

Acidic pH Enhancement of the Fusion of Newcastle Disease Virus with Cultured Cells

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Fusion of the lentogenic strain "Clone 30" of Newcastle disease virus (NDV) with the cell line COS-7 has been studied. Fusion was monitored using the octadecylrhodamine B chloride dequenching assay [Hoekstra, D., de Boer, T., Klappe, K. and Wilschut, J. (1984). *Biochemistry* 23, 5675–5681]. In the present work, fusion of NDV with COS-7 cells was found to occur in a time- and temperature-dependent fashion. Significant dequenching of the probe occurred at temperatures higher than 28°C. A 20-fold excess of unlabeled virus inhibited fusion by about 53% compared with the control, whereas 62% inhibition of fusion was obtained after digestion of viral glycoproteins with trypsin. The data are discussed in terms of the nonfusion transfer of the probe. In addition, preincubation of cells with 50 mM ammonium chloride or 0.1% sodium azide prevented NDV from fusing with COS-7 cells by about 30% in comparison with the control. The cytopathic effect of NDV infection in cell culture in the presence of ammonium chloride was reduced compared with control. Moreover, viral preincubation at pH 5 yielded a mild inhibition of fusogenic activity. Our results suggest that NDV may use the endocytic pathway as a complementary way of entering cells by direct fusion with the plasma membrane. © 1999 Academic Press

Key Words: Newcastle disease virus; viral fusion; virus entry; R18 dequenching; membrane fusion.

INTRODUCTION

Newcastle disease virus (NDV) is an avian enveloped single-stranded RNA virus belonging to the family of Paramyxoviridae, genus *Rubulavirus*. The membrane contains two transmembrane glycoproteins: the hemagglutinin-neuraminidase (HN) and fusion glycoprotein (F) protein (Choppin and Compans, 1975). HN mediates the binding of the virus to cell surface molecules containing sialic acid, and it also displays neuraminidase activity that probably prevents the aggregation of progeny virus. Both sialidase and hemagglutinating activities are affected by the viral membrane composition (Muñoz-Barroso *et al.*, 1997). In addition, a third HN fusion promotion activity has been proposed (Morrison *et al.*, 1991; Horvath *et al.*, 1992; Sergel *et al.*, 1993a, 1993b). This activity seems to be located in the stalk domain of the HN protein (Stone-Hulslander and Morrison, 1999).

F protein is produced as a single inactive peptide, Fo, which once cleaved by a cellular protease (Gotoh *et al.*, 1990; and reviewed in Morrison and Portner, 1991; White, 1990), becomes the active F1—F2 form, with two peptides linked by a disulfide bond (Iwata *et al.*, 1994; McGinnes and Morrison, 1997). As a consequence of the cleavage, a 24-amino acid hydrophobic peptide or fusion peptide is emplaced at the F1 N-terminus (Brasseur *et al.*, 1990). This peptide is thought to play an important

role in fusion mediated by NDV by insertion into the bilayer of the target membrane (White, 1990; Hernandez *et al.*, 1996). The neutral pH mechanism of fusion, mediated by the NDV F protein, remains unknown. Recently, Stone-Hulslander and Morrison (1997) reported an interaction between HN and F proteins in NDV-infected cells. Moreover, the thermal transitions of NDV glycoproteins have been identified (Shnyrov *et al.*, 1997).

After membrane fusion, enveloped viruses enter the cell through two main pathways: direct fusion between the viral envelope and the plasma membrane, and receptor-mediated endocytosis. Togaviruses, rhabdoviruses, and orthomyxoviruses enter cells via endocytosis, and viral membrane fusion with the endosomal membrane is triggered by an acidic pH (reviewed in Lamb, 1993; White *et al.*, 1983; Marsh and Helenius, 1989). Influenza virus, an orthomyxovirus, is the best characterized virus, with low pH fusion activation. Its HA protein undergoes an irreversible conformational change triggered at acidic pH after receptor-mediated endocytosis of the virus (White, 1990, 1992; Wiley and Skehel, 1987). As a consequence of the conformational change, the fusion peptide is exposed close to the target membrane. By contrast, other viruses, such as paramyxoviruses or many retroviruses, can undergo fusion of their envelope with the plasma membrane in a pH-independent mechanism (White *et al.*, 1983; Nagai *et al.*, 1983). For some viruses, fusion can occur at the plasma membrane and also in endosomes (White *et al.*, 1983). The mechanism through which any pH-independent fusion protein func-

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tions remains to be determined. The existence of some conformational changes similar to those of influenza HA-triggered receptor binding in the cell membrane has been proposed (White *et al.*, 1983). NDV, as a typical paramyxovirus, is thought to fuse with a target cell plasma membrane at neutral pH through the combined action of the HN and F glycoproteins, the former involved in virus-target membrane binding and the latter specific for fusion (Morrison and Portner, 1991). However, most fusion events are unknown and remain to be investigated.

The relief of self-quenching of the fluorescent lipid probe octadecylrhodamine B chloride R18 incorporated in the viral membrane has been used as a reliable method for measuring the fusion of enveloped viruses with target membranes, such as liposomes or erythrocyte ghosts (Hoekstra *et al.*, 1984, 1985; Hoekstra and Kok, 1989; Lorge *et al.*, 1986; Cobaleda *et al.*, 1994) or cultured cells (Pedroso de Lima *et al.*, 1991, 1992; Srinivasakumar *et al.*, 1991; Blumenthal *et al.*, 1987; Sinangil *et al.*, 1988). The dependence of fusion on the target membrane such as erythrocyte ghosts, liposomes, or different cell lines has been reported (Pedroso de Lima *et al.*, 1991; Düzgünes *et al.*, 1992). In addition, it has been shown that spontaneous exchange of the probe between membranes can occur, depending on the type of R18 labeling and on the target or effector membranes (Cobaleda *et al.*, 1994; Blumenthal *et al.*, 1987; Stegmann *et al.*, 1993; Ohki *et al.*, 1998).

The fusion activity of NDV has been investigated in terms of its ability to induce cell-cell fusion using the syncytium assay (Sergel *et al.*, 1993b) and its ability to fuse with erythrocyte ghosts (Cobaleda *et al.*, 1994) and liposomes (Lorge *et al.*, 1986). Nevertheless, the potential of NDV to fuse with cultured cells has only been analysed to a limited extent. In the present work we studied the fusion of NDV with the adherent monkey cell line COS-7 by analysing the dequenching of the fluorescent probe R18 inserted into the effector membrane. We examined the temperature and pH dependence of the kinetics and the final extent of fusion. The effects of several agents and conditions on the fusogenic capabilities of NDV were also analysed. Here, we present evidence of a low pH activation of NDV fusion with host cells. We found fusion to be enhanced at acidic pH and that viral fusion activity was inhibited by sodium azide and ammonium chloride together with a reduction of NDV infection in cell culture in the presence of ammonium chloride. We interpret these results as the contribution of endocytosis to the overall fusion events in the interaction of NDV with COS-7 cells. Our results suggest that when fusing with intact cells, NDV can penetrate the host cell through two different ways: fusing with the plasma membrane and through the endocytic pathway.

RESULTS

Effect of cell and virus concentrations on fusion

We used the R18 fluorescence dequenching assay to monitor fusion between NDV and COS-7 cells. Fusion was quantified by continuous fluorometric measurements. To study the effect of target membrane concentration on fusion, 20 μg of R18-labeled NDV was incubated with either 2×10^6 or 3.5×10^6 COS-7 cells at neutral pH, and fusion was monitored continuously at 37°C as described in Materials and Methods. Because no differences were observed in the fusion kinetics after prebinding of NDV to erythrocyte ghosts at 4°C or direct fusion (Cobaleda *et al.*, 1994), no cold preincubation of the samples before the fusion experiments was performed. The extent of fusion was higher with the lower virus/cell ratio because the increased number of cells (approximately twofold) yielded only a slight increase in the extent of fusion. By contrast, when the cell concentration was fixed (3.5×10^6 cells), concentrations of R18-labeled NDV above 20 μg led to lower degrees of fusion (data not shown). This result indicates that most of the binding sites are saturated with 20 μg of NDV at the cell concentration of these assays. In view of the data summarized above, 20 μg of R18-labeled NDV and 3.5×10^6 COS-7 cells were used for most of the experiments reported in this work.

Binding of R18-NDV to COS-7 cells

Experiments on the binding of R18-NDV to COS-7 cells were performed at 4°C. In them, 20 μg of R18-labeled NDV was incubated with 3.5×10^6 COS-7 cells at 4°C from 0 to 60 min under stirring. After incubation, the virus-cell complex was precipitated by centrifugation at 140 *g* for 5 min at 4°C and then washed twice with HEPES buffer, pH 7.4. Total fluorescence was measured at room temperature by the addition of 1% of Triton X-100, final concentration, as described in Materials and Methods. No transfer of R18 to the target membranes was detected after incubation of R18-labeled NDV and COS-7 up to 60 min at 4°C (Fig. 2B). As summarized in Fig. 1, 40% of the viruses added was bound to cells after 30 min of incubation, and no enhancement in this percentage was detected by increasing the time of virus-cell contact at 4°C up to 60 min. This suggests that under our experimental conditions, binding is saturated after 20 min of incubation.

In other sets of experiments, the binding of NDV to cells was analysed after virus treatment with 5% of glutaraldehyde or 7 μg of trypsin/ μg NDV (see Materials and Methods) or in the presence of a 20-fold excess of unlabeled NDV. Compared with the controls, the capacity of glutaraldehyde- or trypsin-treated viruses to bind to target membranes was reduced by about 70% with respect to the control. Similarly, the binding of R18-NDV

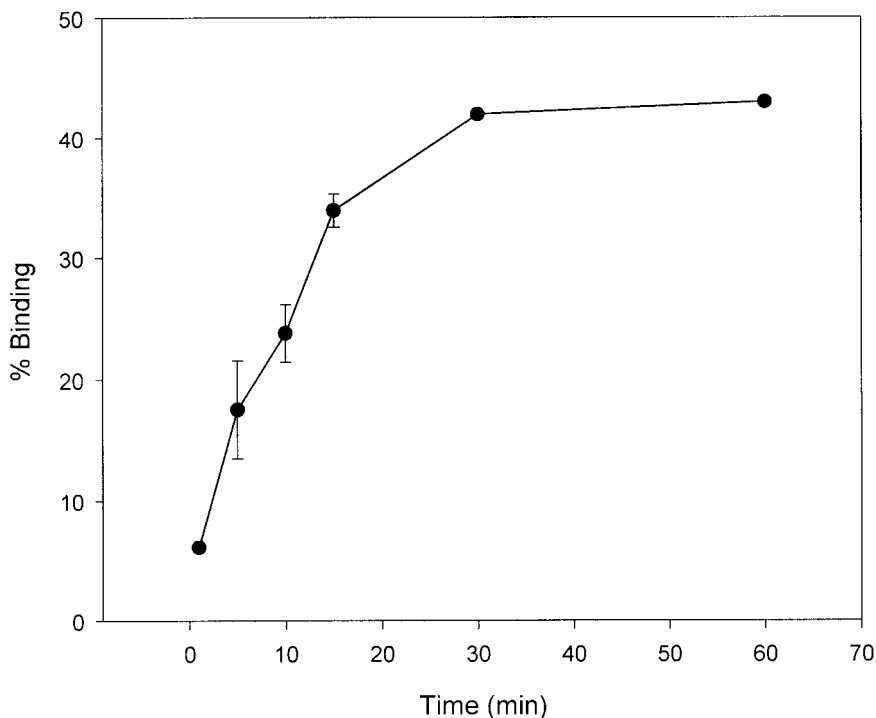


FIG. 1. Binding of R18-NDV to COS-7 cells; 20 μg of R18-labeled NDV was incubated with 3.5×10^6 COS-7 cells at 4°C from 0 to 60 min under stirring. After this, the virus-cell complex was precipitated by centrifugation at 140 g at 4°C for 5 min and then washed twice with 800 μl of ice-cold HEPES buffer, pH 7.4. The supernatant from the washes were pooled, and the distribution of R18 in the cell pellet and supernatant was measured at room temperature by the addition of 1% (final concentration) of Triton X-100, as described in Material and Methods. Data are mean \pm SD of two independent experiments.

was reduced by 60% in the presence of unlabeled effector membranes.

Binding of NDV to COS-7 cells at pH 5 and after treatment of target cells with ammonium chloride or sodium azide (see below) was also measured after 30 min of incubation of virus-cell mixtures at 4°C . Our data indicated no significant differences on the percentage of bound virions to cells under these conditions compared with control (data not shown).

Temperature dependence of fusion

R18-labeled NDV was allowed to fuse with 3.5×10^6 COS-7 cells in HEPES buffer, pH 7.4, at different temperatures. Fusion kinetics were monitored continuously at 37°C and 25°C for 30 min. The results are summarized in Fig. 2A. It is clear from the graph that the kinetics and the extent of fusion are very different at both temperatures assayed, with the final extent of fusion being reduced by more than 75% at 25°C compared with that carried out at 37°C . For 37°C , the fluorescence signal shows an initial sharp increase, with the rate of this increase decreasing with time, whereas the emission of fluorescence dequenching of R18 shows an approximately linear increase at 25°C . By computer fitting, the data on the percentage of fusion versus time at 37°C were adjusted

to the sum of two exponentials (Eq. 2, see Materials and Methods), with the first one representing specific fusion (A1 and K1 parameters), a slow dequenching reaction, and the second one representing nonspecific fusion (A2 and K2 parameters) (Cobaleda *et al.*, 1994; Ohki *et al.*, 1998). The kinetic constants of the fusion reaction at 37°C and pH 7.4 are shown in Table 1. We were unable to use the same approach when trying to fit the experimental data obtained at 25°C . The apparent initial fusion rates calculated from the slope of the steepest part of the curve (Hoekstra *et al.*, 1985) were 5.5 times lower at 25°C than at 37°C (data not shown).

In the next series of experiments, single time points were measured in a final fusion-time experiment (Fig. 2B). R18-labeled NDV (20 μg) were allowed to fuse with 3.5×10^6 COS-7 cells in 2 ml of HEPES buffer, pH 7.4, at several different temperatures. At the end of the incubation time (60 min), Triton X-100 (1% v/v, final concentration) was added. For all the temperatures assayed, the percentage of dequenching was calculated immediately at room temperature. As can be seen from Fig. 2B, the extent of fusion of NDV with COS-7 cells was dependent on temperature, with no probe transfer at 4°C , and increased sharply above 28°C . Therefore, the optimum temperature for fusion between NDV and COS-7 cells is 37°C .

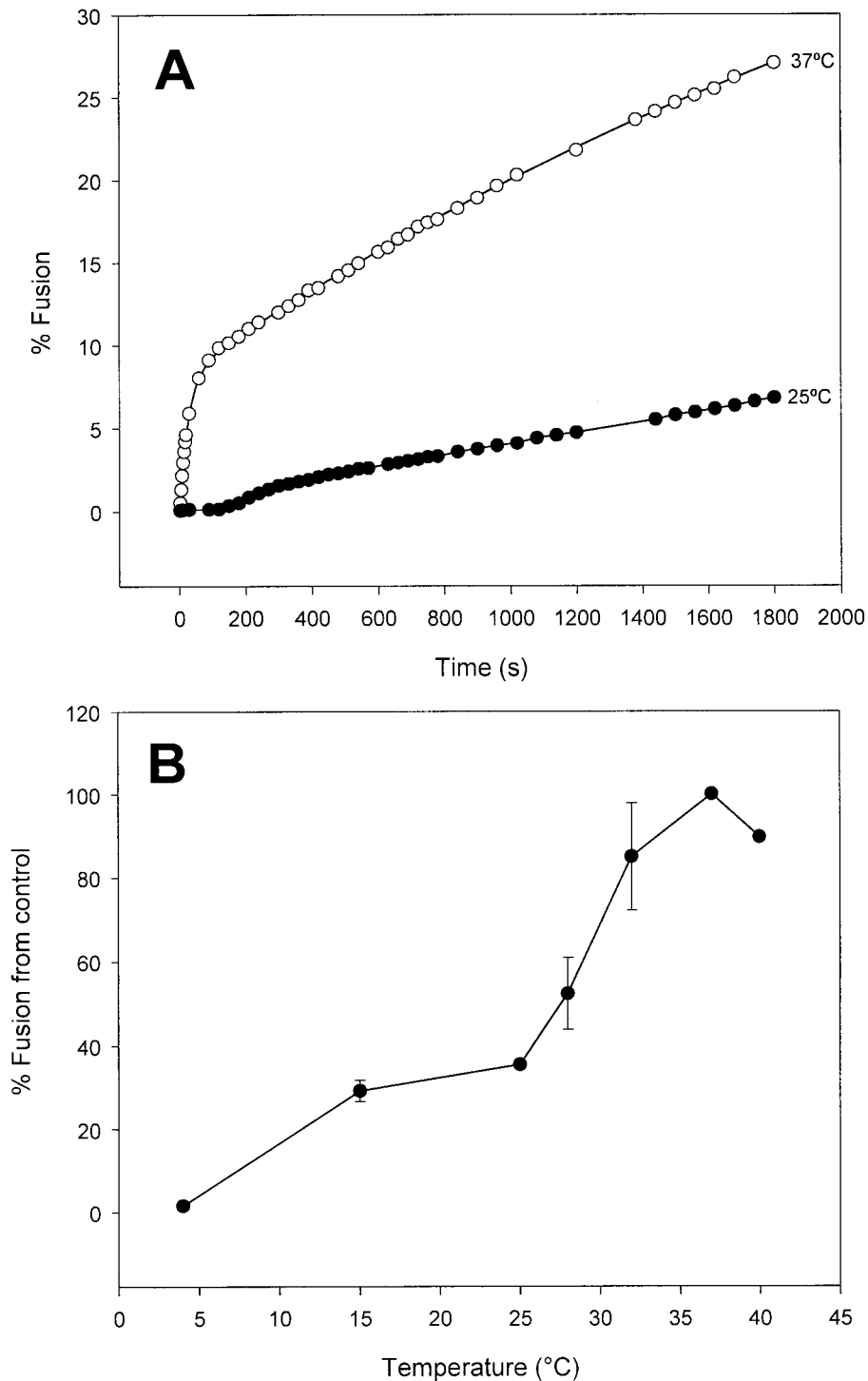


FIG. 2. Temperature dependence of fusion of NDV with COS-7 cells. (A) 20 μg of R18-labeled NDV was incubated with 3.5×10^6 COS-7 cells at 37°C or 25°C for 30 min under continuous stirring in 2 ml final volume. Fusion was monitored continuously as described in Materials and Methods by measuring the dequenching of R18. For both temperatures, data are mean values of two independent experiments; standard deviations were ≤ 0.565 . \circ , Kinetics at 37°C; \bullet , kinetics at 25°C. (B) 20 μg of R18-labeled NDV was incubated with 3.5×10^6 COS-7 cells in 2 ml final volume at the indicated temperatures for 60 min under stirring. Temperature was controlled in a water bath. Then the extent of fusion was calculated according to Eq. 1 (see Materials and Methods) at room temperature after the addition of Triton X-100 (1% v/v, final concentration), taking fusion at 37°C as 100% of fusion. Data are mean \pm SD of two independent experiments.

TABLE 1
pH Dependence of Kinetic Parameters

| pH | A1 | K1 (s ⁻¹) | A2 | K2 (× 10 ⁻⁴ s ⁻¹) |
|-----|--------------------------|--------------------------|---------------|---|
| 7.4 | 4.85 ± 1.17 | 0.041 ± 0.014 | 35.77 ± 9.11 | 5.04 ± 1.9 |
| 5 | 8.87 ± 1.35 ^a | 0.032 ± 0.021 | 25.70 ± 5.65 | 9.3 ± 3.7 |
| 8 | 4.85 ± 0.47 | 0.023 ± 0.003 | 44.67 ± 21.18 | 3.28 ± 2.1 |

Note. R18-labeled NDV (20 μg) was incubated with 3.5 × 10⁶ COS-7 cells at 37°C for approximately 30 min under continuous stirring at different pH values. For pH values 7.4 and 8, HEPES buffer adjusted to the desired pH was used; for pH 5, cell and virus mixtures were incubated in citric-citrate buffer adjusted to pH 5. Fusion was monitored continuously as described in Materials and Methods by measuring the dequenching of R18. Kinetic parameters were determined by adjusting the experimental data to the sum of two exponentials using the SIMFIT simulation package (Bardsley *et al.*, 1995). For pH 7.4, the data are mean values of five independent experiments; for pH 5 and 8, the data are mean values of three and two independent experiments, respectively.

^a Significant difference ($p < 0.001$, in Student's *t* test with 6 degrees of freedom) from pH 7.4.

pH dependence of fusion

To determine the effect of acidic and basic pH on NDV fusion with cultured cells, 20 μg of R18-labeled NDV was

incubated with 3.5 × 10⁶ COS-7 cells at different pH values for approximately 30 min under continuous stirring. We did not detect any increase in dequenching after incubation of the virus in the absence of cells at pH 5 or in the presence of NH₄Cl or sodium azide (see below) up to 30 min at 37°C under continuous stirring. The data are summarized in Fig. 3. Fusion reaction at pH 7.4 and 8 achieved a similar final extent at the time studied; the parameters of the two exponentials were also very similar (Table 1). Nevertheless, it can be seen that the extent of fusion at pH 5 was higher than that in the controls (i.e., pH 7.4). Moreover, the value of the A1 parameter (representing the asymptote of the specific part of the equation) of the two exponential equations fitted to the experimental data of Fig. 3 was twofold higher than those obtained at pH 7.4 and 8 (Table 1). These results indicate that the entry of NDV into COS-7 cells is enhanced at pH 5. To analyse the effect of acidic pH activation on the two components involved in fusion, NDV or COS-7 cells alone were incubated at pH 5 for 30 min before the fusion reaction. After this incubation, preincubated NDV or preincubated COS-7 cells were mixed with their nonincubated partners, and the extent of fusion was monitored continuously as above. This preincubation resulted in an inhibition of fusion of about 10% (preincubated NDV) or 20% (preincubated cells) in comparison with the control.

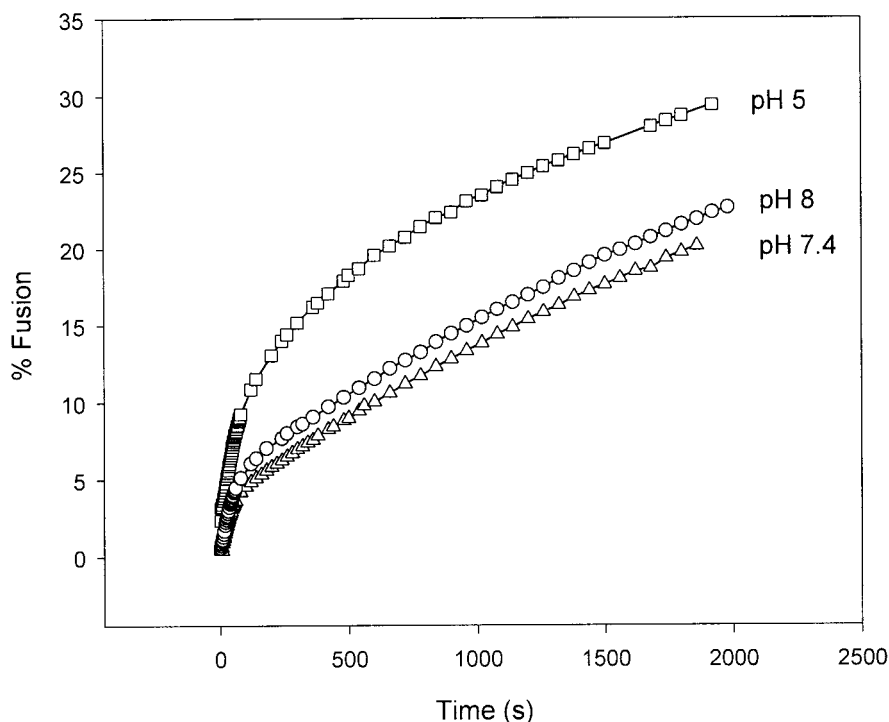


FIG. 3. pH dependence of NDV fusion with COS-7 cells; 20 μg of R18-labeled NDV was incubated with 3.5 × 10⁶ COS-7 cell at 37°C for approximately 30 min under continuous stirring at different pH values. For pH 7.4 (Δ) and 8 (○), HEPES buffer adjusted to the desired pH was used; for pH 5 (□), cell and virus mixtures were incubated in citric-citrate buffer adjusted to pH 5. Fusion was monitored continuously as described in Materials and Methods by measuring the dequenching of R18. For pH 5, the data are mean values of three independent experiments; standard deviations were ≤3.215. For pH 7.4 and 8, the data are mean values of two independent experiments with standard deviations of less than 0.22.

Kinetics of fusion in the presence of the lysosomotropic agent NH₄Cl

To further analyze the activation of fusion between NDV and COS7 cells at pH 5 described above (Fig. 3), the effect of NH₄Cl treatment of cells on virus-cell fusion was investigated. For this purpose, COS-7 cells were preincubated in the presence of 50 mM NH₄Cl for 60 min at 37°C. This reagent has been shown to increase the pH in endosomes (Mellman *et al.*, 1986; Stegmann *et al.*, 1987). After preincubation, R18-labeled NDV virus was added, and fusion was monitored as described above. Analysis of the data revealed that NH₄Cl inhibited fusion by 27% with respect to the control after 30 min of incubation (Table 2). The degree of inhibition was lower after short times of virus-cell contact (Fig. 4; and data not shown). These results were supported by those of fusion inhibition when COS-7 cells were preincubated in the presence of 0.1% of sodium azide, which inhibits endocytosis by blocking the production of the metabolic energy needed for the endocytic activity of the cell (Blumenthal *et al.*, 1987). In this case, the preincubation of the cells resulted in a 27% inhibition of fusion from the control (Table 2), suggesting that the endocytic machinery would be partially necessary.

To test the effect of the salt in the efficiency of NDV infectivity, infection of cell monolayers was studied in the presence of NH₄Cl. After the incubation of Vero cells in the presence of the salt for 1 h at 37°C, virus infection was accomplished as described above. Cytopathic effect was analyzed by microscopy. We observed a reduction of the cell damage in presence of NH₄Cl. Data are summarized in Table 3.

Kinetics of fusion in the presence of unlabeled NDV

To analyze the effect of an excess of unlabeled effector membrane on the extent of fusion between R18 NDV and COS-7 cells as measured by dequenching of the fluorescent probe, we performed competition experiments with unlabeled virus. R18-labeled NDV (20 μg) was incubated with 3.5 × 10⁶ COS-7 cells at 37°C in the presence of a 20-fold excess of unlabeled NDV for approximately 30 min. The initial rate of fusion decreased in the presence of excess of unlabeled NDV (data not shown), and the final extent of fusion was reduced by about 53% in comparison with the control (Table 2). Unlike in the controls, the experimental data obtained for samples in the presence of unlabeled NDV cannot be adjusted to a sum of two exponentials. Binding in the presence of an excess of unlabeled NDV was seen to be inhibited by about 60%. This suggests that the reduction in probe transfer between R18-labeled NDV and COS-7 cells would be due to a competition phenomenon: some of the binding sites are occupied by unlabeled NDV, and hence the number of sites with which the labeled virions are able to fuse is reduced.

TABLE 2

Effect of Different Agents on the Fusion between NDV and COS-7 Cells

| Agent | % Fusion with Respect to Control | % Inhibition |
|--------------------------------------|----------------------------------|--------------|
| 70°C (30 min) | 46.1 | 53.9 |
| 20-fold unlabeled NDV ^a | 47.13 ± 4.9 | 52.87 ± 4.9 |
| NDV + 5% glutaraldehyde ^b | 38.1 ± 2.8 | 62.04 ± 2.8 |
| COS-7 + 5% glutaraldehyde | 0 | 100 |
| Trypsin ^a | 37.75 ± 4.6 | 62.25 ± 4.6 |
| Sodium azide (pH 7.4) ^b | 72.55 ± 0.35 | 27.45 ± 0.35 |
| NH ₄ Cl ^b | 74.03 ± 7.9 | 26.0 ± 7.9 |
| N24 (pH 7.4) ^a | 38 ± 1 | 62 ± 1 |
| N24 (pH 5) ^c | 39 | 61 |

Note. R18-labeled NDV (20 μg) was incubated with 3.5 × 10⁶ COS-7 cells at 37°C under different conditions with continuous stirring for 30 or 60 min, and fusion was monitored continuously according to Eq. 1. For data in the presence of NH₄Cl, COS-7 cells were incubated in HEPES buffer containing 50 mM of NH₄Cl for 60 min at 37°C. Then, cells were mixed with NDV to a final volume of 2 ml, and fusion was continuously monitored for approximately 30 min. For sodium azide data, COS-7 cells were preincubated at 37°C under stirring in the presence 0.1% sodium azide in HEPES buffer, pH 7.4, for 45 min. After incubation, the mixture was transferred to a cuvette, and fusion was initiated by the addition of R18-labeled virus. For N24 data, NDV was incubated in the presence of peptide for 1 h at room temperature and then diluted 10-fold in the suitable buffer to a final peptide concentration of 40 μg/ml (15 μM), and fusion was accomplished as described; the cells were preincubated in the presence of ammonium chloride as described above. For trypsin-treated virus, R18-labeled NDV was incubated in the presence of 1.4 mg/ml trypsin, as described in Materials and Methods. The reaction was stopped by the addition of soybean trypsin inhibitor. Then, COS-7 cells were added, and the fusion reaction was monitored continuously at 37°C. For glutaraldehyde experiments, 20 μg of R18-labeled NDV or 3.5 × 10⁶ COS-7 cells was incubated in the presence of 5% glutaraldehyde (v/v, final concentration) in 1 ml of HEPES buffer, pH 7.4, for 30 min at 37°C with stirring. After adding the target or effector partner, fusion was accomplished as described above. For heat-treated virus, the viral sample was incubated for 30 min at 70°C, and the fusion reaction was accomplished as above. Unless indicated otherwise, for control, 3.5 × 10⁶ COS-7 cells were incubated with 20 μg of R18-labeled NDV in HEPES buffer, pH 7.4

^a Mean ± SD of three determinations.

^b Mean ± SD of two determinations.

^c Control, 3.5 × 10⁶ COS-7 cells incubated with 20 μg of R18-labeled NDV in citric-citrate buffer, pH 5.

Effect of N24 peptide

The inhibitory effect on NDV-mediated fusion of the synthetic peptide N24 was assayed at both pH 7.4 and 5. NDV was incubated in the presence of peptide as described in Materials and Methods, and then the virus-peptide mixtures were diluted 10-fold in the suitable buffer to a final peptide concentration of 40 μg/ml (15 μM). For both pH values, the percentage of fusion inhibition was similar, about 60% of inhibition from control (controls, fusion at pH 7.4 and 5 without peptide, respectively). Data are summarized in Table 2.

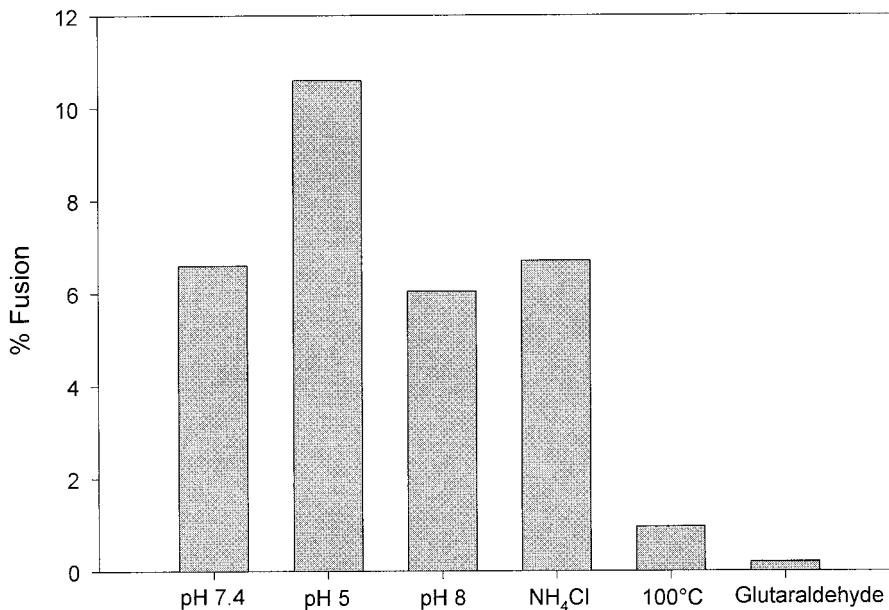


FIG. 4. Extension of fusion after 2 min of virus-cell contact; 20 μg of R18-labeled NDV was incubated with 3.5×10^6 COS-7 cells at 37°C under different conditions and continuous stirring for approximately 30 min, and fusion was monitored continuously according to Eq. 1. The extent of fusion was calculated 2 min after starting the fusion reaction. For the control, the data are mean values of six independent experiments; for pH 5 and 8, see the legend for Fig. 3; for data in the presence of NH₄Cl, COS-7 cells were incubated in HEPES buffer containing 50 mM NH₄Cl for 30 min at 37°C. Then cells were mixed with virus to a final volume of 2 ml, and fusion was monitored continuously. For data on reducing agents, the virus was incubated with 2 mM dithiothreitol or 10 mM mercaptoethanol in 100 μl of KNP buffer, pH 7.4 (final volume), at 37°C for 30 min under stirring. Then fusion was initiated by the addition of COS-7. For trypsin-treated virus, R18-labeled NDV was incubated in the presence of 1.4 mg/ml trypsin as described in Materials and Methods. The reaction was stopped by the addition of soybean trypsin inhibitor. Then COS-7 cells were added, and the fusion reaction was monitored continuously at 37°C. For glutaraldehyde experiments, 20 μg of R18-labeled NDV was incubated in the presence of 5% of glutaraldehyde (v/v, final concentration) in 1 ml of HEPES buffer, pH 7.4, for 30 min at 37°C with stirring. Then after the addition of the target cells, fusion was accomplished as described above. For heat-treated virus, the viral sample was incubated for 5 min at 100°C, and the fusion reaction was accomplished as above.

Effect of other inhibitors

It has been established that the transfer of the probe R18 from one labeled membrane to another as measured by its dequenching can be due to nonspecific transfer (i.e., transfer without fusion) (Cobaleda *et al.*, 1994; Blumenthal *et al.*, 1987; Stegmann *et al.*, 1993; Ohki *et al.*, 1998; Wunderli-Allenspach and Ott, 1990; Wunderli-Allenspach *et al.*, 1993).

To fully characterize our system, we analysed the inhibition of fusion between R18-labeled NDV and COS-7 cells under different conditions. The data are summarized in Table 2. Treatment of COS-7 cells with 5% glutaraldehyde completely blocked fusion, whereas treatment of NDV reduced the extent of fusion up to 62% in comparison with the control.

TABLE 3

Cytopathic Effect of NDV Infection in Cell Culture

| | Virus dilution | | |
|-------------------|------------------|------------------|------------------|
| | 10 ⁻² | 10 ⁻³ | 10 ⁻⁵ |
| Control | +++ | +++ | + |
| Ammonium chloride | ++ | ++ | + |

Note. +, ++, +++, intensity of cytopathic effect. Vero cells monolayers were incubated in the presence of NH₄Cl for 1 h at 37°C. Then, cells were infected with serial dilutions of NDV (initial protein concentration of 1.6 mg/ml, 250 μl final volume of virus inoculum) in the presence or absence (control) of the drug as described in Materials and Methods. At 24 h postinfection, the viral cytopathic effect was analyzed by microscopy.

The effect of proteases on the fusogenic capacity of NDV with COS-7 cells was also studied. For this set of experiments, R18-labeled NDV was incubated in the presence of 1.4 mg/ml trypsin, as described in Materials and Methods. The reaction was stopped by the addition of soybean trypsin inhibitor. Then, COS-7 cells were added, and the fusion reaction was monitored continuously at 37°C. The incubation of virus with trypsin resulted in a 62% inhibition of fusogenic activity compared with the control. SDS-PAGE analysis of the trypsin-treated samples revealed that all the protein bands were smaller than 40 kDa, meaning that the protease had hydrolyzed most of the viral HN and F proteins (data not shown). Because about 40% of probe transfer was detected after trypsin treatment, this finding suggests that only 60% of the total R18 fluorescence dequenching was due to fusion triggered by the viral glycoproteins.

DISCUSSION

In the present work, we characterized fusion of R18-labeled NDV "Clone 30" strain with the adherent cell line COS-7. After 30 min of incubation, the final extent of fusion was between 20% and 30% measured as the percentage of dequenching of R18 (Eq. 1). The incubation of R18-labeled NDV alone at 37°C for 30 min did not result in any increase in dequenching (data not shown). This finding is similar to those reported for other enveloped viruses such as Sendai (Pedroso de Lima *et al.*, 1991; 1992), HIV (Sinangil *et al.*, 1988), or vesicular stomatitis virus (VSV) (Blumenthal *et al.*, 1987) when fused with different cell lines. The fusion rate of NDV with COS-7 cells was affected by the density of virus particles available for binding to the cell surface (data not shown). The inhibition of the extent of fusion found at higher virus/cell ratios could be due to a competition phenomenon similar to that detected in the presence of a 20-fold excess of unlabeled NDV (Table 2). This suggests that fusion sites in the COS-7 membrane are reduced in the presence of high concentrations of virions or that some of the binding sites on the virus particle or on the cell surface are inactive (Ramalho-Santos *et al.*, 1996). Although the binding of paramyxoviruses to cell membranes has been described to be similar at 4°C and 37°C (Hoekstra and Klappe, 1986), we observed differences in the percentage of binding inhibition (80% versus 53% of fusion inhibition). This difference could be due to differences in the binding at 4°C (temperature for binding experiments) and 37°C (fusion assay temperature) of the two R18-labeled and -unlabeled populations of viruses. For some enveloped viruses, two kinds of binding between the viral membrane and receptors of the target cell have been described (reviewed in Haywood, 1994), with one of them being weak at low temperatures, and the other one stable at physiological temperatures. In the case of VSV, the endocytic entry route, but not fusion to the plasma membrane, was inhibited in the presence of a 10-fold excess of unlabeled virus (Blumenthal *et al.*, 1987). By contrast, with Sendai virus fusion to the plasma membrane was reduced in similar conditions (Pedroso de Lima *et al.*, 1992). Regarding the fusion between NDV and COS-7 cells, the inhibition of fusion in the presence of a 20-fold excess of unlabeled effector membranes (Table 2) was higher than the inhibition of the endocytic pathway (discussed below). Thus it may be deduced that fusion to the plasma membrane is indeed inhibited, although effects on the endocytic entry route cannot be ruled out.

Interestingly, we observed that NDV can fuse with COS-7 cells in the pH range of 4–7.4, indicating that NDV has lower requirements for fusion with cells than with erythrocyte ghosts (Lorge *et al.*, 1986; Cobaleda *et al.*, 1994). Different responses to the pH of the medium, depending on the target membrane, have also been

observed for other enveloped viruses (Pedroso de Lima *et al.*, 1991; Düzgünes *et al.*, 1992). The optimum pH for NDV fusion with erythrocyte ghosts has been found to be 7.4 (Cobaleda *et al.*, 1994), whereas here we observed that fusion with COS-7 was highest at pH 5, although binding of NDV to COS-7 cells was similar at pH 7.4 and 5 (not shown). In addition, we found differences in the kinetic parameters at acidic pH. When ghosts were used as the target membrane (Cobaleda *et al.*, 1994), the highest values of A1 and K1 in Eq. 2 (specific fusion) were seen at pH 7.4 and were fivefold lower at pH 5. Despite this, according to the data from the present work, the A1 parameter was about twofold higher at pH 5, meaning a higher value for the asymptotic value of the specific curve, whereas the rate of the reaction, represented by the K1 parameter, was very similar (Table 1) at the pH values studied. Moreover, the parameters A2 and K2 in Eq. 2 were lower at acidic pH, meaning a lower level of nonspecific transfer of the probe. This is additional support to our observation of an enhancement of fusion at acidic pH: if specific fusion is enhanced at acidic pH, there is less chance for nonspecific transfer of the probe.

Ramalho-Santos *et al.* (1996) have shown that influenza and Sendai virions can bind to the cellular surface via "inactive" sites, but after release from the target membrane, they can fuse when contacting new membranes. In other words, not all the bound virus particles can fuse, but they can both be defective particles. In the present work, the percentage of bound NDV to the cell surface was found to be similar at neutral and acidic pH values, whereas the percentage of fusion was greater at pH 5. Several possibilities, which are not excluding, can explain such a difference: (1) the fraction of attached virions to "active" sites is favoured at acidic pH; (2) at pH5, the viral glycoproteins might arrange in a more optimal conformation for fusion; and (3) the intermediate fusion states formed by the viral glycoproteins in the target membrane are favoured to trigger complete fusion at acidic pH.

Viruses that require acidic activation for to enter their host cells, such as orthomyxoviruses and rhabdoviruses, are sensitive to the effect of weak bases, such as ammonium chloride, which act by inhibiting endosomal acidification (Marsh and Pelchen-Matthews, 1994). It is assumed that the entry of NDV into target cells is a pH-independent phenomenon that occurs only by fusion of the viral membrane with the host cell plasma membrane (White *et al.*, 1983; Nagai *et al.*, 1983). Despite this, here we report the inhibitory effect of ammonium chloride and sodium azide (lysosomotropic and an inhibitor of endocytosis agents, respectively) on the fusion between NDV and COS-7 cells. As reported above, we found an increased extent fusion between NDV and COS-7 cells at pH 5. Viruses that use the endocytic pathway show a significant enhancement of fusion at acidic

pH (Blumenthal *et al.*, 1987; Gething *et al.*, 1986; Scheule, 1987). The use of two mechanisms for fusion, direct fusion to the plasma membrane and endocytosis, has been described for some viruses such as Epstein-Barr virus (White *et al.*, 1983); Rous sarcoma virus can fuse through acidic-dependent and pH-independent mechanisms (White *et al.*, 1983), and HIV can fuse either with the cell surface or with internal membranes (Sinangil *et al.*, 1988; Maddon *et al.*, 1988; Stein *et al.*, 1987; Pauza, 1991). Here we report an increase in fusion at acidic pH of 30% over that obtained at neutral pH (Fig. 3) and a similar percentage of inhibition in the presence of sodium azide or ammonium chloride (Table 2). This enhancement of the rate of fusion at acidic pH seems to be due to viral glycoproteins because the extension of fusion was inhibited in a similar rate by an inhibitory peptide derived from the F protein. Moreover, the fusion reaction at pH 5 was faster than that at neutral or basic pH values; after 2 s of virus-cell contact, 2% of fusion was obtained at acidic pH, whereas the same extent of fusion was achieved after 20 s at pH 7.4 and 8. Unlike viruses that require acidic pH triggering, the NDV fusion process was not completely abolished by the weak base ammonium chloride. In addition, and unlike in previous work (Trybala, 1987), the efficiency of virus infection in cell cultures was reduced, but not completely abolished, in the presence of ammonium chloride (Table 3). Taken together, these data suggest that NDV is able to enter the cell by use of the endocytic machinery, although direct fusion of the viral NDV envelope with the host cell plasma membrane would be the main pathway for viral entry. Both mechanisms seem to indeed be functional pathways for NDV entry.

Pretreatment of some enveloped viruses at acidic pH can either inactivate their fusion activity (White, 1983) or cause an enhancement of fusion (Puri *et al.*, 1988). In the present work, we observed that the fusogenic capacity of NDV underwent a slight reduction after preincubation of the virus at acidic pH in the absence of target cells (data not shown). Similar results were found by Pedroso de Lima *et al.* (1992) for Sendai virus when they were fused with cells, although in this system, fusion was pH independent. Because the fusogenic activity of NDV envelope glycoproteins is not strongly affected by low pH pretreatment, the mild inactivation described here could be due to a clustering of viral glycoproteins after the acidic treatment, or an aggregation of viruses, as described before for VSV (Puri *et al.*, 1988). Low pH treatment of NDV would lead to the formation of large clusters of virions that are too large to enter the coated pits. If this were the case, acidic pretreated NDV would enter the cell mainly by direct fusion with the plasma membrane. Despite this, some kind of conformational changes or spatial rearrangements of the viral membrane components at acidic pH cannot be ruled out.

Dequenching of the lipophilic probe R18 has been

widely used to monitor fusion between enveloped viruses and target membranes (Hoekstra *et al.*, 1984, 1985; Hoekstra and Kok, 1989; Lorge *et al.*, 1986; Pedroso de Lima *et al.*, 1991, 1992; Stegmann *et al.*, 1987, 1993; Srinivasakumar *et al.*, 1991; Sinangil *et al.*, 1988; Düzgünes *et al.*, 1992; Ramalho-Santos *et al.*, 1996). The validity of this assay has been questioned on the grounds that nonspecific transfer of the probe (i.e., transfer without fusion) may occur (Cobaleda *et al.*, 1994; Blumenthal *et al.*, 1987; Stegmann *et al.*, 1993; Ohki *et al.*, 1998; Wunderli-Allenspach and Ott, 1990; Wunderli-Allenspach *et al.*, 1993). The magnitude of the effect varies for different membranes. For the same donor membranes but different cell lines, the extent of this phenomenon is difficult to predict, and hence, proper control assays should be performed in which the donor membrane is tightly bound to the target membrane but unable to fuse to it. To analyse the extent of the nonspecific probe transfer during the fusion of R18-labeled NDV with COS-7 cells, we studied the effect of different conditions on the overall dequenching signal (Table 2). The maximum inhibition of fusion was found for trypsin-treated viruses: up to 62% of inhibition. These data differ from those reported for other viruses in which fusion was completely abolished after protease treatment (Hoekstra *et al.*, 1985) but similar to those published for Sendai virus fusion with adherent cells (Pedroso de Lima *et al.*, 1992) and for Rous sarcoma virus (Srinivasakumar *et al.*, 1991). Because in the present work the viral glycoproteins were digested by the protease (checked by SDS-PAGE analysis, data not shown), the 40% of dequenching detected when trypsin-treated NDV was the effector membrane could have been due to probe transfer without fusion. We have previously reported the asymmetric distribution of the anionic phospholipid phosphatidylcholine and the presence of glycolipids in the viral membrane (Muñoz-Barroso *et al.*, 1997). These molecules can facilitate viral membrane-cell interactions, even though most of the viral glycoproteins have been trypsinized.

Recently, Ohki *et al.* (1998) have shown that the initial rate of dequenching (within 2 min) of R18 incorporated in Sendai virus envelope was predominantly due to fusion, with little contribution by nonspecific transfer. Our investigations corroborate this observation (data not shown). On analyzing the data on fusion after 2 min of virus-cell incubation, no differences in the extent of fusion were detected between neutral and basic pH. By contrast, an activation was found at pH 5 (Fig. 4). However, glutaraldehyde-treated virus exhibited 97% of inhibition, and more than 70% of fusion was abrogated in the presence of mercaptoethanol or when heat-treated viruses were used. The inhibitory effect of COS-7 preincubation with NH_4Cl was not observed at 2 min after virus-cell contact. Because of the lag time required for endocytosis and endosome acidification, the time taken for the virus to become internalized through the endocytic pathway

could be slower than direct fusion with the plasma membrane, which could explain the absence of inhibition at 2 min after virus–cell contact. The inhibition of fusion that occurred in the presence of the weak base ammonium chloride after 30 min of the fusion reaction (Table 2) is probably not due to differences in nonspecific probe transfer in the absence or presence of the salt. This therefore provides additional support of the hypothesis presented here to the effect that NDV can enter COS-7 cells through the endocytic pathway in a slower way than direct fusion with the plasma membrane.

For fusion between erythrocyte ghosts and R18-labeled NDV, Cobaleda *et al.* (1994) have shown that 33% of the dequenching detected after 60 min of incubation is due to specific fusion; those authors did not find any variation in nonspecific probe transfer under different conditions of pH or temperature. Nevertheless, on using COS-7 cells as target membranes, we found difficulties in fitting the experimental data to the sum of two exponentials under nonoptimal conditions, similar to the findings of Ohki *et al.* (1998). According to the data presented here, 60–70% of probe transfer inhibition would be considered as the upper limit after 30 min of incubation. This value is close to that calculated by Ohki *et al.* (1998) for fusion between Sendai R18-labeled virosomes with erythrocyte ghosts, where the estimation of nonspecific transfer was 25% of the total dequenching of the probe at 37°C.

To sum up, fusion of NDV with the cell line COS-7 has been characterized. We found that specific fusion was enhanced at acidic pH, and our data suggest that NDV can enter the cells through two different routes: direct fusion with the plasma membrane being the principal mode, and endocytosis accounting for the second route and explaining 30% of the overall phenomenon. Further investigations should be carried out to determine the mechanism and meaning of this dual pathway. In this work, we also quantified the extent of nonspecific transfer of the probe R18 from the donor (R18-labeled NDV) to the target membrane (COS-7 cells). To test the effect of certain inhibitors on the specific fusion undergone by the glycoproteins of NDV, 2 min of incubation should be the optimum time.

MATERIALS AND METHODS

Materials

Octadecylrhodamine B chloride (R18) was a product from Molecular Probes Inc. (Junction City, OR). *N*-Acetyltryptsin (EC 3.4.21.4), Tris, Triton X-100, BSA, SDS, trypsin, soybean trypsin inhibitor, and Sephadex G-75 were from Sigma Chemical Co. (St. Louis, MO). Low melting temperature agarose was from FMC BioProducts (Rockland, ME). Cell culture media were from BioWhittaker (Walkersville, MD). Reagents for SDS–PAGE were of electro-

phoretic grade and purchased from Bio-Rad (Richmond, CA); all other reagents were of analytical grade.

Peptide

Peptide N24, which correspond to a 24-amino acid segment (amino acids 145–168; sequence KQNAANILRLKESIAATNEAVHEV) from the HR1 region of the NDV F protein, was obtained from Tufts University of Medicine Peptide Core Facility (Boston, MA). The peptide was purified by the Tufts facility to a purity greater than 90% through the use of HPLC. This peptide was used without further purification in a freshly made solution of 1 mg/ml in PBS.

Cells and viruses

NDV “Clone 30” was grown and purified mainly as described previously (García-Sastre *et al.*, 1989). COS-7 and Vero cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (580 mg/liter), penicillin–streptomycin (100 U/ml to 100 µg/ml), and heat-inactivated FCS at 10% for COS-7 cells and 5% for Vero cells. For fusion experiments, COS-7 cells grown in monolayers were released with trypsin–EDTA. Trypsin was inactivated by the addition of DMEM. The cells were washed twice with 15 mM HEPES buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose), pH 7.4, and resuspended to 3.5×10^6 cells/200 µl in HEPES buffer, pH 7.4.

Labeling of NDV with R18

The fluorescent probe octadecylrhodamine (R18) was inserted into the viral envelope as described previously (Hoekstra *et al.*, 1984). Briefly, approximately 1 mg of viral protein was suspended in 1 ml of KNP buffer (120 mM KCl, 30 mM NaCl, 10 mM sodium phosphate, pH 7.4). Then 10 µl of an ethanolic solution of R18 containing 25 nmol of the probe was injected into the mixture, under vigorous vortexing. After incubation for 1 h at room temperature, under constant stirring in the dark, the noninserted probe was removed by gel filtration on a Sephadex G-75 column (1 × 25 cm) with KNP as the elution buffer. R18-labeled virus was recovered in the void volume fraction, the amount of virus was determined by protein measurement using the spectrophotometric procedure of Lowry *et al.* (1951) using BSA as standard, and the preparation was stored on ice. This R18-labeled NDV was used for fusion experiments within a maximum of 48 h. The total amount of R18 incorporated into the viral membrane was quantified by measuring the fluorescence after lysing the virus in Triton X-100 1% (v/v, final concentration) and comparing this measurement with a standard R18 curve (Hoekstra *et al.*, 1984). For all the R18-labeled NDV, the R18/viral lipid molar relationship

was less than 10% according to the lipid composition of NDV "Clone 30" (Muñoz-Barroso *et al.*, 1997).

R18 fusion assay

The fusion assay was based on the relief of self-quenching of the probe, which, when diluted into the target membrane as a result of fusion and/or probe transfer, gives rise to an increase in the fluorescence emission signal (Hoekstra *et al.*, 1984). Continuous monitoring of the R18 fluorescence was carried out with an Hitachi F-4010 spectrofluorometer (excitation wavelength, 560 nm; emission wavelength, 590 nm; slit widths, 5 and 10 nm for excitation and emission, respectively). All components in the cuvette were stirred continuously with a magnetic stirrer during the reaction time, and temperature was controlled by a thermostatically controlled water bath. In a typical fusion experiment, 3.5×10^6 COS-7 cells were suspended in a cuvette containing 15 mM HEPES buffer, pH 7.4, at 37°C. Subsequently, 20 μg of R18-labeled virus was added, and the development of R18 fluorescence was monitored continuously for approximately 30 min. The final incubation volume in all fusion experiments was 2 ml. Fusion was stopped by the addition of Triton X-100 to 1% (v/v, final concentration), and the resulting fluorescence was taken as 100%. The extent of fusion and/or probe transfer at any time, which is proportional to the percentage of fluorescence de-quenching, was calculated according to the equation:

$$\% \text{ Fusion} = 100(F_t - F_0/F_{100} - F_0) \quad (1)$$

where F_0 and F_t are the fluorescence intensities at time zero and at the given time point, respectively; and F_{100} is the fluorescence after the addition of Triton X-100, which was considered to result in infinite dilution of the probe (Hoekstra *et al.*, 1984).

Binding studies

Next 20 μg of R18-labeled NDV were allowed to bind to 3.5×10^6 COS-7 cells at 4°C in 400 μl of 15 mM HEPES buffer, pH 7.4, from 0 to 60 min. After incubation, nonbound virus was removed by centrifugation at 140 g for 5 min at 4°C, and the cells were washed twice with 800 μl of ice-cold HEPES buffer. The supernatants were pooled, and the distribution of R18 between the cell pellet and supernatant, corresponding to bound and free virus, respectively, was determined after the addition of Triton X-100 at a final concentration of 1% (v/v).

Effect of pH on fusion

R18-labeled virus was incubated for 30 min with 3.5×10^6 COS-7 cells at 37°C, at different pH values using 0.05 M citrate buffer for acidic pH values, and HEPES buffer for pH 7.4 and 8. After this, fusion percentages were determined as above.

Protease treatment

Aliquots of R18-labeled virus were treated at 37°C with 7 μg of trypsin/ μg of NDV in 100 μl of KNP, pH 7.4, for 1 h under stirring. Then trypsin was inactivated by the addition of 100 μl of KNP containing a twofold excess of soybean trypsin inhibitor (Sigma Chemical Co.). Fusion was quantified by incubation of trypsin-treated NDV with 3.5×10^6 COS-7 cells for 30 min at 37°C, as described above.

Incubation with ammonium chloride or sodium azide

COS-7 cells were preincubated at 37°C under stirring in the presence of 0.05 M ammonium chloride or 0.1% sodium azide in HEPES buffer, pH 7.4, for 1 h and 45 min, respectively. After incubation, the mixture was transferred to a cuvette, and fusion was initiated by the addition of R18-labeled virus. As a control, cells alone were incubated in HEPES buffer, pH 7.4.

Fusion experiments in the presence of N24 peptide

Next 20 μg of R18-labeled NDV in KPN was incubated with 80 μg of N24 peptide in a final volume of 200 μl for 1 h at room temperature under stirring. Then, 3.5×10^6 COS-7 cells were added to a final volume of 2 ml, and fusion was accomplished at 37°C as above.

Glutaraldehyde treatment

Then 20 μg of R18-labeled NDV or 3.5×10^6 COS-7 cells were treated with 5% glutaraldehyde (v/v, final concentration) in 1 ml of HEPES buffer, pH 7.4, for 30 min at 37°C with stirring. Fusion was accomplished as described above.

NDV infection in cell culture

A total of 2.5×10^5 Vero cells were plated onto a 12-well plate. At 16 h after plating, serial dilutions of virus were allowed to infect the cell monolayers. The virus was adsorbed for 30–60 min at 37°C in DMEM containing 2% of FBS. Then 1% of agarose in DMEM with 5% FBS and containing 5 $\mu\text{g}/\text{ml}$ *N*-acetyl-trypsin was overlaid. At 24 h postinfection, the cytopathic effect was evaluated by microscopy after fixing the cells with 4% formaldehyde and staining with 1% crystal violet.

SDS-PAGE

SDS-PAGE was performed essentially by the method of Fairbanks *et al.* (1971) using 5–15% polyacrylamide gradient gels. Gels were stained with Coomassie blue.

Data analysis

To quantify the dequenching due to fusion and due to nonspecific probe transfer between membranes, the analysis of data were essentially as described by Co-

baleda *et al.* (1994). Kinetic data were fitted by nonlinear regression with the SIMFIT computer package version 3.1, developed by W. G. Bardsley, University of Manchester, U.K. (Bardsley *et al.*, 1995) using sum of two exponential terms model:

$$\% \text{ Fusion} = A_1[1 - \exp(-k_1t)] + A_2[1 - \exp(-k_2t)] \quad (2)$$

The goodness of each individual fit was evaluated using the χ^2 test, the run and sign test of residuals, plots of residuals, the magnitude of relative residuals, the *t* test for parameter redundancy, and R^2 .

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