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On the Role of Lysophosphatides in Virus-Induced Cell Fusion and Lysis†

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ABSTRACT: Three strains of Newcastle disease virus (NDV-HP-16, NDV-L-Kansas, and NDV-N) were propagated in chick embryo fibroblasts, equilibrium labeled with $^{32}P_i$, and the composition of phospholipid in the membranous envelope of the virions determined. A phospholipid identified as monoacylphosphatidylserine was consistently observed in the viral strains which are listed as follows in their order of decreasing abundance of lysophosphatidylserine: NDV-HP-16 > NDV-L-Kansas ≫ NDV-N. The phosphatidylserine concentration in the virion envelopes of these strains decreased in proportion to the increase in the monoacylphosphatidylserine concentration. No other lysophosphatide was observed in significant quantity in virions of these strains. The degree of cell fusion induced in mouse fibroblast monolayers by each of the viral strains was independent of the lysophosphatidylserine content of the virions. The ability of the viral strains to induce fusion from within, i.e., that occurring in cells that are actively propagating virus, was: NDV-L-Kansas > NDV-HP-16 ≫ NDV-N. The ability of the viral strains to induce fusion from without, i.e., that occurring in response to incubation of cells with large quantities of irradiated virus, was: NDV-HP-16 > NDV-N ≫ NDV-L-Kansas. On the basis of these findings we conclude that there is no direct correlation between the level of lysophosphatide in the virion and its ability to induce cell membrane fusion. A direct correlation was observed, however, between the presence of high monoacylphosphatidylserine content and the ability of a strain to produce lytic infection.

Cell membrane fusion plays roles in a number of normal physiological processes, e.g., in the fusion of mononuclear myoblasts to form multinucleated fibers during the development of skeletal muscle (Yaffe, 1970) and in the endocytic and exocytic process which lead to pinocytosis and secretion (Poste and Allison, 1973; Korn, 1974; Korn et al., 1974). Membrane fusion leading to the formation of multinuclear cells also occurs in response to viral infection (Kohn, 1965). In recent years considerable attention has been directed to the possibility that monoacyl derivatives of membrane phospholipids might play some central role in membrane fusion processes. This hypothesis was prompted by the observation that lysophosphatides are capable of promoting both homotypic (Lucy et al., 1971) and heterotypic fusion of cells (Keay et al., 1972).

In this report we describe the use of Newcastle disease virus (NDV) to test for lysolipid involvement in membrane fusion occurring in response to paramyxoviruses. NDV provides an excellent biological model since different strains of this virus vary in their abilities to induce cell membrane fusion scored by the formation of multinucleated cells. NDV can cause fusion via two independent routes, classified as fusion from within (Kohn, 1965) and fusion from without (Bratt and Gallaher, 1969, 1970). Fusion from without does not require infectious virus but does require extremely high multiplicities of infection. Whereas fusion from within requires the addition of only small quantities of virus, the added virions must be infectious, and the host cell must be capable of supporting viral protein synthesis.

The present report describes studies in which we have tested for correlations between lysophosphatide concentrations in NDV and the efficiency of the virus in inducing fusion from without. We have also tested for correlations between lysophosphatide levels in newly produced virions and fusion from within occurring at the time of production of these virions.

Materials and Methods

Materials. The following phospholipid standards were

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purchased from Applied Science Laboratories, State College, Pa.: phosphatidylcholine, phosphatidylinositol, phosphatidic acid, sphingomyelin, lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylserine. Cardiolipin and phosphatidylglycerol were products of Supelco, Bellefonte, Pa. Lysophosphatidylinositol was the generous gift of Dr. W. Thompson, Toronto, Canada. Medium 199, chicken serum and dialyzed calf serum were obtained from Gibco, Berkeley, Calif. Tryptose broth was the product of Difco Laboratories, Detroit, Mich.

Cells. Primary cultures of chick embryo fibroblasts were established by plating cells from 10-day old embryos on 100-mm dishes (Falcon Plastics, Oxnard, Calif.) at a density of 5×10^6/plate. Cultures were grown to confluency at 41° in 95% air–5% CO_2 atmosphere. Secondary cultures were prepared from these primary cultures and used throughout these studies. Both secondary and primary cultures were maintained in modified medium 199 supplemented with 4% (v/v) dialyzed calf serum and 10% (v/v) tryptose broth. Dialyzed chicken serum was added to the medium at 1% (v/v) for primary cultures but was omitted in secondary cultures. Modified medium 199, made with Earle's modified salts, contained no sodium phosphate, sodium acetate, cholesterol, adenosine, or adenylic acid. In order to achieve equilibrium labeling of cellular and viral lipids, 10 μCi/ml of $^{32}P_i$ was included in the culture medium for at least four generations of growth prior to infection with virus.

Virus. Newcastle disease virus strains NDV-HP-16 and NDV-N were obtained from M. Bratt and NDV-L-Kansas, from W. S. Robinson. All virus stocks were maintained by propagation in the chorioallantoic cavities of 10-day old chick eggs. To obtain monolayer-grown virions the egg-grown stocks were diluted in 0.025 *M* Tris-HCl buffer (pH 7.4) containing 0.14 *M* NaCl (Tris-saline solution) and adsorbed to chick cell monolayers for 30 min at 37°. The multiplicity of infection was unity. After unadsorbed virus was removed by washing with Tris-saline solution, fresh growth medium was added and the cultures were incubated at 41°. At specified times the medium was collected and the virus purified by previously described methods after cell debris and mitochondria were sedimented by centrifugation at 10,000*g* for 10 min (Samson and Fox, 1973). The plaque forming assay used for determining virus titer was that described by Samson and Fox (1973).

Lipid Extraction and Analysis. Purified virus was transferred into 20 volumes of chloroform–methanol 2:1 (v/v) and the lipids were extracted by shaking for 2 hr at room temperature. The extract was subsequently washed with 0.9% saline (Folch et al., 1957), and the lipids were applied to the origin of a thin-layer plate. Phospholipids were separated by two-dimensional chromatography (Parkes and Thompson, 1973) and localized by autoradiography. Radioactive lipids were then scraped into vials for counting. Authentic lipid standards, cochromatographed with the labeled lipids or processed under identical conditions, were utilized to identify the radioactive lipids. For confirmation of their identity, certain lipids were eluted (Arvidson, 1968) and subjected to milk alkaline hydrolysis (Dawson, 1967). Alkali-labile material was localized using ninhydrin and phosphate-specific sprays (Dawson, 1967).

Fusion Studies. Chick fibroblast secondaries used in fusion from within studies were prepared in multidish trays each containing six 35-mm diameter wells (Linbro Plastics, Van Nuys, Ca.). Virus diluted to 2 hemagglutination units/ml (Shibuta et al., 1971) was added to each culture. After a 60-min period for virus adsorption at 41°, unadsorbed virions were removed by aspiration, and fresh medium was added. The infected cells were then incubated at 41° for various time intervals. The number of fusion events per cell was determined after fixing and staining (Kohn, 1965) by subtracting the average number of nuclei per uninfected cell from the average number of nuclei per infected cell (Bratt and Gallaher, 1972).

Microtest plates with 6-mm diameter wells (Falcon Plastics) were employed for the FFWO investigation to conserve virus. The virus stocks were inactivated by a 10-min ultraviolet irradiation under a Mineralite lamp. In this assay 0.1 ml of virus suspension (40 hemagglutination units/ml) was added to each well and the system incubated for 5 hr at 41°. The extent of fusion was estimated by the procedure described for assessment of fusion from without.

Results

Viral Lipid Composition. Conventional techniques are not sufficiently sensitive for analysis of phospholipid composition in virus preparations purified from a monolayer culture. A system that provides this level of sensitivity involves the use of cultured cells grown in the presence of $^{32}P_i$ for at least four generations to provide a uniform labeling of all cellular phosphate-containing compounds. A method similar to that employed here has been described by Quigley et al. (1972). The distribution of $^{32}P_i$ among the phospholipids of virus grown in cells labeled in this manner was shown by them to be equal to the mass distribution of the lipids. The percentage distribution of phospholipids from three strains of NDV, harvested at selected time intervals after infection, is described in Table I. Each strain is distinguished by the relative content of a phospholipid identified as lysophosphatidylserine. NDV-HP-16 has the highest level of this monoacyl lipid, accounting for approximately 17% of total phospholipids in virions produced between 8 and 12 hr postinfection, while the maximal lysophosphatidylserine level displayed by NDV-N was less than 2% for all time intervals tested. An intermediate concentration of monoacylphosphatidylserine was observed in the phospholipids of NDV-L-Kansas. In addition, the phosphatidylserine content appears to complement the lyso derivative concentration, the sum of serine-containing phospholipids being a relatively constant percentage of the total phospholipid radioactivity. The phospholipid composition of uninfected chick fibroblasts as revealed by the equilibrium labeling method is shown in Table II. The level of lysophosphatidylserine is very nearly the same following isolation of lipids from both NDV-HP-16 infected and uninfected cells.

Identification of Monoacylphosphatidylserine. The identification of radioactively labeled lysophosphatidylserine from viral strain NDV-HP-16 was based on criteria as follows. Under the condition utilized for two-dimensional thin-layer chromatography, it migrated with authentic monoacylphosphatidylserine and not with any diacyl lipid tested including phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, or cardiolipin. Monoacyl derivatives of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol also displayed unique mobilities unlike that of the labeled viral lipid. Phospholipase A (*Crotalus adamanteus*) digestions resulted in a product which, in the thin-layer system utilized, did not migrate from the origin, indicating the presence of an acyl ester in the starting material. After mild

Table I: Phospholipid Composition[a] of Three Strains of Newcastle Disease Virus Grown in Chick Embryo Fibroblasts.

		Percent Distribution of $^{32}P_i$ among Viral Phospholipids[c]						
		Time Interval (hr)						
Strain	Lipid[b]	0–4	4–8	8–12	12–16	16–20	20–24	24–36
NDV-HP16	LPS	11.4	15.4	17.3	15.1	12.7	15.1	
	SM	16.3	25.3	26.1	29.6	28.1	31.2	
	PC	35.7	24.0	18.8	16.2	17.6	9.1	
	PE	27.1	28.7	31.1	32.7	33.8	32.9	
	PS	<0.3	<0.3	1.0	0.8	<0.3	2.5	
	PI	4.6	1.6	2.0	1.6	2.7	3.6	
	Unident.[d]	4.9	5.0	3.7	4.0	5.1	5.6	
NDV-L-Kansas	LPS		12.8	8.9	8.2		7.9	
	SM		11.3	26.5	27.6		19.2	
	PC		23.9	21.2	22.6		26.4	
	PE		30.8	30.8	27.1		31.7	
	PS		5.4	4.4	3.3		2.7	
	PI		4.6	1.0	2.0		4.0	
	Unident.		11.2	7.2	9.2		8.1	
NDV-N	LPS			1.8	0.7		0.7	1.0
	SM			22.8	22.7		31.0	32.9
	PC			17.0	21.5		11.9	12.6
	PE			32.3	31.3		35.3	33.2
	PS			11.2	13.1		14.0	13.2
	PI			4.1	1.1		2.0	2.5
	Unident.			10.8	9.6		5.1	4.6

[a] Secondary cultures of chick embryo fibroblasts, equilibrium labeled with $^{32}P_i$ (Materials and Methods), were infected with each strain of virus at time 0. Every 4 hr the medium was collected and replaced with fresh medium. Lipids from the virus recovered and purified from the culture medium were separated by two-dimensional thin-layer chromatography and localized by autoradiography. [b] Abbreviations used are: LPS, lysophosphatidylserine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. [c] For NDV-HP-16 the 0–4 and 20–24-hr samples are values from single determinations. For NDV-L-Kansas and NDV-N all the values are from single determinations. The independently determined values for phospholipids for the (4–8), (8–12), (12–16), and (16–20) hr intervals for NDV-HP-16 were respectively as follows: LPS, (13.7, 17.2), (18.1, 19.9, 13.9), (12.4, 17.8, 15.2), and (12.0, 13.5); SM, (25.6, 24.8), (22.1, 26.5, 29.8), (28.3, 27.3, 33.3), and (34.2, 21.9); PC, (25.1, 23.0), (20.9, 16.9, 18.4), (20.6, 14.7, 12.9), and (13.6, 21.6). [d] Unidentified lipid comprises radioactive material at the solvent front and the origin of the thin-layer chromatograms.

Table II: Phospholipid Composition of Chick Embryo Fibroblasts[a] before and after Infection with Newcastle Disease Virus, Strain HP-16.

	Percent Distribution of ^{32}P in Phospholipids			
		Duration of Infection (hr)		
Phospholipid[b]	Uninfected	0–4	0–12	0–20
LPS	4.6	6.7	4.9	5.2
LPC	1.6	2.5	1.7	5.6
SM (+PI)	13.5	16.2	16.1	17.1
PC (+PS)	45.2	40.0	40.6	39.2
PE	31.3	31.1	31.8	29.7
Unident.	3.7	3.5	4.9	3.4

[a] Cells removed from culture dishes by a 5-min treatment with 0.12% trypsin in Tris-saline solution at 41° were harvested by centrifugation for 5 min at 1500*g* and transferred directly to chloroform–methanol 2:1 for lipid extraction. Phospholipids were separated by thin-layer chromatography in one dimension using chloroform–methanol–ammonium hydroxide–water (120:70:4:6 v/v). [b] See Table I.

alkaline hydrolysis over 97% of the label was rendered water soluble whereas only 20% of the label was found in the aqueous phase prior to hydrolysis. This change in partitioning after alkaline hydrolysis is characteristic of the presence of an ester bond (Dawson, 1967). The water-soluble product from mild alkaline hydrolysis, when subjected to paper chromatography and electrophoresis (see Materials and Methods) yielded a radioactive species with a R_f of 0.28. Glycerophosphorylserine in the same system has a relative mobility of 0.30 (Dawson, 1967). The radioactive material obtained from lysophosphatidylserine after mild alkaline hydrolysis and chromatography also cochromatographed with ninhydrin-positive glycerophosphorylserine, derived by the same procedure from authentic phosphatidylserine. Thus the accumulated evidence strongly favors the identification of monoacylphosphatidylserine as a major phospholipid present in NDV-HP-16, grown on chick secondary monolayers.

Lysophosphatide Content and Cell Fusion. The amounts of virus used to produce cell fusion from within and without are expressed as hemagglutination units per milliliter as described in Materials and Methods. The ratios of infectious particles (plaque forming units) to hemagglutination units were approximately the same for the lytic strains used. The number of plaque forming units per hemagglutination units for NDV-HP-16 and NDV-L-Kansas were 9.2×10^5 and 5.5×10^5, respectively. The fraction of added virions adsorbing to cell monolayers was also nearly constant. Approximately 20–30% of the input NDV-L-Kansas, NDV-N, and NDV-HP-16 virions, as determined either by infectivity (strains L-Kansas and HP-16) or viral $^{32}P_i$ content (all three strains) adsorbed to the cell monolayer. The methodology used for assaying viral adsorption was that described by Li et al. (1975). These findings indicate that differences in the abilities of viral strains to cause fusion from within and without are unlikely to arise from differences in either the efficiency of infection or adsorption.

Table III: Fusion of Chick Embryo Fibroblasts Induced by Three Strains of Newcastle Disease Virus Propagated in Monolayer Cultures.[a]

Virus	Fusion Events/Cell[b]							
	Fusion from within							
	Time after Infection (hr)							Fusion from without
	4	8	12	16	20	24	30	
NDV-HP-16	0.02	0.08	0.17	0.13	0.43	0.65	0.71	0.33
NDV-L-Kansas	0.08	0.05	0.33	>1	>1	>1	>1	<0.01[c]
NDV-N	0.01	0.05	0.08	0.25	0.28	0.53	>1	0.24

[a] Virus propagated in chick embryo fibroblast secondary cultures and harvested from culture medium at 20 hr postinfection. [b] Average number of nuclei per infected cell minus average number of nuclei per uninfected cell (Bratt and Gallaher, 1972). [c] No fusion from without was observed when cells were challenged with a tenfold higher virus concentration than that described in Materials and Methods.

Since the membrane lipids of paramyxoviruses are derived from the host cell surface membrane (Choppin et al., 1972), the viral lipid compositions described in Table I provide a good estimate of the host cell surface lipid compositions at different periods of virion production. It is therefore possible to test for correlations between host surface membrane lysophosphatidylserine content and fusion from within by comparing the lysophosphatidylserine content of virions produced during a short time interval with fusion from within occurring during this same interval. Comparisons of the lysophosphatidylserine data in Table I with the fusion from within data in Table III indicate that there is no correlation between the extent or rate of appearance of cell fusion from within and the level of lysophosphatidylserine in virus produced at the time of fusion assay.

The data in Table III indicate that no correlation exists between the lysophosphatidylserine content of the *added* virions and their potential for inducing cell membrane fusion from without. Although NDV-HP-16, which had the highest concentration of lysophosphatidylserine, was the most effective strain in inducing fusion from without, NDV-L-Kansas, which contains nearly four times the amount of lysophosphatidylserine as NDV-N, was completely inactive in promoting fusion from without. Furthermore, NDV-N induces fusion from without to the same extent as NDV-HP-16 even though the monoacylphosphatidylserine content of NDV-N was about 1/15th that found in NDV-HP-16. These data thus provide no indication of a role for viral lysophosphatidylserine and the ability of a virus to cause fusion from without.

The viral content of lysophosphatidylserine does correlate with the ability of an NDV strain to cause lytic infection. Strains HP-16 and L-Kansas produced plaques on chick embryo fibroblast monolayers (data not shown) and contained high concentrations of lysophosphatidylserine (Table I), whereas little lysophosphatidylserine was observed in the phospholipids of NDV-N, a strain which produced no plaques on chick embryo fibroblast monolayers.

Discussion

This appears to be the first report of the presence of a lysolipid in appreciable levels in a paramyxovirus. The usefulness of equilibrium labeling to study viral lipid composition was borne out by the excellent correlation between phospholipid composition of Rous sarcoma virus as determined by quantitative phosphate analysis and by distribution of radioactive $^{32}P_i$ in lipids (Quigley et al., 1972). These workers also examined the phospholipids of NDV using this same technique. While the proportions of lecithin, phosphatidylethanolamine, and sphingomyelin were similar to those described in Table I, no lysolipids were detected (Quigley et al., 1971). Since the strain used was not specified, the apparent absence of lysophosphatidylserine in their study could be attributable either to analytical or strain differences. However, the phospholipid composition of chick embryo fibroblasts (Table II) is in good agreement with that reported by these workers (Quigley et al., 1971). The phospholipid composition of another strain of NDV (NDV-B1) resembles the distribution reported here for NDV-N (Table I) in that lecithin and phosphatidylserine each account for approximately 12% of the total phospholipid (Blough and Lawson, 1968). These findings further emphasize the variability between strains with respect to phospholipid composition. Tiffany and Blough also observed clearly different fatty acid compositions in three strains of NDV (Tiffany and Blough, 1969). The data in Table I indicate that strain specificity with respect to fatty acid patterns could arise from a variation in phospholipid distribution, where the different phospholipids have varying fatty acid composition.

Two schools of thought have emerged regarding the mechanism underlying cell membrane fusion. These emphasize the role of lysolipids on the one hand (Lucy et al., 1971; Ahkong et al., 1972) and the topographical distribution of protein on the other (Poste and Allison, 1973; Satir, 1975). The lack of a correlation between fusion and lysolipid content shown here provides no support for the former hypothesis. The HP-16 strain of NDV, which contains the highest level of lysophosphatidylserine, does not induce fusion from within to as great an extent as either NDV-N or NDV-L-Kansas. Similarly, NDV-N is nearly as effective as NDV-HP-16 in inducing fusion from without, though far less lysophosphatidylserine is detectable in the former. The proposed role of lysolipids in membrane fusion first gained attention when the Lucy group reported that erythrocytes can be induced to fuse by the addition of exogenous lysolecithin (Poole et al., 1970). This proposal was further supported by subsequent reports describing lysolecithin-induced fusion of fibroblasts (Keay et al., 1972). However, considerable lysis was observed at the lysophosphatide concentration required to stimulate polykaryocyte formation, raising serious doubts about the physiological role of lysolipids in inducing fusion. Chicken erythrocytes have been shown to fuse at pH 10.5 in the presence of 40 m*M* Ca^{2+} ion with a concomitant breakdown of phosphatidylethanolamine and lecithin to their monoacyl derivatives (Toister and Loyter, 1973). However, there has been no confirmation of this finding, and others have not observed fusion under these conditions (Poste and Allison, 1973). Thus in view of these conflicting observations, the fusogenic capability of lysolipids cannot be assigned a sine qua non role in the mechanism of cell fusion.

Though there appears to be no correlation between viral monoacylphosphatidylserine content and cell fusion, a high concentration of lysophosphatidylserine does correlate with the cytopathogenicity of the virus strain. Thus strains that undergo a lytic (velogenic) cycle of infection, NDV-L-Kansas and NDV-HP-16, contain monoacylphosphatidylserine in much higher concentrations than NDV-N, a strain with no detectable cytolytic (lentogenic) effect (Shloer and Hanson, 1968). The correlation also applies to another lentogenic strain of NDV, B1, in which no significant level of lysolipid has been detected (Blough and Lawson, 1968).

Inspection of Tables I and II indicates that lysophosphatidylserine (as a percent of total phospholipid) was higher in lipid extracts of cells than it was in NDV-N. This circumstance could result from a release of a specific phosphatidylserine acyl hydrolase from a cellular organelle during lipid extraction. This hydrolytic activity might then also be responsible for the production of monoacylphosphatidylserine in cells infected with NDV strains that give rise to lytic infection. We do not know if the production of the monoacyl derivative occurs prior to or after cellular lysis. If phosphatidylserine of NDV is localized on the inner monolayer of cell surface (and viral) membranes as it is in certain erythrocyte membranes (Bretscher, 1973, Verkleij et al., 1973), the conversion of diacylphosphatidylserine to the monoacyl derivative might well occur prior to the budding of newly formed virions from the cell surface.

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