

FACTORS AFFECTING THE CELLULAR SPECIFICITY OF VESICULAR
STOMATITIS VIRUS MEDIATED CELL FUSION

By

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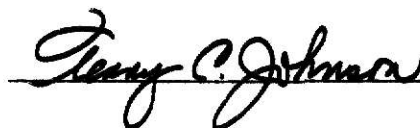
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SECTION I

ACKNOWLEDGMENTS

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SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

INTRODUCTION

Although vesicular stomatitis virus (VSV) is not generally regarded as a virus with fusion properties, ts G31 VSV a temperature sensitive mutant of vesicular stomatitis virus induced extensive cell fusion in mouse neuroblastoma cells when incubated at the nonpermissive temperature of 39°C. Ts G31 VSV has also been employed as a model system for studying slow viral diseases such as Creutzfeldt-Jakob disease and Kuru in humans, and Scrapie and Transmissible mink encephalopathy in animals.

Many of the atypical infectious agents of the slow viral diseases have a neuropathology that is remarkably similar to the pathology induced in the Central Nervous System of mice inoculated with ts G31 VSV. These similarities include a spongiform encephalopathy known as status spongiosus and the ability to induce cell fusion, both in vitro and in vivo. In fact, the ability of these agents to induce cell fusion has been taken advantage of and is used as a monitoring tool to diagnose slow virus diseases.

The ability of ts G31 VSV to promote neuroblastoma cell fusion at 39°C appears to be the result of an intracellular defect in maturation and/or assembly of the virions. The production and release of infectious virions is not necessary for cell fusion to occur. However, polykaryocyte formation has been shown to require the synthesis and expression of the viral glycoprotein at the surface of infected cells.

Some initial work has suggested a cellular specificity for vesicular stomatitis virus mediated cell fusion. It was found that neuroblastoma cells fused very rapidly when infected with ts G31 VSV, followed by

incubation at the nonpermissive temperature. While BHK-21, mouse 3T3 and many other cell lines did not fuse. The inability of these cell lines to fuse is not a result of either a decreased expression of the glycoprotein or a lack of sufficient cell to cell contact. The viral glycoprotein, as processed by nonfusible cell lines, appears capable of initiating the fusion event and, therefore, cannot explain the observed differences in fusion ability.

Although the stages of virus-induced cell fusion have been previously described, the mechanism(s) or properties of the cell membrane responsible for the induction of fusion remains unclear. This study compares the membrane properties of the fusion competent neuroblastoma cells with various nonfusing cell types, using liposomes as a model system. Liposomes are particularly applicable for this approach since membrane properties of liposomes can be easily manipulated. Fluorescein diacetate was employed as a probe, to monitor liposome-cell fusion, since it does not fluoresce until hydrolyzed by cytoplasmic esterases, resulting in low backgrounds of fluorescence when liposomes only bind to target cells without fusion.

GENERAL PROPERTIES OF VESICULAR STOMATITIS VIRUS

Vesicular stomatitis virus (VSV), the experimental prototype of the rhabdoviruses, has been shown to have a bullet shaped morphology as revealed by electron microscopy (82). The typical infectious virion (B particle) is 180 ± 10 nm in length and 65 ± 10 nm in width at the blunt end. The virion is composed of two distinct and separate components, the ribonucleocapsid (RNC) and the envelope.

The RNC can be obtained by solubilizing the envelope with detergents such as deoxycholate, leaving the RNC completely free of envelope material (55,132). The RNC has a bouyant density of 1.31 g/ml in CsCl and contains only RNA and three of the five viral proteins (L, N, NS). The intact RNC when completely void of envelope is infectious, although, the efficiency of infection is considerably lower than that of the intact virion (12,122). The RNA, absent of protein is not infectious because the single stranded RNA molecule is anti-polar to the VSV mRNAs and transcription is required to initiate the infection process (78).

The RNA of VSV is a covalent, linear, single stranded molecule with negative polarity and has a molecular weight of $3.8-4.0 \times 10^6$ daltons (14,58,100). The RNA of VSV has sufficient genetic information to code for all five viral proteins.

The L protein (mw 190,000) is present in the nucleocapsid at approximately 60 molecules/nucleocapsid and possess the transcriptase activity (30). The NS protein (mw 32,000), a phosphoprotein, has been shown to function as a modulator for transcriptase action and there appears to be a requirement for high levels of covalently bound phosphate for full activity of the NS protein in enhancing transcription (59). The N protein (mw 52,000) is the major protein component of the nucleocapsid and is

tightly complexed with the RNA (55,132). The N protein affords a significant amount of stability to the nucleocapsid and appears to protect the viral RNA from the degradative effects of enzymes (8).

The viral envelope is a lipid bilayer and has two of the viral proteins, the G glycoprotein (mw 69,000) and the matrix (M) protein (mw 29,000). The lipid composition of the envelope has been studied extensively (95), revealing that virions contain approximately 20% lipid. Analysis by thin layer chromatography suggests that no unusual neutral lipids or fatty acids were present in the infectious virion (74). In fact it appears that the lipid composition of the purified virion resembles closely the lipid composition of the host cell plasma membrane. It also has been reported that cellular proteins can be incorporated into the virion envelope during the budding process that allows the release of mature progeny virions (15). Cholesterol is an important component of the viral membrane and represents 35 mol % of the total viral lipid (95). Evidence from several laboratories has suggested that cholesterol is distributed asymmetrically within the viral envelope and may play a significant role in determining viral infectivity (77,88,94).

The G glycoprotein (500 molecules/virion), the only glycoprotein present in the intact virion, is found inserted in the outer surface of the viral envelope and produces the characteristic spikes that are associated with VSV morphology (18,133). The G glycoprotein when incorporated into lipid vesicles exerts a stabilizing effect and in conjunction with cholesterol is responsible for the organization of the virion envelope (when the M protein is not present) (89). Evidence indicates that the G glycoprotein represents the attachment moiety of the virion for cell surfaces (127). It has been reported that treatment of cell surfaces with neuraminidase and trypsin did not alter infection (127). Due to the wide

host range observed with VSV it has been difficult to determine the nature of the surface receptor associated with virus attachment. It has been suggested that there could be a wide range of surface components that can serve as a receptor for virus attachment (127). Phosphatidylserine has been suggested as the surface receptor for VSV and liposomes loaded with phosphatidylserine have been shown to bind VSV while liposomes containing phosphatidylcholine do not bind VSV (28), however, this is in direct conflict with the results of Moore et al. (77). These investigators showed that incubation of intact VSV virions with phosphatidylcholine vesicles resulted in a substantial inhibition of infection.

The M protein (mw 45,000) is a nonglycosylated component of the viral membrane where it appears to form a close association with lipids on the inner membrane where it forms the lipoprotein envelope (14,133). It is believed that the M protein functions in the maturation of the virus by acting as a bridge between cytoplasmic nucleocapsids and the regions of the cellular plasma membrane into which the G glycoprotein has been inserted (28). It has also been suggested that the interaction occurs through an attraction between the highly positively charged M protein and negatively charged phospholipids in the inner leaflet of the plasma membrane (28). In fact purified M protein can be reconstituted only within lipid vesicles that contain acidic phospholipids (138) and it has recently been observed that the M protein increases the order of bilayer lipids (135).

Infection of cells by enveloped viruses, such as VSV, involves the transfer of genetic information across the lipid bilayer of the host cell. This transfer of genetic material across a bilayer can occur through fusion of the viral envelope with the hosts lipid bilayer or by phagocytosis of the virion at the cell surface and movement of the intact virus particle across the membrane in an intracellular vacuole (20). Evidence for VSV

exists for both fusion at the plasma membrane and for endocytosis of the intact virion (19,72,120). The controversy has not been resolved completely since data suggesting fusion at the cellular bilayer involves electron microscopic studies and the data lending evidence for entry via phagocytosis has involved the use of inhibitors of lysosomal function that have been shown to alter cellular functions involved in virus maturation (25). However, many different cell lines have been used in these studies and the mechanism of VSV entry may be host specific. It is likely that the mechanism(s) of VSV entry, that leads to a productive infection, will become less confusing when experiments are performed to resolve the importance of the nature of the host cell in the initial infection process.

After gaining entry into the cell as an intact virion (phagocytosis), uncoating must occur inside phagocytic vesicles prior to transcription. However, if fusion occurs, uncoating may be accomplished right at the cell surface. After the nucleocapsid is released, by either method, VSV transcription is initiated in the host cell cytoplasm. Transcription proceeds from the 3' terminus to the 5' terminus. Processing enzymes cleave the mRNA into 4 size classes of mRNA's which are complementary to the viral genome. Following cleavage the mRNA's are polyadenylated at the 3' end and are capped at the 5' end (2). The 4 size classes are revealed by sucrose gradient ultracentrifugation, are 28S and three smaller RNA species with sedimentation coefficients of 17, 14.5, and 12 respectively (9). The 28S mRNA has been translated into the L protein (79). From the smaller mRNA species the 17S band codes for the G protein, the 14.5S band codes for the N protein and the 12S band codes for both the NS and the M protein (19,80).

The G glycoprotein is synthesized on membrane-bound polyribosomes (78) and differs from the primary polypeptide. The microsomal G glycoprotein

has missing an NH_2 -terminal hydrophobic sequence of 16 amino acids (128)², it is nonglycosylated, and has a transmembrane configuration in the rough endoplasmic reticulum (56,128). The G protein is then transported from the rough endoplasmic reticulum to the Golgi system, possibly through intermediate clathrin coated vesicles (112). In the Golgi system the carbohydrate side chains present on the G glycoprotein are modified resulting in what has been termed the complex glycopeptide form (7,51,109). It is at this step, or even a later step of maturation, that 1-2 molecules of fatty acid are covalently attached (acylation) to the G glycoprotein (117,118). It is thought that acylation may play an important role in the insertion of the G glycoprotein into the plasma membrane (117,118). Once inserted into the plasma membrane, assembly and budding of virus particles can take place (1).

The N, L, NS and M proteins are synthesized as soluble proteins in the cytoplasm. The N protein is initially found associated with the newly synthesized 40S viral RNA (64). The interaction between the N protein and the 40S RNA results in stabilization of the viral RNA and eventually forms the nucleocapsid (64). The M protein functions, as stated earlier, as a bridge between the newly formed nucleocapsid and the G glycoprotein at the cell surface.

Temperature sensitive mutants of VSV have proven invaluable in understanding the genetics and virus-host cell interactions of VSV. These temperature sensitive mutants of VSV have properties that allow normal replication at 31°C, while production of progeny virus is reduced, or nonexistent, at the nonpermissive temperature of 39°C. Classification of the temperature sensitive mutants can be accomplished by infection of cells by two of the mutants at the same time. When cells are infected with two temperature sensitive mutants at the same time and incubated at the

nonpermissive temperature normal virus progeny may be produced if the two mutants are defective in different genes. That is, a normal protein of one virus may be supplied by the other and, therefore, the two viruses complement each other.

Six complementation groups have been demonstrated with VSV (99). Complementation group I has neither viral RNA synthesis or protein synthesis at the nonpermissive temperature. The alteration can be traced to the production of a thermolabile transcriptase (L) protein. At 39°C, the nonpermissive temperature, the L protein is incapable of making any mRNA or viral RNA, resulting in the observed lack of protein synthesis.

Complementation groups II and VI are not well characterized and at elevated temperatures some of the mutants are RNA- and others are RNA+. The site of the genetic defect has not been identified for complementation group II and the phenotypic and genotypic properties of complementation group VI have not been identified (118).

Complementation group III mutants make both RNA and proteins at the nonpermissive temperature, however, the M protein is labile at elevated temperatures. Hughes et al (50) working with temperature sensitive mutant G31 VSV showed that this mutant synthesized all five viral protein at 39°C, but the M protein was extremely labile and did not accumulate in the infected cell to appreciable amounts.

Mutants of complementation group IV all have in common a defective N protein. This defect has been shown by Dille et al. (117), using ts G41 VSV, to result in synthesis of all five viral proteins, but at 10% of the amount produced at permissive temperatures. Complementation group V mutants are all defective in G protein production or maturation and results in the lack of production of G glycoprotein or the production of G protein that is unable to be an effective substrate for host cell glycosylation.

VIRUS-INDUCED CELL FUSION

A number of animal viruses have been associated with polykaryocyte formation both in vivo and in vitro. The ability of these viruses to induce polykaryocyte formation, as a result of cell fusion, has been studied extensively although the precise mechanism of membrane fusion still remains unclear. Two mechanisms of virus-induced cell fusion have been proposed by Bratt and Gallaher (11), fusion from within, and fusion from without.

Fusion from without is characterized by the addition of infectious or UV-inactivated virus at high multiplicity of infection and occurs only rarely with VSV (11). Some of the most studied viruses that promote fusion from without include Sendai virus (85,96), Newcastle disease virus (52, 65), mumps virus (43) and several DNA viruses, including herpes simplex virus (45,83) and vaccinia virus (54).

Sendai virus is probably the best characterized virus with fusion from without properties. Early work has shown that treatment of Sendai virus with ultraviolet light or β -propiolactone destroys infectivity but has no effect on the ability of the virion to promote cell fusion (65). Fusion from without by Sendai virus requires a viral glycoprotein denoted F protein for its fusion properties (17,46). Scheid and Chopin (114) revealed a cellular involvement in the fusion process involving the activation of the Fo protein, through proteolytic cleavage, to form the activated F protein. Studies involving electron microscopy have suggested that Sendai virus particles form a bridge between two cells, fuses with the plasma membrane from both cells, and eventually results in a binucleated cell.

In contrast, fusion from within generally occurs at low multiplicity of infections, at a later step in maturation and requires active protein

synthesis. Fusion from within is the predominant form of fusion in certain VSV infected cells. Fusion from within is characterized by several RNA and DNA viruses, including Newcastle disease virus (29), vaccinia virus (54) and respiratory syncytial virus (16).

A few reports have shown a relationship between cell fusion and virion production (106,107). Several Newcastle disease virus strains have shown that an imbalance between the production of viral components and the production of virions can have a significant impact on cell fusion. Other studies have implicated cell fusion with the inability of virions to promote cell lysis (43,129). Another cytopathic effect, cell rounding, also has been implicated in the inability of virions to promote cell fusion (110,111).

Probably the least studied area of virus-induced cell fusion deals with the host cell susceptibility to fusion. Poste (96) has shown the differences in fusion of two different cell types in response to the same virion. While both HEp-2 and mouse L cells replicate herpes type II virus to the same degree, only HEp-2 cells show any significant fusion. This same phenomena was also shown with many other virus systems, including mumps (10), measles (81), visna (40) and vesicular stomatitis virus (26).

Differences in the host cell susceptibility to cell fusion can possibly be explained by the lipid composition of the cell. An initial report by Dingle (27) suggested that a high proportion of short-chain fatty acids and the presence of polar groups in hydrocarbons result in an unstable membrane that can fuse. Others have suggested that the cholesterol to phospholipid ratio is important in determining whether cell fusion will occur (63,130). Since these early observations were reported little, if any, further work has been done on the host cell membrane involvement in the fusion process.

Although little is understood about the precise mechanisms of virus mediated cell fusion, several models have been described that can account for this process. Poste (96) has described a general scheme for fusion involving at least three prerequisite stages. Stage one depicts the displacement of Ca^{2+} and ATP from the membrane. This is thought to result in an increase in the permeability of the membrane, a reduction in the electrical resistance of the membrane and a transformation of the lipid bilayer to a micellar form. Stage two involves the formation of intermediate linkages between the two opposing membranes. This results in an increase in the molecular freedom of a membrane and produces conformational changes in membrane macromolecules. Stage three suggests the stabilization of the membrane after fusion with the return of Ca^{2+} and ATP.

Another model system was developed after reviewing the literature dealing with liposome fusion. Bangham (3) theorized that, in order for two membranes to fuse, water molecules must be eliminated from the fusion site. Three possible mechanisms exist for the elimination of water from the fusion site. Water can be removed by binding with salts, polyethelene-glycols and dimethylsulphoxides. Divalent ions can crosslink complementary membrane ions to form an insoluble area, this can occur through an interaction between calcium and phosphatylserine, for example. The third mechanism involves a mechanism by which nonpolar regions of membranes exceed their solubility resulting in its accumulation as a discrete liquid.

LIPOSOME PREPARATION AND CHARACTERIZATION

Procedures and methods for the preparation of liposomes vary widely depending upon experimental design. Phospholipids are most often used for liposome preparation, but vesicles can be prepared from lysophosphatides, cholesterol and single chain amphiphiles (35,36,39,62,126). A commonly accepted nomenclature for lipid vesicles classifies them according to their number of lipid layers and then according to their size. The different categories of liposomes are: multilamellar vesicles (MLV); small unilamellar vesicles (SUV, smaller than 100 nm); and large unilamellar vesicles (LUV) those with diameters greater than 100 nm.

Large unilamellar vesicles (greater than 100 nm in diameter) can be prepared by reverse-phase evaporation. Liposomes prepared by this technique encapsulate a high percentage of the initial aqueous solution and have a high aqueous space to lipid ratio. To prepare reverse-phase evaporation vesicles, lipids are dissolved in organic solvents, such as chloroform, followed by the addition of the aqueous suspension in a ratio of 3:1, respectively. The lipid solution is then sonicated to produce a homogeneous solution which is then subjected to a low vacuum in a rotary evaporator. Evaporation under reduced pressure removes the organic solvent and results in a homogeneous suspension of liposomes. Reverse-phase evaporation techniques are particularly useful in encapsulating compounds of a high molecular weight, such as SV 40 DNA, 25 S RNA, and proteins such as ferritin (33). Reverse-phase evaporation has also been used extensively to reconstitute membrane proteins such as rhodopsin (21) and the Semliki Forest virus fusion protein (73).

Solvent injection is the most common, and simplest, method of liposome preparation available (5,22,23,115). This technique involves the injection

of organic solvents containing phospholipids into a large volume of buffer. Organic solvents such as ethanol and ether are often used. Liposomes formed from the rapid injection of phospholipids dissolved in ethanol are small unilamellar vesicles and encapsulate only a small fraction of the aqueous volume. Due to its simplicity, and inability to encapsulate large volumes, ethanol injection primarily is used for fluorescent polarization studies involving fluorescent phospholipid analogues. Solvent injection can also be used for encapsulation of hydrophobic drugs, since the properties of these probes will allow them to remain with the liposome.

The detergent removal technique for preparing liposomes was first developed by Kagawa and Racker (53) to reconstitute membrane proteins and has widely been accepted as the method of choice for the reconstitution of cell membranes (66,105). This technique is relatively simple and depends upon the removal of the detergent from a detergent/lipid/protein suspension. Various methods for detergent removal have been developed, including ultracentrifugation (134), gel filtration (13,32), and dialysis (49). The detergent of choice appears to be β -octyl glucopyranoside (OG), although deoxycholate is still widely used.

Because of its high critical micellular concentration (~ 25 mM), which allows for easy removal, β -octyl glucopyranoside has been extremely important in membrane reconstitution studies (119). In addition to having a high critical micellular concentration, β -octyl glucopyranoside, unlike many other non-ionic detergents does not denature membrane proteins (121). Therefore, β -octyl glucopyranoside provides an excellent model system for membrane reconstitution studies that include proteins. Liposomes prepared by detergent extraction techniques can vary between 34 to 130 nm in diameter depending upon the percentage of cholesterol present or the pH of the dialysis solution (108).

A method for preparing large unilamellar vesicles from acidic phospholipids has been described extensively (90-93). The procedure involves the fusion of small unilamellar vesicles containing acidic phospholipids by the addition of calcium (as calcium chloride). Calcium can be added either through dialysis or by direct addition, and this procedure results in the formation of large cylindrical, folded, multilamellar structures having a spiral configuration which are referred to as cholate cylinders. Large closed, spherical unilamellar vesicles can be prepared from the above liposome population by the addition of calcium chelators, such as sodium (tetra) ethylenediamine tetraacetate (EDTA).

Knowledge of the size distribution of lipid vesicles is often important in evaluating the results of biochemical, biophysical, drug delivery, and fusion experiments. Most of the physical techniques for measuring liposome size require a homogeneous population with a well defined shape. These techniques are covered in a review by Mason and Huang (71).

Sizing heterogeneous liposome populations is often more difficult than sizing homogeneous populations and can be accomplished using electron microscopic techniques. Negative staining of liposomes (smaller than 5×10^3 nm in diameter) using molybdate and phosphotungstic acid has often proved of value in size determinations. These techniques have given results that are within 10 to 20% of the values determined by freeze fracture techniques (49).

The difficulty in obtaining good negative staining of liposome populations results from their poor spreading properties on carbon coated grids. Several techniques have been employed to solve this problem; including treating the grid with 0.1 mg/ml solution of bacitracin (38), or coating the support film with silica by the evaporation of silica monoxide

(68). Another method for reducing the hydrophobicity of the carbon coated grids has employed wetting agents such as a 1% solution of bovine serum albumin or a 0.4% sucrose solution (113).

Several other methods for determining the size distribution of liposome populations have been introduced and are widely used today. Some of these techniques include thin-layer chromatography using agarose beads (131), exclusion chromatography (48) for vesicles smaller than $1-3 \times 10^3$ nm in diameter, light scattering (4) and field flow fractionation (60,61).

In addition to sizing liposome populations, methods for preparing liposomes with a defined size have been developed. Small unilamellar vesicles of defined size can be prepared by modifications of ethanol injection (67) and detergent dialysis procedures (76). Recently a method for preparing large unilamellar vesicles of defined size have been described by Olson et al. (86). This method involves the extrusion of large unilamellar vesicles through polycarbonate membranes of defined size and results in the removal of large vesicles and absorption of smaller vesicles. Small unilamellar vesicles that adsorb to the filters can then be completely removed by subsequent washing and used for in vivo drug delivery experiments (for example).

Phospholipid vesicles can interact with cells in vitro by four well characterized ways: 1) stable absorption, 2) endocytosis, 3) fusion and, 4) lipid exchange. Stable absorption is a non-specific electrostatic or hydrophobic interaction between various components on the vesicle/cell surface. Intact vesicles can be taken up into endocytic vesicles through specific or non-specific mechanisms. Fusion is the result of a merging between the lipid layer of a vesicle and the plasma membrane of a cell. Fusion results in the incorporation of vesicle lipid components into the plasma membrane of the target cell with a concomitant release of vesicle

contents into the cell cytoplasm. Lipid transfer is the exchange of specific lipid molecules between the vesicle and the cells surface.

Fluorescent probes, such as carboxyfluorescein and fluorescein isothiocyanate-dextran have been commonly employed as tools to monitor liposome-cell fusion. These fluorescent probes are generally incorporated inside the liposome during preparation. Fusion is then monitored by measuring cellular fluorescence with the use of a fluoremeter or fluorescent microscope. To avoid nonspecific fluorescence the concentration of carboxyfluorescein and fluorescein isothiocyanate-dextran within liposomes must be high enough to result in quenching which allows the investigator a semi-quantitative assay for measuring fusion. Several researchers have shown that only a small percentage of the quenched fluorescent probe need leak out to result in cellular fluorescence (136). Fluorescent probes such as fluorescein diacetate and 6-carboxyfluorescein diacetate appear to be more reliable when studying liposome-cell fusion since these probes are only fluorescent after enzymatic cleavage by esterases contained in the target cell cytoplasm and, therefore, can be loaded into liposomes at a relatively low concentration.

The problems associated with the use of soluble fluorescent probes can be circumvented by using fluorescence energy transfer methods to study vesicle-cell fusion. This method simply follows (with the use of a phospholipid analog fluorescent probe) the mixing of vesicle lipid with cellular membranes. It is unfortunate, however, that suitable methods for quantitating the dilution of vesicle lipids with cellular lipids have not been developed and, therefore, cannot be used as a quantitative assay (24,57).

Properties and components of liposomes and their correlation to liposome-cell fusion have been studied extensively, although the data is

controversial (87,91). Several investigators have shown a greater extent and efficiency of fusion with a decrease in vesicle size (37,73,84). It appears that a decrease in liposome size leads to the bilayer of the liposome becoming strained and this results in a tendency of the vesicles to fuse with cells.

A correlation between liposome membrane fluidity and liposome-cell fusion has been attempted by Poste and Papahadjopoulos (97) and by Pagano and Huang (87). Poste and Papahadjopoulos observed, using 3T3 fibroblasts as target cells, that negatively charged liposomes in a "fluid" state favor uptake by a fusion process while neutral "fluid" and negative "solid" liposomes did not favor fusion (103). These results contrast those of Pagano and Huang (87) and Weinstein et al. (136) who found that neutral "fluid" vesicles favored uptake by fusion. The role that vesicle charge and target cell type have on vesicle fusion remains unclear. It does appear, however, that "fluid" liposomes tend to favor fusion.

Several reports have tried to determine the effect of vesicle charge on subsequent liposome-cell fusion although, once again, a great deal of controversy exists. Many early reports demonstrated that positively charged liposomes had a greater affinity for cells than did neutral and negatively charged liposomes (6,69,70,123,124,125). These experiments were performed using suspension cell cultures and the results contrast sharply with the results of experiments employing monolayer cell cultures (34,44,87). Negatively charged liposomes have been shown to have a greater number and a higher affinity for monolayer cell cultures than positively charged liposomes (34). It has also been shown that negatively charged liposomes and positively charged liposomes do not compete for the same binding sites and that this may be a reason for the observed differences in the way these two liposome populations interact with cells (34).

The ability of certain populations of lipid vesicles to fuse with monolayer cells has been used to the advantage of several researchers. Liposomes have been used extensively for the delivery of compounds that normally do not cross the cellular membrane. Intact poliovirus has been encapsulated into large unilamellar vesicles prepared from phosphatidylserine by Ca^{2+} and sodium (tetra) ethylenediamine tetraacetate (EDTA). These liposome-encapsulated polioviruses have been used to infect cells that normally lack the proper receptor (rodent cells). Incubation of these liposomes with Chinese Hamster Ovary cells has resulted in infection of these normally nonpermissive cells with an infection efficiency of 10 plaque forming units/ 10^6 cells (137). Liposomes can also deliver poliovirus RNA to cells, without the protection of the virus coat. These studies by Papahadjopoulos et al. (93) revealed that foreign RNA can be delivered to cells in a form that is readily expressible. The obvious genetic, and financial, implications are enormous and is possibly the reason for a patent being drawn up on this technique.

Little correlation has been drawn between events as they occur in a model liposome membrane and events as they occur in a much more complicated membrane system such as what occurs in a eukaryotic membrane. Overall, liposomes can be used to understand membrane functions, including membrane fusion requirements. The ease at which manipulations of membrane phenotypes can occur suggests that liposome technology will continue to be very important to membrane biology.

SECTION III

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SECTION IV

MANUSCRIPT: FACTORS AFFECTING THE CELL-SPECIFICITY OF
VESICULAR STOMATITIS VIRUS MEDIATED
CELL FUSION

ABSTRACT

Temperature-sensitive (ts) mutant G31 of vesicular stomatitis virus (VSV) induces mouse neuroblastoma (N-18) cells to undergo marked fusion during infections at 39°C which is nonpermissive for viral maturation. In contrast to neuroblastoma cells, BHK-21 cells and other cell lines not derived from the nervous system do not fuse under these conditions. To determine the molecular interactions responsible for cell fusion, liposomes were prepared from ts G31 VSV infected neuroblastoma cells with octylglucoside, loaded with fluorescein diacetate as a fluorescent probe, and added to noninfected N-18 cells to which they rapidly fused. Fusion required liposomes, prepared from ts G31 VSV infected N-18 cells, and the presence of the VSV glycoprotein. The same liposomes, however, would not fuse with BHK-21 cells nor would liposomes prepared from ts G31 VSV infected BHK-21 cells fuse with noninfected N-18 or BHK-21 cells. Hybrid liposomes (N-18 x BHK-21) were prepared to determine if N-18 cell membranes contained components that promoted fusion or if BHK-21 cell membranes contained components that were inhibitory to fusion. Membrane properties of both BHK-21 and N-18 liposomes were examined with respect to membrane fluidity, liposome size and phospholipid composition. It was determined that the high level of phosphatidylserine present in N-18 membranes (2.5 to 4 fold high than in any other cell line examined) was the primary factor leading to the ability of N-18 cell membranes to participate in fusion.

INTRODUCTION

The neuropathology observed in the central nervous system (CNS) of mice 4 days after intercerebral inoculation with a temperature sensitive (ts G31, complementation group III) mutant of vesicular stomatitis virus (VSV) closely resembles the status spongiosus induced by the atypical infectious agents associated with slow viral diseases such as kuru and Creutzfeldt-Jakob disease in humans, and scrapie and transmissible mink encephalopathy in animals (9,25). In addition to the status spongiosus produced during these slow viral diseases, fusion of neurons and glial cells has been observed in the CNS (24). In vitro fusion of neuroblastoma cells has also been observed after the addition of brain homogenates from scrapie and Creutzfeldt-Jakob disease victims (23,27). We have found that ts G31 VSV induces extensive cell fusion in mouse neuroblastoma cells when incubated at 39°C, which is non-permissive for viral maturation (12,21).

The ability of ts G31 VSV to promote neuroblastoma cell polykaryocyte formation at 39°C appears to be the result of an intracellular defect in maturation and/or assembly of the virions and the production and release of infectious virions is not necessary for cell fusion to occur (18,20). Since ts G31 VSV is an M protein mutant (complementation group III) and G glycoprotein production is not altered, the newly synthesized glycoprotein, under nonpermissive incubation conditions, is almost entirely localized in the plasma membrane (21). Polykaryocyte formation has been shown to require the synthesis and expression of this viral glycoprotein at the surface of infected cells (7,10,18,20,28).

In contrast to the fusion observed in mouse neuroblastoma cells infected with ts G31 VSV and incubated at 39°C, CHO, 3T3, and BHK-21 cells do not fuse when infected and incubated at this temperature (12). Fusion

is also not common to all nervous system derived cells, since primary astroglial cells do not fuse under these conditions (12). The inability of these cell lines to fuse was shown not to be the result of either a decreased expression of the viral glycoprotein, or a lack of sufficient cell to cell contact (12).

Infected BHK-21 cells can fuse with uninfected N-18 neuroblastoma cells although the formation of the initial BHK-21 x N-18 cell hybrid is followed with continued fusion of only neuroblastoma cells (12). These observations suggested that the viral glycoprotein, as processed by BHK-21 cells, is at least capable of initiating the fusion process (12). There are at least two factors, therefore, necessary for ts G31 VSV mediated polykaryocyte formation. The first is an accumulation of the viral glycoprotein at the surface of infected cells, and the second requirement appears to be related to the physical/chemical nature of the host cell membrane (18,20). However, the properties of the cell membrane that promote cell fusion are unknown. It has been suggested that a specific interaction between the viral glycoprotein and cellular membrane phospholipid may contribute to ts G31 VSV induced polykaryocyte formation (40).

In this study we examined the properties of mouse neuroblastoma and BHK-21 host cell membranes that may be associated with their ability to participate in ts G31 VSV-induced fusion. Liposomes from ts G31 VSV infected cells were prepared and loaded with fluorescein diacetate to provide a sensitive and quantitative measure of fusion. Fluorescein diacetate was particularly applicable since it does not fluoresce until hydrolyzed by cytoplasmic esterases (37), providing low backgrounds of fluorescence when liposomes only bind to target cells without fusion.

MATERIALS AND METHODS

CELL CULTURE AND VIRUS STOCKS: Mouse neuroblastoma N-18 cells were grown in F-12 medium and BHK-21 cells were grown in DMEM as previously described (21). Cell cultures were maintained routinely at 37°C in a 5% CO₂:95% air atmosphere. Fetal calf serum was purchased from Dutchland Laboratories Inc. (Denver, PA) and all other media components were purchased from Grand Island Biological Company (Grand Island, NY). Sucrose gradient purified stocks of ts G31 VSV, provided by M. Reichmann (University of Illinois, Urbana), were prepared from infected BHK-21 cells as previously described (11).

LIPOSOME PREPARATION: Liposomes were prepared as described by Huang et al. (19) with the following modifications. Confluent flasks of N-18 or BHK-21 cells were infected with ts G31 VSV (MOI 10) at room temperature, the virus was allowed to adsorb for 15 min., fresh medium was then added, and the cells were incubated at 39°C for 18 h. Following the incubation period, cells were washed 3 times with phosphate buffered saline (PBS) (140 mM NaCl, 5 mM KCl, 10 mM Na₂HPO₄, 10 mM KH₂PO₄, 10 mM CaCl₂, 10 mM MgCl₂; pH 7.2), scraped and pelleted at 1,000 x g for 15 min. Octylglucoside (10% w/v) was then added to the cell pellet, followed by incubation at 37°C for 15 min. with occasional mixing. Nonsolubilized cellular debris was then removed by ultracentrifugation at 100,000 x g for 1 h. The supernatant fluid (~ 4.0 ml) was dialyzed against 500 ml PBS (2 changes) containing fluorescein diacetate (0.01 µg/ml) for 18 hrs at 37°C. Following dialysis the fluorescein diacetate-loaded liposomes were pelleted at 100,000 x g for 1.0 h, resuspended in PBS, and repelleted by centrifugation. The resulting liposome pellets were then resuspended in 1.0 ml of PBS.

FUSION ASSAY: Confluent monolayers of N-18 or BHK-21 cells were washed three times with sterile PBS. Liposomes containing the fluorescent probe were then incubated at 39°C for various lengths of time with the cells (200 mM liposome phospholipid/ 10^6 cells). Following the incubation period the cells were quickly washed with ice-cold PBS, removed by scraping, maintained at 0°C at a final concentration of 1×10^6 cells/ml. Fluorescence was measured in a Spex Fluorolog spectrofluorometer with excitation at 478 nm and emission at 516 nm. Background fluorescence was determined by measuring fluorescence of 1×10^6 cells to which no liposomes were added. Liposomes, loaded with fluorescein diacetate, but not added to cells, did not fluoresce above background levels. All experiments presented in this paper have been repeated a minimum of three times.

MEMBRANE FLUIDITY ASSAY: Membrane fluidity was measured using the fluorescent probe trans-parinaric acid (9,11,13,15 all trans octadecatetraenoic acid) with a Spex Fluorolog spectrofluorometer and data analyzed according to the method of Rintoul et al. (35). Sample heating and cooling was regulated by a Neslab RT-5 circulating water bath and an ETP-3 temperature programmer. Liposomes were prepared for fluidity analyses as described above except following the final wash the liposomes were resuspended in Ca^{2+} - and Mg^{2+} -free PBS; pH 7.2.

PHOSPHOLIPID EXTRACTION AND ANALYSIS: Cells from monolayer cultures were removed by scraping, washed 3 times with ice-cold PBS (pH 7.2) and resuspended in 1-2 ml water. Methanol, containing 10 mg/1 butylated hydroxytoluene (BHT), was then added to the cell suspension and the mixture was vigorously mixed by vortexing. Chloroform, containing 10 mg/1 BHT, was then added, followed by the addition of 0.5 ml of a saturated KCl solution,

and the final ratio of solvents was adjusted to 2:1:1 (chloroform:methanol:water). The mixture was centrifuged at 1,000 x g for 5 min., the lower chloroform phase was removed and set aside, and the aqueous phase was re-extracted with 6.0 ml chloroform. The resulting two chloroform phases were combined and dried under a nitrogen stream (4). The extracted lipids were resuspended in 1.0 ml chloroform and the various lipid classes were separated on a 200-325 mesh silicic acid column (Clarkson Chemical Co. Inc., Williamsport, PA) as described by Rintoul et al. (35). Phospholipid phosphate was assayed after ashing with $\text{Mg}(\text{NO}_3)_2$ by the method of Ames (2). Two-dimensional thin layer chromatography of membrane phospholipids was carried out with Silica G gel plates (Supelco Inc., Belfonte, PA) as described by Freter et al. (16). Quantitation of phospholipids, following thin layer chromatography, was achieved by first visualizing the lipids by spraying with 50% H_2SO_4 , charring overnight at 130°C, scraping the developed areas, and assaying for phosphate as described by Rintoul et al. (36).

RESULTS

Liposomes prepared from N-18 cells, previously infected with ts G31 VSV (MOI 10) and loaded with fluorescein diacetate as described in the Materials and Methods section, fused rapidly with uninfected N-18 cells with maximum fusion observed at 20-25 min. after their addition (Fig. 1). The viral glycoprotein was essential to the fusion process since liposomes prepared from ts G31 VSV infected neuroblastoma N-18 cells and pre-incubated for 15 min. at 37°C with antibody (IgG) directed against the G glycoprotein resulted in a marked decrease (~ 85%) in liposome-cell fusion (Fig. 1). While the liposomes prepared from infected N-18 cells fused rapidly with the N-18 target cells, liposomes prepared from VSV infected BHK-21 cells fused to N-18 cells only to a small degree (20% of that measured with N-18 liposomes) (Fig. 1). These results indicated that the cell type from which the liposomes were prepared was important in the fusion process and was consistent with earlier reports of fusion with intact cells infected with ts G31 VSV (12). In addition to the cell source for liposome preparation, the target cell to which the liposomes were added was also important since liposomes prepared from either VSV infected BHK-21 or N-18 cells did not readily fuse with BHK-21 cells (Fig. 2).

Several conditions could have existed that would have biased our observations. As an example, the lack of fusion (fluorescence) with BHK-21 cells could have resulted from an absence of cytoplasmic esterases to activate the fluorescein diacetate. Preliminary experiments, however, revealed that in the presence of 0.1 µg/ml fluorescein diacetate both N-18 and BHK-21 cells (1×10^6 cells) were able to convert fluorescein diacetate to its fluorescent analogue (relative fluorescence 1.313 and 1.223, respectively). The lack of fluorescence observed in BHK-21 cells,

therefore, was not due to the absence of cytoplasmic esterases. There also was little, if any, non-specific leaking of fluorescein diacetate from liposomes since fluorescence was only observed when liposomes prepared from infected N-18 cells were added to N-18 monolayers. It was possible that liposomes prepared from BHK-21 cells, added to N-18 or BHK-21 cells, did not fuse simply because the liposomes were not intimately associated with the cells. Additional studies with VSV infected BHK-21 cells, loaded with fluorescein isothiocyanate-dextran, showed that the liposomes bound tightly to both BHK-21 and N-18 target cells although subsequent fusion was rare (data not shown).

Covalent attachment (acylation) of fatty acids to the VSV glycoprotein, as well as glycoproteins of other viruses, has been suggested to play a role in the process of cell fusion (24). Analysis of the fatty acids covalently attached to the VSV G glycoprotein, processed by BHK-21 and N-18 cells, revealed no significant differences with respect to the amount of acylation or the species of fatty acids present (Table 1). Therefore, differences in host acylation of the VSV glycoprotein cannot account for the marked differences in fusion potential by liposomes prepared from BHK-21 and N-18 cells (Figs. 1 and 2).

Post-translational modifications of the VSV glycoprotein are directed by the host cell (22) and the inability of BHK-21 membrane liposomes to efficiently fuse to the target cell surface membrane could have been related to the presence of a VSV G glycoprotein that did not efficiently participate in this membrane-membrane interaction. This possibility was examined by preparing hybrid liposomes by mixing solubilized membrane of VSV infected BHK-21 cells with increasing ratios of solubilized membrane from noninfected N-18 cells. The resulting hybrid liposomes would, therefore, all possess the VSV glycoprotein as processed by the BHK-21

cells. Although the increase in the proportion of uninfected N-18 membrane in the resulting hybrid liposomes diluted the VSV glycoprotein, an increase in the proportion of N-18 membrane in the hybrid liposome resulted in an increase in fusion (Fig. 3). These results clearly indicated that the VSV glycoprotein, as processed by BHK-21 cells, is capable of initiating the fusion process.

Although the results in Fig. 3 indicated that the VSV glycoprotein synthesized in BHK-21 cells was capable of initiating fusion, the extent of fusion with the hybrid liposomes remained less than that measured with homologous N-18 x N-18 cell interactions. It was possible that the BHK-21 membrane components were inhibitory for optimal fusion or that the BHK-21-processed VSV glycoprotein could only initiate, and not efficiently promote, membrane-membrane fusion. In order to discriminate between these possibilities hybrid liposomes were prepared from VSV infected N-18 neuroblastoma cell membranes and uninfected BHK-21 cell membranes. In contrast to the results in Fig. 3, an increase in the proportion of uninfected BHK-21 membrane in the hybrid liposomes markedly decreased fusion (Fig. 4). This decrease in fusion was independent of a dilution of the viral glycoprotein since hybrid liposomes prepared by combining membrane from VSV infected N-18 cells with increasing amounts of membrane from uninfected N-18 cells resulted in only a slight decrease in fusion (Fig. 4).

An analysis of the kinetics of fusion shown in Figs. 3 and 4 indicated that when the percentage of N-18 membrane (infected or uninfected) reaches ~37.5% the fusion index of the two types of hybrid liposome populations overlap. This indicates that the VSV glycoprotein, as processed by N-18 and BHK-21, cells are approximately equal in their ability to induce fusion. The limiting component to the fusion process, therefore, can only be due to the membrane properties of the BHK-21 cell. The kinetics also

indicated that neither the neuroblastoma nor the BHK-21 cell membrane preferentially associated with the hybrid liposomes during the dialysis step to remove the detergent.

One potential artifact that could arise from liposome preparation that could influence relative fusibility potential of neuroblastoma N-18 and BHK-21 liposomes was the relative size of the liposomes themselves. Studies by Weinstein et al. (42) have shown that the relative size of liposomes can influence their ability to promote fusion. Electron microscopic examination of liposomes that we prepared from neuroblastoma and BHK-21 cells, however, showed that they were similar in size having an average diameter of $96 \pm 4 \text{ \AA}$ and $98 \pm 3 \text{ \AA}$, respectively.

Since fluidity and membrane phospholipid composition can influence membrane-membrane fusion (30,31), it was possible that the differences between neuroblastoma and BHK-21 liposome fusion was a result of their physical and/or chemical properties. Fluidity measurements were made with liposomes prepared from both N-18 and BHK-21 cells infected with ts G31 VSV and cells that were not infected. This fluorescence technique measures the rotational diffusion of a free fatty acid probe in membrane bilayers; high polarization ratios indicate less lipid motion (less fluid), and lower polarization ratios indicate increased lipid mobility (more fluid). When examined with the fluorescent probe transparinaric acid, the fluidity of liposomes prepared from ts G31 VSV-infected N-18 and BHK-21 cells was quite similar and no differences in phase transitions could be measured that could explain the marked differences in their fusibility (Fig. 5a and 5b). In both cases, however, the membranes from VSV infected cells were slightly less fluid, as indicated by a higher polarization ratio, than the membranes from uninfected cells. Although, between 40°C to 45°C, there appears to be a large difference in the fluidity of VSV infected N-18 liposomes when

compared with uninfected N-18 liposomes this proves to be insignificant since relatively small changes in fluorescence intensity at this temperature can give differences in the polarization ratio (13).

Although the total phospholipid content of BHK-21 and N-18 cells was similar, the phospholipid composition was quite different in neuroblastoma, BHK-21, CHO and LM-22 cells. Of particular interest was the marked difference in phosphatidylserine content where liposomes prepared from N-18 neuroblastoma cells was 2.5 to 4.0 higher than that measured in BHK-21 cells and the other nonfusible cell types (Table 2). In light of previous reports that phosphatidylserine content significantly influences the fusibility of liposome-membrane interactions (30,22), and that the proportion of phosphatidylserine is in direct correlation with fusion (15), the high phosphatidylserine content of N-18 neuroblastoma cells most likely is the fundamental reason for their liposomes to readily fuse. In addition, the reduced fusion by dilution of phosphatidylserine in hybrid liposomes (BHK-21 x N-18), but not (N-18 x N-18), is consistent with this observation.

DISCUSSION

Liposomes have been used extensively to study the factors necessary for membrane fusion, and liposome size has been shown to be important to the ability of vesicles to fuse. Several investigators using vesicle-vesicle fusion have shown a greater extent and efficiency of fusion with a decrease in vesicle size (17,26). The data presented in this paper indicates that there is no significant difference in vesicle size when comparing fusible and nonfusible liposome preparations as revealed by electron microscopy. Negative staining of vesicle preparations has been shown by Olson et al. (29) to give similar size distributions as those observed by scanning electron microscopy methods and therefore is an acceptable method for sizing liposomes.

The importance of membrane fluidity has been studied by Poste and Papahadjopoulos (34) and by Pagano and Huang (30) with conflicting results. Poste and Papahadjopoulos (34) observed, using 3T3 fibroblasts as target cells, that negatively charged liposomes in a "fluid" state favored uptake by a fusion process while neutral "fluid" and negative "solid" liposomes did not favor fusion. These results contrast with the work of Pagano and Huang (30) who found that neutral "fluid" vesicles favored uptake by fusion. Although the exact nature and role that charge may have on fusion remains unclear, it is certain that "fluid" liposomes tend to favor uptake via a fusion process. However, the observed differences in fusion of the N-18 liposomes and the BHK-21 liposomes cannot be explained on the basis of membrane fluidity (Fig. 5A and 5B).

Acylation has been shown to be a common link between the fusion glycoproteins of several viruses, including VSV (26). Other evidence suggesting that acylation may play a role in fusion is the finding by Schimdt et al.

(39) that oleic acid could be found in the fatty acid fraction released from the Sindbis virus glycoprotein E1. Oleic acid has long been known as a fatty acid with fusogenic properties (1). It also has been suggested that the fatty acid binding site present on VSV G glycoprotein is located at the external boundary or just outside the lipid bilayer and that oleic acid binding in such a position could disrupt two closely associated lipid bilayers and thus promote fusion. Whatever the relationship between acylation and the fusogenic properties of some virus glycoproteins are, there appears to be no significant difference in acylation of the G glycoprotein of VSV as processed by BHK-21 and N-18 cells. Acylation, therefore, cannot account for the observed differences in cell fusion with liposomes from these two infected cell lines. The lack of any discernable difference in the acylation of the G protein supports the observation that the glycoprotein, as processed by nonfusible BHK-21 cells, appears equal in its ability to promote fusion when compared with the glycoprotein processed by the fusion competent N-18 cells.

Poste and Papahadjopoulos (34) have studied the effects of lipid composition on vesicle fusion and have shown that little if any vesicle-cell fusion occurs when vesicles contain neutral lipids, however, fusion is enhanced by the presence of phosphatidylserine. Phosphatidylserine has also been shown by Fraley *et al.* (15) to enhance delivery of SV-40 DNA to target cells, and that the extent of delivery was proportional to the percentage of phosphatidylserine present in the vesicle preparation. The data presented in Table 2 shows that phosphatidylserine represents 28-30% of the total phospholipid present in N-18 cells, while phosphatidylserine represents only 5-10% of the total phospholipid in BHK-21 cells and the other cell lines that do not fuse readily.

Although vesicle-cell fusion has been studied extensively, the majority of the work has examined the properties of the liposome vesicle

and little work has been focused on determining the factors required for participation of the target cell (32). From the observations presented in this study it is evident that both the liposome and the target cell play an important role in determining whether fusion will ultimately occur. Although ts G31 VSV has been shown to be an M protein mutant, the viral M protein is synthesized at the nonpermissive temperature of 39°C at a normal rate, but the M protein is unstable at 39°C and is eventually degraded (21). The M protein of VSV has been shown to bind with phosphatidylserine on the inner leaflet of plasma membranes and exert a stabilizing effect. However, this association results in a localized high concentration of phosphatidylserine (43). It appears likely that the M protein of ts G31 VSV at 39°C functions in this same respect on a temporary basis. Following the initial binding the M protein is degraded, at the nonpermissive temperature leaving an unstable area of highly concentrated phosphatidylserine. The observations by Handa et al. (18) that the G glycoprotein diffuses from membrane to membrane through intracellular connections and the observation by Schlegel et al. (38) that phosphatidylserine appears to be a VSV binding site suggests the following hypothesis. A specific interaction between the G glycoprotein and phosphatidylserine first leads to a partial phase separation which has been shown with other systems (5,6). Following this initial interaction, a membrane area that has a localized high concentration of phosphatidylserine, and which is unstable, then triggers a partial phase separation between two opposing membranes which Portis et al. (33) suggested may be a prerequisite for membrane-membrane fusion.

The literature is lacking any attempt to correlate the properties present in liposomes that might be the basis for virus-induced cell fusion. The liposomes that we prepared from whole cells retained the same fusion

specificity with respect to the cells from which the vesicles were derived. Therefore, we conclude that the presence of unusual high levels of phosphatidylserine in N-18 cell membranes (8,14) exerts a destabilizing effect in the N-18 cell membrane during nonpermissive infections with ts G31 VSV and this promotes fusion with liposomes and intact cells.

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Table 1. Profile of fatty acid covalently attached to ts G31 VSV G glycoprotein.

FATTY ACID	N-18 (% of total)	BHK-21 (% of total)
Myristate	6.0 $\pm$ 1.1	2.4 $\pm$ 1.6
Palmitate	2.4 $\pm$ 0.5	17.5 $\pm$ 4.8
Stearate	49.7 $\pm$ 3.3	35.0 $\pm$ 2.5
Oleate	20.7 $\pm$ 7.2	23.4 $\pm$ 4.7
Linoleate	21.2 $\pm$ 3.9	21.7 $\pm$ 1.6

Purified G glycoprotein (41) was deacylated and transesterified in 1.0 ml of a 0.1N KOH solution in 100% methanol for 20 min. at room temperature. Following the 20 min. incubation the sample was acidified with 0.1 ml of 1M HCl and extracted with chloroform, methanol and water (final ratio of 8:4:3 respectively). The lower phase was collected and washed with 1.0 ml of a chloroform, methanol and water solution (1:10:10 respectively) and dried under a nitrogen stream. Hepadecanoic acid (50 nM) was added as an internal standard to the dried lower phase prior to the addition of 1.5 ml methanolic HCl (60 min. at 70°C). Water (1.5 ml) was added following the incubation period and the entire sample was extracted 2x with pentane. The sample was then dried and analyzed on a Hewlett-Packard, series 5790A, gas chromatograph using a 6' CS-10 (10% on Chromosorb WP 100/120) column at 170°C (average of three separate determinations $\pm$ S.D.).

Table 2. Phospholipid composition of fusible and nonfusible cell lines.

	CELL LINE						
	<u>*IN-18</u>	<u>n-18</u>	<u>#IBHK</u> (% of total phospholipid)	<u>BHK</u>	<u>@ICHO</u>	<u>CHO</u>	<u>LM-22</u>
SM	2 ± 1	1 ± 1	8 ± 3	4 ± 3	4 ± 3	6 ± 2	12 ± 3
PC	22 ± 2	33 ± 1	19 ± 4	36 ± 5	34 ± 2	34 ± 2	45 ± 5
PS	30 ± 1	28 ± 2	5 ± 3	5 ± 2	7 ± 4	7 ± 1	10 ± 2
dPG	4 ± 1	4 ± 2	4 ± 2	4 ± 3	2 ± 1	2 ± 1	-
PG	9 ± 1	4 ± 3	16 ± 2	14 ± 1	4 ± 2	4 ± 1	-
PA	2 ± 1	2 ± 1	6 ± 3	6 ± 4	3 ± 1	6 ± 2	-
PE	9 ± 2	10 ± 3	23 ± 2	10 ± 2	19 ± 2	15 ± 3	30 ± 3
PI	5 ± 3	5 ± 3	12 ± 3	13 ± 3	10 ± 2	7 ± 1	-
LYSO	12 ± 2	8 ± 4	3 ± 2	6 ± 2	14 ± 3	12 ± 2	-
OR	5 ± 2	15 ± 3	3 ± 2	2 ± 1	3 ± 2	7 ± 1	-

Phospholipid profiles were obtained on the fusion competent (N-18) and non-fusible (BHK-21 and CHO) cell types as described in Materials and Methods. Briefly, cells were grown as monolayer cultures and infected or mock infected with ts G31 VSV. Spingomyelin, SM; phosphatidylcholine, PC; phosphatidylserine, PS; dPG; phosphatidylglycerol, PG; phosphatidic acid, PA; phosphatidylethanolamine, PE; phosphatidylinositol, PI; lysophospholipids, LYSO; or., nonmigrating material (average of 3 separate determinations +/- S.D.).

* - Phospholipid profile of ts G31 VSV infected N-18 cell line.

- Phospholipid profile of ts G31 VSV infected BHK-21 cells.

@ - Phospholipid profile of ts G31 VSV infected CHO cells.

Fig. 1. Fusion kinetics of liposomes prepared from ts G31 VSV infected N-18 cells. Liposomes prepared from ts G31 VSV infected N-18 cells were incubated with monolayer N-18 (●) or BHK-21 cells (▲) at 39°C for various lengths of time as described in the Materials and Methods. Prior to incubation with N-18 cells one set of liposomes was incubated with a neutralizing antibody, specific for the G glycoprotein, for 15 min. at 37°C (○).

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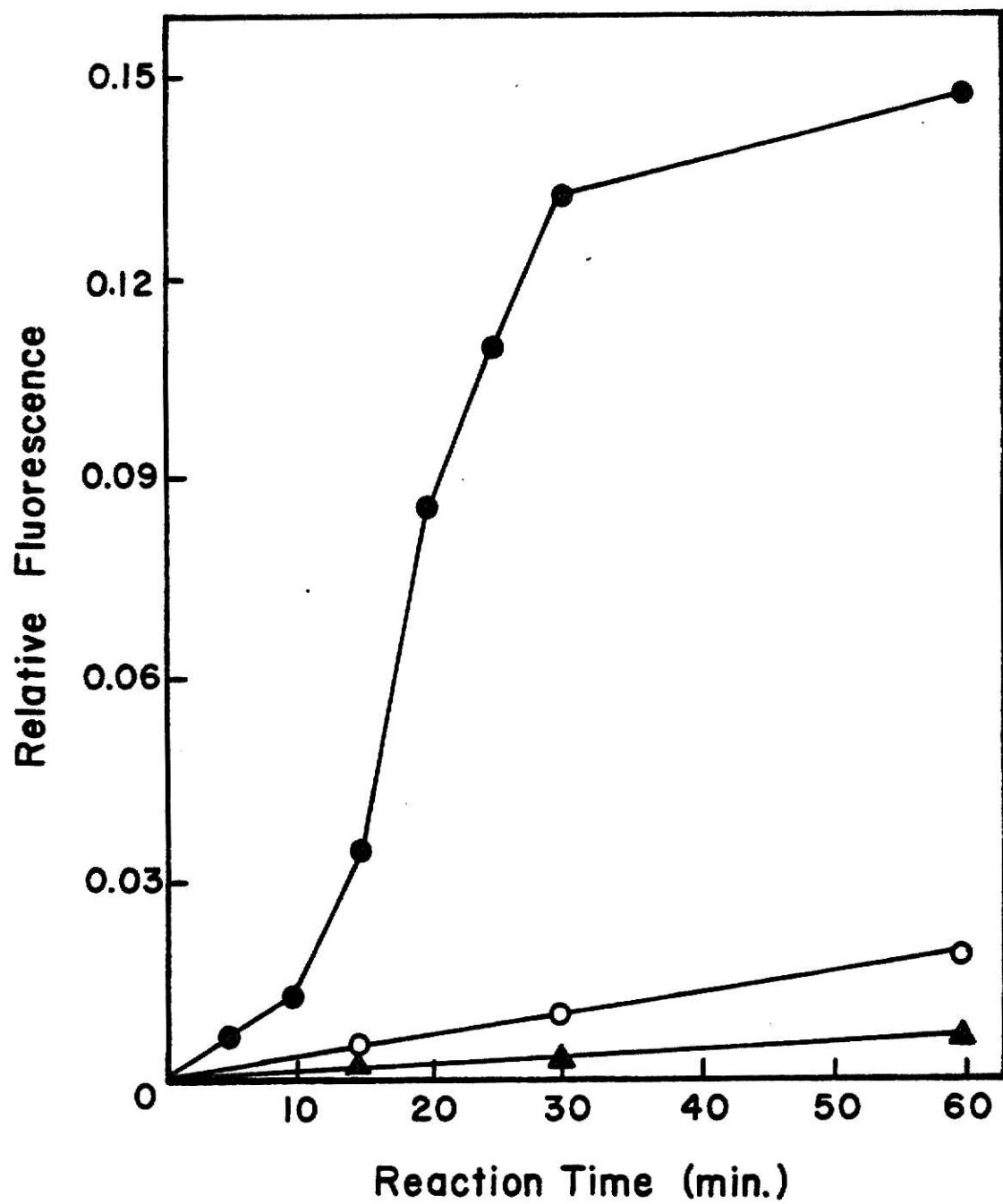


Fig. 2. Fusion kinetics of liposomes prepared from ts G31 VSV infected BHK-21 cells. Liposomes prepared from BHK-21 cells previously infected with ts G31 VSV were incubated with monolayer N-18 (○) or monolayer BHK-21 cells (●) as described in Materials and Methods. The dashed line (----) indicates the extent of fusion that would occur if a similar amount of liposomes prepared from ts G31 VSV infected N-18 cells were incubated with monolayer N-18 cells as indicated in Fig. 1.

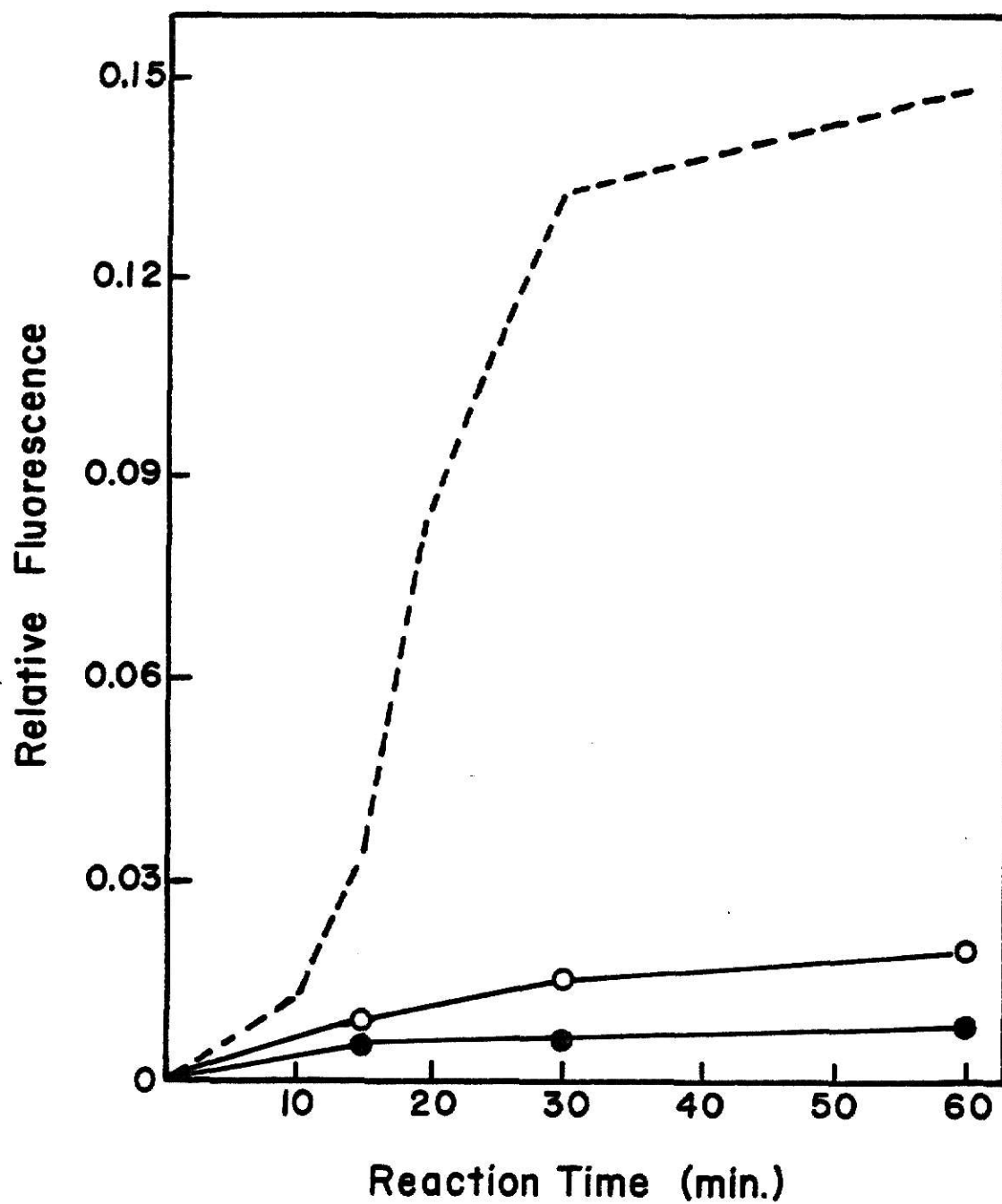


Fig. 3. Fusion kinetics of hybrid liposomes. Hybrid liposomes were prepared by mixing solubilized membrane from ts G31 VSV infected BHK-21 cells with increasing amounts of solubilized membrane from uninfected N-18 cells.

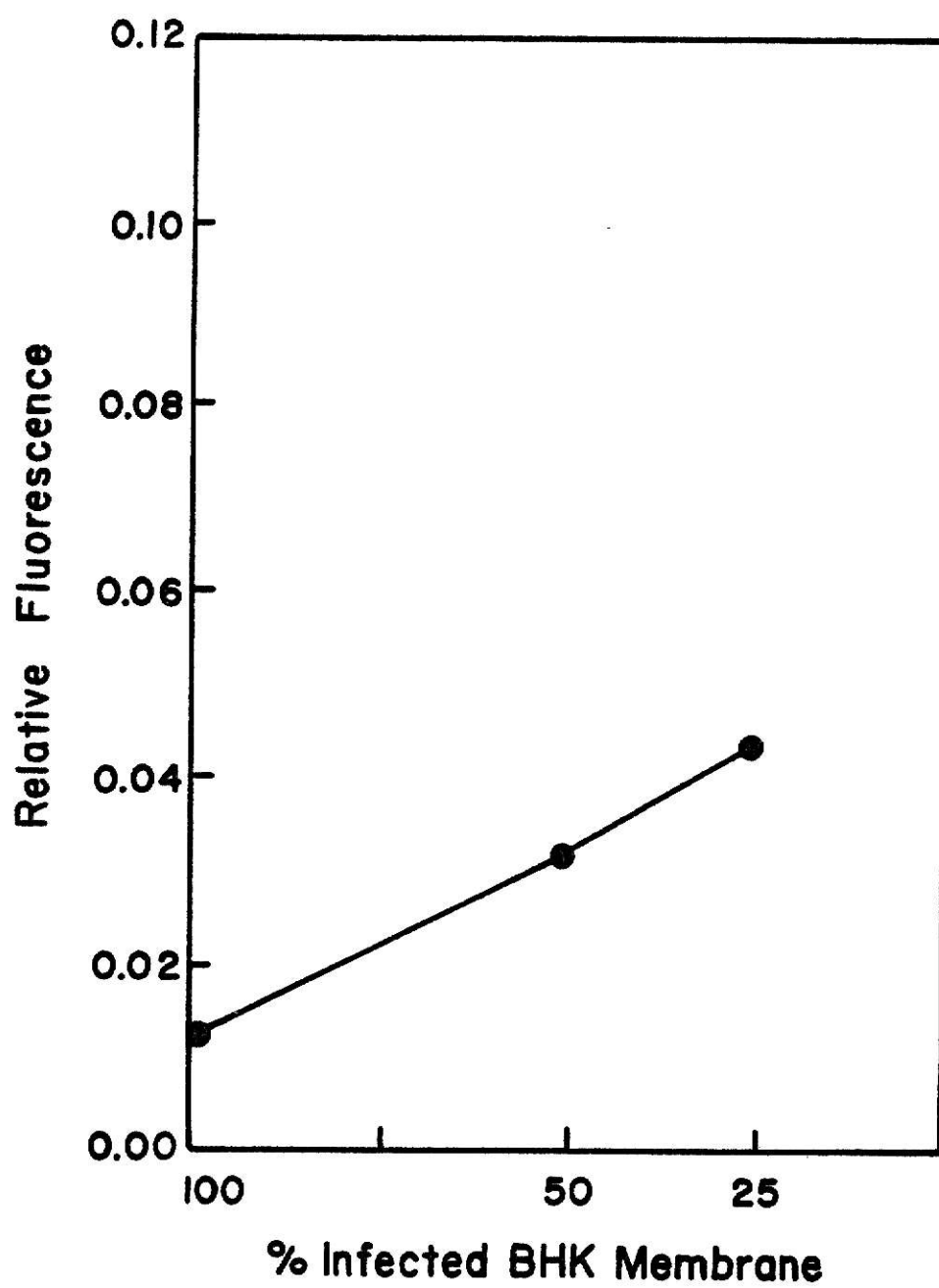


Fig. 4. Fusion kinetics of hybrid N-18 x BHK-21 liposomes. Liposomes were prepared by mixing membrane from ts G31 VSV infected N-18 cells with increasing amounts of solubilized membrane from uninfected BHK-21 cells (○) or increasing amounts of solubilized membrane from uninfected N-18 cells (●). Liposomes were added to monolayer N-18 neuroblastoma target cells and fusion was assayed as described in Materials and Methods.

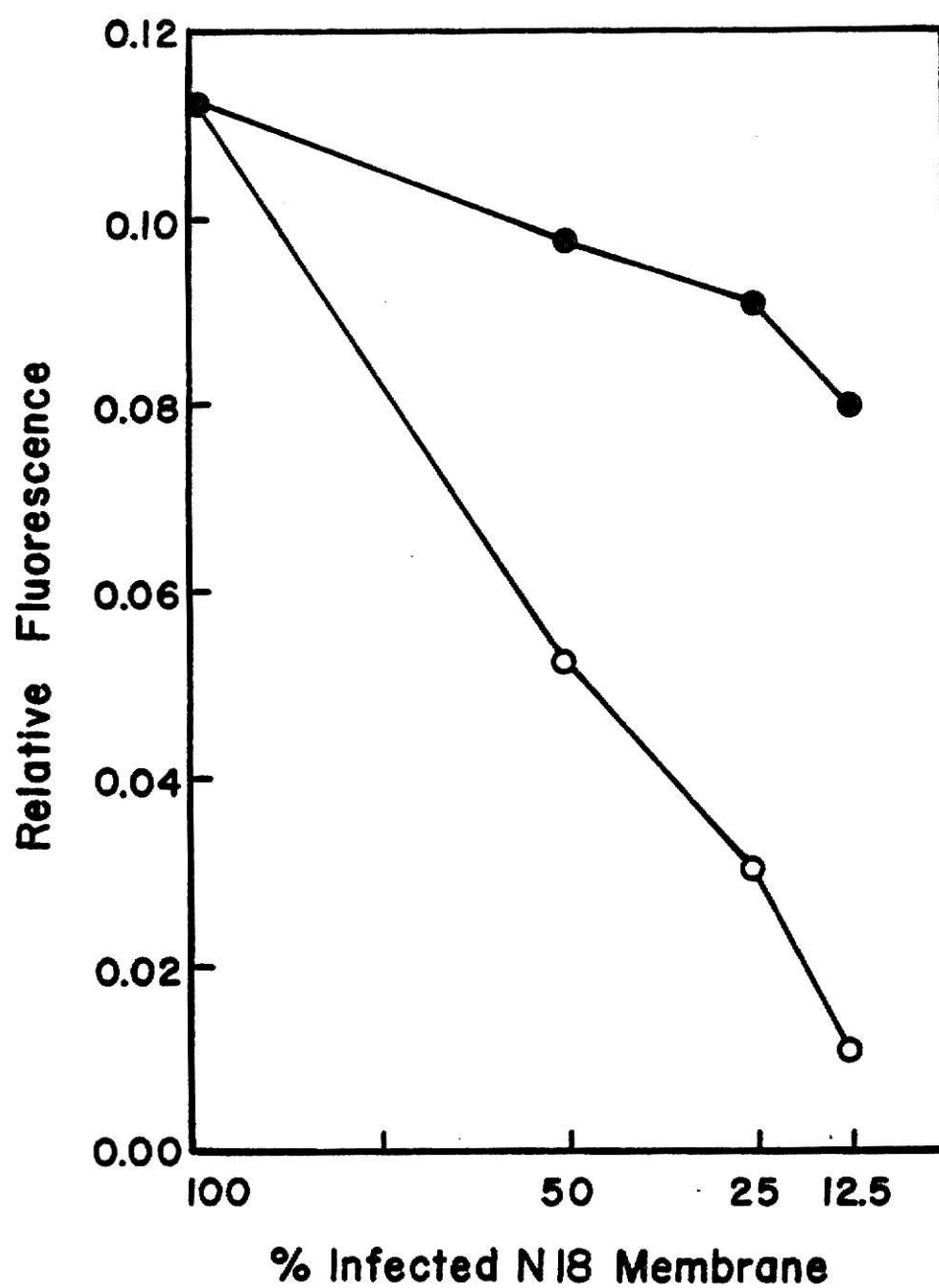
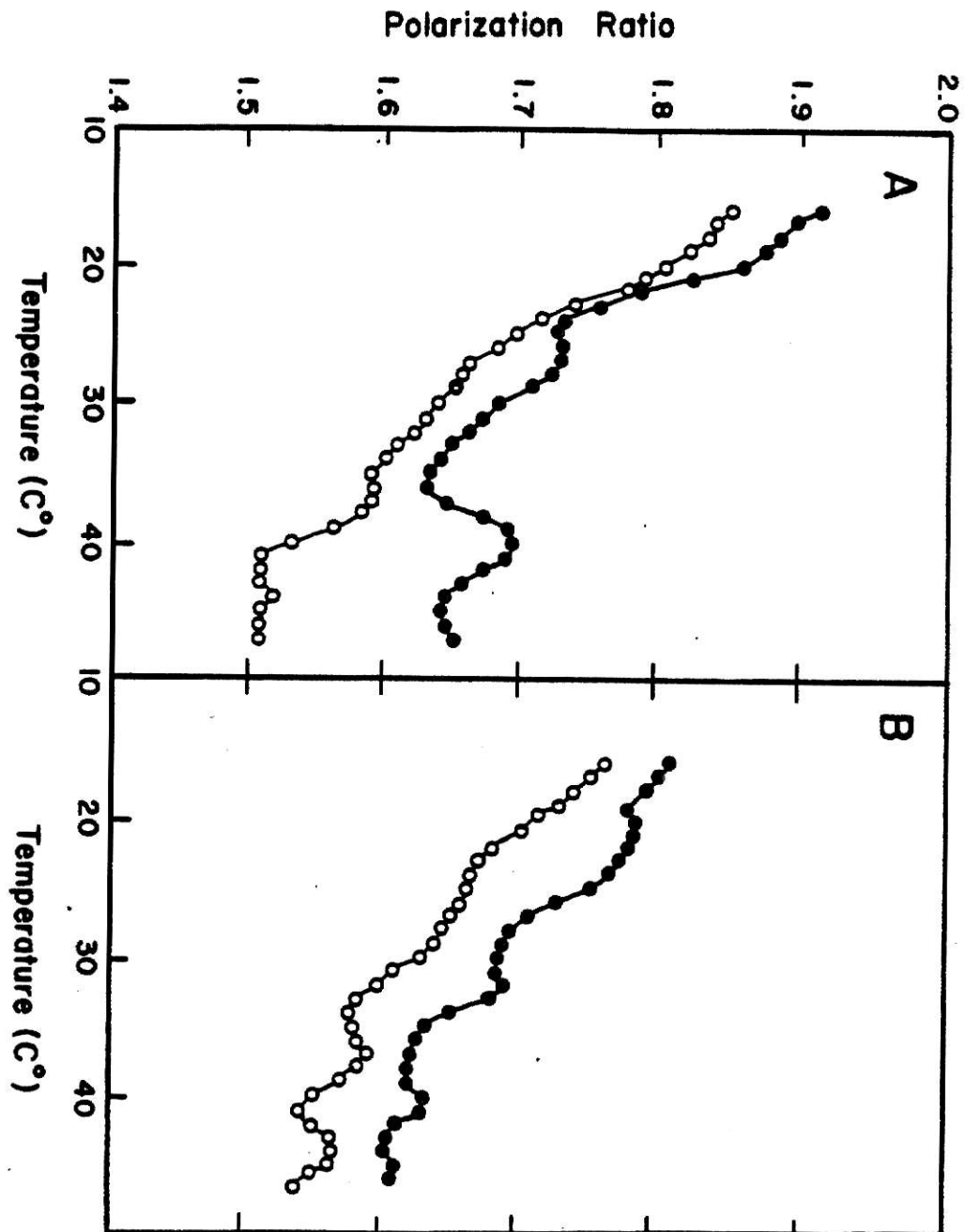


Fig. 5. Membrane fluidity of uninfected and infected N-18 and BH-21 liposomes:

A. Assay was performed in Ca^{2+} -, Mg^{2+} -free PBS using the fluorescent probe trans-paranaric acid. Data was computer analysed as described by Rintoul et al. (35). (○) N-18 and (●) ts G31 VSV infected N-18 liposomes.

B. Experiment was performed as described in Fig. 5A. (○) BHK-21 and (●) ts G31 VSV infected BHK-21 liposomes.



FACTORS AFFECTING THE CELLULAR SPECIFICITY OF VESICULAR
STOMATITIS VIRUS MEDIATED CELL FUSION

By

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ABSTRACT

Temperature-sensitive (ts) mutant G31 of vesicular stomatitis virus (VSV) induces mouse neuroblastoma cells to undergo marked fusion during infections at 39°C which is nonpermissive for viral maturation. In contrast to neuroblastoma cells, BHK-21 cells and other cell lines not derived from the nervous system do not fuse under these conditions. To determine the molecular interactions responsible for cell fusion, liposomes were prepared from ts G31 VSV infected neuroblastoma cells (N-18) with octylglucoside, loaded with fluorescein diacetate as a fluorescent probe, and added to noninfected N-18 cells to which they rapidly fused. Fluorescein diacetate is only fluorescent after enzymatic cleavage by esterases contained in target cell cytoplasm, which allows a quantitative assay of fusion. Fusion required liposomes prepared from ts G31 VSV infected N-18 cells, and the presence of the VSV glycoprotein. The same liposomes, however, would not fuse with BHK-21 cells nor would liposomes prepared from ts G31 VSV infected BHK-21 cells fuse with noninfected N-18 or BHK-21 cells. Acylation profiles of the G glycoprotein processed by both BHK-21 cells and by N-18 cells revealed no significant differences and thus could not account for the observed differences in fusion. Hybrid liposomes (N-18 x BHK-21) were prepared to determine if N-18 cell membranes contained components that promoted cell fusion or if BHK-21 cell membranes contained components that were inhibitory to fusion. Membrane properties of both BHK-21 and N-18 liposomes were examined with respect to membrane fluidity, liposome size and phospholipid composition. It was determined that liposome size and membrane fluidity were similar in fusible and nonfusible liposome preparations and that the high levels of phosphatidylserine present in N-18 membranes (2.5 to 4 fold higher than in any other cell line examined) correlated with the ability of N-18 cell membranes to promote membrane fusion.