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Characteristics of Fusion of Respiratory Syncytial Virus with HEp-2 Cells As Measured by R18 Fluorescence Dequenching Assay

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The characteristics of fusion of respiratory syncytial virus (RSV) with HEp-2 cells were studied by the R18 fluorescence dequenching assay of membrane fusion. A gradual increase in fluorescence intensity indicative of virion-cell fusion was observed when R18-labeled RSV was incubated with HEp-2 cells. Approximately 35% dequenching of the probe fluorescence was observed in 1 h at 37°C. Fusion showed a temperature dependence, with significant dequenching occurring above 18°C. The dequenching was also dependent on the relative concentration of target membrane. Thus, increasing the concentration of target membrane resulted in increased levels of dequenching. In addition, viral glycoproteins were shown to be involved in this interaction, since dequenching was significantly reduced by pretreatment of labeled virus at 70°C for 5 min or by trypsinization of R18-labeled virions prior to incubation with HEp-2 cells at 37°C. The fusion of RSV with HEp-2 cells was unaffected over a pH range of 5.5 to 8.5, with some increase seen at lower pH values. Treatment of HEp-2 cells with ammonium chloride (20 and 10 mM), a lysosomotropic agent, during early stages of infection did not inhibit syncytium formation or progeny virion production by RSV. At the same concentrations of ammonium chloride, the production of vesicular stomatitis virus was reduced approximately 4 log_{10} units. These results suggest that fusion of the virus with the cell surface plasma membrane is the principal route of entry.

Respiratory syncytial virus (RSV) is a member of the family *Paramyxoviridae* and the genus *Pneumovirus*. The family also includes the genera *Paramyxovirus* and *Morbillivirus*. RSV differs significantly from the other viruses of the family in that it has a more complex genome, coding for 10 proteins (4, 20), instead of the 6 or 7 proteins in other viruses (22). It also lacks hemagglutinating and neuraminidase activities (28), which are common to the others. It has a lipid envelope that bears two surface glycoproteins, called G (attachment) (25) and F (fusion) (7), and possibly a third protein (SH for small hydrophobic) (5, 33). RSV is a significant cause of severe infections, such as bronchiolitis and pneumonia in newborns and infants (21), and it is one of the agents of the common cold syndrome in adults. Since RSV appears to differ significantly from other paramyxoviruses, it is important to delineate various aspects of its pathogenesis, including the entry of the virus into host cells. In this context, the role of the surface glycoproteins must be defined.

Fusion of viral and cell membranes has been studied by several techniques: polykaryon formation in virus-infected cell culture, hemolysis of erythrocytes by hemagglutinating viruses, electron microscopy, and newer methods employing probes for the mixing of membrane lipids have been used. While spin-labeled probes (27) and radioactive labels (14) have been used by several investigators for the detection of fusion between membranes, of late, fluorescence methods have gained popularity. Fluorescence methods allow real-time monitoring of fusion, and therefore the process can be studied both qualitatively and quantitatively. Two methods have been described: relief of self-quenching of fluorescent probes (16) and resonance energy transfer between fluorescent probes (13, 36). In the former method, one of the membranes is labeled with a fluorescent lipid probe at self-quenching concentrations. As a consequence of fusion with unlabeled target membrane, mixing and dilution of the fluorescent probe occur in the target membrane, resulting in the dequenching of the probe and an increase in fluorescence emission. The fluorescent lipid probe octadecylrhodamine (R18) can be readily inserted into preformed lipid bilayers at concentrations that result in self-quenching (10, 16), apparently without significantly altering viral infectivity (30).

Recently, some reports suggested that virus-cell fusion and cell-cell fusion were separable events (15). It may therefore be more appropriate to use methods that detect virus-cell fusion rather than cell-cell fusion to determine the role of surface glycoproteins in the initial interactions of viruses with host cell membranes. The R18 fluorescence dequenching assay, because of its simplicity and applicability, has been used by several investigators for the study of the interaction of enveloped viruses with target membranes and to define the role of surface glycoproteins in this regard (1, 3, 6, 10, 12, 16, 17, 19, 26, 34, 35). Here, we report on the characteristics of fusion of RSV with HEp-2 cells as measured by the R18 fluorescence dequenching assay.

MATERIALS AND METHODS

Virus and cells. The A_2 strain of RSV was used in this study. HEp-2 cells were passaged in Eagle's minimal essential medium (EMEM) containing 10% newborn calf serum (gamma globulin free; GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (cEMEM). Virus stocks were prepared by infecting HEp-2 cells at low multiplicities of infection (MOIs) of approximately 0.001 to 0.05 PFU/cell. After incubation of infected cells at 37°C for 48 h, the supernatant was harvested and clarified by centrifugation at 500 × *g* for 10 min. Stock virus pools were stored in liquid nitrogen and had titers of ~2 × 10^6 PFU/ml.

Virus purification. Virus was purified by the method of

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Ueba (39) with slight modifications. Briefly, supernatants from virus-infected HEp-2 cells were pooled and clarified at 5,000 × *g* at 4°C for 20 min. Polyethylene glycol 8000 (Fisher Scientific, Fairlawn, N.J.) was added to the clarified supernatant to 10% (wt/vol) final concentration and stirred at 4°C for 1 h. The polyethylene glycol precipitate was collected by centrifugation at 5,000 × *g* at 4°C for 10 min. The precipitate was resuspended in 20% (wt/wt) sucrose in NTE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA) by Dounce homogenization. The virus was pelleted through 30% sucrose–NTE buffer at 23,000 rpm in an SW41 Ti rotor at 4°C for 1 h. The virus pellet was resuspended in 20% sucrose–NTE buffer and centrifuged on a discontinuous sucrose gradient (35, 50, and 60%) at 36,000 rpm in an SW41 Ti rotor for 2 h at 4°C. The virus band at the interface of 35 and 50% sucrose was harvested and in aliquots stored at −70°C. The protein content was determined by a dye-binding assay (2). The PFU-protein ratio was ~1 × 10^8 PFU/mg of protein for different purified stocks of the virus.

Plaque assay. Viral infectivity was measured by a plaque assay carried out in 24-well plates (Costar, Cambridge, Mass.) as described previously (38).

Effect of ammonium chloride on infectivity of RSV. The infectivity assay was carried out as described previously by Gilbert et al. (10). Briefly, HEp-2 cells in 24-well Costar plates were treated with ammonium chloride (20 or 10 mM) in EMEM with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.4, for an hour at 37°C. Virus adsorption was carried out at 37°C in the presence of the drug. After 90 min, unbound virus was washed away with EMEM containing the drug. After further incubation for 5 h in the presence of the drug, medium was replaced with drug-free medium. Cultures were examined for syncytium formation and progeny virion production at 40 h postinfection. Parallel cultures were examined for neutralization of intracellular granular pH with 1.5 μM acridine orange as described previously (10). In addition, as a positive control, vesicular stomatitis virus, serotype Indiana, was tested in this system, with the difference that progeny virion production and cytopathic effect were examined at 18 h postinfection. The differences in the time at which samples were tested for progeny virion production was due to known differences in the growth cycles for the two viruses.

Fluorescent labeling of RSV. The virus envelope was labeled with the fluorescent lipid probe octadecylrhodamine B (R18; Molecular Probes Inc., Eugene, Oreg.) as described by Hoekstra et al. (16), with slight modifications. Briefly, virus (250 μg) in 500 μl of Hanks's balanced salt solution (HBSS; GIBCO) was labeled with R18 (3 μg) in 5 μl of ethanol and incubated for 1 h at room temperature. Unincorporated probe was removed by gel filtration on Sephadex G-75 (1 by 20 cm) equilibrated with HBSS. Labeled virus was recovered in the void volume. Approximately 70% of the original amount of virus was recovered (as determined by a mock labeling experiment with purified ^{35}S-labeled virus) with 10% of the input infectivity.

Target cells for fusion. HEp-2 cells in monolayers were released with trypsin-EDTA (GIBCO). Trypsin was inactivated by adding cEMEM. The cells were washed once with 100 volumes of HBSS and resuspended to 2 × 10^7/ml in HBSS. Cell viability, as determined by the trypan blue exclusion method, was usually about 90%.

Binding studies. Aliquots of R18-labeled RSV were allowed to bind to HEp-2 cells (2 × 10^6) at 4°C. After incubation, free virus was separated from cell-bound virus by centrifugation at low speed. The cells were washed twice with 750 μl of HBSS. The supernatants were pooled, and the distribution of R18 between the cells and supernatant, corresponding to bound and free virus, respectively, was determined after addition of Triton X-100. The effect of incubating different amounts of R18-labeled virus (30 to 180 μg) with HEp-2 cells and the time course of binding were also examined in this system.

R18 fluorescence dequenching assay of membrane fusion. The dequenching assay was modeled on the method of Nussbaum et al. described previously (31). R18-labeled virus (5 μg) was mixed with 2 × 10^6 cells in 200 μl of HBSS (with or without 20 mM NaN_3) and incubated in a 37°C water bath for various periods of time. R18-labeled virus alone in 200 μl of HBSS was included as control and for determining baseline levels of fluorescence. At the end of the incubation period, the reaction volume was increased by addition of HBSS to 1.8 ml, and the contents of the tube were transferred to a cuvette. Fluorescence intensity was measured in a Perkin-Elmer L-5 spectrofluorometer with an excitation wavelength of 560 nm and an emission (Em) wavelength of 584 nm. Each experiment was run in duplicate, and the percent dequenched (DQ) was calculated for each sample after addition of Triton X-100 to 1%, which was considered to result in infinite dilution of the probe. The percentage dequenched (%DQ) was calculated as follows (26, 31): %DQ = $100 \times (F_d - F_0)/(F_t - F_0)$, where F_0 = Em 584 nm of R18-labeled virus, F_d = Em 584 nm after incubation with target membrane, and F_t = Em 584 nm after addition of Triton X-100.

To monitor fusion continuously, 5 μg of R18-labeled virus was incubated with 2 × 10^6 HEp-2 cells in 1.8 ml of HBSS. At the end of the observation period, Triton X-100 was added to 1% final concentration. After incubation for 10 min, the level of dequenching was determined as above. This formula does not take into account any background signal that may arise due to light scattering on addition of cells which may spuriously contribute to the final fusion estimation. We have estimated that this may vary between 10 and 15% DQ. Except where indicated, the data have been corrected for this by using appropriate controls.

Glutaraldehyde treatment of HEp-2 cells. Aliquots of HEp-2 cells (2 × 10^7) were treated with different concentrations of glutaraldehyde (0.025 to 0.1% [vol/vol] final concentration) in 1 ml of HBSS for 30 min at 37°C, washed twice with HBSS, and tested for binding capacity and fusion competence with R18-labeled RSV.

Effect of pH on fusion. R18-labeled virus was allowed to bind to HEp-2 cells in HBSS (pH 7.5) at 4°C for 1 h in a 195-μl reaction volume. Then 5 μl of a 1 M stock solution of citrate buffer (for pH range 4 to 6) or HEPES buffer (for pH range 6.5 to 9.0) preadjusted to various pHs was added (final molarity, 0.175 M). NaN_3 was included in the reaction mixture at a final concentration of 20 mM. The mixture was then incubated at 37°C for 30 min prior to determination of %DQ. As a control, virus alone was incubated at the various pHs.

Trypsin treatment. Aliquots of R18-labeled RSV (~7 μg each) were treated with different concentrations of trypsin (2×, lyophilized; 3,400 U/mg; from bovine pancreas, catalog no. 840-706811; GIBCO) in 50 μl of HBSS at 37°C for 15 min. Trypsin was inactivated by addition of 10 μl of HBSS containing twice the minimum amount of soybean trypsin inhibitor (Sigma, St. Louis, Mo.). Virus was then incubated with 2 × 10^6 HEp-2 cells in the presence of 20 mM NaN_3 for 1 h at 37°C. Final levels of dequenching were calculated. As controls, R18-labeled virus was treated with inhibitor alone

TABLE 1. Effect of ammonium chloride on infectivity of RSV and vesicular stomatitis virus

Ammonium chloride treatment (mM)	Titer (log PFU/ml) at indicated MOI[a]			
	VSV		RSV	
	0.2	0.02	0.1	0.01
None	7.7	7.8	5.0	3.3
10	6.5 (93.2)	5.8 (98.8)	5.1	3.4
20	3.7 (99.9)	4.2 (99.9)	5.3	3.5

[a] Values are averages of two determinations. Numbers in parentheses indicate percent reduction in PFU. VSV, vesicular stomatitis virus.

or with trypsin-inhibitor complexes prior to incubation with target cells.

RESULTS

Effect of ammonium chloride on infectivity of RSV. Inclusion of the lysosomotropic agent ammonium chloride during the early stages of infection did not appear to have any effect on the infectivity of RSV, as measured by syncytium formation and progeny virion production (Table 1). That the concentration of ammonium chloride used in the experiment actually caused an increase in intracellular granular pH was confirmed by staining parallel cultures with acridine orange. Also, at these concentrations of ammonium chloride, no cytopathic effects were observed, and supernatant titers of vesicular stomatitis virus (which is known to enter by the endocytic pathway) were reduced by approximately 4 $\log_{10}$ units.

Binding studies. While increasing amounts of R18-labeled RSV bound to HEp-2 cells with time (Fig. 1) or with increasing amounts of input virus (Fig. 2), only 2 to 4% of input virions bound to HEp-2 cells under the conditions used. Under either condition, saturation of binding could not be demonstrated.

Time course of fusion of RSV with HEp-2 cells. The fusion of the RSV membrane with the host cell plasma membrane was studied with a fluorescence dequenching assay (16, 26). Incubation of 5 μg of R18-labeled virus with 2×10^6 HEp-2 cells (~0.25 PFU/cell) at 37°C resulted in a gradual increase

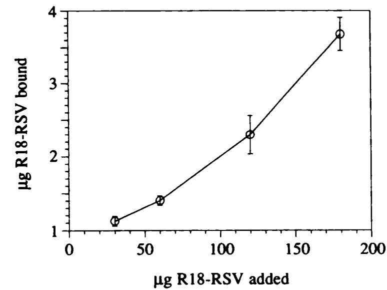

FIG. 2. Aliquots of R18-labeled RSV (30 to 180 μg) were incubated with 2×10^6 HEp-2 cells for 2 h at 4°C. The distribution of R18 between cell pellet and supernatant was determined as described in the legend to Fig. 1. Error bars indicate ±1 standard deviation of the mean.

in fluorescence (Fig. 3). Incubation of virus alone in buffer at 37°C for extended periods of time (1 to 2 h) did not result in any significant degree of dequenching. Under the standard conditions of the assay, between 35 and 45% dequenching was seen at the end of 1 h of incubation. Sodium azide was previously shown by other investigators to interfere with the entry of influenza virus (32) and adenovirus (37) into cultured cells. Dequenching of RSV was therefore measured in the presence and absence of 20 mM NaN_3 (Fig. 3). While the %DQ was somewhat higher in the presence of NaN_3, the differences were found to be within experimental error ($P > 0.05$).

Effect of target membrane concentration on fusion. Experiments were conducted to determine the effect of the concentration of target membrane on fusion. When 5 μg of R18-labeled RSV was incubated with increasing numbers (0.06×10^6 to 4×10^6) of HEp-2 cells (Fig. 4) at 37°C, there was an enhancement of %DQ. Thus, increasing the relative concentration of unlabeled target membranes resulted in an enhancement of the dequenching signal, as anticipated. This also suggested that the dequenching signal was probably a result of fusion of the viral membrane with the target membrane and not an artifact. At the highest cell concentration used in this study (4×10^6), ~50% dequenching was obtained at the end of 1 h of incubation at 37°C.

Temperature dependence of fusion. If the fusion is protein mediated, one can expect a threshold temperature above

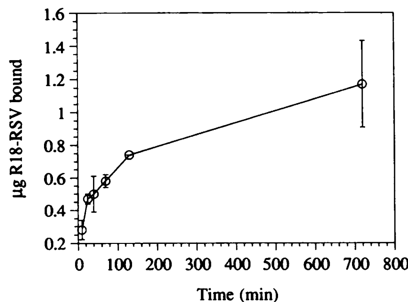

FIG. 1. Aliquots of R18-labeled RSV (30 μg each) were incubated with 2×10^6 HEp-2 cells at 4°C in 400 μl of HBSS. At the indicated times, bound virus was separated from free virus by centrifugation, and the cells were washed twice with 750 μl of HBSS. The supernatants from the washes were pooled, and the distribution of R18 in the cell pellet and supernatant was determined after addition of Triton X-100 to 1% final concentration. Each datum point represents two determinations. Error bars indicate ±1 standard deviation of the mean.

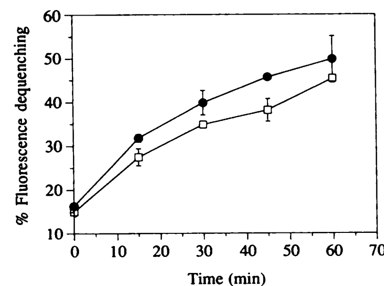

FIG. 3. Time course of fusion of RSV with HEp-2 cells. Aliquots of R18-labeled RSV (~5 μg each) were allowed to fuse with 2×10^6 HEp-2 cells at 37°C. Fusion was measured in both the presence (●) and absence (□) of 20 mM sodium azide. Replicates were removed at various intervals, and final levels of fluorescence dequenching were measured as described in Materials and Methods. Data were not corrected for light scattering. Error bars indicate ±1 standard deviation of the mean.

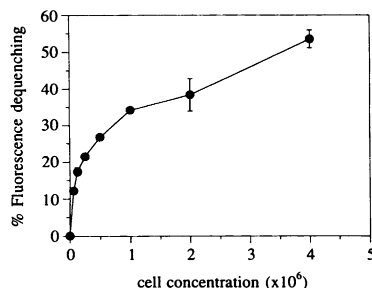

FIG. 4. Effect of relative target cell concentration on fusion of R18-labeled RSV with HEp-2 cells. Aliquots of R18-labeled RSV (~5 μg each) were incubated with different concentrations of target cells at 37°C for 1 h. Final levels of fluorescence dequenching were determined as described in Materials and Methods. Data were not corrected for light scattering. Error bars indicate ±1 standard deviation of the mean.

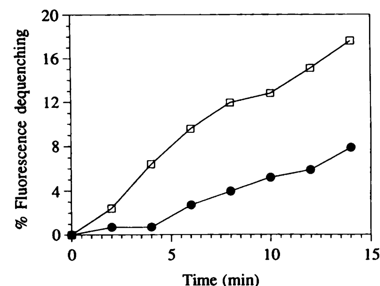

FIG. 6. Effect of heat treatment of virus on fusion. R18-labeled virus (5 μg) was treated at 75°C for 5 min in 875 μl of HBSS. Virus was then allowed to fuse with 2 × 10^6 HEp-2 cells in 1.8 ml of HBSS for 14 min (●). The fluorescence was monitored continuously as described in Materials and Methods. Untreated virus was used as a control (□).

which fusion can be discerned (10, 14, 18). R18-labeled RSV (5 μg) was allowed to fuse with 2 × 10^6 HEp-2 cells in 1.8 ml of HBSS at various temperatures (Fig. 5). The temperature of the fluorescence cuvette holder was controlled with a circulating water bath. At the end of the incubation period (14 min), Triton X-100 was added to 1% final concentration. The 100% dequenching at any particular temperature was determined at the same temperature at which the fusion was done to compensate for temperature effects on fluorescence. Dequenching of R18-labeled RSV with HEp-2 cells was strictly dependent on temperature. Significant levels of dequenching were noted only above 18°C, as has been noted for Sendai virus (14, 18) and Rous sarcoma virus (10).

Treatment of R18-labeled virus at 75°C for 5 min resulted in a reduction in levels of dequenching (Fig. 6). These results also suggested that the dequenching was protein mediated.

Effect of treatment of target cells with glutaraldehyde on fusion. Treatment of target cells with more than 0.05% glutaraldehyde caused a greater than 80% decrease in fluorescence dequenching when incubated with R18-labeled RSV at 37°C for 1 h (Table 2). This was not a result of decreased binding of the virus, since glutaraldehyde appeared to increase the percentage of bound virions (Table 2).

Effect of pH on fusion. In order to determine whether RSV was capable of fusing at an acidic pH, the effect of pH on fusion of RSV with HEp-2 cells was studied. Figure 7 shows the effect of pH on fusion of R18-labeled RSV with HEp-2 cells. Incubation of R18-labeled virus alone at various pHs (4.5 to 8.5) did not result in any dequenching. Thus, R18 fluorescence per se was not affected by pH. When incubated with HEp-2 cells at 37°C for 30 min, no difference in the amount of fusion (%DQ) was seen over a pH range of 5.5 to 8.5. Below pH 5.5, there was an increase in %DQ.

Effect of removal of envelope glycoproteins by trypsin on fusion. The effect of trypsinization on the ability of RSV to fuse with HEp-2 cells was studied. Virus labeled with R18 was treated with different concentrations of trypsin (100 to 0.1 μg) at 37°C for 15 min. Trypsin was inactivated by the addition of twice the minimal amount of soybean trypsin inhibitor (200 to 0.2 μg) necessary to inhibit enzyme activity. Virus was then allowed to fuse with HEp-2 cells at 37°C, and the %DQ was determined as described earlier. The results are shown in Table 3. In the presence of soybean trypsin inhibitor or trypsin-inhibitor complexes (not shown), no reduction in fusion was observed. Trypsin treatment of the virus resulted in a concentration-dependent decrease in fusion efficiency. At 100 and 10 μg of trypsin, dequenching was approximately 15 and 25%, respectively, corresponding to a 56 and 27% reduction in the efficiency of fusion, respectively. No significant decrease in the levels of dequenching was noted with trypsin concentrations of 1 and 0.1 μg. These results suggested that the surface glycoproteins participated in the fusion process, as has been noted for other enveloped viruses (12, 14, 16, 27, 32, 34, 35).

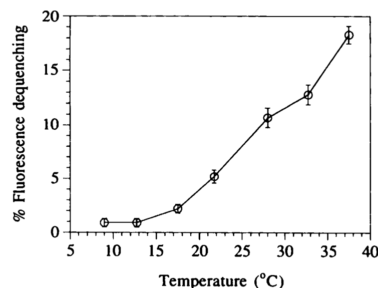

FIG. 5. Temperature dependence of fusion of RSV with HEp-2 cells. R18-labeled RSV (5 μg) was allowed to fuse with 2 × 10^6 HEp-2 cells in 1.8 ml of HBSS at the indicated temperatures for 14 min. Fluorescence was monitored continuously as described in Materials and Methods. Error bars indicate ±1 standard deviation of the mean.

TABLE 2. Binding and fluorescence dequenching of R18-labeled virus with glutaraldehyde-treated HEp-2 cells[a]

Glutaraldehyde treatment (%)	% Bound[b]	%DQ[c] (% reduction)
None	6.7	24.7 (0)
0.025	13.4	16.7 (24)
0.05	19.6	1.9 (92.3)
0.1	20.4	4.1 (83.4)

[a] Values are averages of two determinations.

[b] Aliquots of R18-labeled virus (15 μg each) were incubated with 2 × 10^6 HEp-2 cells (untreated or glutaraldehyde treated) for 1.5 h at 4°C. The percent bound was determined as described in Materials and Methods.

[c] Aliquots of R18-labeled virus (7.5 μg each) were incubated with 2 × 10^6 HEp-2 cells (untreated or glutaraldehyde treated) for 1 h at 37°C. Final levels of fluorescence dequenching were determined as described in Materials and Methods.

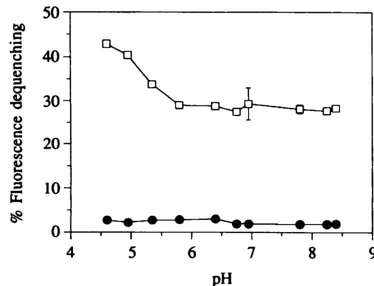

FIG. 7. Effect of pH on fusion of RSV with HEp-2 cells. Aliquots of R18-labeled RSV (~6 μg each) were allowed to bind to HEp-2 cells at 4°C for 30 min in HBSS buffered with 5 mM HEPES, pH 7.5. The pH was then changed by addition of 5 μl of a 1 M stock buffer adjusted to various pHs (citrate buffer for pH range of 4 to 6, HEPES buffer for pH range of 6.5 to 9.0). The mixtures were then incubated at 37°C for 30 min. Final levels of fluorescence dequenching were measured (□). The control was R18-labeled virus incubated at various pHs (●). Data were not corrected for light scattering. The error bar indicates ±1 standard deviation of the mean.

DISCUSSION

It has been tacitly assumed that the entry of RSV into target cells is similar to that of other paramyxoviruses, i.e., it occurs by fusion of the viral envelope with the host cell plasma membrane. However, no direct evidence for this is available. Here we report on the effect of ammonium chloride, a lysosomotropic agent (known to inhibit entry of viruses, such as orthomyxoviruses and rhabdoviruses, that use the endocytic route), on the infectivity of RSV. We have also directly measured the fusion activity of RSV with a fluorescence dequenching assay. The R18 dequenching assay of membrane fusion developed by Hoekstra et al. (16) was adapted for this purpose.

A model system for the study of membrane fusion was established with R18-labeled RSV and HEp-2 cells. Incubation of labeled virus with unlabeled target membrane at 37°C and pH 7.5 resulted in a gradual increase in fluorescence. The fluorescence was quantitated as %DQ by comparison with fluorescence in the presence of 1% Triton X-100, which was assumed to result in complete dissolution of membranes, resulting in infinite dilution of the probe (100% DQ).

Several lines of evidence indicate that the dequenching was a result of fusion of viral and target membranes and was not due to spontaneous transfer of the fluorescent probe.

TABLE 3. Effect of trypsin treatment on fusion of R18-labeled RSV with HEp-2 cells[a]

Trypsin (μg)	Mean %DQ ± SD		% Reduction in DQ with trypsin[c]
	Control[b]	After trypsin treatment	
100	28.1 ± 3.6	14.8 ± 0.1	56
10	33.7 ± 1.9	24.4 ± 0.2	27.7
1	34.4 ± 1.6	32.1 ± 0.7	4.9 (NS)[d]
0.1	38.9 ± 1.6	32.2 ± 0.2	4.6 (NS)

[a] Data not corrected for light scattering.
[b] R18-labeled virus was treated with soybean trypsin inhibitor alone prior to incubation with target cells.
[c] Arithmetic mean of all the controls (column 2) was considered 100% for calculation of percent reduction.
[d] NS, not significant ($P > 0.05$).

First, fluorescence dequenching was proportional to the concentration of the target membrane. Incubation of labeled virus in the absence of target membrane did not result in any dequenching. Second, glutaraldehyde treatment of target cells drastically reduced the dequenching signal even though virus binding appeared to be actually enhanced by the treatment. In addition, no transfer of probe between viral and target membrane occurred under conditions that precluded fusion but allowed binding of virus to target cells, such as incubation at 4°C for several hours (data not shown). Taken collectively, these results suggest that the major signal of dequenching can be attributed to the fusion of membranes (viral and target membranes).

Hoekstra and coworkers (18) and others (10, 14) have shown that the dequenching signal of viral fusion with target membranes shows a temperature dependence. The temperature dependence of fusion has been attributed to the constraints on the mobility of viral glycoproteins at lower temperatures (18). Our results showing that the dequenching occurred only above 18°C are in agreement with previously published results of others. Our results also show that the viral glycoproteins participated in the fusion process, since heat treatment of virus caused a reduction in dequenching. Also, trypsinization of R18-labeled virus prior to incubation with target cells resulted in a significant decrease in the levels of dequenching in a trypsin dose-dependent manner (Table 3). It should be noted that the assay as described here measures both binding and fusion steps. Therefore, the effects of these various treatments may be due to interference with either of these two steps. Considering the low level of binding at 4°C, the eventual levels of dequenching observed after an hour at 37°C suggest the possible recycling of receptors. The existence of virus-receptor interactions of various affinities is also a possible factor in the entry of the virus.

Viruses that fuse at the cell surface plasma membrane can be expected to fuse over a broad pH range (3, 10, 14), whereas viruses that enter by the endocytic route (1, 8, 27, 29, 31, 32, 34, 35) show significant fusion only at acidic pH. When the effect of pH on fusion was investigated in the presence of sodium azide, no significant difference in fusion between pH 5.5 and 8.5 was seen. However, there was some increase in fluorescence dequenching at pHs below 5.5. This suggests that RSV would be able to enter by the endocytic pathway even though RSV produces polykaryons in infected HEp-2 cells at neutral pH. Sodium azide at concentrations of 15 to 50 mM was shown by other investigators to block the entry of influenza A virus (32) and adenovirus (37) into cultured cells (presumably by inhibiting endocytosis). Inclusion of sodium azide in the fusion mixture (20 mM final concentration) did not appear to have any effect on fusion of RSV with HEp-2 cells. From the results of the pH experiment, one would expect to see an increase in fusion in the absence of the inhibitor if endocytosis were a significant process in viral entry. The reason for the increased dequenching at lower pHs is not known but may be due to enhanced binding at these pHs.

The results suggesting that fusion occurs at the plasma membrane were confirmed by studying the effect of ammonium chloride, a lysosomotropic agent, on the infectivity of RSV and vesicular stomatitis virus (which is known to enter by the endocytic route). While the infectivity of vesicular stomatitis virus was almost completely abolished by ammonium chloride, that of RSV was unaffected. These results suggest that RSV does not require low pH for infectious entry. However, recent reports on in vitro antibody-medi-

ated enhancement of RSV infection of macrophage cell lines (11, 24) support the hypothesis that an endocytic mode of entry is possible for RSV.

The fusion characteristics of RSV with HEp-2 cells appear to resemble those of Sendai virus (17). The establishment of an assay to determine the direct interaction of RSV with target cells allows us to design experiments to define the role of RSV glycoproteins in its entry.

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