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Comparison of the Lipids of Intracellular and Extracellular Rabies Viruses

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The lipid composition of both intracellular and extracellular forms of the ERA strain of rabies virus grown in BHK/21 cells was determined. The lipids from purified preparations of both intracellular and extracellular virus yielded 57 and 58% neutral lipid, respectively. The phospholipids of the intracellular and extracellular virus constituted 43 and 42%, respectively. Triglyceride and cholesterol appear to be the major neutral lipids, whereas sphingomyelin, phosphatidylethanolamine, and phosphatidylcholine comprise the major bulk of phospholipid in both virus types. The molar ratio of cholesterol to phospholipid was 0.87 (intracellular) and 0.92 (extracellular). On the basis of the data presented, it is reasonable to assume that the lipids of both intracellular and extracellular rabies virus are similar.

The various strains of rabies virus show differences in the morphogenesis of their envelopes (7). Viral particles may arise solely by "budding" from a newly synthesized patch of viral membrane which is continuous with the cell membrane. The viral envelopes may also be synthesized de novo in the cytoplasm, i.e., or on the edge of characteristic ribonucleoprotein inclusions, without involvement of the cell membrane. The former mode of synthesis predominates among vaccine, whereas street viruses show the latter (7). Certain vaccine strains, however, do exhibit both types of membrane formation in tissue culture, with intracellular de novo synthesis preceding budding. Both virus populations can be separated easily because the intracellular virus is not released until a total breakdown of the parasitized cell occurs.

These observations led to comparative studies of the lipid contents of the viral envelope of intracellular and extracellular rabies virus progeny.

MATERIALS AND METHODS

Virus strain. The ERA strain of rabies virus was used in this study.

Tissue culture. The BHK/21 cells were grown as a monolayer culture in 32-oz (0.946 liter) prescription bottles with modified Eagle medium containing 10% fetal calf serum.

Infection of cells and virus propagation. Monolayers of BHK/21 cells were inoculated at a multiplicity of 10 PFU/cell and incubated for 1 h at 35 C. After absorption, the cell monolayers were washed three times with phosphate-buffered saline (PBS), refed with modified Eagle medium containing 0.2% bovine serum albumin (BSA; Fraction V; General Biochemicals Corp., Chagrin Falls, Ohio), and then incubated at 35 C for 72 h.

Viral purification: extracellular virus. Following incubation at 35 C for 72 h, the medium was clarified at 500 × *g* for 10 min at 4 C, the pellet was discarded, and, the supernatant fluid, containing virus, was centrifuged at 90,000 × *g* at 4 C for 60 min in a SW 25.1 rotor of a Spinco ultracentrifuge. The pellet was suspended in phosphate-buffered saline and exposed to sonication for 1 min (Di Sontegrator, Ultrasonic Industries, Inc., Albertson, Long Island, N.Y.). The suspension was then clarified by centrifugation at 6,000 × *g* for 10 min. The virus containing supernatant fluid was centrifuged two times in sucrose density gradients (15 to 55%, wt/vol) at 90,000 × *g* at 4 C for 90 min in the SW 25.1 rotor of a Spinco ultracentrifuge. The virus containing fractions was collected, checked for purity by electron microscopy, and frozen at −24 C until used.

Intracellular virus. The infected monolayers were washed three times with phosphate-buffered saline and then scraped from the glass. The cell suspension was exposed to three alternate cycles of freezing and thawing, followed by sonication for 3 min. The virus containing suspension was then treated as previously described under the purification of extracellular virus.

Preparation of lipid extracts. Lipids were isolated

from lyophilized material by multiple extractions with chloroform-methanol (2:1) mixtures according to the method of Weinstein et al. (14).

Thin layer chromatography. Samples of total lipid were spotted on plates coated with Silica Gel G 0.25 mm thick (Brinkmann Instruments, Inc., Westbury, N.Y.). After air drying, the plates were activated for at least 1 h at 100 C before use. Neutral lipids were separated by development with either ethylene chloride or petroleum ether-ethyl ether-acetic acid (90:10:1, vol/vol/vol) (13). Iodine was used to visualize the lipids. Identification of the neutral lipid classes was made by comparison of the R_f values of the unknowns with that of the known standards run on the same chromatogram. The area corresponding to neutral lipid classes was scraped from the plate and the lipid removed by extraction with chloroform. Quantitation was accomplished by placing the samples in aluminum trays. The solvents were evaporated in a stream of nitrogen and weighed with a microbalance (Cahn M-10 Electrobalance, Ventron Instruments Corp., Paramount, Calif.) (12).

The phospholipids that remained at the origin were scraped from the plate and eluted from the Silica Gel G with chloroform-methanol (2:1). After centrifugation at 500 × *g*, the chloroform-methanol was removed with a Pasteur pipette, and the phospholipids were recovered by evaporation of the chloroform-methanol under a stream of nitrogen. Phospholipids were separated by thin-layer chromatography by using the Silica Gel G and chloroform-methanol-ammonium chloride (17:7:1 vol/vol/vol) as a solvent system (4). Identification of the phospholipids was made, after exposure to iodine, by comparison to known standards. Quantitation was done by determining the phosphorus content in each fraction (1).

Determination of cholesterol and cholesterol esters. The colorimetric method of Zlatkis et al. (14) was used to determine the amount of cholesterol and cholesterol esters present.

RESULTS

Comparison of the lipid composition of whole BHK/21 cells and the intracellular and extracellular rabies virus grown in BHK/21 cells. In repeated experiments, the distribution of lipid components of the intracellular and extracellular rabies virus and the BHK/21 cells were compared. The purified virions and cellular material were tested simultaneously on thin layer plates as described in Materials and Methods. Table 1 compares the lipid composition of intracellular virus, extracellular virus, and the BHK/21 cells used for their production. The data indicate that no significant differences exist between the amount of total neutral lipid and total phospholipid in both virus types. The viruses, however, showed a higher content of total neutral lipid and phospholipid when compared to the whole cell material. The neutral lipid pattern and the cholesterol to phospholipid ratio of both virus types were not significantly different. The value for the cholesterol to phospholipid ratio found in whole cell preparations was similar to that previously reported for hamster cells (9). McSharry and Wagner (11) have shown a similar relationship between viral and host cell lipids for vesicular stomatitis virus.

The three major phospholipids present in both intracellular and extracellular virus were phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, whereas triglyceride and cholesterol comprise about 50% of the neutral lipid.

DISCUSSION

The lipids of the membranes of enveloped viruses closely resemble those of the cell membranes from which the viruses emerge. The vaccinia virus envelopes, in contrast, are formed de novo in the cytoplasm of infected cells and contain fatty acids and phospholipids other than those found in the host cell (5).

TABLE 1. *Lipid composition of BHK/21 cells and intracellular and extracellular rabies virus grown in BHK/21 cells*

Component	Whole cells (%)	Intracellular virus (%)	Extracellular virus (%)
Total lipid			
Neutral	16.3	57.0	58.0
Phospholipid	83.7	43.0	42.0
Cholesterol to phospholipid molar ratio	0.20	0.87	0.92
Neutral lipids			
Free fatty acids	9.1	12.2	14.7
Monoglycerides and diglycerides	17.1	17.4	10.1
Triglycerides	36.5	25.9	26.4
Cholesterol	26.2	18.2	22.7
Cholesterol esters	11.0	16.4	16.7
Phospholipids			
Lysophosphatidylcholine	4.2	7.0	8.3
Sphingomyelin	16.0	24.3	19.8
Phosphatidylcholine	20.3	24.2	21.7
Phosphatidylethanolamine	30.5	20.8	20.2
Phosphatidylinositol and phosphatidylserine	9.6	15.3	19.8
Unknowns	9.6	11.3	13.2

Certain strains of rabies virus, an enveloped rhabdovirus, show both modes of morphogenesis (6, 7). Viral particles are formed de novo in the cytoplasm, and also by budding from newly formed viral membranes which are continuous with the preexisting cell membrane. The two virus populations, when physically separated, are biologically and morphologically indistinguishable with the exception that the intracellular virus seems to contain less hemagglutinin (10). It seemed reasonable to investigate if the contents of the viral envelopes exhibited any qualitative and/or quantitative differences.

The present investigation of the viral lipids revealed essentially no qualitative differences between viruses produced by the two forms of replication. All viral lipids appear to be derived from the host cell. Quantitatively, a difference was found in the total neutral lipid and total phospholipid content of both virus populations when compared to the host cell. The neutral lipid and phospholipid content was greater in the viral particles than in the uninfected cells.

The mechanism for the de novo formation of membranes within or near the matrices is unknown. In the case of the myxoviruses and paramyxoviruses, it has been shown that the performed nucleocapsid lines up under the cell membrane, initiating antigenic changes in the membranes with subsequent formation of the virus particle (2, 3). The same mechanism was observed for rabies virus particles (8). It must be assumed that with some rabies strains, the nucleocapsid in the matrix is able to elicit proper membrane formation in the absence of the cell membrane by utilizing cellular lipids and proteins specific for the virus.

A comparison of the proteins of the two virus populations similarly did not show appreciable qualitative or quantitative differences. The reduced content of hemagglutinin in the intracellular virus population seems to be due to the partial breakdown of the glycoproteins during the extraction procedure (F. Sokol, personal communication).

Despite the difference in their morphogenesis, the two virus populations do not appear to be biologically or chemically different.

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