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MOLECULAR EVENTS DURING THE INTERACTION OF ENVELOPES OF MYXO- AND RNA-TUMOR VIRUSES WITH CELL MEMBRANES

A 270 MHz ^{1}H NUCLEAR MAGNETIC RESONANCE STUDY

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Summary

The nuclear magnetic resonance (NMR) spectra of chick embryo cells have been analyzed after exposure to Newcastle disease virus (NDV). Virions that contained the envelope glycoproteins in the cleaved form and, thus, had full biological activity have been compared to virions that had reduced infectivity due to the presence of uncleaved glycoprotein F. After exposure to infectious virus, drastic changes occurred in the signals assigned to choline and the hydrocarbon chains of fatty acids. These observations are interpreted to demonstrate alteration of the fluid lipid bilayer structure of the cell membranes. This is compatible with the concept of membrane fusion as a penetration mechanism for NDV. Virus containing uncleaved F glycoprotein did not alter the NMR spectra. This indicates that infection is blocked at the stage of penetration.

Similar, though less pronounced, differences have been observed when the effects of highly infectious influenza virus containing the hemagglutinin in the cleaved form were compared to the effects of virus which had a lower infectivity due to the presence of uncleaved hemagglutinin. Thus, it appears that the hemagglutinin of influenza virus is involved in penetration and that cleavage is necessary for this function.

Alterations of the NMR spectra of the membrane lipids have also been observed when susceptible chick embryo cells (C/E) were infected with Rous sarcoma virus of subgroup B. Such alterations did not occur when non-susceptible cells (C/B) were used. Thus, infection appears to be blocked again at the stage of penetration.

Abbreviations: NDV, Newcastle disease virus; RSV-PrB, Rous sarcoma virus Prague B strain; FPV, fowl plague virus; p.f.u., plaque-forming units.

Introduction

In order to establish infection of a cell, virus particles have to penetrate the plasma membrane. With enveloped viruses two different mechanisms have been proposed for penetration. One of them is viropexis, which has been imagined to be a pinocytotic process whereby whole virus particles are incorporated into a pinosome that subsequently fuses with lysosomes, the lysosomal enzymes causing uncoating of the virus [1]. A number of other studies, however, suggest that penetration of enveloped viruses may be the result of fusion of the viral envelope with the host cell membrane [1,2]. In any case, penetration involves a close interaction between the viral envelope and the cell surface which might result in physical and chemical alterations of the plasma membrane.

The purpose of the present paper was 2-fold: (1) We wanted to know whether such alterations could be measured by comparing the nuclear magnetic resonance (NMR) spectra of infected and uninfected cells. (2) We were interested to know whether this technique could provide information on virus-cell systems in which virus replication is inhibited at a very early stage of the infection process. The systems analyzed were influenza A virus, Newcastle disease virus (NDV), and Rous sarcoma virus (RSV). With influenza virus, particles can be obtained in which the hemagglutinin glycoprotein of the viral envelope is present either as a larger polypeptide chain or as fragments which are derived from the large form by limited proteolytic cleavage. Similarily, NDV may contain either the cleaved or the uncleaved form of an envelope glycoprotein responsible for cell fusion and hemolysis. In previous studies it has been clearly shown that cleavage is necessary for infectivity of these viruses, but there is only circumstantial evidence that the primary function of these glycoproteins is a direct involvement in the penetration process [3—7].

In the avian RNA tumor virus system it is well established that the virus susceptibility of chicken embryo cells is under genetic control and that cells obtained from a given embryo have a certain phenotype with respect to their susceptibility to the various virus subgroups [8]. These viruses are also classified according to their host range which in turn is defined by the type of their envelope glycoprotein antigen [9]. Therefore, this system should allow us to compare the interaction of one and the same virus strain with susceptible and resistant cells.

Materials and Methods

Viruses. Strain Italien and strain Ulster of NDV, two avian strains of influenza A virus: virus N [A/chick/Germany/49 (Hav2Neq1)] and fowl plague virus [A/FPV/Rostock (Hav1N1)], and the Prague strain, subgroup B, of Rous sarcoma virus (RSV-PrB) have been used.

Seed stocks of the myxoviruses were grown in the allantoic cavity of embryonated eggs and were stored as infected allantoic fluids at —80°C. RSV-PrB has been passaged and cloned in chick embryo cells.

Virus growth. Influenza virus was grown in embryonated eggs and in chick embryo fibroblasts as described previously [4]. NDV was grown in embryonated eggs and MDBK cells [6]. Virus titres have been determined by hemagglutinin and plaque assays.

The B virus was grown in chick embryo fibroblast cultures from fertilized eggs and stored as tissue culture supernatant at −80°C. The eggs were from a leukosis-virus-free flock and kindly supplied by Dr. E. Vielitz, Lohmann-Tierzucht GmbH., Cuxhaven.

Virus purification. Virus was purified from cell culture medium or allantoic fluid by procedures described previously for myxoviruses [10] and for RSV-PrB [11], respectively.

NMR measurements. A Bruker WH 270 NMR spectrometer, with an internal deuterium lock and operating in the Fourier Transform mode was used to obtain the spectra. It is equipped with a Nicolet BNC 12 Computer with a 32 K memory and a Diablo Disk-System. A time delay of 41 μs between the end of the transmitter pulse and the beginning of the data acquisition was chosen automatically by software under our measuring conditions. All measurements were carried out at 37°C in buffered saline ^2H_2O, p^{2}H 7.4 (^2H_2O "100" Merck Sharp and Dohme) in 5-mm sample tubes with a spinning rate of about 30 s^{-1}. The cells were harvested either by mild trypsinization (0.025% trypsin, 2 min at 37°C) or by scraping with a rubber policeman. No difference in the cell NMR spectra were observed between the trypsinized cells and those mechanically harvested. The purified virus was washed twice with deuterated phosphate buffer, saline (p^{2}H = 7.4), sedimented at 150 000 × *g* and then added to the cell suspension. No attempts were made to add the virus before harvesting the cells. In a typical experiment $60 \cdot 10^6$ cells were used and the multiplicities used for virus infection varied from 0.1 to 10 plaque-forming units (p.f.u.)/cell. Experiments were carried out under sterile conditions, in order to prevent bacterial contamination. 2000 transients with a repetition rate between the pulses of 1.1 s were used to record a cell membrane lipid spectrum. 20 spectra were recorded over 15 h and stored on the disk. For intensity determinations a lipid extract in deuterated $CHCl_3/CH_3OH$ (20 : 1, v/v) was used as a standard sample. The chemical shifts, δ, were measured with respect to the ^{1}H NMR signal of sodium 2,2'-dimethyl-2-silapentane-5-sulfonate, used as external reference [12], in deuterated phosphate-buffered saline (δ = 0). The deuterated buffer was used as internal lock.

Results

^{1}H NMR spectra of intact chick embryo fibroblasts

Unlike L-cells, human lymphocytes, pig alveolar macrophages, 3T3 and 3T3 SV 40 cells, the chick embryo fibroblasts were, in our experience, among the few cell types which gave rise to proton spectra of the type observed for isolated membranes like sarcoplasmic reticulum [12,13] and rod outer segment vesicles [14]. The spectra consist of a broad line on which the "high resolution" signals of the mobile $-\overset{+}{N}(CH_3)_3$, $-(CH_2)_n-$, and $-CH_3$ groups are superimposed, with chemical shifts of δ = 3.3, 1.2, 0.9 ppm [12,13]. In order to get information about the percentage of lipid protons in the spectra of the cells, the intensities of the resolved peaks were compared with the corresponding peaks in the total cell lipid extract in $C^2HCl_3/C^2H_3O^2H$ (20 : 1, v/v). 4 mM sodium maleate solution, contained in a coaxially fixed capillary, was used as intensity standard for both samples. A curved baseline was chosen to approxi-

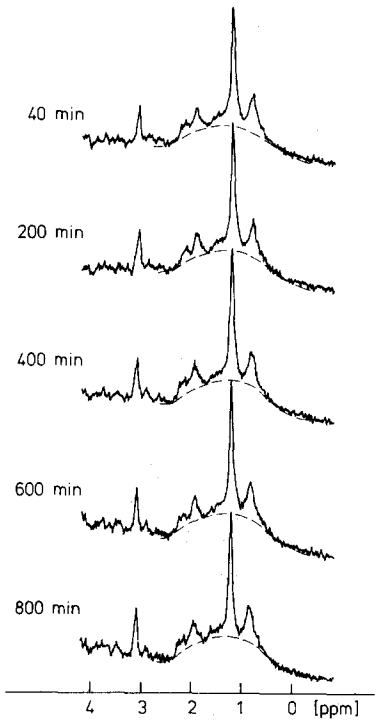

Fig. 1. 270 MHz ^1H NMR spectra of chick embryo cells suspended in deuterated phosphate-buffered saline, p^2H 7.4; $T = 37°C$. 2000 scans; $60 \cdot 10^6$ cells in 2 ml buffer. Spectra recorded during ≈14 h and stored on disk memory, every 40 min. Delay time: 41 μs. Time points refer to the period of incubation.

mate the broad, underlying, unresolved component (Fig. 1). The $-\dot{N}(CH_3)_3$, $-(CH_2)_n-$ and $-CH_3$ resonances were cut and weighed. The results are shown in Table I.

The cell spectra were also compared with the spectra of a sonicated suspension of the cell lipids in deuterated phosphate-buffered saline, $p^2H = 7.4$. The lipid suspension in deuterated phosphate-buffered saline was sonicated for

TABLE I

PERCENTAGE OF TOTAL LIPID PROTONS GIVING RISE TO SHARP RESONANCES IN THE ^1H NMR SPECTRA OF INTACT CHICK EMBRYO FIBROBLASTS AND IN THEIR LIPID VESICLES IN ^2H_2O AT 37°C

Reference: the total lipid cell extract in deuterated $CHCl_3/CH_3OH$.

Resonance	Percent of lipid protons observed in the high resolution part of the spectrum	
	Intact cells	Lipid vesicles
Terminal $-CH_3$	21 ± 2	100 ± 10 *
$-(CH_2)n-$	10 ± 1	30 ± 6
$-\dot{N}(CH_3)_3$	39 ± 4	100 ± 10

* The motion of the terminal methyl group is not restricted by cholesterol [17], in contrast to that of the methylene groups.

45 min in a bath type ultrasonic disintegrator, Schwoler 25 TG under N_2, at about 50°C. As seen in Table I, an increased percentage of the proton lipids are detected in the high resolution part of the lipid vesicle spectrum, but even there, only 60% of the hydrocarbon-chain methylene groups are observed.

It is possible to obtain ^1H NMR cell spectra with poor signal-to-noise ratio within 2 min; no changes appear to take place within the 40 min of incubation. Similar results were obtained by Gaffney et al. [15] using spin labels to investigate normal and RSV-PrB-transformed chick embryo fibroblasts. Over 60 min no change was observed in the order parameter of a fatty acid spin label incorporated in these cells.

Newcastle disease virus

Examination of Fig. 1 shows that no changes occur in the chick embryo fibroblast spectra over a period of 14 h, indicating that the organization of the lipids yielding the NMR spectra does not change.

Fig. 2a shows the time dependence of the NMR spectra of the fibroblast membranes when the cells are incubated with Newcastle disease virus (NDV) obtained from eggs, i.e. virus with cleaved glycoproteins, displaying full biological activity. Incubation was carried out at a multiplicity of 10 p.f.u., corresponding to about 1000 physical particles, per cell. As can be seen, profound modifications are observed in the ^1H NMR spectra (Table II). After 40 min of contact a very weakened choline signal is observed; a significant loss of intensity of the $-(CH_2)_n-$ and $-CH_3$ signals take place. These changes are accentuated at longer times, and after 14 h the spectrum consists of a broad

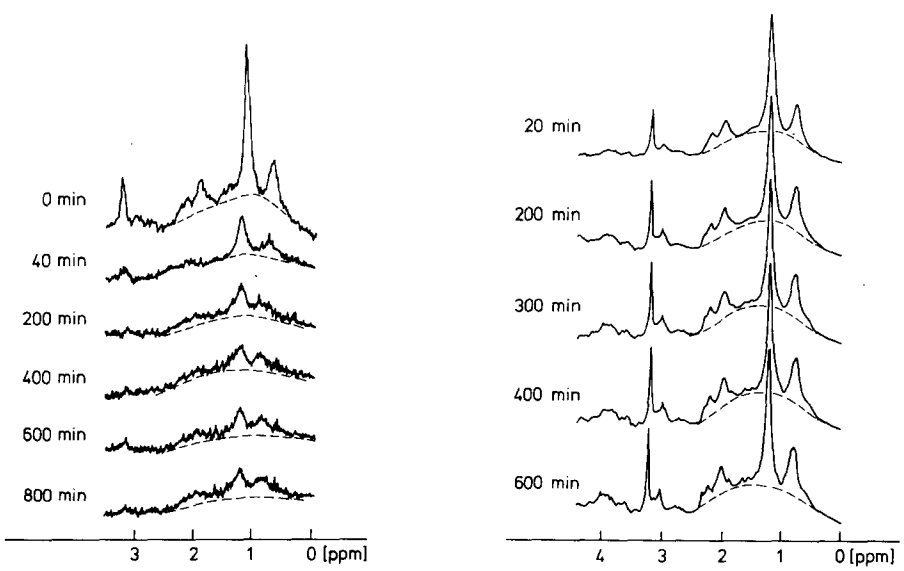

Fig. 2. (a) 270 MHz ^1H NMR spectra of chick embryo cells incubated with infectious NDV (strain Italien, grown in egg). Deuterated phosphate-buffered saline, p^2H 7.4; T = 37°C. 2000 scans. Multiplicity of infection 10 p.f.u./cell; $60 \cdot 10^6$ cells in 2 ml buffer. Spectra recorded during ≈14 h and stored on disk memory every 40 min. (b) 270 MHz ^1H NMR spectra of chick embryo cells incubated with non-infectious NDV (strain Ulster, grown in MDBK cells). All parameters as before.

TABLE II

PERCENTAGE OF TOTAL LIPID PROTONS GIVING RISE TO SHARP RESONANCES IN THE ^1H NMR SPECTRA OF THE CHICK EMBRYO FIBROBLASTS INFECTED WITH NDV

Number of experiments in parentheses.

Time of incubation (min)	Percent of $-(CH_2)_n-$ protons observed	Percent of the $-CH_3-$ protons observed	$\Delta\nu_{1/2}$ (Hz) * for the $(CH_2)_n$ peak
0	10 ± 1 (3)	21 ± 2 (3) *	34 ± 8
20	4 ± 0.4 (2)	10 ± 1 (2)	
40	3 ± 0.3 (3)	8 ± 0.8 (3)	48 ± 8
200	2.5 ± 0.25 (3)	6 ± 0.6 (3)	48 ± 8
400	2.5 ± 0.25 (3)	6 ± 0.6 (3)	60 ± 8
600	1.0 ± 0.1 (3)	4 ± 0.4 (3)	60 ± 8
800	1.0 ± 0.1 (3)	4 ± 0.4 (3)	60 ± 8

* The half-line widths were estimated by the procedure of Davis et al. [13]. A smooth baseline, corresponding to the line shape of the underlying broad component was interpolated. The height of the sharp signals was measured from the underlying line up to the peak maximum and the line width at half this height was taken as $\Delta\nu_{1/2}$ of the sharp line.

unresolved resonance. It is significant, however, that no new signals belonging to potential lipolysis products are observed.

In a second and a third run, it was observed that after 20 min of incubation, almost the same percentage of lipid protons were detected in the $-CH_3$ and $-(CH_2)_n$-resonances as after 40 min of incubation.

Similar results have been obtained using egg-grown virions of NDV strain Ulster which also contain cleaved glycoproteins and were fully infectious. However, when NDV particles of strain Ulster grown in MDBK cells, which are non-infectious due to the presence of uncleaved glycoproteins, have been used no significant changes in the ^1H NMR spectra of the cell membranes could be observed (Fig. 2b).

Influenza virus

Similar experiments were performed on fibroblasts exposed to influenza virus. Again, incubation was carried out at a multiplicity of about 10 p.f.u. (i.e. 1000 physical particles) per cell, and NMR spectra were recorded over a period of 10 h. Virus particles of strain N which, after growth in chick embryo fibroblasts, have the hemagglutinin in the uncleaved form and have a reduced infectivity failed to produce any alterations of the fibroblast membrane lipids over 600 min. However, after exposure to infectious virus, represented by strain N derived from embryonated eggs or by FPV, both having the hemagglutinin in the cleaved form, a small but distinct broadening of the methyl and methylene resonances occurs. In a typical experiment, for instance, the 'high resolution' methylene resonance of the fibroblasts had a half-line width of approx. 35 ± 8 Hz, (for half-line width estimation of high-resolution membrane signal see ref. 13) which after exposure to infectious virus N, increased to approx. 45 ± 8 Hz and, after exposure to FPV, to approx. 60 ± 8 Hz. In contrast to NDV, modification of the choline resonance was not observed. The broadening of the methyl and methylene resonances is indicative of membrane stiffening as in the case of NDV.

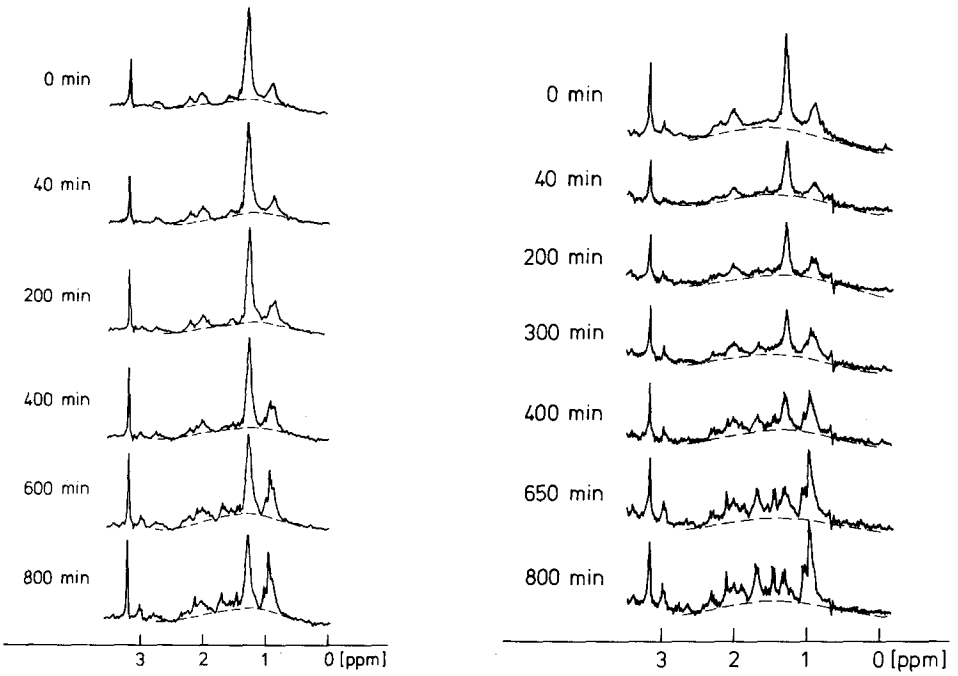

Fig. 3. (a) 270 MHz ^{1}H NMR spectra of chick embryo cells, C/B line (non-susceptible for RSV-PrB) incubated with 10 p.f.u./cell of RSV-PrB in deuterated phosphate-buffered saline, p^{2}H 7.4; T = 37°C; 60 · 10^6 cells in 2 ml buffer. Spectra recorded during ≈14 h and stored on memory disk, every 40 min. (b) 270 MHz ^{1}H NMR spectra of chick embryo fibroblast C/E line (susceptible for RSV-PrB) incubated with 10 p.f.u./cell of RSV-PrB in deuterated phosphate-buffered saline, p^{2}H 7.4; T = 37°C; 60 · 10^6 cells in 2 ml buffer. Spectra recorded during ≈14 h and stored on memory disks, every 40 min.

Rous sarcoma virus

When the Prague B strain of RSV was used with two genetically different chick embryo fibroblast lines, C/B barring the penetration into the cell and C/E permissive for this virus, striking differences were observed in the time evolution of the NMR spectra (Figs. 3a and 3b). After about 650 min, the NMR spectrum of -$(CH_2)_n$- and -CH_3 groups in the membranes of the permissive cell

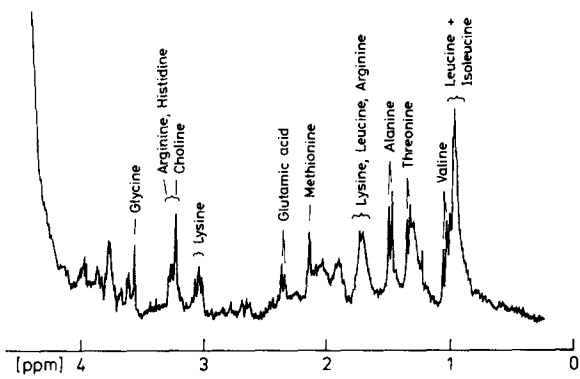

Fig. 4. Assignments [22] in the fully resolved 270 MHz ^{1}H NMR spectrum of the amino acids appearing after 6 h of contact of chick embryo fibroblasts C/E with RSV-PrB.

line (Fig. 3b) has been replaced by the amino acid pattern, whereas it was still intact in the non-permissive cells (the $\overset{+}{N}(CH_3)_3$ signal is not influenced). The measurements were carried out for about 14 h, and while the spectrum of the C/E cells consisted entirely of the resolved amino acid lines, that of the C/B cells also showed the characteristic resonances of the normal lipid bilayer, even after 800 min of contact, although some weak amino acid resonances are also detectable in this system. Under the experimental conditions used, no ^1H NMR spectra of any of the virus particles could be detected.

Discussion

The results shown above indicate that myxoviruses containing biologically active glycoproteins perturb the fluid lipid bilayer in cellular membranes. The fact that a considerable change occurs in the spectrum of the cells after only 20 min of incubation and before any biochemical changes could be detected strongly supports this claim.

The perturbation is most pronounced in fibroblasts incubated with NDV. The changes in the ^1H NMR spectra observed upon virus addition point to a considerable rigidization of the lipid bilayer. The loss of intensity and broadening of the $-(CH_2)_n-$signals when cholesterol was added to phospholipids were reported by Chapman and Penkett [18] to be indicative of mobility changes in the phospholipid hydrocarbon chain. Radioactivity measurements indicated a rather rapid migration of [^{14}C]cholesterol from the virus envelope into the cell plasma membrane [19]. After 30 min of contact about 10% of ^{14}C-labeled cholesterol as compared to about 2% of [^{3}H]choline lipids had been transferred from the virus into the cell. The cholesterol enrichment of the plasma membranes, however, cannot be the only factor responsible for this broadening of the resonances of the $-CH_3$ and $-(CH_2)_n-$ groups observed in the NMR spectra of these systems; besides the cholesterol transfer other causes combine to produce the observed effects.

Interestingly, no new signals indicative of cleaved phospholipids, of free fatty acids or the diglycerides or triglycerides can be detected, so that we conclude that the extensive alteration of the cell membrane lipid bilayer fluidity by the infective viruses is of a physical rather than an enzymic nature. We attribute these observations to the fact that an intermixing of the lipid bilayers of the NDV envelope and the plasma membrane takes place. Such intermixing is compatible with fusion between both membranes as has been proposed as a penetration mechanism for paramyxoviruses [1,2].

Although the membrane alterations induced by influenza virus are qualitatively similar to those observed with NDV, there is one aspect which distinguishes the behaviour of the NDV-chick embryo fibroblast system from that of the influenza virus-chick embryo fibroblast system: the loss of area under the peaks corresponding to the terminal $-CH_3$ and $-(CH_2)_n-$ groups in the lipid hydrocarbon chain is much more dramatic than it is with the infectious influenza viruses, which points to quantitative differences in the mechanism of cell-virus interaction in these systems.

NDV containing uncleaved fusion glycoprotein and influenza virus containing uncleaved hemagglutinin glycoprotein do not seem to affect the NMR

spectra of the fibroblast membranes markedly over a period of up to 15 h. Since these viruses adsorb to host cells [4–6] but have no infectivity, do not replicate and, according to the data presented here, do not perturb the membrane spectra, it is suggested that infection is blocked at the stage of penetration.

The changes observed in the NMR spectra of the C/E (susceptible) in comparison to the C/B (non-susceptible) fibroblast lines when exposed to RSV-PrB were quite dramatic. Whereas with the insusceptible system C/B + RSV-PrB the cell membrane lipid bilayer resonances are observed during the whole 800 min of the experiment, with the susceptible C/E + RSV-PrB system the cell membrane lipid bilayer resonances collapse after less than 400 min. Simultaneously, well-resolved resonances belonging to amino acids appear, more markedly in the C/E + RSV-PrB system, but they are detectable in the system C/B + RSV-PrB as well. The reasons for this appearance of resolved amino acid resonances observed with RSV might be: (1) Since the PrB strain of RSV has been isolated from transformed cells notably richer in cell proteases than their normal counterparts [20], the virus may have dragged on its surface some host-cell proteolytic enzymes which then are active on the new host-cell membranes. (2) The cell becomes leaky for internal small peptides (or amino acids) upon virus adsorption. Pasternak and Micklem [21], in a study of the Sendai virus-induced agglutination and fusion of Lettre and P815Y cells, observed that the interaction of this virus with the cells caused a weakening of the membrane integrity so that intracellular metabolites leaked out. This alternative is currently being studied in our laboratories with chick embryo fibroblast plasma membrane vesicles.

It appears that with the non-susceptible C/B cells, infection with RSV-PrB does not affect the membrane lipid fluid bilayer NMR spectrum even after long exposures, though some amino acid signals can be detected in the spectrum. On the other hand, with the susceptible C/E cells, infection with RSV-PrB leads to the disappearance of the lipid bilayer spectrum of the cell membrane(s) within about 5 h and the appearance of completely resolved amino acid (or oligopeptide) NMR spectra. This indicates extensive perturbation of the lipid organization in the cell membrane under the influence of virus in the susceptible cells only. This perturbation does not seem to be of enzymatic nature, since no free fatty acid or di- or triglyceride NMR signals could be detected.

The data presented here are compatible with the common concept that infection by paramyxoviruses is initiated by a penetration mechanism involving fusion of the viral envelope with the plasma membrane of the host cell. Furthermore, our data suggest that influenza and RNA tumor viruses penetrate by a similar mechanism.

Endocytosis of the virus, not leading to the alteration of the membrane lipid composition and/or to the protein : lipid ratio in the membrane, would presumably not lead to the signal broadenings which were observed. At most, digestion by the lysosomal enzymes of plasma membrane fragments incorporated into the cell during the endocytosis process would give rise to high resolution NMR signals, which were not observed with the myxo- and paramyxoviruses.

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