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Quantitative determination of virus-membrane fusion events

Fusion of influenza virions with plasma membranes and membranes of endocytic vesicles in living cultured cells

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Incubation of fluorescently labeled influenza virus particles with living cultured cells such as lymphoma S-49 cells or hepatoma tissue culture cells resulted in a relatively high degree of fluorescence dequenching. Increase in the degree of fluorescence (35–40% fluorescence dequenching) was observed following incubation at pH 5.0 as well as at pH 7.4. On the other hand, incubation of fluorescently labeled influenza virions with erythrocyte ghosts resulted in fluorescence dequenching only upon incubation at pH 5.0. Only a low degree of fluorescence dequenching was observed upon incubation with inactivated unfusogenic influenza or with hemagglutinin$_0$-influenza virions. The results of the present work clearly suggest that the fluorescence dequenching observed at pH 5.0 resulted from fusion with the cells' plasma membranes, while that at pH 7.4 was with the membranes of endocytic vacuoles following endocytosis of the virus particles. Our results show that only the fluorescence dequenching observed at pH 7.4 – but not that obtained at pH 5.0 – was inhibited by lysosomotropic agents such as methylamine and ammonium chloride, or inhibitors of endocytosis such as EDTA and NaN_3.

Virus-membrane fusion; Fluorescence dequenching; (Influenza virion)

1. INTRODUCTION

A membrane fusion step is involved in the penetration of many animal viruses into living cells [1,2]. Indeed, fusion between enveloped viruses and isolated biological membranes or phospholipid vesicles has served as one of the most studied systems for elucidating the molecular mechanism of membrane fusion [1]. Two main steps are involved in the early stages of virus infection: (a) binding of the virus particles to specific cell receptors, and (b) fusion of the viral envelope with the plasma membranes of cells or with membranes of endocytic vesicles [1].

Influenza virus is an enveloped virus whose binding to cell membrane sialoglycolipids and sialoglycoproteins is mediated by the viral hemagglutinin glycoprotein [2]. Following endocytosis, the fusogenic ability of the viral HA glycoprotein is activated by the intraendosomal, low pH environment [1]. Fusion of enveloped viruses with membranes of endosomes may serve as an ex-

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DQ, fluorescence dequenching; FCS, fetal calf serum; HA, hemagglutinin; HEG, human erythrocyte ghost; HSV-1, *Herpes simplex* virus; HTCs, hepatoma tissue culture cells; NA, neuraminidase; PBS, phosphate-buffered saline; EIVEs, reconstituted influenza virus envelopes; R_{18}, octadecylrhodamine-B chloride; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus

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cellent model system to study fusion events occurring within living cells. The lack of an assay system for a quantitative estimation of fusion events taking place within living cells, hampered accurate biochemical and biophysical studies of such processes. Many of the past studies have been focused on fusion between viral envelopes and liposomes [3,4] or isolated erythrocyte membranes [5,6]. Evidently, questions related to the correlation between the cell cycle, cell metabolic state, as well as the cell's endocytic activity and susceptibility to fusion with enveloped virions could not be studied with the above systems.

Recently, fluorescently labeled enveloped viruses and fluorescence dequenching methods have been used to study fusion between enveloped viruses and isolated biological membranes or liposomes [3-5]. In the present work, we show that fluorescence dequenching methods can also be used to follow fusion between influenza virions and cultured cells. The present system may be extremely useful to follow fusion events occurring in intact living cells, especially between envelopes of animal viruses and membranes of intracellular organelles.

2. MATERIALS AND METHODS

2.1. *Materials*

Neuraminidase (*Vibrio cholera*, 1 unit/ml) was obtained from Boehringer (FRG), and octadecylrhodamine-B chloride from Molecular Probes (USA). Trypsin from bovine pancreas (type III) was obtained from Sigma (USA). All other chemicals used were of analytical grade.

2.2. *Virus*

Influenza virus (A_oPR_8 strain) was isolated from the allantoic fluid of fertilized chicken eggs [7]. The viral hemagglutinating units were determined essentially as described [8]. Influenza A, possessing uncleaved HA_o [7] (virus $N_1H_{10}N_7$) was a generous gift from Dr R. Rott of the Institute of Virology, Giessen (FRG). Trypsinization of HA_o virions was performed by incubating 200 μg of virus with 3 μg of trypsin, as described [7]. Fusogenic RIVEs were prepared following incubation of intact virions with Triton X-100 (Triton X-100:viral proteins, 2:1, w/w) as described before for Sendai [9] or influenza [10] virions.

2.3. *Cells*

Mouse lymphoma S-49 cells were grown in DMEM + 10% horse serum, as described elsewhere [11]. Prior to use, the cells were washed twice with DMEM without serum. HTCs were grown in Swimm's 77 medium + 5% FCS, as described [12].

2.4. *Preparation of fluorescently labeled, intact influenza virions*

Intact virions were labeled with R_{18} essentially as described before for Sendai virus [5,13]. Briefly, 2-3 μl of 1 mg/ml ethanolic solution of R_{18} were rapidly injected into 250 μl PBS (150 mM NaCl and 10 mM phosphate buffer, pH 7.4), containing 400 μg of viral protein. After 15 min of incubation at room temperature in the dark, the viral preparations were washed with 6 vols of PBS in an Eppendorf centrifuge (15 min). Under such conditions, the R_{18} was inserted into the viral membranes at self-quenching surface density (about 3 mol% of

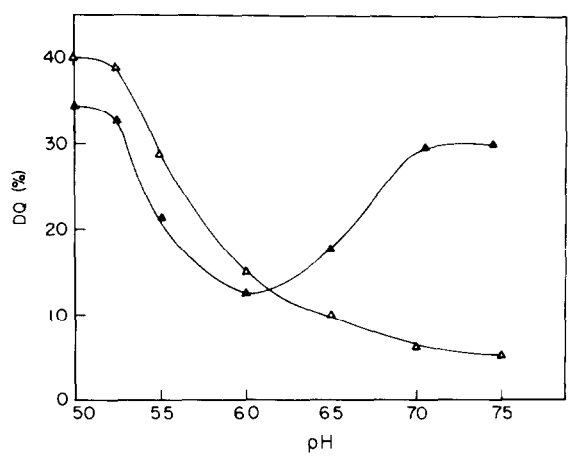

Fig.1. Interaction of fluorescently labeled influenza virions with HEGs and mouse lymphoma S-49 cultured cells: pH profile. Fluorescently labeled influenza virions (5 μg) were added to either HEGs (△) (150 μg protein), giving a final volume of 200 μl PBS, pH 7.4, or to lymphoma S-49 cultured cells (5×10^6) (▲), suspended in a final volume of 200 μl DMEM, without serum, at pH 7.4. Following 10 min of incubation at 4°C, a volume of 50 μl of sodium acetate (0.5 M), adjusted to the indicated pH values, was added. At the end of 30 min of incubation at 37°C, the DQ degree was determined as described in section 2. HEGs were prepared from outdated, washed, intact human erythrocytes, as described in [5,6].

total viral phospholipids), and its decrease was shown to be proportional to the DQ [5,13].

2.5. *Fluorescence measurements*

Fluorescent influenza virions and RIVEs (5 μg protein of each) were incubated with HEGs or living cultured cells, in a final volume of 200 μl of PBS (pH 7.4) or DMEM (without serum, pH 7.5), respectively. Following 10 min of incubation at 4°C, the pH of the medium was adjused to the desired pH values by the addition of 50 μl sodium acetate (0.5 M of the appropriate pH value), and the suspension obtained was then incubated at 37°C. At the end of the incubation period, a volume of 1 ml PBS, pH 7.4, was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was estimated before and after solubilization with 0.1% Triton X-100. The extent of fluorescence obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe [5,13]. All fluorescence measurements were carried out with a Perkin-Elmer MFP-4 spectrofluorimeter. Virus preparations were also incubated under the same experimental conditions in the absence of recipient

Table 1

Interaction of fluorescently labeled influenza virus with lymphoma S-49 cultured cells

Virus treated with	pH of incubation			
	7.4		5.0	
	DQ (%)	Inhibition (%)	DQ (%)	Inhibition (%)
Expt I				
None	33	0	38	0
Glutaraldehyde	7	78	13	70
NH_2OH	10	70	15	60
Low pH	8	76	12	70
85°C	10	70	13	70
Expt II				
(i) Virus strains				
A_oPR_8	36		42	
HA_o	9		12	
Trypsinized HA_o	29		36	
(ii) System				
Lymphoma S-49 at 37°C	34		40	
Neuraminidase-treated lymphoma S-49 at 37°C	9		13	
Lymphoma S-49 at 4°C	9		12	

Expt I: Influenza virions were treated with glutaraldehyde (0.1%, 30 min, 37°C), NH_2OH (1.5 M, 30 min, 37°C, pH 6.5), or incubated at 85°C (30 min) or at low pH (0.5 M of acetate buffer, pH 5.0, 30 min, 37°C), essentially as described in [5,10,14,15]. At the end of the incubation period, the virus in the various systems was washed with 10 vols PBS, pH 7.4, resuspended in 250 μl PBS, and labeled with R_{18}. Untreated and treated fluorescently labeled viruses (5 μg) were incubated with lymphoma S-49 cultured cells (5×10^6) as described in fig.1 and in section 2. Expt. II: Wild type (A_oPR_8), HA_o ($N_aH_{10}N_7$) and trypsinized HA_o (5 μg of each) were incubated with lymphoma S-49 cultured cells (5×10^6, 200 μl DMEM without serum), first at 4°C and then at 37°C, as described in fig.1. Lymphoma S-49 cultured cells were desialized by treatment with NA (35 mU), as described before for HTCs [16]. The lymphoma S-49 cells (5×10^6 in 200 μl of DMEM) were incubated with influenza virions (A_oPR_8, 5 μg protein). After 10 min of incubation at 4°C and after adjusting the medium pH, the systems were either incubated for 30 min at 37°C or at 4°C. At the end of the incubation period, the degree of DQ was estimated as described in fig.1 and above

membranes. DQ was calculated as described before [5,6].

3. RESULTS

3.1. *Fusion of intact influenza virions with cultured cells: quantitative determinations*

Incubation of fluorescently labeled influenza virions with erythrocyte ghosts at low pH values resulted in a relatively high degree of DQ (fig.1). Under the conditions used, the DQ reflects a process of virus-membrane fusion [3,6]. Very little, if any DQ was observed following incubation of influenza virions with erythrocyte ghosts at neutral pH values such as between 7.0 and 7.5 (fig.1). It has been well established that the fusion activity of influenza virions is stimulated and expressed at low pH values [1].

DQ has also been observed following incubation of intact virions with cultured cells such as mouse lymphoma S-49 (fig.1). However, in this case an increase in the degree of fluorescence was also observed at neutral pH values. As can be seen (fig.1), the lowest degree of DQ (12%) was observed at pH 6.0, while at higher or lower pH values the degree of fluorescence increased. This is in contrast to the observations with erythrocyte ghosts, the incubation with which led to an increase in fluorescence only at low pH values (fig.1).

Essentially the same results were obtained with another two cultured cell lines, i.e., HTCs and BSC_1 (not shown). An increase in the degree of DQ was observed following incubation at either pH 5.0 or 7.4.

The view that the DQ observed at both pH values, namely pH 5.0 and 7.4, indeed results from a process of virus-membrane fusions, is strengthened by the results summarized in table 1, expt I, which shows that the degree of DQ observed with treated virions was much lower than that obtained with untreated virus particles. Treatment of influenza virions with glutaraldehyde, NH_2OH or incubation at low pH (5.0) or high temperatures (85°C), has been shown to inactivate the viral fusogenic and hemolytic activities [10,14,15]. As can be seen (table 1, expt I), almost the same degree of inhibition was observed following incubation of lymphoma S-49 cells with treated, unfusogenic virions at either pH 5.0 or 7.4. It is noteworthy that glutaraldehyde- or hydroxylamine-treated influenza virions are able to agglutinate and bind to cultured cells but are unable to induce cell-cell fusion [10,14].

The results in table 1, expt II, show that incubation of HA_o-influenza virions with lymphoma S-49 cells resulted in a relatively low degree of DQ (9–12%). It has been well established that trypsinization of HA_o-influenza virus activates its fusogenic polypeptide and, therefore, its ability to penetrate and infect living cells [7]. Indeed, the degree of DQ observed with trypsinized HA_o virions was much higher than that obtained with untreated HA_o virus (table 1, expt II).

Fusion with and infection of influenza virus is a temperature-dependent process and has been shown to be mediated by virus receptors, i.e., membrane sialoglycolipids [1,2]. Indeed, the results in table 1, expt II, also show that a low degree of DQ was obtained upon incubation of the fluorescently labeled influenza virions with lymphoma S-49 cells at 4°C, as well as following incubation at 37°C with neuraminidase-treated cells.

3.2. *Fluorescence dequenching at pH 7.4 reflects fusion with membranes of endocytic vesicles*

The degree of DQ observed following incubation of fluorescently labeled influenza virions with lymphoma S-49 cells was dependent on the number of cells present in the incubation medium, reaching a maximum value with $6–8 \times 10^6$ cells per system (fig.2A,B). In the presence of a lysosomotropic agent such as methylamine, a low degree of DQ was obtained when the incubation was performed at pH 7.4 (fig.2B). Incubation at pH 5.0, however, in the absence or presence of methylamine, resulted practically in the same degree of DQ (fig.2).

Kinetic studies revealed that the increase in DQ was faster upon incubation at pH 5.0 than at pH 7.4 (fig.3). As can be seen (fig.3), a maximum degree of DQ was reached within 15–20 min of incubation at 37°C. However, a half-maximal degree of DQ ($\tau_{1/2}$) was observed at pH 5.0 (21%) within 2.5 min, while that observed at pH 7.4 (18%) was reached only after 5 min of incubation at 37°C. The results in fig.3 also show that the presence of methylamine drastically reduced the degree of DQ observed at pH 7.4 but not that at pH 5.0.

In addition to methylamine, also the presence of

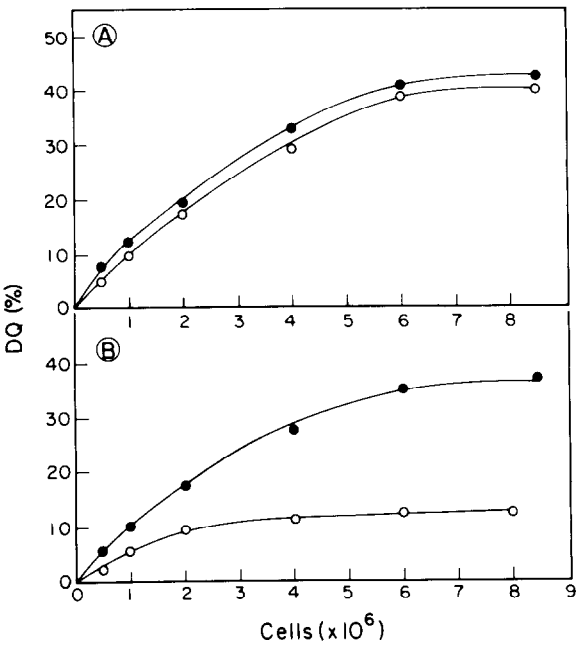

Fig.2. Dependency of the extent of DQ on cell concentration: effect of methylamine. Influenza virus particles (5 μg) were incubated with increasing concentration of lymphoma S-49 cells, at pH 5.0 (A) and at pH 7.4 (B), in the presence (○—○) or absence (●—●) of 50 mM methylamine. All other experimental conditions were as in fig.1. The degree of DQ was monitored after 20 min of incubation at 37°C.

ammonium chloride as well as EDTA or NaN_3 markedly reduced the degree of DQ obtained following incubation with influenza virions at pH 7.4 (fig.4). These compounds did not have any effect when influenza virions were incubated at pH 5.0 with lymphoma S-49 or HTC cells (fig.4A and B, respectively). Studies with both cell lines revealed that lymphoma S-49 were more susceptible to the inhibitory effect of the lysosomotropic agents (fig.4A), while the HTCs were more susceptible to the effects of EDTA and NaN_3 (fig.4B).

3.3. *Fusion of reconstituted influenza virus envelopes with lymphoma S-49 cells*

We have recently shown that influenza reconstituted vesicles, bearing the HA and NA glycoproteins, are as fusogenic as intact virions when incubated with HEGs [10]. The results in table 2 (expts I and II) show that incubation of RIVEs with lymphoma S-49 cells at pH 5.0 or 7.4, resulted

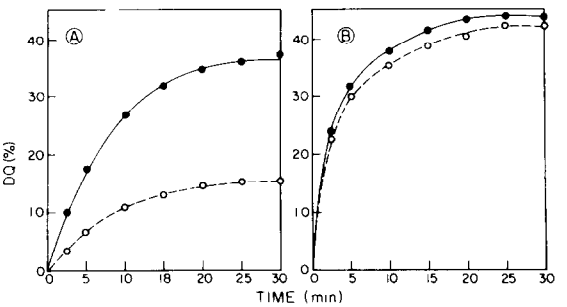

Fig.3. Fusion of influenza virions with lymphoma S-49 cultured cells: kinetic studies. Several systems, each containing 5 μg of fluorescently labeled influenza virions and 5×10^6 lymphoma S-49 cells, in a final volume of 200 μl DMEM, pH 7.4, were incubated in parallel for 10 min at 4°C, with (○—○) or without (●—●) 50 mM methylamine (see also fig.2). At the end of the incubation period, a volume of 50 μl of warm sodium acetate (0.5 M, 80°C), adjusted to pH 7.4 (A) or 5.0 (B) (with or without 50 mM methylamine), was added, and the suspension obtained was immediately incubated at 37°C. At the indicated periods, samples were withdrawn, and the degree of DQ was monitored as described in fig.1 and in section 2.

in DQ to an extent very close to that observed with intact influenza virions. Significantly less DQ was observed following incubation with RIVEs which were treated with NH_2OH, glutaraldehyde, or incubated at low pH or at 85°C (table 2, expt I). Similarly, a low degree of DQ was observed following incubation of RIVEs with neuraminidase-treated lymphoma S-49 cells or when incubation was carried out at pH 7.4 in the presence of methylamine, ammonium chloride, EDTA or NaN_3. Almost no inhibition was observed upon incubation with these compounds at pH 5.0 (table 2, expt II).

4. DISCUSSION

The results of the present work show that incubation of fluorescently labeled influenza virions with erythrocyte ghosts at pH 5.0, or with living cultured cells at pH 5.0 or 7.4, resulted in a relatively high degree of DQ. We suggest that most of the DQ observed reflects a process of virus-membrane fusion. This is based on our results showing that incubation of inactivated, non-infective fluorescently labeled virions with living

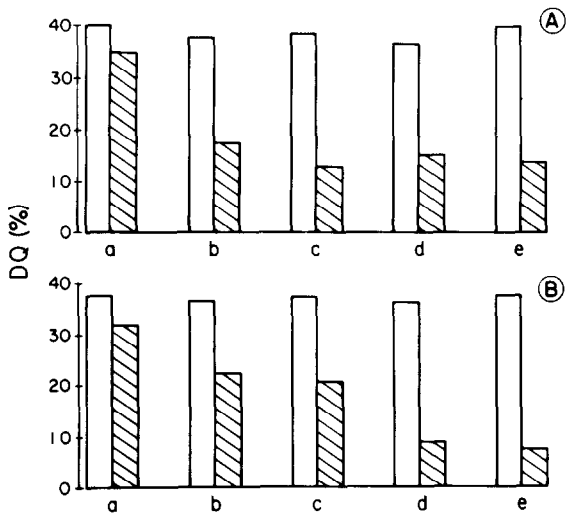

Fig.4. Fusion of influenza virions with lymphoma S-49 and HTC cells: effect of lysosomotropic agents and inhibitors of endocytosis. Influenze virions (5 μg) were incubated with 5×10^6 lymphoma S-49 cells (A) or HTC cells (B), at pH 5.0 (□) or 7.4 (□). The incubation media contained the following compounds: (a) none; (b) 50 mM NH_4Cl; (c) 50 mM NH_2CH_3; (d) 5 mM EDTA; and (e) 50 mM NaN_3. Before incubation with the virus, the lymphoma S-49 cells and HTCs were washed once in a medium containing the appropriate compounds. DQ was monitored after 20 min of incubation at 37°C. All other experimental conditions were as described in section 2.

cultured cells resulted in a low degree of DQ, the extent of which reached only about 20–30% of that found with active viruses. Similar results have been obtained previously following incubation of inactive Sendai [5,17] or influenza [10] virions with erythrocyte ghosts. It is conceivable that the residual dequenching observed with treated virions and cell membranes at pH 7.4 and 5.0, or with untreated virions and HEGs at pH 7.4, is due to lipid-lipid exchange occurring between the viral envelopes and the recipient cell membranes [17]. The maximum value of fluorescent DQ determined following virus-cell fusion is about 40–50%. This may indicate that only about 40% of the labeled virions are capable of binding to or fusing with the cell membrane, as described for Sendai virus [5] or reconstituted Sendai virus envelopes [6].

Our results showing that the degree of dequenching observed with HA_o-influenza virions also reached only 30% of that obtained with trypsinized HA_o virions, further support the view that most of the dequenching (70% of its maximal value) observed should result from virus-membrane fusion events. The possibility that the low degree of DQ observed with HA_o-influenza or with the inactivated virions is due to some residual fusogenic activity left in the preparations, cannot be excluded.

From the present results, it should be inferred that the DQ observed at pH 5.0 is due to fusion of the virions with the cell's plasma membrane, while that at pH 7.4 is caused by fusion with the membrane of endocytic vesicles. At pH 7.4, the virus is

Table 2

Interaction of RIVEs with lymphoma S-49 cells: fluorescence dequenching studies

Experiment	pH of incubation			
	7.4		5.0	
	DQ (%)	Inhibition (%)	DQ (%)	Inhibition (%)
I. RIVEs treated with				
None	37	0	43	0
85°C	7	81	11	75
Low pH	8	78	11	75
NH_2OH	10	73	16	63
Glutaraldehyde	7	81	10	77
II. Cells incubated with				
None	39	0	46	0
Neuraminidase	7	82	14	70
NH_2CH_3	15	60	42	9
NH_4Cl	18	55	44	4
EDTA	22	42	40	13
NaN_3	20	50	44	4

RIVEs were prepared following solubilization of intact virions with Triton X-100, as described [9,10]. RIVEs were fluorescently labeled with R_{18} as described for intact virions. Fluorescent RIVEs were incubated at 85°C or at low pH, or treated with NH_2OH or glutaraldehyde, as described for intact virions in table 1. Lymphoma S-49 cells were desialized by treatment with NA, as described in table 1. RIVEs (5 μg) were incubated with lymphoma S-49 cells at pH 7.4 or 5.0, as described in fig.1 and in section 2 for the incubation with intact influenza virions. In expt II, the incubation was performed in the presence of methylamine, EDTA or NaN_3, as described in fig.4. DQ was monitored following 20 min of incubation at 37°C

first attached to its membrane receptor, after which it is taken into endocytic vesicles [1]. The intraendosomal low pH environment activates the viral fusogenic activity. The dependency of the DQ on the pH of the medium probably reflects the change from fusion with the cell's plasma membrane at low pH values to fusion with endosomal membranes following endocytosis at neutral pH. Since fusion with endosomes is limited by the rate of endocytosis, it appears from our kinetic studies that, indeed, it was a somewhat slower process as compared with that observed at pH 5.0.

Inhibitors of endocytosis such as NaN_3 or EDTA [18] strongly reduced the DQ observed at pH 7.4 but not that obtained at pH 5.0. Moreover, lysosomotropic agents which are known to locally increase the intraendosomal low pH [1], reduced the DQ observed at pH 7.4. These observations further support the view that penetration of the influenza strain used by us depends, under physiological conditions, upon the recipient cells' endocytic activity as well as on acidification of the intraendosomal space. It seems that virus penetration via endocytosis is not completely blocked by the reagents described above; the fluorescent DQ detected after using those reagents (13%) is still higher than that detected with HEGs at pH 7.4 (7%). This observation was also detected using radioactivity or electron microscopy to study virus penetration into cultured cells [18,19].

The use of the present system allows one, for the first time, to study questions related to the quantitative aspects of virus-cell fusion and the dependence of such processes on the specificity of the recipient cells and their methanolic activity. Our results show that various cell lines may respond differently to inhibitors of endocytosis or lysosomotropic agents, thus exhibiting a different degree of susceptibility to influenza viruses.

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