Cholesterol Enhances Mouse Hepatitis Virus-Mediated Cell Fusion

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Mouse hepatitis virus (MHV) infection of the L-2 subline of mouse fibroblasts results in acute infection characterized by extensive cell fusion. In contrast, infection of the LM-K subline leads to virus persistence with reduced cell fusion. We undertook studies designed to elucidate the role of host cell membrane lipid composition and the cytoskeleton in modulating the fusion process and the resultant effect(s) on virus persistence. MHV-induced cell fusion proceeded normally in cells treated with cytoskeleton-disrupting drugs, cytochalasin B and colchicine. Modification of cell membrane fatty acid composition by supplementation of LM-K cells with arachidonic (C-20:4) or palmitic (C-16:0) acids had little effect on the extent of MHV-induced cell fusion or on virus replication. However, supplementation of both cell types with cholesterol (resulting in increased membrane cholesterol/fatty acid ratio) resulted in marked enhancement of virus-mediated cell fusion. The increase in cell membrane cholesterol did not enhance internalization of MHV suggesting that cholesterol primarily modulates a later event. This suggestion was confirmed by demonstrating cholesterol-enhancement of fusion in a contact fusion assay. Cholesterol-supplemented L-2 cells were less productive for virus replication than unsupplemented cells, in agreement with our previous observations that MHV replication is compromised by extensive cytopathic effect. Although cholesterol-supplemented LM-K cells showed increased susceptibility to MHV-mediated cell fusion, the extent of such susceptibility did not approach that observed in L-2 cells. Also, the property of LM-K cells to support MHV persistence was not abolished by cholesterol supplementation. Thus membrane fusion resistance and MHV persistence are modulated but not alleviated by cell membrane cholesterol content. © 1988 Academic Press, Inc.

INTRODUCTION

Mechanisms which underly virus persistence remain incompletely understood. Among persistent infections of murine coronaviruses, infections of mouse hepatitis virus (MHV) in rodent hosts have provided useful information for the investigation of animal models for virus-mediated chronic neurological disease (Cheever *et al.*, 1949; Weiner, 1973; Haspel *et al.*, 1978; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980). Typical of such infections is a state of prolonged virus persistence in the central nervous system in which discrete pockets of virus-infected cells coexist among areas of apparently normal, uninfected tissue. While the JHM strain of MHV has been the virus of choice in such experimental systems, there is evidence that other MHV strains, such as MHV-3 (LePrevost *et al.*, 1975) and A59 (Hirano *et al.*, 1980), also readily establish infections of a chronic nature in mice or rats.

Besides their ability to give rise to various degrees of virus persistence, many MHV strains are also able to produce infections of an acute, lytic nature (reviewed by Wege *et al.*, 1982). The elucidation of the conditions which determine the outcome of infection is accordingly important to the understanding of MHV pathogenicity. Within the central nervous system, various cell types appear to behave differently with respect to either the replication or dissemination of MHV (Sorensen *et al.*, 1980; Dubois-Dalcq *et al.*, 1982; Buchmeier *et al.*, 1984). In particular, cell-type differences have been noted in the expression of virus-induced cell–cell fusion (Dubois-Dalcq *et al.*, 1982; Buchmeier *et al.*, 1984) which is an important mechanism of virus spread.

We have previously described an *in vitro* model for MHV persistence (Mizzen *et al.*, 1983). In these studies we defined two categories of mouse fibroblast sublines (*L-2* and LM-K) which differ in initial susceptibility to MHV infection and in the expression of virus-induced cell fusion. The outcome of infection is at least in part dependent upon fusion susceptibility such that fusion-sensitive L-2 cells succumb to acute, lytic infection, whereas the relatively fusion-resistant LM-K cells support a persistent infection in which a steady-state balance between virus replication and cell survival is maintained. In the present communication we present further studies emphasizing the role of MHV-induced cell fusion in determining the severity of MHV infection and document the importance of host cell membrane lipid composition in modulating the fusion process and the resultant effect on virus persistence.

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MATERIALS AND METHODS

Cells, virus, and culture conditions

Mouse fibroblast sublines L-2 (Rothfels et al., 1959) and LM-K (Kit et al., 1963) were routinely propagated in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS), except where modified as described below. All virus inoculations were performed with the A59 strain of MHV (Manaker et al., 1961). Virus was adsorbed onto cell monolayers for 30 min at 4° and then incubated at 37° in one of the media described below. Virus titrations were determined by plaque assay on L-2 cells. To score cell fusion, cultures were stained with Giemsa and examined by phase-contrast microscopy in order to obtain fusion index values (Fusion index = 1 − [(mononuclear cells in infected culture)/(mononuclear cells in control culture)]; Mizzen et al., 1987).

Treatment of cells with cytoskeleton-disrupting drugs

Confluent L-2 cell monolayers in wells of 24-well plates were inoculated with MHV (m.o.i. 3), adsorbed as above and subsequently incubated at 37° for 6 hr in medium containing either cytochalasin B (0, 0.5, 1.0, or 2.5 μg/ml) or colchicine (0, 0.1, 0.2, or 0.3 μg/ml). Cultures were subsequently stained with Giemsa and scored for fusion.

Fatty acid supplementation

A procedure modified from those of Spector and Hoak (1969) and Doi et al. (1978) was used. Cultures of LM-K cells were grown to confluency in MEM supplemented with 5% FCS, and then the medium was replaced with MEM supplemented with 0.25 μM bovine serum albumin (BSA) complexed to either palmitic acid or arachidonic acid (Sigma). Cells were further cultured for 24 hr and then processed for gas chromatographic analysis or virus inoculations.

Cholesterol supplementation

The procedure of Shinitsky (1978) for the incorporation of cholesterol into red blood cells was employed. Briefly, a solution of 1 mg cholesterol (Sigma) in 1 ml tetrahydrofuran and 0.2 ml 0.6% KCl was added dropwise to a vigorously stirring solution (10 ml) of MEM containing 10% FCS. The mixture was lyophilized and reconstituted to 10 ml with water and designated "cholesterol medium." Gas chromatographic analysis of this cholesterol medium yielded a cholesterol content of 75 μg/ml. Cultures of L-2 or LM-K cells, grown to confluency in MEM containing 5% FCS, were main-tained in normal medium (MEM + 10% FCS) or cholesterol medium for a further 24 hr and subsequently processed for gas chromatography or virus inoculations. To generate the various cholesterol concentrations used for the study shown in Fig. 1, dilutions of cholesterol medium were made in normal medium.

Infectious center (IC) assays

IC assays were performed as described previously (Mizzen et al., 1985) except that proteinase K (0.5 mg/ml) replaced trypsin. In order to examine the effect of cholesterol supplementation on early events of MHV replication, cultures of unsupplemented or cholesterol-supplemented (24 hr) L-2 and LM-K cells were inoculated with MHV in the presence of appropriate medium (unsupplemented or cholesterol-supplemented) and adsorbed for 30 min at 4°. Monolayers were subsequently washed three times and incubated in the appropriate medium for 2 hr in order to permit virus internalization and uncoating (Mizzen et al., 1985). Cells were subsequently treated with proteinase K to remove externally bound virus and assayed for infectious centers by plating on L-2 cells (Mizzen et al., 1985) in the appropriate medium.

Cell harvesting

For subsequent gas chromatographic analysis, cell monolayers were washed five times with cold phosphate-buffered saline (PBS), trypsinized, spun into pellets, and washed twice with PBS. Cells were lysed by swelling in 10 mM NaCl, 10 mM Tris, pH 7.4, and syringing through a 26-gauge needle; nuclei were removed by centrifugation (2 min × 650 g) and the cytoplasmic supernatants were centrifuged for 20 min at 13,000 g to obtain whole-cell membrane pellets for gas chromatographic analysis.

Gas chromatographic analysis of fatty acids and cholesterol

Samples of media or of whole-cell membranes were extracted with chloroform/methanol 1:1 and the filtered extracts were blown down under a nitrogen stream. The residues were heated for 2 hr at 70° with 0.5 ml of 5% methanolic HCl in sealed glass tubes to convert total fatty acids to their methyl esters. After evaporation of solvent under nitrogen, the residual material was mixed overnight at room temperature with 200 μl of a mixture of acetic anhydride/pyridine (1:1) in order to acetylate cholesterol. After evaporation of solvents under nitrogen, the residues were dissolved in chloroform and subjected to analysis at 295° on a Hewlett–Packard 5890 Gas Chromatograph equipped

with a 25-m crosslinked 5% phenylmethyl silicone capillary column. Fatty acid methyl esters and cholesterol acetate were identified by their retention times as compared with authentic standards. Quantitation was performed using a Hewlett–Packard Integrator.

RESULTS

Biological properties of cell membranes have been shown to be profoundly influenced by changes in membrane "fluidity." Fluidity has been shown to be largely determined by characteristics of membrane constituent lipids, particularly the cholesterol content and the degree of fatty acid unsaturation.

MHV infection of cholesterol-supplemented LM-K cells

In a modification of a procedure originally used to introduce cholesterol into erythrocytes (Shinitsky, 1978), cholesterol was complexed to serum lipoproteins present in FCS-supplemented medium. Confluent cultures of L-2 and LM-K cells were grown in the presence of normal and cholesterol-supplemented medium for 24 hr and the cholesterol content of whole-cell membranes was determined. As shown in Fig. 1, membrane cholesterol contents could be dramatically elevated by this procedure as reflected by an increase in the cholesterol/fatty acid ratio from 0.14 to 0.48.

As shown in Fig. 2, L-2 cells supplemented with cholesterol showed a marked enhancement in their ability to undergo virus-induced fusion in comparison to unsupplemented cells. In the former case, fusion appeared earlier and developed much more rapidly than in the latter. Thus, although both cultures eventually became completely fused, the progression of syn-
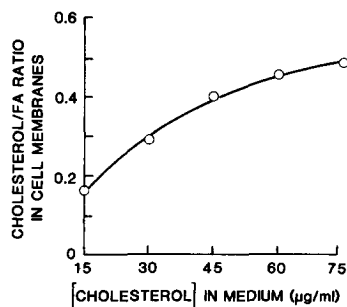

FIG. 1. Uptake of cholesterol into L-2 cell membranes. Confluent monolayers of L-2 cells were maintained for 24 hr in cholesterol-containing medium at the concentrations indicated, and subsequently harvested for determination of membrane cholesterol and total fatty acid.

cytial spread occurred much more quickly as a result of cholesterol supplementation.

In contrast to the enhancing effect of cholesterol on MHV-induced L-2 cell fusion, no such enhancing effect was observed on virus replication. Production of progeny virus was actually somewhat depressed in cholesterol-supplemented cultures, particularly at relatively early time points (6–8 hr p.i.). However, by 9 hr p.i., virus titers released from both cholesterol-supplemented and control cultures were fairly similar. It is possible that MHV replication is slightly compromised in cholesterol-supplemented cells as a result of the more extensive cytopathic effect.

LM-K cells, in contrast to L-2 cells, have been shown previously to undergo a longer-term, persistent type of infection with MHV. The persistence is at least partly related to the ability of the LM-K plasma membrane to resist virus-mediated cell–cell fusion (Mizzen et al., 1983). The hypothesis was accordingly examined that LM-K resistance to MHV-induced fusion might be related to a membrane lipid-controlled parameter such as membrane cholesterol content. As shown in Fig. 2, MHV-inoculated LM-K cells in which membrane cholesterol levels had been elevated indeed showed a dramatic enhancement of cell fusion.

Over the course of several experiments, virus titers from both control and cholesterol-enriched LM-K cultures were found to be variable, probably as a result of asynchrony arising after the first replication cycle (12–16 hr). However, the characteristic long-term and cyclical nature of MHV replication in this cell line (Mizzen et al., 1983) was still maintained in cholesterol-supplemented cultures. The results of a single LM-K culture experiment which is representative of this phenomenon is shown in Fig. 2. Thus, despite greater manifestation of MHV-induced cell fusion, cholesterol supplementation did not overcome the fundamental property of the LM-K cell to support a persistent, weakly fusogenic infection with MHV.

Cells with elevated membrane cholesterol content do not enhance early events of MHV infection

On the basis of current knowledge there are two events within the MHV replication cycle which involve obvious membrane interactions and could therefore be perturbed by alteration of membrane cholesterol content. These include the interaction of the viral envelope and the membrane of the endocytic vesicle/endosome in the process of uncoating (early event) and the interaction of the infected cell plasma membrane (expressing MHV fusion protein E_2) with neighboring cells resulting in cell–cell fusion (late event). These
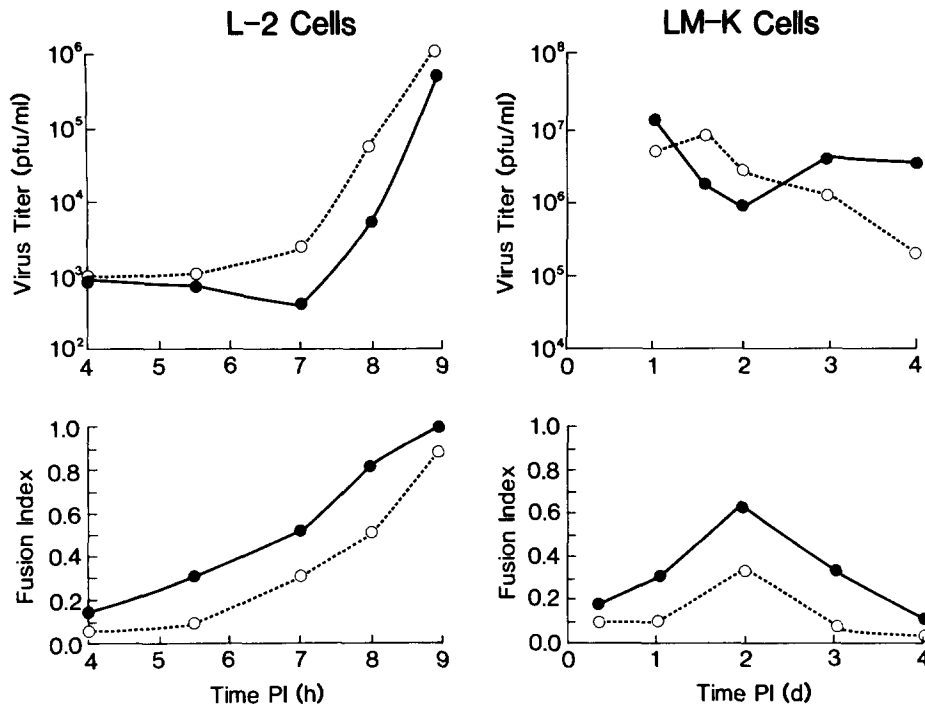
FIG. 2. Effect of cholesterol supplementation on MHV replication and cell fusion. Confluent cultures (10^6 cells) of unsupplemented (O) and cholesterol-supplemented (●) L-2 and LM-K cells were inoculated with MHV (10^6 plaque-forming units, PFU) and assayed for virus replication (supernatant media) and fusion index at various times p.i.

events are shown schematically in Fig. 3. Thus, the enhancement of membrane fusion by cholesterol (Fig. 2) could be a result of enhancement of either or both of the above events. In order to examine the early event, an infectious center assay was performed on unsupplemented and cholesterol-supplemented cells at 2 hr p.i. [following adsorption, penetration, and uncoating (Mizzen et al., 1985)]. Cells were then dispersed with proteinase K and seeded onto normal (unsupplemented) L-2 cell monolayers and the resultant numbers of infectious centers were taken as a measure of cells which had internalized virus inoculum. It was found that cholesterol-supplemented cells were somewhat less efficient at internalizing MHV than were unsupplemented cells (Table 1). Thus, cholesterol-supplemented cells do not facilitate early events of MHV infection. It is therefore apparent that the enhancement of MHV-induced cell fusion observed in Fig. 2 is more likely due to an effect at a later stage in MHV infection.

Cells with elevated membrane cholesterol content show increased susceptibility to MHV-mediated cell fusion

To determine directly whether cholesterol-supplemented cell membranes were more susceptible to

MHV-mediated fusion, a contact fusion assay (Mizzen et al., 1983) was performed. A low number of MHV-infected L-2 cells was seeded in 35-mm plates and subsequently (3 hr p.i.) overlaid with a 10-fold excess of either unsupplemented or cholesterol-supplemented uninfected L-2 cells. Contact fusion was monitored after 2 hr. As shown in Fig. 4, contact fusion was markedly enhanced in the cholesterol-supplemented cells, providing direct evidence for cholesterol-dependent facilitation of MHV-mediated cell fusion. Using a variety of membrane cholesterol/fatty acid ratios, fusion appeared to increase directly with increasing cholesterol content (Fig. 5).

MHV infection in fatty acid-supplemented LM-K cells

Confluent cultures of LM-K cells were cultured in media containing fatty acid/BSA complexes in order to modify their membrane lipid composition according to the procedure of Spector and Hoak (1969) as modified by Doi et al. (1978). As shown in Table 2, this procedure resulted in major alterations in membrane fatty acid composition, as reflected by individual fatty acid content as well as by the total degree of fatty acid unsaturation. In particular, supplementation with C-20:4 resulted in apparently compensatory declines

in cellular content of unsaturated C-18:1 and C-18:2 fatty acids, concomitant with increased C-20:4 and C-22:4. The overall effect of either C-20:4 or C-16:0 supplementation, nevertheless, was to significantly alter the saturated/unsaturated fatty acid balance as measured by the average number of double bonds/mole fatty acid (Table 2).

Inoculation with MHV of fatty acid-supplemented and unsupplemented LM-K cells gave rise to infections which, after 24 hr, differed only slightly in terms of virus replication and expression of cell fusion (Table 2).

Cytoskeletal involvement

The influence of the cytoskeleton in determining the severity of MHV-3 infection of peritoneal macrophages has been documented (Mallucci and Edwards, 1982). It was shown that impairment of the actomyosin system using subcytotoxic concentrations of cytochalasin B changed the outcome of infection so that a normally acute infection was converted to one with characteristics of a long-term steady-state infection in which cell fusion was relatively restricted.
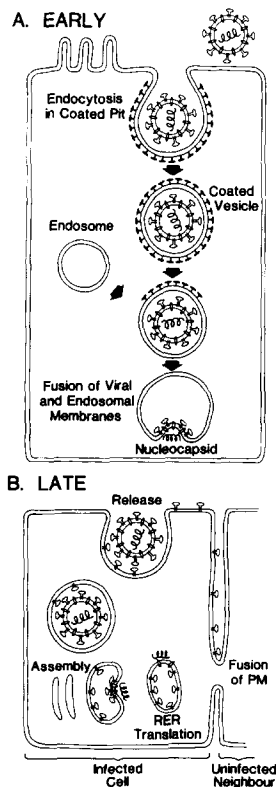

Fig. 3. Schematic representation of MHV interactions with host cell membranes at "early" and "late" times of the virus infection cycle, with particular emphasis on membrane fusion events.

TABLE 1

INCREASED CELL MEMBRANE CHOLESTEROL CONTENT DOES NOT ENHANCE MHV EARLY EVENTS

Cholesterol/FA ratio	Infectious centers	
	L-2	LM-K
0.17	n.d.	73
0.18	1200	n.d.
0.45	610	n.d.
0.48	n.d.	39

Note. Cultures (10^6 cells) of L-2 or LM-K cells were incubated for 24 hr in unsupplemented or cholesterol-supplemented medium. Cultures were either harvested at this time for determination of membrane cholesterol/fatty acid ratios or inoculated with MHV. L-2 cells were inoculated with 10^3 PFU/culture while LM-K cells were inoculated with 10^5 PFU/culture. Following 2 hr incubation at 37° (to permit internalization and uncoating) in the respective media, cultures were treated with proteinase K (to strip externally bound virus) and assayed for infectious centers by plating on L-2 cells and incubation in the respective media for 24 hr.

In order to elucidate any role of the cytoskeleton in MHV-induced cell fusion of mouse fibroblasts, L-2 cells were cultured in the presence of subcytotoxic amounts of cytochalasin B or colchicine starting at 1 hr p.i. It was found that cytochalasin B treatment altered the syncytial shape (long filamentous processes extending from a central region) but without altering significantly the fusion index. Likewise, in the presence of colchicine, syncytial formation proceeded to levels similar to those seen in controls, except that the normal aggregation of nuclei did not occur and that, instead, nuclei were dispersed throughout the syncytial body. It is thus concluded that while the cytoskeleton is involved in morphological changes associated with MHV infection (e.g., syncytial morphology, nuclear aggregation), it does little to either facilitate or restrict the extent of syncytial formation. Moreover, in contrast to the situation described for MHV-infected macrophages (Mallucci and Edwards, 1982) treatment of L-2 cells with cytoskeleton-disrupting drugs did not in any way attenuate MHV infection.

DISCUSSION

The effects of lipid composition on membrane fusion have best been studied in model systems using liposomes of defined lipid content. Cholesterol, in particular, has been shown to affect the fluidity of lipid membranes (Oldfield and Chapman, 1972; Papahadjopoulos et al., 1972) with the generalized effect that lateral mobility of fatty acid chains is increased below the transition temperature but reduced above it. In
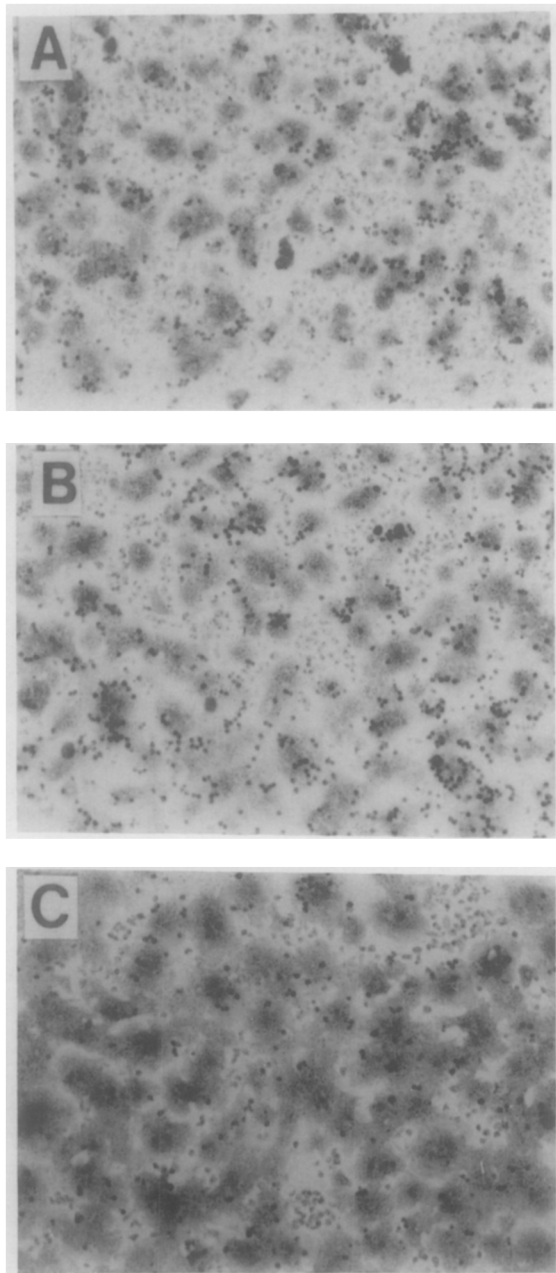
FIG. 5. Dependence of MHV-induced cell fusion on cholesterol content of "target" cell membrane. Contact fusion assays were performed as in the legend to Fig. 4 and fusion indices were determined as a function of membrane cholesterol/fatty ratio in the target cell.

therefore be expected to affect membrane properties as a consequence of both direct alteration of membrane fluidity as well as indirect perturbation of the membrane organization of functional proteins.

Evidence has been presented that membrane fusion is inhibited by increased membrane cholesterol content (Papahadjopoulos *et al.*, 1973, 1974; Breisblatt

FIG. 4. Increased cholesterol in "target" membrane enhances contact fusion. MHV-infected L-2 cells were mixed with a 10-fold excess of unsupplemented (A) or cholesterol-supplemented (B, C) L-2 cells at 3 hr p.i. Cultures were stained with Giemsa after 2 hr at 37°. Dark areas are syncytia, containing numerous nuclei (black dots). Because the syncytia are often slightly raised relative to the plane of uninfected cells, the nuclei of the latter appear lighter and unfocused. Membrane cholesterol/fatty acid ratios in the target cells used for (A), (B), and (C) were determined on parallel cultures to be 0.18, 0.25, and 0.46, respectively.

erythrocytes, cholesterol supplementation has been shown to influence the surface distribution of integral membrane proteins (Borochov *et al.*, 1979) and would
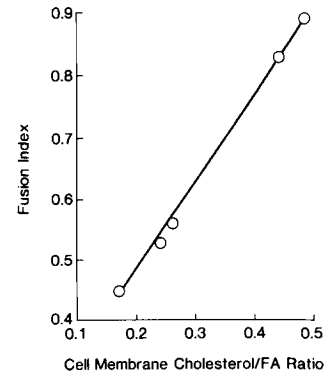
TABLE 2

MHV REPLICATION AND CELL FUSION IN FATTY ACID-MODIFIED LM-K CELLS

	% w/w fatty acid in cell membranes		
Fatty acid	Unsupplemented	C-20:4 supplemented	C-16:0 supplemented
C-14:0	4.5	3.4	2.3
C-16:0	27.2	28.4	37.5
C-16:1	7.9	1.2	10.5
C-18:0	23.6	30.5	22.0
C-18:1	9.2	1.7	6.9
C-18:2	18.5	4.1	13.8
C-20:4	8.0	26.4	5.7
C-22:4	1.2	4.3	1.3
Double bonds/ mol FA	0.87	1.30	0.71
Virus titer (PFU/ml)	1.6×10^6	2.4×10^6	4.2×10^5
Fusion index	0.10	0.10	0.10

Note. Cultures of LM-K cells (10^6 cells) were incubated in the appropriate medium (unsupplemented, C-20:4 supplemented, or C-16:0 supplemented) for 24 hr prior to inoculation with MHV (10^6 PFU). After a further 24 hr incubation in the respective media, cultures were assayed for virus titers (supernatant media) and fusion index.

and Ohki, 1976). However, studies with fusogenic agents (e.g., polyethylene glycol and Sendai virus) have demonstrated a requirement for cholesterol in the fusion process (Hope et al., 1972; Hsu et al., 1983). When studying cell membranes it is also important to consider that concomitant with cholesterol enrichment there is a consequent dilution of other membrane lipids, some of which may be inhibitory to fusion (Papahadjopoulos et al., 1974).

Membrane cholesterol appears to have different effects on fusion depending on the system studied. Fusion of Semliki Forest virus (White and Helenius, 1980) and Sendai virus (Hsu et al., 1983) required cholesterol in the target liposome, although in another study cholesterol appeared to decrease Sendai virus fusion (Haywood and Boyer, 1984). In contrast, the rate of liposome fusion mediated by VSV G protein reconstituted into liposomes (Eidelman et al., 1984) or the fusion of influenza virus with liposomes (Maeda et al., 1980) was not affected by cholesterol.

To the best of our knowledge, the present report represents the first directly documented instance in which host cell membrane cholesterol content modulates cell fusion induced in any virus system. It is evident from our study that MHV-induced cell fusion is affected much more strongly by membrane cholesterol content than by membrane fatty acid composition. We have been able to show that a major effect of cholesterol supplementation is to render neighboring (uninfected) cell membranes more susceptible to MHV-mediated cell fusion. This observation is significant to questions of viral pathogenesis in view of the importance of cell fusion as a mechanism of MHV dissemination. Furthermore, it is evident that the putative early event in MHV replication involving fusion of virus envelope and endosomal membrane is, in contrast to cell–cell fusion, not enhanced by an increase in host membrane cholesterol.

In a persistent infection, that of MHV-infected LM-K cells, cell fusion was enhanced by cholesterol supplementation. However, fusion was not as extensive as that observed in MHV-infected L-2 cells and the LM-K culture remained persistently infected. Thus, membrane fusion resistance and MHV persistence are modulated but not alleviated by cell membrane cholesterol content.

Finally, we note that MHV-induced cell fusion of L-2 cells is not significantly modulated by such membrane-associated structures as the cytoskeleton. Our finding is thus comparable to a report by Holmes and Choppin (1968) in which treatment of BHK 21F cells with cytochalasin B had no effect on Simian virus-5-induced cell fusion. However, it is in contrast to the

reported situation of MHV-3 infection of macrophages (Mallucci and Edwards, 1982). Conceivably, the role of the cytoskeleton could vary among different cell types.

ACKNOWLEDGMENTS

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