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Conditional Dependence of Fusion from Within and Other Cell Membrane Alterations by Newcastle Disease Virus

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Fusion from within (FFWI) by Newcastle disease virus occurs optimally in medium maintained at pH 8.2, whereas fusion from without is relatively insensitive to the pH of the medium in the range of 7.0 to 8.3. The pH-sensitive events in FFWI take place in the synthesis of the hypothetical fusion factor rather than in the response to it. pH pulse and pH shift experiments have localized the pH-sensitive events between 4 and 6.5 h postinfection (a period of synthesis of proteins required for FFWI), but before the fusion process. The pH sensitivity is not due to a pH-sensitive interference phenomenon. Virus production and the appearance of hemadsorbing cell surfaces are also pH sensitive, but for these functions the pH optima depend upon the virus strains tested. The independence of FFWI, hemadsorption, and virus production is discussed. Also discussed are the possible roles of virus-specific proteins in the fusion process.

Several years ago we reported that the cell fusion which results from productive infection by Newcastle disease virus (NDV), termed fusion from within (FFWI), is sensitive to the pH of the culture medium, whereas fusion which occurs as a direct response to high concentrations of input NDV particles, termed fusion from without (FFWO), is not sensitive to pH (3, 4). This difference suggested the possibility that FFWI is sensitive to pH at a step in the synthesis of a hypothetical fusion factor rather than in the interaction of this factor with other cells. If this were the case, the pH dependence could be exploited as a conditional system for studying FFWI and the relationship between this fusion process and other viral functions that occur late in infection.

In this communication, we report that the pH dependence of FFWI is indeed associated with synthesis of the fusion factor and that the pH effect is exerted during a period when proteins and viral antigens necessary for FFWI are being synthesized. In addition, two other surface properties of infected cells, production of hemadsorbing surfaces and release of progeny virus, are also sensitive to pH. Thus, pH sensitivity can be used as a conditional system for studying the mechanism of FFWI and other late viral functions within the genetically homogenous background of a single virus strain and cell type.

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MATERIALS AND METHODS

Cell culture. Primary and secondary chicken embryo cell cultures were prepared and grown in supplemented Eagle minimal essential medium as previously described (3). Standard medium for all experiments consisted of minimal essential medium supplemented with 2.5% calf serum and 2.5% tryptose phosphate broth. Secondary cultures were used for all experiments 24 h after plating 2.0×10^6 cells per 60-mm tissue culture plate (Falcon). Culture medium pH was controlled by varying the concentration of $NaHCO_3$ as previously described (3).

Virus strains, purification, and assay. NDV strains AV (Australia-Victoria, 1932), HP (Israel-HP, 1935), L (L-Kansas, 1948) and N (N. J. LaSota, 1946) were grown in eggs and purified as previously described (8). Infectivity titers (PFU per milliliter) were determined as previously described (3, 8). Hemagglutination titers were determined as described by Clavell and Bratt (8) and are given in hemagglutinating units per milliliter.

Viral growth curves. Virus released from infected cells was measured using slight modifications of the techniques of Rubin et al. (21). After adsorption of virus at 23 C for 1 h, cultures were washed 3 to 5 times with standard medium at 42 C to remove unadsorbed virus. Then 5 ml of medium at the appropriate pH was added and the cultures were incubated at 42 C. Culture medium samples (0.1 ml) were taken at various times after infection, diluted 1:10 in standard

medium, and frozen at −20 C for subsequent assay by plaque titration.

Fusion and hemadsorption experiments. Procedures for fusion experiments and for the hemadsorption assay were as previously described (3, 10). All experiments were done at 42 C rather than 38 C because the extent of fusion varies considerably in the range of 35 to 38 C but not in the range of 40 to 43 C. This procedure minimizes temperature-dependent variation during the different manipulations of the cultures. Similar results were obtained at 38 C, but with greater statistical variation (W. R. Gallaher, Ph.D. thesis, Harvard University, Cambridge, Mass., 1971). Fusion was quantitated as fusion events per cell (10; W. R. Gallaher, Ph.D. thesis). Hemadsorption was quantitated as the average number of erythrocytes absorbed per microscope field of 70 to 100 cells (400×). When first presented in tabular form, the standard deviation from the mean (σ) is included as an estimate of the reproducibility of these assays.

Transfer of infected cells to uninfected monolayers. Infected cells were transferred to monolayers of uninfected cells by a procedure similar to the transfer of cells as infectious centers as previously described (6). Cells were infected at an input multiplicity of 5 PFU/cell and then incubated at 42 C at either high or low pH, as described for fusion experiments. At 5.5 to 6 h after infection, the medium was replaced with 1 ml of 0.1% crystalline trypsin and the cultures were incubated at 37 C for 30 min. Trypsinized cells were collected, centrifuged, and resuspended in medium equilibrated at the pH to be used for adsorption to uninfected monolayers. The cells were washed once in this medium and then resuspended in concentrations of 10^6 to 3.0×10^6 cells/ml. Volumes (0.2 to 0.4 ml) of this suspension were then added to uninfected monolayers containing 2.0×10^6 cells per 60-mm plate (which had been incubated at the appropriate pH for several hours) and allowed to adsorb for 1 h. After adsorption, 5 ml of medium was gently added to these mixtures of infected and uninfected cells, and the cultures were then incubated at 42 C for the time indicated. Since cycloheximide was to be used after transfer, it was included in the first wash as well.

The uninfected monolayers were also treated with cycloheximide for 1 h before the addition of the infected cells and until fixation. At the concentrations used (10 μg/ml), cycloheximide inhibits amino acid incorporation by more than 95% within 10 min.

Antiserum. Antiviral antiserum was prepared by infection of chickens with the N strain of NDV as previously described (6). Under the conditions used, a 1:200 dilution of antiserum reduces the titer of free AV by more than 10^2 in 10 min.

RESULTS

pH dependence of FFWI, hemadsorption, and virus release. We showed previously that FFWI was much more extensive at high pH (8.0 to 8.4) than low pH (7.0 to 7.4), whereas FFWO was similar in both ranges (3, 4). Figure 1 shows the effect on fusion of varying pH over the whole

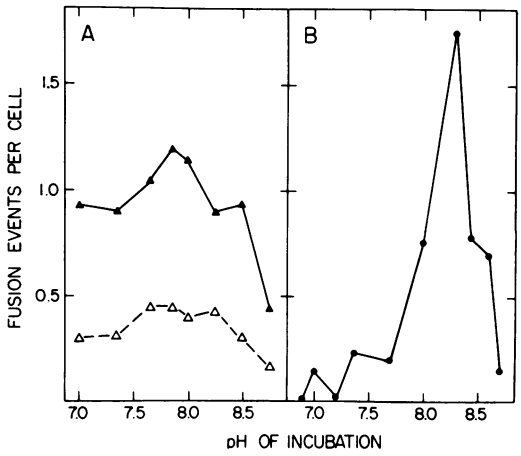

FIG. 1. *pH dependence of fusion. (A) FFWO. Cells were treated for 1 h at 4 C with either 1, (△) or 7,500 hemagglutinating units/ml (▲) of the N strain at pH 7.0. Inocula were then removed, and medium prewarmed to 42 C at each pH was added. All cultures were fixed after 2.5 h at 42 C. (B) FFWI. Cells infected at pH 7.0 with the AV strain were treated with the same prewarmed media as in (A) and then incubated at 42 C until fixation at 7.5 h postinfection.*

range from pH 6.9 to 8.7 (the range within which standard medium can be varied by changes in $NaHCO_3$ concentration). Figure 1A shows a slight optimum at pH 7.8 for FFWO by our best inducer of FFWO, strain N. However the extent of FFWO varied by less than 30% over the whole range except above pH 8.5, where a decrease in FFWO accompanied the degeneration of infected cells. In contrast, FFWI by strain AV (Fig. 1B) showed a sharp optimum at pH 8.2 which was 8 to 10 times higher than at pH 7.0.

The effect of pH became even more pronounced later in infection since FFWI increased only slightly at pH 7.0, whereas it increased at pH 8.2 until it was no longer quantifiable, and cells containing as many as 50 and more nuclei detached from the plate (4; W. R. Gallaher, Ph.D. thesis).

Release of progeny virus could also vary considerably with the pH of incubation as shown by the growth curves for three NDV strains at low and high pH in Fig. 2. Strain HP (Fig. 2A), which caused little if any FFWI (3–5), grew almost equally well at the two pH values. Strains AV (Fig. 2B) and L (Fig. 2C), which both caused FFWI optimally at high pH (3–5), gave maximal virus release at low and high pH, respectively.

The appearance of hemadsorbing surfaces on infected cells can also be affected by the pH of incubation. Figure 3A shows a small number of

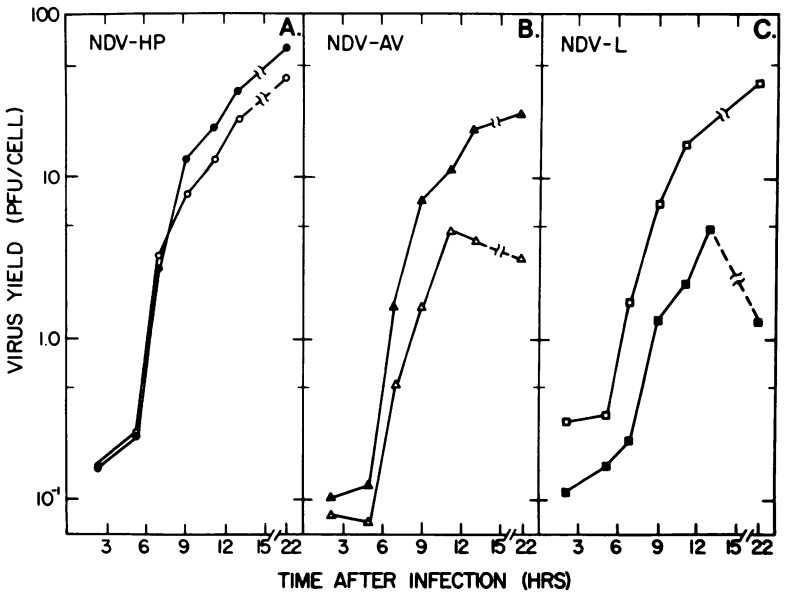

FIG. 2. *Virus production. Cultures infected with the indicated viruses at an input multiplicity of 5 to 10 PFU/cell were incubated at 42 C at pH 7.0 (closed symbols) or 8.4 (open symbols). Samples were taken as described in Materials and Methods.*

erythrocytes nonspecifically adsorbed to the mononucleate cells of uninfected control cultures. Figure 3B shows extensive hemadsorption to the mainly mononucleate cells of AV-infected cultures incubated at pH 7.2. Figure 3C shows only minimal hemadsorption to the polykaryocytes of parallel AV-infected cultures incubated at pH 8.3. Table 1 contains quantitative data for the pH sensitivity of the appearance of hemadsorbing surfaces during infection by strains AV, HP, and L. Cells of AV-infected cultures incubated at low pH adsorbed 10 times as many erythrocytes as those of parallel cultures incubated at high pH. HP-infected cultures showed the opposite pH dependence, whereas L-infected cultures showed no pH dependence for this property.

These results show that three properties of the surfaces of NDV-infected cells, FFWI, progeny virus release, and the appearance of hemadsorbing cell surfaces, can be affected by the pH of incubation. Furthermore, although there is a tendency for maximal virus release to occur under conditions where considerable hemadsorption was detected, the pH dependence of each property varies independently for each strain.

Time course of the pH-dependent events. To determine whether pH sensitivity was exerted at a particular time or throughout infection, the effects of pH shifts and pulses on FFWI were measured. Figure 4A shows that if AV-infected cultures initially incubated at low pH were shifted to high pH by 4 h postinfection maximal FFWI occurred. When shifted after that time, fusion was considerably decreased. Conversely, shifts from high to low pH suppressed FFWI only until 4 h postinfection. The pH-sensitive step in FFWI could be more precisely localized by measuring the effects of high pH incubation during 1- or 2-h pulses. High pH pulses around 5 h postinfection enhanced FFWI the most (Fig. 4B). Conversely, low pH pulses to cultures otherwise incubated at high pH suppress FFWI most when given in the range of 5 h (Gallaher, unpublished data). In other experiments where the extent of FFWI was measured at 10 h postinfection rather than at 8 h, results identical to those described here were obtained (not shown).

Figure 5 compares the kinetics of the pH-dependent step with other events in FFWI. As previously described (3, 4), FFWI was first observed in cultures continuously incubated at pH 8.3 at 6.5 h, and then it increased rapidly. Approximately 30 min before detection, fusion became resistant to the addition of antiviral antiserum, suggesting that cellular interaction had occurred. Two to three hours before detection, FFWI became insensitive to cycloheximide, indicating that any proteins required for FFWI were synthesized. As indicated by the curves of pH shifts (low pH to high pH and high pH to low pH), the pH-dependent step occurred

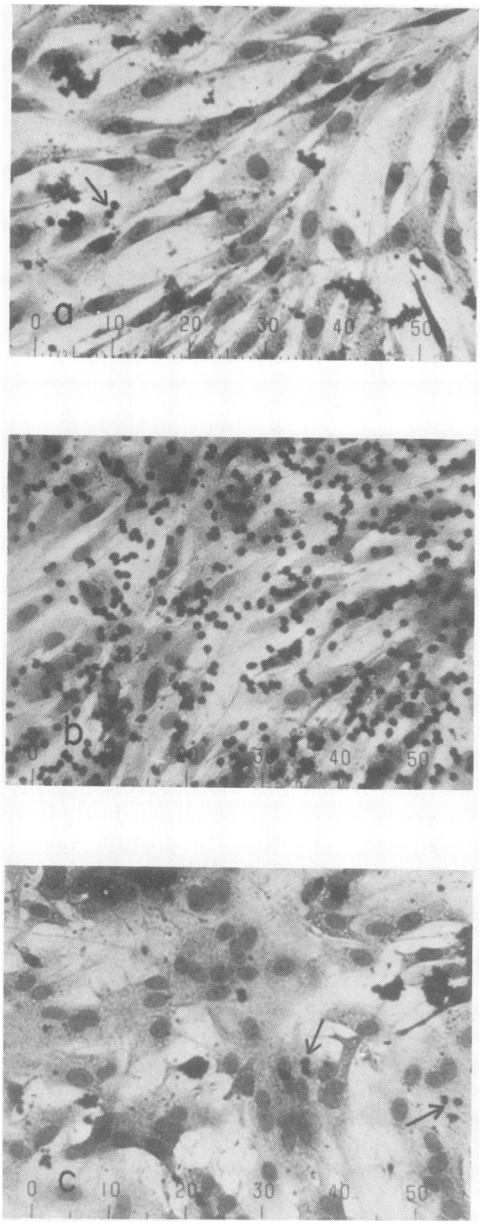

FIG. 3. *Hemadsorption to AV-infected cells. Uninfected cultures (A) and cultures infected with AV and incubated at pH 7.2 (B) or 8.3 (C) were tested for ability to adsorb erythrocytes at 8 h postinfection. Erythrocytes are indicated by arrows.*

during the period of prerequisite protein synthesis but before the cellular interaction which led to fusion.

Figure 6 shows the effects of pH shifts on the appearance of hemadsorbing cell surfaces and virus release in AV-infected cultures. Although the effects of pH shifts were the opposite of those on FFWI, it was obvious that the pH-dependent step in the appearance of hemadsorbing cell surfaces (Fig. 6A) also occurred during the period between 4 and 6.5 h. In contrast, virus yield was affected by pH later in infection (Fig. 6B). pH shifts in the range of 4 to 6.5 h had very little effect on virus yield, and the complete reversal seen with FFWI was not seen.

For both virus release and the appearance of hemadsorbing cell surfaces, it was obvious that the pH-dependent step occurred in some process of synthesis since released virus and hemadsorption were always assayed at a standard pH of 7.4. For FFWI, however, the experiments described thus far do not distinguish between a pH-dependent step in production of the fusion factor or in its interaction with other cells. To distinguish between these possibilities, we separated the processes of synthesis and interaction by transferring infected cells to fresh monolayer cultures and determining the effects of pH before and after transfer. Transfers were done before detectable fusion (5.5 h postinfection) and in the presence of cycloheximide, so that any fusion observed could be attributed to those prerequisite proteins already synthesized in the originally infected cell. Fusion was two- to threefold greater when the originally infected cells had been incubated at pH 8.2 rather than 7.2, and was only slightly affected by the pH after transfer (Table 2). In other experiments (Gallaher, Ph.D. thesis), the pH after transfer was shown to be a factor only if protein synthesis were allowed to continue (no cycloheximide) for several hours after transfer. Therefore, for

TABLE 1. *pH dependence of hemadsorption to cells infected by different NDV strains*[a]

Virus strain	pH	Erythrocytes/ microscope field[b]	Ratio pH 7.2/8.3
AV	8.3	52 ± 20	
	7.2	492 ± 85	9.48
HP	8.3	303 ± 70	
	7.2	96 ± 35	0.32
L	8.3	186 ± 31	
	7.2	202 ± 22	1.09

[a] Cultures infected at an input multiplicity of 5 PFU/cell were incubated at 42 C at the appropriate pH and tested for ability to adsorb erythrocytes at 8 h postinfection. Standard deviation from the mean is indicated (± σ).

[b] Erythrocytes adsorbed to uninfected cultures and infected cultures before 6-h postinfection never exceeded 10 per field.

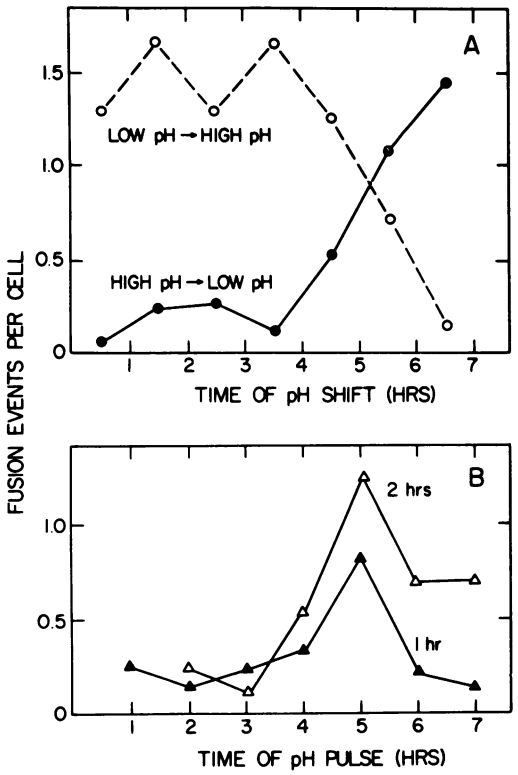

FIG. 4. *Effect of pH shifts and pulses on FFWI. (A) Cultures infected with AV were incubated at 43 C at either pH 7.2 or 8.3 and then at the indicated times, shifted to the other pH by a medium change. All cultures were incubated at the second pH until fixation at 8.5 h. (B) As in (A), cultures incubated at pH 7.2 were shifted for a 1- (▲) or 2- (△) h pulse to pH 8.3 and then shifted back to pH 7.2 for incubation until 8.5 h. The extent of fusion at 8.5 h is plotted at the midpoint in time for each high pH pulse.*

FFWI also, the pH dependence occurs in a step in the synthesis of the fusion factor rather than its interaction with other cells.

Multiplicity of infection and the pH dependence of FFWI. Our previous studies have shown that FFWI is inhibited by high multiplicity infection, and that this inhibition is greater at low than at high pH (3, 4). We therefore determined whether the pH dependence results from a pH-dependent auto interference phenomenon (6, 7). The ratio of fusion caused by an input multiplicity of 10 PFU/cell to that caused by 2 PFU/cell was constant over the range of pH from 7.1 to 7.9 (Table 3). Therefore, the effects of pH and multiplicity of infection appear to be independent of each other. Finally, to eliminate the possibility that pH affects FFWI by increasing an auto interference phenomenon by a constant factor at each multiplicity, the effect of pH was studied under conditions designed to prevent auto interference. This was accomplished by infecting cultures at an input multiplicity of 0.01 PFU/cell. The number of multinucleate cells (efficiency of infection) was independent of pH, but the average number of fusion events per multinucleate cell (size) was almost three times as great after incubation at pH 8.3 rather than pH 7.2 (Table 4). We therefore conclude that the pH dependence of FFWI was not due to a pH-dependent interference phenomenon.

DISCUSSION

Three properties of NDV-infected chicken embryo cells (and BHK-21 cells [Gallaher, Ph.D. thesis]) are affected by the pH of the culture medium. These include FFWI, the appearance of hemadsorbing cell surfaces, and virus production. FFWI occurs maximally at high pH (8.1 to 8.3) rather than low pH (7.0 to

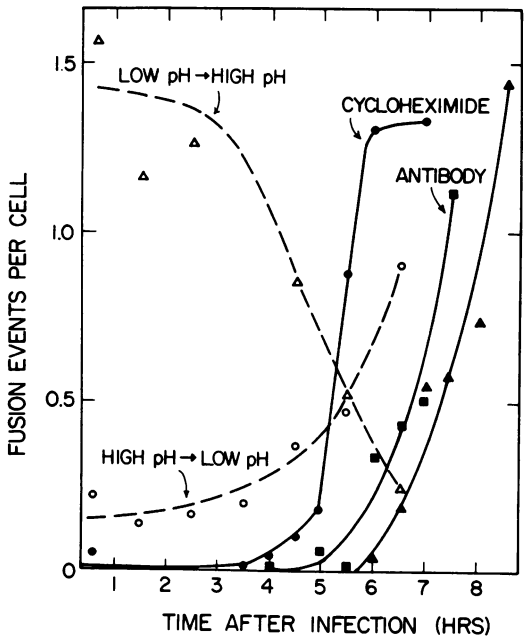

FIG. 5. *Localization of the pH-dependent step in FFWI. Cultures infected with AV in the usual way were divided into four series and treated in the following way. The normal time course of fusion was determined by incubating cultures at pH 8.2 and fixing duplicate cultures at the times indicated (▲). Two other series were incubated at pH 8.2 and then treated at the times indicated with either cycloheximide at 10 μg/ml (●) or antiviral antiserum at a 1:200 dilution (■) until fixation at 8.5 h. As in Fig. 4A, another series was incubated at pH 7.2 or 8.2 and then shifted to the other pH at the times indicated, and then fixed at 8.5 h postinfection (open symbols).*

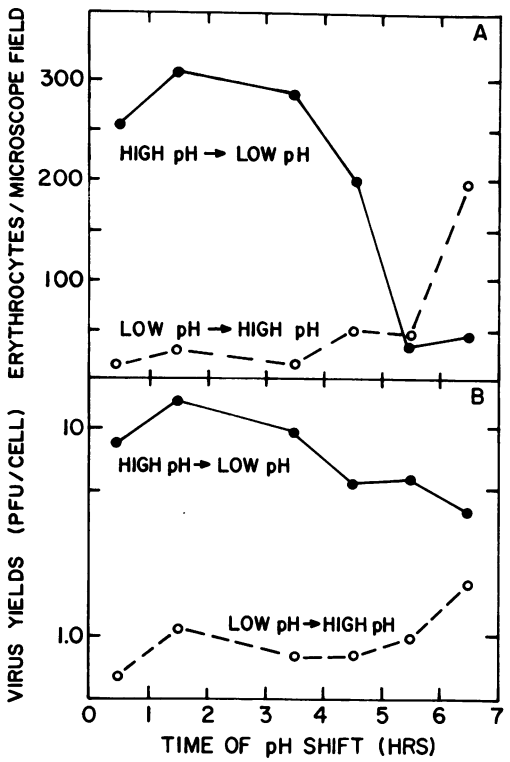

FIG. 6. *Comparison of effects of pH shifts on hemadsorption and virus production. Cultures were infected with AV and subjected to pH shifts as in Fig. 4A. After washing with phosphate-buffered saline, pH 7.4 (9), at 8.5 h postinfection half of the cultures were tested for ability to absorb erythrocytes (A). Virus production (B) was measured at 12 h postinfection.*

TABLE 2. *Effects on FFWI of pH of incubation before and after cell transfers*[a]

pH before transfer	pH after transfer	Fusion events/cell
7.2	7.2	0.16 ± 0.08
7.2	8.2	0.18 ± 0.01
8.2	7.2	0.38 ± 0.05
8.2	8.2	0.48 ± 0.06

[a] Cultures infected with the AV strain under the usual conditions were incubated at 42 C at either pH 7.2 or pH 8.2 until 5.5 h postinfection and then trypsinized and, in the presence of cycloheximide, transferred (5×10^5 cells previously incubated at low pH and 3.5×10^5 cells previously incubated at high pH) to fresh monolayer cultures at the same or opposite pH. Statistical variation shown is the standard deviation from the mean ($\pm\sigma$).

7.2) for all NDV strains capable of inducing this type of fusion. For the other two properties, however, the pH optimum depends on the NDV strain used. For instance, strains AV and L produce hemadsorbing cell surfaces optimally at low and high pH, respectively. It is, therefore, becoming increasingly clear that for NDV, FFWI and ability to hemadsorb are far more independent than the studies of Reeve and Alexander (18) would suggest. Other evidence for the independence of these properties is to be found in Bankowski's (2) decade-old observation of FFWI in the absence of hemadsorption, and Tsipis and Bratt's (*in* B. H. Mahy and R. D. Barry, ed., *The negative stranded viruses*, in press) isolation of mutants of the AV strain which are independently temperature sensitive for these two properties. For Sendai virus too, these properties are probably independent since each appears to involve a different glycoprotein (12, 23, and A. Scheid and P. W. Choppin, *in* B. H. Mahy and R. D. Barry, ed., *The negative stranded viruses*, in press). The independence of these properties appears to be a common phenomenon since Ichihashi and Dales (14) have shown that certain pox virus strains will fuse and not produce hemadsorbing surfaces, or vice versa.

In a similar manner, virus production and FFWI are independent of each other. For instance, strains AV and L, which share a high pH optimum for FFWI, release virus optimally at low and high pH, respectively. In addition, pH shifts do not affect these two properties coordi-

TABLE 3. *Effects of pH and multiplicity on FFWI*[a]

pH	Input multiplicity		Ratio 10/2
	10 PFU/cell	2 PFU/cell	
6.8	0.03	0.22	0.14
7.1	0.13	0.46	0.35
7.4	0.27	0.97	0.36
7.6	0.45	1.66	0.37
7.9	1.11	3.50	0.32

[a] Cultures infected at pH 7.0 with AV at either 10 or 2 PFU/cell were incubated at 42 C at various pH values and fixed 9 h after infection.

TABLE 4. *pH dependence of FFWI under conditions of minimal interference*[a]

pH	Multinucleate cells/100 original cells	Fusion events/multinucleate cell
7.2	4.0	2.7
8.3	4.3	7.5

[a] Cultures infected with AV at a multiplicity of 0.01 PFU/cell were incubated at pH 7.2 or 8.3 and fixed at 11 h postinfection. Only multinucleate cells with three or more nuclei were counted.

nately. The temperature-sensitive mutants of Tsipis and Bratt (*in* B. H. Mahy and R. D. Barry, ed., *The negative stranded viruses*, in press) also provide evidence for the independence of these properties. These results and the findings with canine distemper virus (17) support the hypothesis that FFWI is a function of the infected cell membrane and is not dependent on progeny virus production. Nevertheless, the extensive membrane changes which are reflected in massive fusion may be responsible for low virus yields in certain cases. This seems particularly likely in the case of SV5 infection of different cell types (11, 15), where differences in cytopathology are great and severe cytopathology might be expected to inhibit either the rate or duration of virus release.

Analyzing FFWI. The approach we have adopted is applicable to the analysis of viral and cellular factors involved in FFWI in other systems. Cellular susceptibility to fusion can be evaluated by determining susceptibility to FFWO by the same or a closely related virus. Transferring infected cells to uninfected cultures in the presence of cycloheximide provides a means of determining whether cells which do not show FFWI are blocked in synthesis or expression. We successfully used this approach to show that MDBK cells are susceptible to fusion and that the failure of NDV to cause FFWI in these cells lies in synthesis rather than expression of a fusion factor (5). Rankin et al. (17) recently used a similar approach to analyze FFWI by canine distemper virus.

Involvement of virus-specific proteins. Our ability to control these properties by adjusting pH provides a means of controlling infection within a framework of constant cellular and viral genetic input. It is clear that a pH-dependent step occurs during a period of synthesis of proteins required for FFWI, but considerably before FFWI takes place. The experiments we described do not distinguish between a pH-dependent step in the synthesis of components required for functions such as FFWI and hemadsorption, and the pH-dependent synthesis of inhibitors of these functions. Nor do they distinguish between quantitative requirements for specific molecules on the infected cell surface, on the one hand, and critical local densities, perhaps analogous to the requirement of critical localized receptor densities for cell agglutination by plant lectins (16), on the other. Furthermore, we did not determine whether the reduction in total cell surface mucoproteins reported to accompany FFWI (20) is pH dependent.

Although it is obvious that virus-specific proteins must be synthesized for FFWI to take place (3, 4, 19; Gallaher, Ph.D. thesis), the role of these proteins is unclear. Even in FFWO there is no direct proof that virus-specific proteins are involved since neither virus particles nor viral antigens are detected at fusion sites (1, 14). However, indirect evidence is increasing for the involvement of virus-specific proteins, specifically, the glycoproteins which constitute the major protein components of the surface of paramyxovirus particles (22). For NDV, both FFWI and hemadsorption require glycoprotein synthesis as suggested by their inhibition in the presence of sugar analogues such as 2-deoxy-D-glucose (10). In addition, Homma and Ohuchi (12) and Scheid and Choppin (Virology, in press) showed that Sendai virus grown in L or MDBK cells requires protease treatment to activate its fusion factor. Concomitant with this activation is cleavage of one glycoprotein to another which is normally found in virus with the ability to fuse.

We are currently studying protein and lipid metabolism by using the conditional system employing pH variation, as well as the temperature-sensitive mutants and the behavior of the different NDV strains in different cell types, in an attempt to determine the role of both cellular and viral components in the development of surface membrane alterations during NDV infection.

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