusogenic Sendai or influenza virions (Fig. 5).

**FIG. 4**. Effect of CCCP on fusion between influenza virions and *M. capricolum*. A, influenza virions (6 μg) were incubated with *M. capricolum* (300 μg) in the absence (O) and presence (●) of 5 μM CCCP at pH 7.4 for 10 min at 4 °C as described under "Materials and Methods." At the end of the incubation period, the pH of the suspension was adjusted with 50 μl of 0.5 M sodium acetate, pH 5.0-7.5, 0.5 M Tris-HCl, pH 7.0-9.0, and glycine/NaOH, pH 9.0-10.0. Following incubation at 37 °C for 30 min, the degree of fluorescence dequenching was estimated as described under "Materials and Methods." B, incubation of Sendai virions (6 μg of protein) at pH 7.4 (open bars) or influenza virions (6 μg of protein) at pH 7.4 (closed bars) or pH 5.2 (hatched bars) with *M. capricolum* (300 μg of protein) was performed as described for A and under "Materials and Methods." In experiments with CCCP, cells of *M. capricolum* were incubated with 5 μM CCCP for 20 min at 37 °C prior to the addition of Sendai or influenza virions. It should be noted that at the concentrations used, CCCP, by itself, slightly reduced the degree of fluorescence. Therefore, CCCP was also added to control systems incubated in the absence of cells to estimate its effect on the degree of fluorescence. The use of higher concentrations of CCCP was avoided in order to minimize its effect on the fluorescence measurements.

The results in Table III clearly indicate that: (a) almost the same decrease in the degree of fluorescence dequenching (percent of inhibition) was observed by incubating treated virions with either human erythrocyte ghosts or *M. capricolum* (Table III). Influenza virions were rendered nonfusogenic by treatment with hydroxylamine (Schmidt and Lambrecht, 1986) or with glutaraldehyde or by preincubation at low pH (pH 5.2) or at high temperatures (Sato et al., 1983).

The results in Fig. 4A show that almost the same high degree of fluorescence dequenching (40-45%) was observed following incubation of influenza virions and *M. capricolum* between pH 6.0 and 10.0. The degree of fluorescence (fluorescence dequenching) observed at pH 5.2 was always higher than that at pH 7.4 (Fig. 4, A and B). The addition of CCCP (5 μM) markedly decreased the extent of fluorescence observed at pH values between 6.0 and 10.0, but had very little effect on that observed at pH 5.2 (Fig. 4, A and B). No effect of CCCP could be detected on the fluorescence dequenching at pH 7.4 following incubation of Sendai virions and *M. capricolum* (Fig. 4B).

**FIG. 5**. Fusion of Sendai and influenza virions with *M. capricolum*: effect of cholesterol. *M. capricolum* cells with different contents of membrane cholesterol were prepared by growing them in the presence of 0.5% (a), 2% (b), or 4% (c) horse serum (Razin and Rottem, 1976). The amount of cholesterol in the cell membranes was determined as previously described (Rudel and Morris, 1975). A, fusion with Sendai virus. R18-labeled, intact (closed bars) or trypaninized (open bars) virions (2 μg) were incubated with 300 μg of cell protein as described for Fig. 1, B, fusion with influenza virus. R18-labeled, intact virions (6 μg) were incubated with 300 μg of cell protein either at pH 7.4 (closed bars) or at pH 5.2 (hatched bars) as described for Table I. Also, hydroxylamine-treated virions (open bars) were prepared (see Table II) and incubated at pH 5.2 with *Mycoplasma* cells as described above for intact virions. Cholesterol content of *M. capricolum* membranes (μg/mg of cell protein) was 7 (bar a), 30, (bar b), and 43 (bar c).

**DISCUSSION**

Fusion of enveloped viruses with recipient animal cells was studied and demonstrated mainly by following the ability of such viruses either to cause infection or, alternatively, to promote cell-cell fusion and induce lysis of recipient cells. The development of energy transfer and fluorescence dequenching methods allowed the investigation, on a quantitative basis, of fusion of animal viruses either with lipid vesicles or with biological membranes lacking virus receptors (Hoekstra et al., 1984; Citovsky and Loyter, 1985; Citovsky et al., 1985). Utilising these methods, the results of this work demonstrate that fusogenic animal enveloped viruses are able to fuse with the plasma membranes of prokaryotic cells. Fusion was inferred from experiments showing a relatively high degree of fluorescence dequenching following the incubation of...
fluorescently labeled Sendai and influenza virions with two species of Mycoplasma. Much less fluorescence dequenching was observed with virions whose fusogenic activity had been inactivated. Sendai virions were rendered nonfusogenic by treatment with trypsin, DTT, or PMSF. It is noteworthy that PMSF-treated virions, similarly to viruses bearing the uncleaved fusion factor, are able to attach to but not to fuse with animal cells (Israel et al., 1983).

The present results also showed that fusion of Sendai virus with mycoplasmata (similarly to its fusion with animal cultured cells (Fukami et al., 1980)) required the presence of the two viral envelope glycoproteins. Membrane vesicles bearing only the Sendai virus-binding (NH) or the fusion (F) glycoprotein failed to fuse with M. capricolum. This was inferred from the experiments showing that only a low degree of dequenching was observed following incubation of Mycoplasma cells with NH- or F-vesicles, as opposed to a high degree of dequenching obtained with co-reconstituted F-NH-vesicles. These results further support the view that the increase in the extent of fluorescence resulted from fusion between the virus envelopes and the Mycoplasma cells. Using different experimental systems, it has been demonstrated (Miura et al., 1982; Heath et al., 1983; Gitman et al., 1985) that the HN glycoprotein, besides being the viral binding protein, also plays an active role in the fusion process itself. Its presence, together with the F glycoprotein, was required even for fusion of Sendai virus envelopes with membrane vesicles lacking virus receptors. Had the fluorescence dequenching observed been caused by lipid transfer or by lipid-lipid exchange processes, it should have also occurred following incubation of the fluorescent NH- or F-vesicles with Mycoplasma cells.

The degree of fluorescence dequenching is a quantitative measure of the extent of virus-membrane fusion (Citovsky and Loyter, 1985; Chejanovsky and Loyter, 1985); therefore, it should be inferred that about 50–60% of the virus particles in the population fused with the mycoplasmata. This percentage is very close to that observed for fusion of Sendai virions with cultured cells and was also confirmed by electron microscopy. Essentially, the same micrographs were also obtained using influenza virions (data not shown).

Enveloped virions belonging to the Orthomyxovirus group require a low pH environment to activate their fusogenic glycoprotein (Choppin and Scheid, 1980; White et al., 1983). Based on fluorescence dequenching measurements, the results of this work show that influenza virions fuse with erythrocyte membranes only at pH 5.2, but not at pH 7.4. This is expected from previous observations (Huang et al., 1981) showing that influenza virions induced hemolysis at low pH values only. On the other hand, fusion of influenza virions with mycoplasmata was observed also at neutral and high pH values. This may indicate the existence of a local, low pH environment at the outer surface of the Mycoplasma membrane, which may be attributed to a potent proton pump known to be present in these cells (Linker and Wilson, 1985).

This view is supported by the experiments showing that the [H+] ionophore CCCP which causes the collapse of a pH potential across biological membranes (Harold, 1982) strongly inhibits influenza-Mycoplasma fusion occurring at high pH values, although practically without any effect on the fusion observed at pH 5.2. Furthermore, under the conditions used, fusion of Sendai virions (whose fusogenic protein is also active at pH 7.4) with mycoplasmata was not affected by the addition of CCCP. Thus, it appears that a local, low pH environment may activate the fusogenic activity of those influenza virions which are in close proximity to the Mycoplasma membranes. However, alternative explanations cannot as yet be excluded.

Evidently, the fusion observed in this work cannot be mediated by membrane-associated virus receptors because the prokaryotic cells used lack any sialic acid residue-containing components (Razin, 1975). In addition, it should be mentioned that the same degree of fluorescence dequenching was obtained following incubation of Sendai or influenza virions with untreated or neuraminidase-treated Mycoplasma cells (not shown). Hence, the process observed should be attributed to a direct association and interaction between the viral glycoproteins and the Mycoplasma membrane, especially its phospholipid bilayer. In this respect, virus-Mycoplasma fusion resembles the previously reported fusion between Sendai or influenza virions and phospholipid vesicles lacking any specific virus receptors (Citovsky and Loyter, 1985; Citovsky et al., 1985). It is noteworthy that fusion with liposomes composed of only phosphatidyicholine and cholesterol and lacking virus receptor required the presence of two Sendai viral glycoproteins, namely the HN and F polypeptides (Citovsky and Loyter, 1985). As opposed to fusion with cultured cells bearing virus receptors, fusion of Sendai virions with liposomes composed of only phosphatidyicholine and cholesterol was found to be a nonleaky process (Citovsky and Loyter, 1985). The same results were observed using influenza virions (Schmidt and Lambrecht, 1985). Indeed, preliminary results in our laboratory have also indicated that fusion of Sendai and influenza virions with Mycoplasma cells does not result in the release of the Mycoplasma content as was monitored by following leakage of intracellular enzymes or [3H]thymidine (not shown). These results may indicate that under the conditions used (about one virus particle/one to two cells of Mycoplasma), the Mycoplasma cells remained intact. Recent results in our laboratory also showed that under the conditions used, fusion with Sendai virions caused inhibition in the growth rate of mycoplasmata. Experiments are under way to study whether this is due to virus-induced cell death or to a decrease in the growth rate of cells fused with virus envelopes.

The failure of Sendai as well as influenza virions to fuse effectively with cells of A. laidlawii can be attributed to the relatively low amount of cholesterol present in the membranes of these cells (Razin and Tully, 1970). The presence of a certain amount of cholesterol has been found to be obligatory to allow fusion of Sendai virions as well as other enveloped viruses with phospholipid vesicles (White et al., 1983; Citovsky et al., 1985). The ability to change the lipid composition of the Mycoplasma cells by modifying the lipid precursors present in the growth medium (Rottem, 1980) makes these cells an exceptionally excellent tool to study the relationship between the lipid composition of biological membranes and their ability to interact functionally with fusogenic animal virions. In this work, we have altered the cholesterol content of M. capricolum by culturing these cells in a medium containing increasing amounts of serum. Our results indeed show that almost a linear correlation exists between the cholesterol content of M. capricolum and the susceptibility of these cells to fusion with Sendai and influenza virions. Our results demonstrate, for the first time, that cholesterol is required to allow fusion between enveloped viruses and biological membranes.

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