EFFECTS OF TEMPERATURE AND HOST LIPID COMPOSITION ON THE INFECTION OF CELLS BY NEWCASTLE DISEASE VIRUS

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Received December 4,1974

SUMMARY. Newcastle disease virus was adsorbed to BHK21 monolayers at 4°, the excess virus removed, and the cells incubated at various temperatures from 0-40°. Loosely associated virus was then eluted from the cells which were subsequently incubated for assay of infectivity. A critical temperature for optimal infectivity was observed at approximately 15°, a critical temperature which has been detected for a number of other surface phenomena in cultured mammalian cells. Alteration of fatty acid composition in cellular phosphoslipids changed the critical temperature for infectivity indicating that the physical state of host cell membrane lipids is a determinant of some step in the infection of cells by paramyxoviruses.

The penetration of cells by paramyxoviruses has been shown to be a temperature-dependent process on the basis of cytological observation, whereas viral adsorption to the cell surface is reported to occur independently of incubation temperature (1-2). A number of cell surface phenomena have critical or characteristic temperatures which respond to the beginning or end of a membrane lipid phase transition, i.e., the temperatures at which the bulk membrane lipid phase of one or both membrane monolayers becomes totally melted or frozen (3-5). In animal cell membranes, these phenomena include binding of the lectin concanavalin A (Con A) (6-7), Con A-mediated agglutinability (6-7), the transport of metabolites (8) and the lateral mixing of cell surface antigens (9). In this paper we report observations which indicate that a virus-cell interaction leading to optimal infectivity is critically dependent upon the physical state of the membrane lipids.

METHODS. Newcastle disease virus, strain HP-16, was propagated in embryonated chick eggs and purified by published procedures (10). Media and culture procedures for the growth of BHK21 cells and procedures for the preparation of salt fractionated serum (SFS) are described in the companion paper (11). Procedures for lipid extraction and analysis and for the prepa-

ration of the fatty acid supplements Tween-nonadecanoic acid (Tw-19:0) and Tween-linolenic acid (Tw-18:3) are those used previously (12-13). The Tweens were employed at a concentration of 8 µg/ml of the fatty acid moiety.

RESULTS. In order to study the effects of temperature and membrane lipid composition on virus-cell interactions leading to infectivity, it was first necessary to develop a procedure for removing loosely associated virus from the cell surface. The experiment described in Figure 1 shows that this

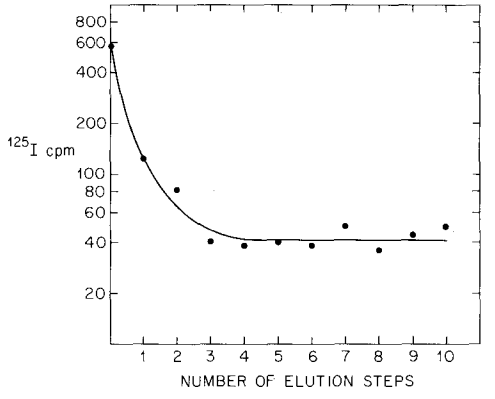

FIGURE 1. Elution of ^{125}I-labeled NDV from cells. Virus remaining attached to monolayers was estimated by dissolving the monolayer in 2.5 ml of aqueous 5% Triton X-100 and by assaying an aliquot for radioactivity by scintillation counting (10). Radioiodinated NDV (approximately 2 x 10^7 plaque forming units (PFU) in 0.1 ml of MEM + 5% calf serum and 10% tryptose phosphate broth) was incubated for 30 min at 4° with confluent BHK21 monolayers in 35 mm diameter dishes (Linbro). Unadsorbed virus was removed by aspiration. Reversibly adsorbed virus was then eluted with 2.5 ml portions of cold Tris•saline (11). The duration of each incubation for elution was 15 min, and each point represents the average of three determinations. For radioiodination, 1-2 mg (as protein) of purified virus was suspended in 0.5 ml of a solution containing 0.01 M NaCl, 0.01 M sodium phosphate buffer (pH 7.4), 5 µg of lactoperoxidase (Sigma) and 100 µCi of carrier-free ^{125}I-NaI (Schwartz/Mann). Iodination was initiated at 30° by the addition of 5 µmoles of H_2O_2, and additional 5 µmole portions of peroxide were added at 1 min intervals for 5 min, at which time the reaction was terminated by the addition of excess mercaptoethanol. The specific activity of the virus was approximately 1500 cpm/10^7 PFU.

can be achieved by successive stepwise elution with Tris•saline solution. A steady state level of cell-associated virus was consistently observed after 4 elution steps. Identical results were obtained when infectivity (plaque forming units, PFU) was the measured parameter.

Figure 1 describes the infectivity obtained when monolayers to which virus had been attached at 4° were incubated at various temperatures from 0-40° prior to the elution procedure. The cells used in this experiment were grown under three different conditions to modify the fatty acid composition of phospholipids (Table 1). These are: A) growth with a nonadecanoic acid (19:0) supplement to increase the saturated fatty acid content, B) growth in the absence of fatty acid supplement, and C) growth with a polyunsaturated fatty acid supplement (18:3) to increase the double bond content. With cells grown under condition B, the critical temperature for optimal infectivity was between 12 and 16°. A number of other cell surface

TABLE 1. Fatty acid composition of phosphatides[a] from BHK21/13 cells grown in control medium and in medium containing Tween-fatty acid supplements.

Fatty acid	19:0 supplement[b]	Control[c]	18:3 supplement[b]
14:0	0.5	2.1	0.1
15:0	0.8	1.9	0.2
16:0	5.0	17.9	7.8
16:1	1.0	15.7	1.7
17:0	4.4	0.2	0.7
17:1	3.7	0.4	0.5
18:0	6.4	10.8	20.0
18:1	30.3	43.6	39.0
18:2	3.3	1.7	6.2
18:3	-0-	-0-	15.7
19:0	37.9	-0-	-0-
19:1	6.0	-0-	-0-
20:1	0.6	1.2	3.7
20:4	-0-	2.5	4.2
Others	0.1	2.0	0.2
Unsaturates	45.0	67.1	71.2
Polyunsaturates	3.3	4.2	26.1

[a]Phosphatidylcholine plus phosphatidylethanolamine, which constitute approximately 80% of the total cellular membrane phospholipids (12).

[b]BHK21/13 cells grown in minimal essential medium (MEM) + 15% SSF + 8 μg/ml of either Tw-19:0 or Tw-18:3. The cells grown with the Tw-19:0 supplement were those described in Table 4 of the companion paper (11).

[c]BHK21/13 cells grown in MEM + 5% calf serum + 10% tryptose phosphate broth (11).

membrane phenomena have a critical temperature at approximately 15° (6-9), which appears to be the temperature at which the freezing process for surface membrane lipids is completed in mammalian cells cultured in the absence of a fatty acid supplement (8). With cells grown under condition A to enrich for saturated fatty acids, the critical temperature was increased; and in cells grown under condition C to enrich for polyunsaturated fatty acids, the critical temperature was decreased. These are the results predicted on the basis of the modifications in lipid composition described in Table 1. Furthermore, the shifts in critical temperature are similar to those observed for lectin binding and lectin-mediated hemadsorption by cultured mouse fibroblasts where procedures similar to those reported here were used to modify fatty acid composition (6). These data indicate that some step in the infectivity process is sensitive to the physical state of membrane lipids.

The susceptibility of viral infectivity to treatment with neutralizing antibody was determined under a number of the conditions encountered in the experiment described in Figure 1. Nearly all the virus which attached to cells at 4° was neutralized when antibody was added at 4° at the end of the attachment period (Table 2, condition 2). When antibody was added at the end of the attachment period, and the monolayers were eluted prior to assay, a 5-fold increase in infectivity was observed (Table 2, condition 3). No further increase in infectivity was detected when the cells were incubated at 37° after antibody addition, but prior to elution, indicating that this increase in infectivity was affected during the elution procedure. The data in Table 2, conditions 4-5 indicate that antibody has no effect when added at the end of the elution period.

DISCUSSION. The experiment described in Figure 2 gives clear indication that some step in the infection of cells by NDV has a critical temperature which is determined by the physical state of the membrane lipids. The data described in Table 2 show that little infectivity observed either above or below this critical temperature can be blocked by neutralizing antibody.

TABLE 2. Effects of neutralizing antibody* on virus adsorbed to cells at 4°.

	Condition	Temperature	PFU ± S.D.
1.	Plaque directly at 37°		190 ± 15
2.	Add antibody at 4° and incubate for 30 min, then plaque at 37°		4 ± 1.6
3.	Add antibody at 4° and incubate for 30 min at the indicated temperatures, elute, and plaque at 37°	4° 37°	20 ± 3.4 21 ± 4.1
4.	Incubate at the indicated temperatures for 30 min, elute, then plaque at 37°	4° 37°	34 ± 5.0 56 ± 4.1
5.	Incubate at the indicated temperatures for 30 min, elute, add antibody and incubate for 30 min at 4°, then plaque at 37°	4° 37°	26 ± 2.5 59 ± 5.3

*Neutralizing antibody to Newcastle disease virus was obtained by injecting 5 x 10^9 PFU of purified virus (10) into rabbits subcutaneously at one week intervals for a minimum of six weeks. The antiserum was heated for 30 min at 56° to inactivate complement and diluted 100-fold with MEM prior to use.

In all the experiments, virus (190 PFU when plaque directly) was added to the BHK21 monolayers ("control" conditions, Table 1) at 4° in a volume of 0.1 ml, and the mixture incubated for 30 min at the same temperature. Excess fluid was aspirated from the plates and the experiments were conducted as described above. Addition of antibody was in a 1.0 ml volume of Tris·saline, and excess antibody solution was removed by aspiration after the 30 min incubations at 4°. Incubations in the absence of antibody were likewise conducted as described for Figure 1. All experiments were in triplicate, and results are expressed as the mean ± the standard deviation.

Since the critical temperature corresponds to what is probably the point where the bulk membrane lipid phase becomes frozen (8), it is worthwile to list the most likely processes in infectivity which might be influenced by the freezing of membrane lipids. These are: 1) inhibition of lateral diffusion of host or viral membrane components, leading to cell-virus association not subject to disruption by elution, 2) inhibition of engulfment (but not of penetration) of the virions owing to reduced flexibility of the host cell membrane and 3) inhibition of penetration by infecting virions. Though our data do not pinpoint the precise event which is restricted by a change in state of membrane lipids, they make some of these possibilities appear more likely than others.

With respect to the first process, a requirement of lateral motion of

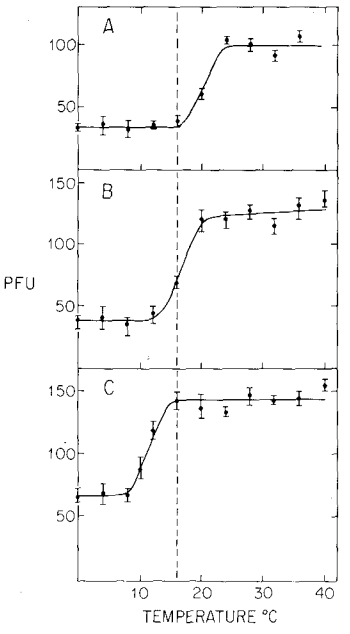

FIGURE 2. Effect of lipid composition and temperature on infectivity by NDV. Host cells in A were grown with a Tw-19:0 supplement, cells in B were grown without a supplement and cells in C were grown with a Tw-18:3 supplement (see METHODS and Table 1 for details). The dashed verticle line drawn arbitrarily at approximately 17° is to assist in visualization of the shifts in critical temperature for infectivity. Viral adsorption (approximately 750 PFU per monolayer, a multiplicity of approximately 10^{-3}) was at 4° for 30 min as described for Figure 1. After unattached virions were removed by aspiration, culture medium which had been equilibrated at various temperatures (0-40°) was added for a 30 min incubation with constant gentle agitation. Incubation medium was then removed and four viral elution steps were conducted at 4° as described for Figure 1. Cell monolayers were then overlayed with 1.0% agar-medium and incubated for three days at 37° under humidified air containing 6% CO_2. Cell monolayers were stained with 0.5% Neutral Red in Tris•saline for 4 hr at room temperature to allow for visualization of plaques. Each point represents the average PFU from three determinations, and the range of values is indicated by a bar.

virion proteins for infectivity seems unlikely. The membranous envelope of NDV, like the surface membrane of cultured mammalian cells, undergoes a change of state in lipids at approximately 15° (8). Were both cellular and membrane "fluidity" required for optimal infectivity, enrichment of the host membrane with polyunsaturated fatty acids would probably not have given rise to the observed decrease in critical temperature. This same argument applies to process 3, i.e., penetration. An intriguing debate resides with

the question of whether the infectious virion penetrates by cell-virus membrane fusion or by phagocytosis (2). Our data indicate that a fluid membrane lipid phase in the virus is not a prerequisite for infectivity. Thus if the observed critical temperature does result from an effect on penetration, it is likely that the virion must either enter the cell by phagocytosis, or by a cell-virus membrane fusion process that requires fluidity in only the host cell membrane lipids.

Morgan and Howe have shown by electron microscopy that paramyxovirions appear to bind to cells at low temperature (0-4°), but do not penetrate into the cell under this condition (1). Our data, when considered together with those of Morgan and Howe, might indicate that separate processes are responsible for the different efficiencies in infectivity observed with cells incubated on different sides of the critical temperature prior to the elution procedure (Figure 2). For example, infectivity observed below the critical temperature might be due to virions which attach with sufficient strength to cells to resist elution. The increased increment of infectivity observed above the critical temperature could then arise from additional virions becoming firmly attached or engulfed, from virions becoming internalized within the cells, or from a combination of these effects.

ACKNOWLEDGMENTS. This work was supported in part by USPHS grants AI-10733 and GM-18233. CFF is the recipient of USPHS Career Development Award GM-42359.

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