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Control of Virus-Induced Cell Fusion by Host Cell Lipid Composition

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Virus-induced cell fusion has been examined in a series of stable cell lines which were originally selected for resistance to the fusogenic effects of polyethylene glycol (PEG). For a wide variety of viruses, including murine hepatitis virus (a coronavirus), vesicular stomatitis virus (a rhabdovirus), and two paramyxoviruses (Sendai virus and SV5), susceptibility to virus-induced fusion was found to be inversely correlated with susceptibility to PEG-induced fusion. This phenomenon was observed both for cell fusion occurring in the course of viral infection and for fusion induced "from without" by the addition of high titers of noninfectious or inactivated virus. The fusion-altered cell lines (fusible by virus but not by PEG) are characterized by their unusual lipid composition, including marked elevation of saturated fatty acids and the presence of an unusual ether-linked neutral lipid. To test the association between lipid composition and fusion, acyl chain saturation was manipulated by supplementing the culture medium with exogenous fatty acids. In such experiments, it was possible to control the responses of these cells to both viral and chemical fusogens. Increasing the cellular content of saturated fatty acyl chains increased the susceptibility of cells to viral fusion and decreased susceptibility to PEG-induced fusion, whereas lowering fatty acid saturation had the opposite effect. Thus, parallel cultures of cells can be either driven toward the PEG-fusible/virus-fusion-resistant phenotype of the parental cells or rendered susceptible to viral fusion but resistant to PEG-induced fusion, solely by the alteration of cellular lipids. The ability of cellular lipid composition to regulate virus-induced membrane fusion suggests a possible role for lipids in viral infection and pathogenesis.

INTRODUCTION

Cell fusion in virus-infected tissues has been described for over 100 years, and numerous viruses are capable of inducing polykaryocytosis under experimental conditions (reviewed by Roizman, 1962; Spear, 1987). In particular, most enveloped viruses are capable of inducing membrane fusion as a consequence of their infectious strategy: successful delivery of the viral genome into the host cell requires fusion either with the surface membrane of the cell (Dales, 1973) or with internal vesicles following endocytosis (White *et al.*, 1983). Later in infection, the appearance of viral fusion proteins on the surface membranes of infected cells may cause the fusion of adjacent cells with each other, but for reasons not yet entirely understood, the extent of such fusion can vary considerably among different cell types infected with the same virus (Holmes and Choppin, 1966; Wang *et al.*, 1982).

We have previously studied chemically induced cell fusion in cultured mouse fibroblasts treated with polyethylene glycol (PEG). A series of cell lines with reduced susceptibility to PEG-induced fusion was selected by repeated cycles of PEG treatment (Roos and Davidson, 1980). The most highly PEG-resistant mutants exhibit <20% fusion under conditions where virtually 100% of the parental cells fuse (Roos *et al.*, 1983). These cells lines are altered with respect to other membrane-associated phenomena as well, including the frequency of spontaneous hybridization (Roos and Choppin, 1985b), nonspecific adsorptive endocytosis and fluid-phase pinocytosis (Li *et al.*, 1986; Ryser *et al.*, 1988), and tumorigenicity and metastatic spread *in vivo* (Roos and Choppin, 1984).

Several biochemical alterations have been noted in the PEG-resistant mutants, including much more highly saturated fatty acyl chains than found in the parental cells from which they were derived, and the presence of an unusual ether-linked neutral lipid (Roos and Choppin, 1985a). Through the manipulation of cellular lipid content, we have directly demonstrated a link between fusion response and lipid composition in the PEG-fusion system: alteration of acyl chain saturation

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in these cells allows complete control over susceptibility to PEG-induced fusion (Roos and Choppin, 1985b).

In the experiments described here, virus-induced fusion has been examined in these cells resistant to PEG-induced fusion. Surprisingly, increasingly PEG-resistant cells are increasingly sensitive to fusion induced by a wide range of viruses. Nevertheless, virus-induced fusion in this system can also be controlled by the manipulation of cellular acyl-chain saturation. These findings have interesting implications for host and tissue specificity of viral pathogenesis.

MATERIALS AND METHODS

Cells and viruses

The cell lines used in this study have previously been described and characterized (Roos and Davidson, 1980; Roos *et al.*, 1983). The parental cell line was originally isolated as a 5-BrdU-resistant mouse L-cell clone (Kit *et al.*, 1963) and is referred to as Clone 1D. PEG-resistant derivatives of Clone 1D were isolated by repeated cycles of treatment with fusogenic concentrations of PEG followed by outgrowth of the remaining unfused cells. Cells isolated in this manner are designated F_1, F_2, . . ., where the subscripted number refers to the number of cycles of selection used to produce that cell line. Previous studies have demonstrated that each of these lines is homogeneous in its response to PEG and that its fusion phenotype is stable for several hundred generations in continuous culture (Roos and Davidson, 1980). All cell culture was performed in plastic tissue culture ware (Falcon Labware, Oxnard, CA) using Dulbecco's reinforced Eagle's medium supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Inc., McLean, VA). Cells were maintained at 37° in a humid environment containing 7% CO_2.

The "Z" strain of Sendai virus was grown in the allantoic sac of 10-day-old embryonated eggs by inoculation with a 10^{-4} dilution of stock virus ($\sim 10^{6.5}$ 50% egg-infective dose) in 100 μl Eagle's medium containing 1% bovine serum albumin (BSA). Virus (~1024 HAU/ml) was harvested 48 hr after infection and concentrated as previously described (Hsu *et al.*, 1979). Inactivated virus was prepared by irradiating a 2-mm pool of virus for 15 min at a distance of 15 cm from a short-wave ultraviolet lamp (Model UVSL-58; Ultraviolet Products, San Gabriel, CA).

All other viruses were grown in tissue culture. Cell monolayers (grown as described for Clone 1D cells, above) were rinsed with PBS without divalent cations (rinse omitted for murine hepatitis virus (MHV)), inoculated with virus, and incubated for 1–2 hr at 37° with occasional rocking, after which the monolayers were again aspirated and rinsed with PBS, and fresh growth medium was added. The W2 strain of simian virus 5 (SV5; Holmes and Choppin, 1966) was grown in CV-1 African green monkey kidney cells inoculated with a 1000-fold dilution of seed stock (~20 PFU/cell) in Dulbecco's reinforced Eagle's medium containing 1% BSA. Hemagglutination titers of the supernatant medium were measured periodically, and virus was harvested 24–36 hr postinfection. Further concentration and inactivation of SV5 was performed as for Sendai virus. Vesicular stomatitis virus (VSV, Indiana serotype) was grown in BHK-21 cells by inoculation at a multiplicity of 1 PFU/cell and harvested 20 hr postinfection. Murine hepatitis virus (MHV, strain A59) was grown in 17-Clone 1 cells inoculated with 1 PFU/cell in Dulbecco's reinforced Eagle's medium containing 10% FBS and harvested 16–24 hr postinfection as previously described (Holmes *et al.*, 1981).

Viral assays

Hemagglutination assays were performed using serial twofold dilutions of virus stock in PBS (without divalent cations). To a final volume of 100 μl of these dilutions was added an additional 100 μl of a 0.5% solution of chicken red blood cells in PBS. Plates were incubated at 4° for 1–2 hr and the titer was read as the reciprocal of the highest dilution of virus yielding definite agglutination of erythrocytes.

Plaque assays were performed in 35-mm petri dishes using confluent monolayers of BHK-21 cells (for Sendai, SV5, and VSV) or 17-Clone 1 cells (for MHV). Cells were inoculated with serial 10-fold dilutions of virus in 0.5 ml and incubated for 1–2 hr with occasional shaking. After infection, residual medium was aspirated and replaced with 3 ml of a 1:1 mixture of 1.9% bactoagar and 2× Eagle's medium containing 8% calf serum and 4% tryptose phosphate broth (omitted for MHV). Plates were incubated at 37° until cytopathic effects were observed, stained by the addition of 1 ml agar solution containing 0.01% neutral red dye, and plaques were scored the next day.

Cell fusion and quantitative analysis

Petri dishes (35 mm) containing 25 mm round No. 1 glass coverslips were inoculated with 1.5×10^6 Clone 1D cells, 1.3×10^6 F_{16} cells, or 1.1×10^6 F_{40} cells in 3 ml medium and incubated ~24 hr prior to fusion. These procedures produce confluent monolayers of cells for treatment with virus or PEG. (The different cell lines are initially plated at differing densities to compensate for inherent differences in growth rate; Roos, 1984.) In experiments where lipid supplements were added, fatty acids were prepared as 10^{-2} *M* stock solu-

tions in 3×10^{-2} *M* NaOH, and 300 μl of this solution was added to the culture medium ~12 hr after plating (final concentration 10^{-4} molar lipid). Cells were incubated with lipid for 18 hr prior to fusion (Roos and Choppin, 1985b).

Two procedures were followed for viral fusion assays. Viruses which are incapable of productive growth in these cells (Sendai and SV5) were concentrated and inactivated as described above, and 500 μl of high-titer virus (>5000 HAU/ml, >1000 PFU/cell) was allowed to attach to cells at 4°. Cultures were then rapidly warmed to 37°, allowing fusion to proceed. Under these conditions, maximal fusion was seen within 4 hr of virus addition. For MHV and VSV, cells were inoculated with stock virus (1–5 PFU/cell) and infection was allowed to proceed for 16–20 hr before fixation.

Fusion with polyethylene glycol was accomplished as previously described (Robinson *et al.,* 1979). PEG-1000 (J. T. Baker Chemical Co., Phillipsburg, NJ) was prepared as a 50% solution in Dulbecco's reinforced Eagle's medium without serum. Cells grown on coverslips were removed to a fresh dish where they were treated with 2 ml PEG solution at 37° for 60 sec, followed by a series of rapid rinses through several containers of warm medium without serum. Finally, the coverslips were transferred to a fresh dish and incubated in fresh medium for 2–3 hr in order to allow fusion to proceed.

Cells were prepared for microscopic evaluation by rinsing in PBS, fixation in methanol, and staining with Giemsa (Fisher Scientific Co., Fair Lawn, NJ). Coverslips were mounted on glass slides using Gurr's aqueous mountant (Bio/medical Specialties, Santa Monica, CA) and observed under a Zeiss Photomicroscope III. For quantitative analysis, confluent fields were selected randomly at low magnification where cellular morphology could not be distinguished. The objective was switched to high magnification (400×) and all nuclei within a 200-μm square were scored as present in mononucleate or fused cells without further selection of field. At least 10 fields were counted per sample, and the results were corrected for spontaneously occurring fusion, determined from parallel plates of uninfected cells (Roos and Choppin, 1985b).

Fatty acid analysis and preparation of lipid supplements

Cellular fatty acid composition was determined by gas–liquid chromatography as previously described (Roos and Choppin, 1985a), using cultures prepared in parallel with those used for fusion.

Procedures for the preparation of fatty acid supplements have previously been described (Roos and Choppin, 1985b). Fatty acids, pure by thin-layer chromatography, were obtained from Nu-chek prep (Elysian, MN) either as sodium salts or as free acids which were converted to salts by dissolution in a threefold molar excess of NaOH. In some experiments, fatty acid salts were conjugated to bovine serum albumin according to Spector *et al.* (1979) using "essentially fatty acid-free" BSA obtained from Sigma Chemical Co. (St. Louis, MO). Supplements were added directly to the growth medium from 10^{-2} *M* stocks (5×10^{-3} *M* stocks for BSA conjugates). Lipid stocks were stored in the dark at −20° under N_2 in acid-washed glass tubes sealed with Teflon-lined caps, and periodic analysis by gas–liquid chromatography revealed no detectable breakdown over the course of this study.

Immunostaining

Cells grown on coverslips were infected with MHV, fixed at various times in 2% paraformaldehyde in PBS, permeabilized with 0.2% Nonidet-P40 for 4 min, and washed overnight in PBS at 4°. Fixed cells were prepared for staining by incubation for 3 min in 0.3% H_2O_2 in methanol, followed by 20 min in 1% BSA (in PBS), and blocking of nonspecific binding with normal goat serum. MHV antigens were labeled using a 1:100 dilution of ascites fluid from mice infected with the JHM strain of MHV (prepared by Dr. S. G. Robbins, Uniformed Services Univ., Bethesda, MD). This antiserum recognizes all structural proteins of strain A59. Staining was accomplished using the Vecta-stain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

RESULTS

Effects of fusogenic viruses on PEG-resistant cells

We have previously described the process of PEG-mediated cell fusion (Robinson *et al.,* 1979; Roos *et al.,* 1983) and the genetic analysis of the PEG-resistant cell lines used in this study (Roos and Davidson, 1980). Because of the striking differences in susceptibility to PEG-induced fusion between Clone 1D (the parental mouse L-cell line) and the various mutants derived from it (such as our most highly PEG-resistant line, designated F_{40}), it was of interest to examine the behavior of these cells in response to fusogenic viruses. The morphology of Clone 1D cells and the F_{40} mutants treated with various enveloped viruses is illustrated in Fig. 1. Quantitative data on cell fusion are presented in Fig. 2. Less than 5% fusion was seen when Clone 1D cells were incubated with high titers of uv-inactivated Sendai virus (Fig. 1A), but significantly more fusion was observed in F_{40} cells under these conditions (Fig. 1B).

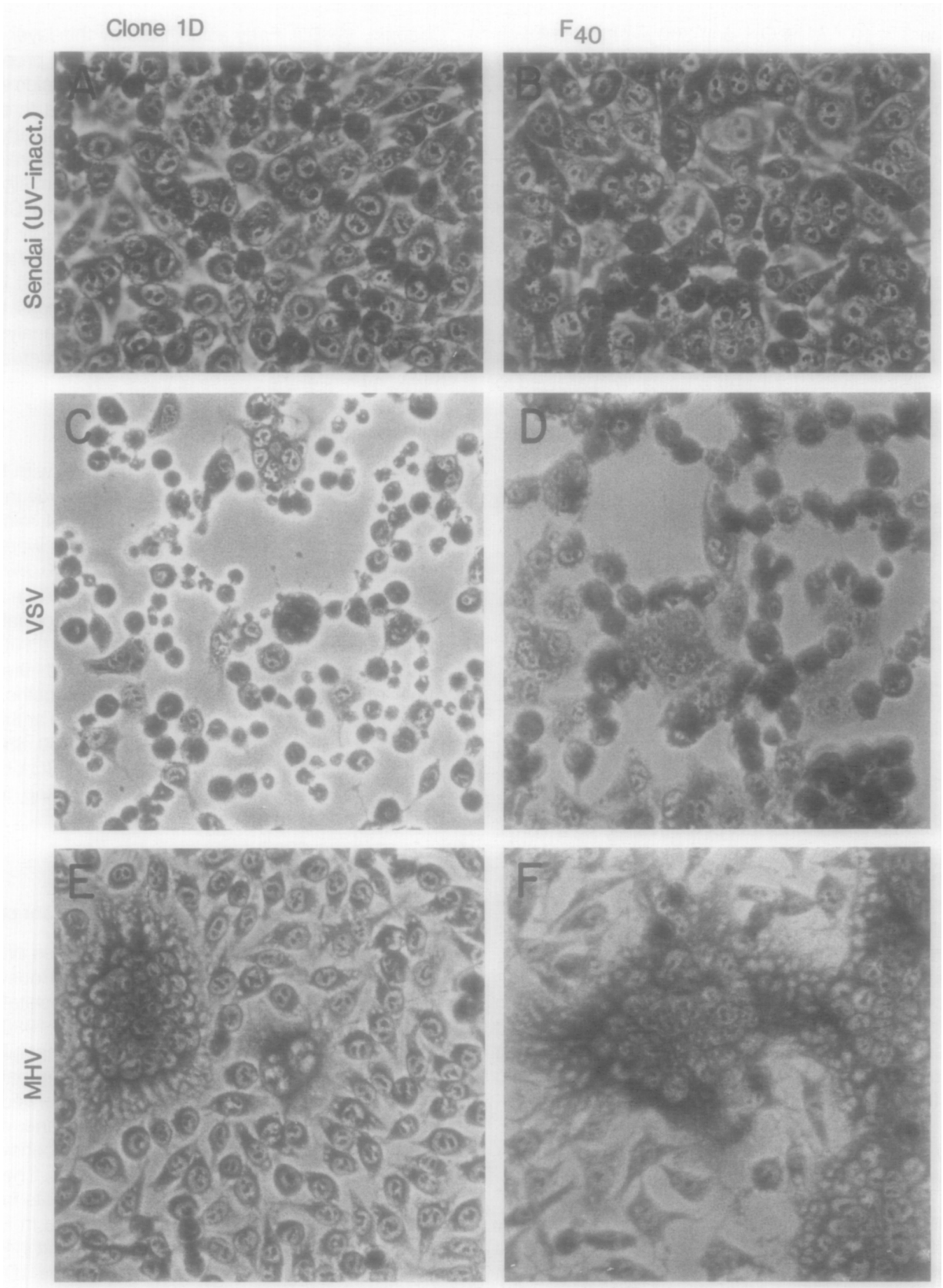

FIG. 1. Fusion of cells induced by various enveloped viruses. Confluent cultures of Clone 1D (A, C, E) or F_{40} cells (B, D, F) were incubated with highly concentrated (5000 HAU/ml), uv-inactivated Sendai-Z virus (A and B), or infected with vesicular stomatitis virus (C and D) or murine hepatitis virus (E and F) and prepared for microscopy at 4 hr (Sendai) or 16 hr (VSV and MHV) after inoculation. In each case, F_{40} cultures (right panels) were more highly susceptible to virus-induced fusion than Clone 1D (left panels).

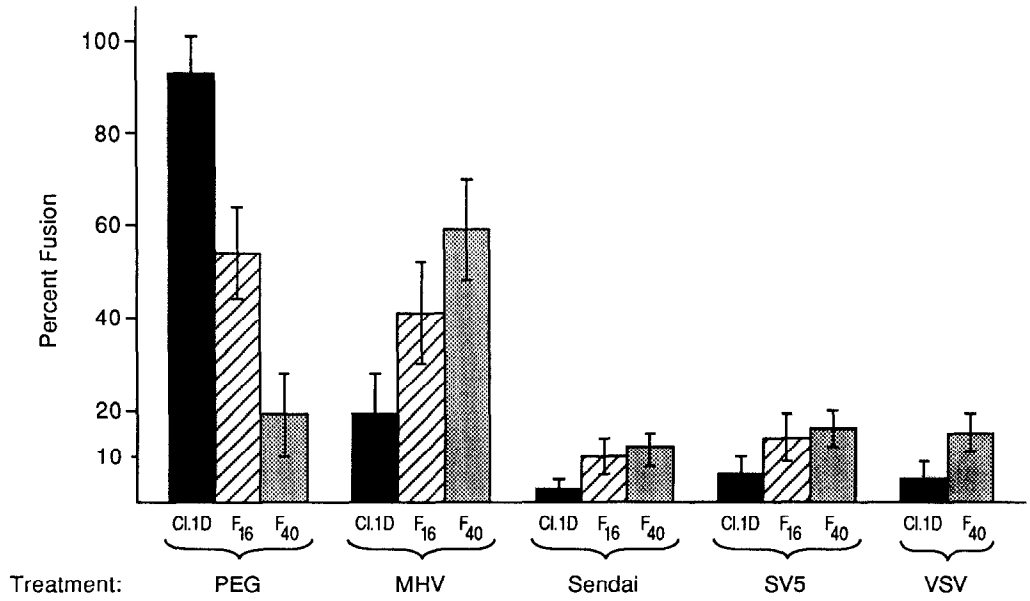

FIG. 2. Fusion of Clone 1D, F_{16}, and F_{40} cells induced by PEG and fusogenic viruses. Parallel cultures of Clone 1D, F_{16}, or F_{40} cells were treated with polyethylene glycol, infected with virus (VSV, MHV), or exogenously treated with high titers of uv-inactivated virus (Sendai, SV5), as described under Materials and Methods. Fusion was scored as the percentage of all nuclei which were present in fused cells of any size after treatment (after correction for the low levels of spontaneously occurring multinucleates found in control cultures).

Mouse L cells do not support productive Sendai virus infection, and hence these experiments were performed using high concentrations of exogenously added virus, (termed "fusion from without"; Bratt and Gallaher, 1969). VSV will replicate in L cells, and the morphology of VSV-infected Clone 1D and F_{40} cells is illustrated in Figs. 1C and 1D. Infected cells detach from the culture surface and eventually die when infected with VSV, but it is clear that more cell fusion was induced in F_{40} than in Clone 1D. (Quantitation of fusion in Fig. 2 was carried out before the cytolytic effects of VSV were as evident as shown in Fig. 1.)

The most effective virus in causing fusion of these cells was MHV, which induced 60% fusion in F_{40} cells, but only 19% fusion in Clone 1D (average of five experiments). Representative micrographs of MHV-infected cells are shown in Figs. 1E and 1F. Similar results have also been obtained for SV5 (Fig. 2) and several other enveloped viruses, including visna virus (a sheep lentivirus; Harter and Choppin, 1967) and influenza virus (an orthomyxovirus; fusion performed at pH 5 according to the procedures of Matlin *et al.*, 1981), although fusion induced by these viruses was less extensive than with MHV (data not shown). In all cases Clone 1D cells were less susceptible to fusion than the F_{40} mutants. This finding is precisely the opposite of the responses observed for chemical fusogens: Clone 1D cells are highly fusible with PEG (93% fusion), while F_{40} cells were isolated by virtue of their resistance to PEG-induced fusion (19%). Thus, susceptibility to chemical- and virus-induced fusion is inversely correlated in these cells.

Differences in the nature of fused syncytia (giant cells) formed following infection of Clone 1D or F_{40} cells with MHV versus treatment with PEG can be seen in Table 1. While PEG treatment caused nearly all Clone 1D cells to fuse, most of the fusion products formed were of moderate size (median size of giant cells = 8 nuclei). PEG-resistant cell lines F_{16} and F_{40} formed only small multinucleates. By contrast, most virus-induced syncytia were very large, even for Clone 1D, where the

TABLE 1

SIZE ANALYSIS OF SYNCYTIA FORMED DURING PEG VS MHV-INDUCED FUSION

Treatment	Cell line	Fusion response (% nuclei in fused cells)	Mean size of fusion product (No. nuclei)	Average size of fusion product (No. nuclei ± SD)[a]
PEG	Clone 1D	94	8	13.7 ± 15.5
	F_{16}	46	3	3.1 ± 1.6
	F_{40}	10	2	2.4 ± 0.7
MHV	Clone 1D	12	45	47.6 ± 20.7
	F_{40}	52	25	29.4 ± 19.9

[a] The standard deviation presented indicates the range of fusion product sizes obtained, *not* variation between experiments. The average size of fusion products, range of sizes, and overall extent of fusion was highly reproducible (cf. error bars in Fig. 2).

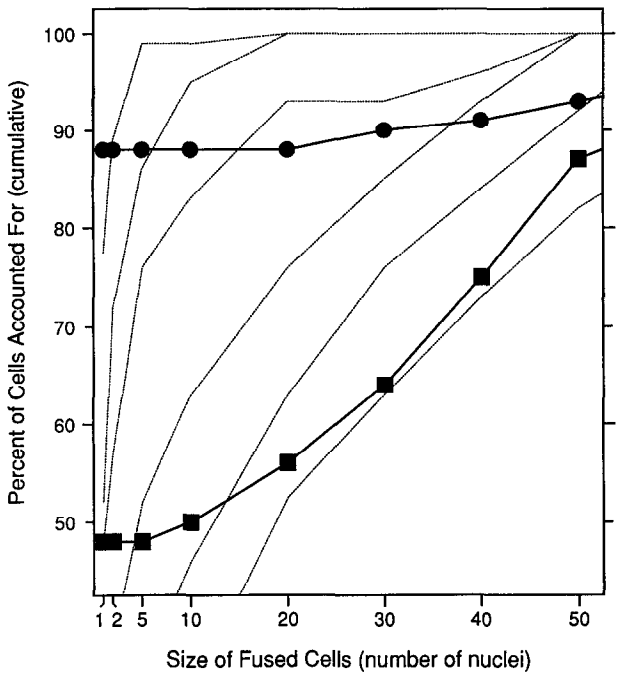

FIG. 3. Analysis of the size of fused syncytia produced by infection with murine hepatitis virus. Cultures of Clone 1D (●) or F_{40} (■) cells were infected with MHV, and the size of syncytia formed was scored 16 hr later. Results are plotted as the cumulative percentage of nuclei incorporated into fused cells of a given size or smaller. For example, the left-most solid square indicates that 48% of all MHV-infected F_{40} cells remained unfused (mononucleate) in this experiment; the right-most solid square shows that 88% of all F_{40} nuclei were incorporated into syncytia containing ≤50 nuclei. The lines without symbols illustrate the very different pattern of size distributions found when cells were treated with PEG. Progressing from bottom to top, each curve represents the PEG fusion response of an increasingly fusion-altered cell line: Clone 1D, F_4, F_8, F_{12}, F_{16}, and F_{24} (uppermost curve without symbols). Note the relatively high percentage of large fusion products in MHV-infected cells.

overall level of fusion was not very extensive (12% fusion; median size of fusion products = 45 nuclei).

Figure 3 presents a more detailed analysis of the size of syncytia formed during PEG- or MHV-induced fusion. We have previously reported on the spectrum of sizes of fused cells formed following PEG treatment (Roos and Davidson, 1980). In general, PEG-induced fusion can be viewed as a stochastic process, with the probability of fusion between any two adjacent cells being determined by the cell lines involved and the conditions of treatment (Röhme, 1981; Röhme and Thorburn, 1981). Such conditions produce a smooth distribution in the sizes of syncytia formed, as illustrated by the lines without symbols in the background of Fig. 3 representing the PEG-fusion response of Clone 1D and several PEG-resistant cell lines. For example, PEG treatment of F_{24} cells (uppermost background line) left 77% of all cells as unfused mononucleates, 88% as mono- or dinucleates, 99% as syncytia containing five or fewer nuclei, and no fused cells containing more than 20 nuclei. Extensive fusion, as found in PEG-treated Clone 1D cells, produced a broad spectrum of fusion products of all sizes (standard deviation >100%; Table 1).

This picture is very different from the spectrum of syncytia observed in virus-infected cells, as shown by the lines with symbols in Fig. 3. Slightly less than 50% of all cells in MHV-infected F_{40} cultures remained as unfused (mononucleate) cells, similar to the effects of PEG on F_{16} cells. But while only 7% of the PEG-treated F_{16} cells formed giant cells containing more than 20 nuclei, the great majority of fused cells in virus-infected F_{40} cultures were found in large syncytia—84% of all those F_{40} cells which fused at all fused into giant cells with ≥20 nuclei. The average size of fusion products in PEG-treated F_{16} cultures contained only 3 nuclei; average size in MHV-infected F_{40} was 29 nuclei (Table 1). Fusion was rare in MHV-infected Clone 1D cells (12% in the experiments from which this figure was compiled), but virtually all of those fusions which did occur produced very large multinucleates (as seen in Fig. 1E). Overall, while PEG fusion produces a wide spectrum of fused cells of many sizes, most fusion observed in MHV-infected cells tends to be in the form of large syncytia.

Fusion response of lipid supplemented cells

F_{40} cells and other fusion-altered cell lines are characterized by several peculiarities in their lipid content (Roos and Choppin, 1985a). In particular, the ratio of saturated to polyunsaturated fatty acyl chains in Clone 1D is ~1:1, while this ratio is ~3.5:1 in F_{40}. F_{40} cells also contain unusually high concentrations of neutral ether-linked lipid, which may serve to deliver saturated fatty acids to the cell membrane (Roos, 1988). The acyl-chain composition of these cells has proved susceptible to manipulation in culture, demonstrating a direct link between lipid saturation and susceptibility to PEG-induced fusion (Roos and Choppin, 1985b). We have used the same procedures of lipid alteration which were effective in controlling PEG-induced fusion to determine the effects of fatty acyl-chain saturation on coronavirus-induced fusion. Figure 4 shows cultures of MHV-infected Clone 1D or F_{40} cells grown in normal medium or in medium supplemented with 10^{-4} *M* linolenic (18:3[5]) or nonadecanoic (19:0) acids.

[5] This notation is a shorthand abbreviation for fatty acid structure, where the first number indicates chain length, and the single digit following the colon indicates degree of unsaturation. Unless otherwise indicated, all double bonds are in the *cis* configuration.

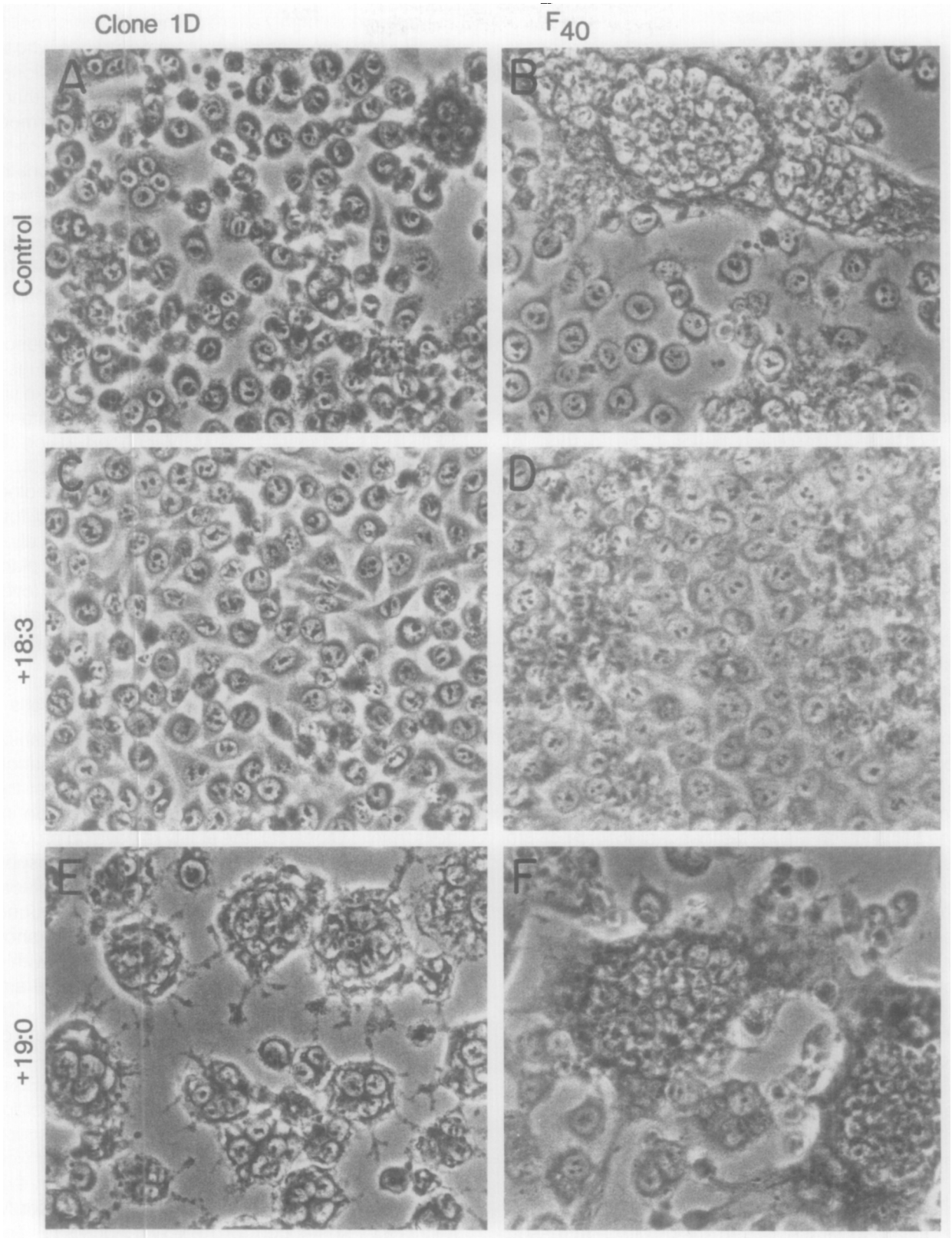

FIG. 4. Control of virus-induced fusion by fatty acid supplements. Clone 1D (A, C, E) and F_{40} (B, D, F) cells were infected with MHV after growth in control medium (A, B) or medium supplemented with polyunsaturated (C, D) or saturated (E, F) fatty acyl chains. Untreated Clone 1D cells were highly resistant to MHV-induced fusion (A), while F_{40} cells fused extensively (B). When F_{40} cells were grown in 10^{-4} *M* linolenic acid (18:3; (D)), altering their lipid composition to resemble that of Clone 1D, these cultures became fusion resistant. Conversely, growth of Clone 1D in saturated fatty acid (19:0; nonadecanoic acid) allowed extensive fusion by MHV (E).

TABLE 2

FATTY ACID CONTENT AND FUSION RESPONSE OF LIPID SUPPLEMENTED CELLS

	Ratio of saturates to polyunsaturates			MHV-induced fusion (%)		
Cell line	+19:0[a]	None	+18:3[a]	+19:0[a]	None	+18:3[a]
Clone 1D	3.0	0.9	0.3	61	24	15
F_{40}	2.8	2.9	0.5	70	56	9

[a] 10^{-4} molar lipid supplement.

As already seen, MHV infection of control cells produces little fusion in cultures of Clone 1D and extensive fusion of F_{40} cells (cf. Figs. 4A and 4B). However, when the acyl chains of F_{40} cells were induced to become more like Clone 1D by preincubation in the presence of 10^{-4} *M* polyunsaturated fatty acid (18:3) for 18 hr prior to virus infection, these lipid-altered F_{40} cells became highly resistant to MHV-induced fusion (Fig. 4D). Conversely, Clone 1D cells which were preincubated in saturated fatty acid (19:0) were extensively fused by MHV (Fig. 4E), as normally seen only in F_{40} cells.

To more directly explore the relationship between fatty acid supplements and virus-induced fusion, parallel dishes of cells were supplemented with 10^{-4} *M* fatty acid and either infected with MHV or extracted for lipid analysis. Quantitative data on the lipid composition and fusion-response of lipid-supplemented cultures and unmodified controls are presented in Table 2. Acyl-chain composition of both Clone 1D and F_{40} cells grown with added 18:3 became highly unsaturated, and the MHV-fusion response changed accordingly, to levels even lower than normally seen in untreated Clone 1D cells. The addition of 19:0 to Clone 1D cells increased both chain saturation and cell fusion to levels observed in untreated cultures of F_{40}. More detailed study has indicated that fatty acid supplements are incorporated into both neutral and phospholipids throughout the cell, including the plasma membrane, and that there is little metabolic alteration of the incorporated acyl chains over the course of these experiments (Roos and Choppin, 1985b). We have achieved similar results using other saturated and polyunsaturated fatty acids (including palmitic [16:0], stearic [18:0], and linoleic [18:2] acids; data not shown). In every case, manipulation of the ratio of saturated to polyunsaturated acyl chains produced a parallel change in susceptibility to virus-induced fusion; i.e., increased saturation stimulated virus-induced fusion, while decreased saturation inhibited the fusion response.

The procedure employed for the selection of PEG-resistant cells produced a series of cell lines of which F_{40} is the most highly altered. The intervening cell lines (F_4, F_8, etc.) are stable, homogeneous lines whose fusion responses are intermediate between Clone 1D and F_{40}, each slightly more resistant to PEG than the previous ones. These cell lines are also intermediate between Clone 1D and F_{40} in their response to fusogenic viruses, as seen in Fig. 2. Figure 5 demonstrates that parallel cultures of F_{16} cells can be supplemented with lipids so as to control their fusion response at will. Unsupplemented cultures of F_{16} exhibit moderate fusion following treatment with either PEG or MHV (Figs. 5A and 5B, respectively). By the addition of appropriate lipid supplements to the medium, parallel cultures of F_{16} can be made either to resemble the PEG-susceptible/virus-resistant phenotype of Clone 1D (Figs. 5C and 5D; treated with PEG or virus after growth in linolenic acid [18:3]), or to resemble F_{40} in their resistance to PEG and susceptibility to MHV-induced fusion (Figs. 5E and 5F; grown in nonadecanoic acid [19:0]). This figure also illustrates the characteristic morphological differences between PEG- and MHV-induced fusion. PEG-treated cells migrate and fuse extensively, but most of this rearrangement occurs within the plane of the culture plate. Virus-infected cells round up (eventually falling off the plate), and viral fusion products form large balls of cells extending far out of the plane of focus.

Virus growth in parental and fusion-altered cells

It was of interest to determine the relationship between the ability of these various viruses to induce fusion and their ability to infect cells. While all of the viruses studied produced more extensive fusion in F_{40} cells than in Clone 1D, the ability to develop productive infection did not always parallel the fusion response. Neither Clone 1D nor F_{40} cells produced infectious Sendai virus or SV5 (although both cell lines bound radiolabeled Sendai virus equally well; data not shown). Both cell lines were susceptible to infection with VSV and MHV, however, and each virus exhibited similar plaquing efficiencies on both Clone 1D and F_{40}. Titers of virus harvested from the supernatant medium of infected cultures are shown in Table 3.

In cultures without added lipid supplement, F_{40} cells produced VSV titers of 6.9×10^8 PFU/ml (average of five experiments). Clone 1D cells were also susceptible to VSV infection, producing a somewhat lower yield of virus (7×10^7 PFU/ml). Lipid supplementation experiments analogous to those described above for MHV also allow some control of VSV-induced fusion (although the degree of control allowed in these experiments is difficult to assess because overall fusion induced by VSV is so low), but these supplements had little effect on virus titer.

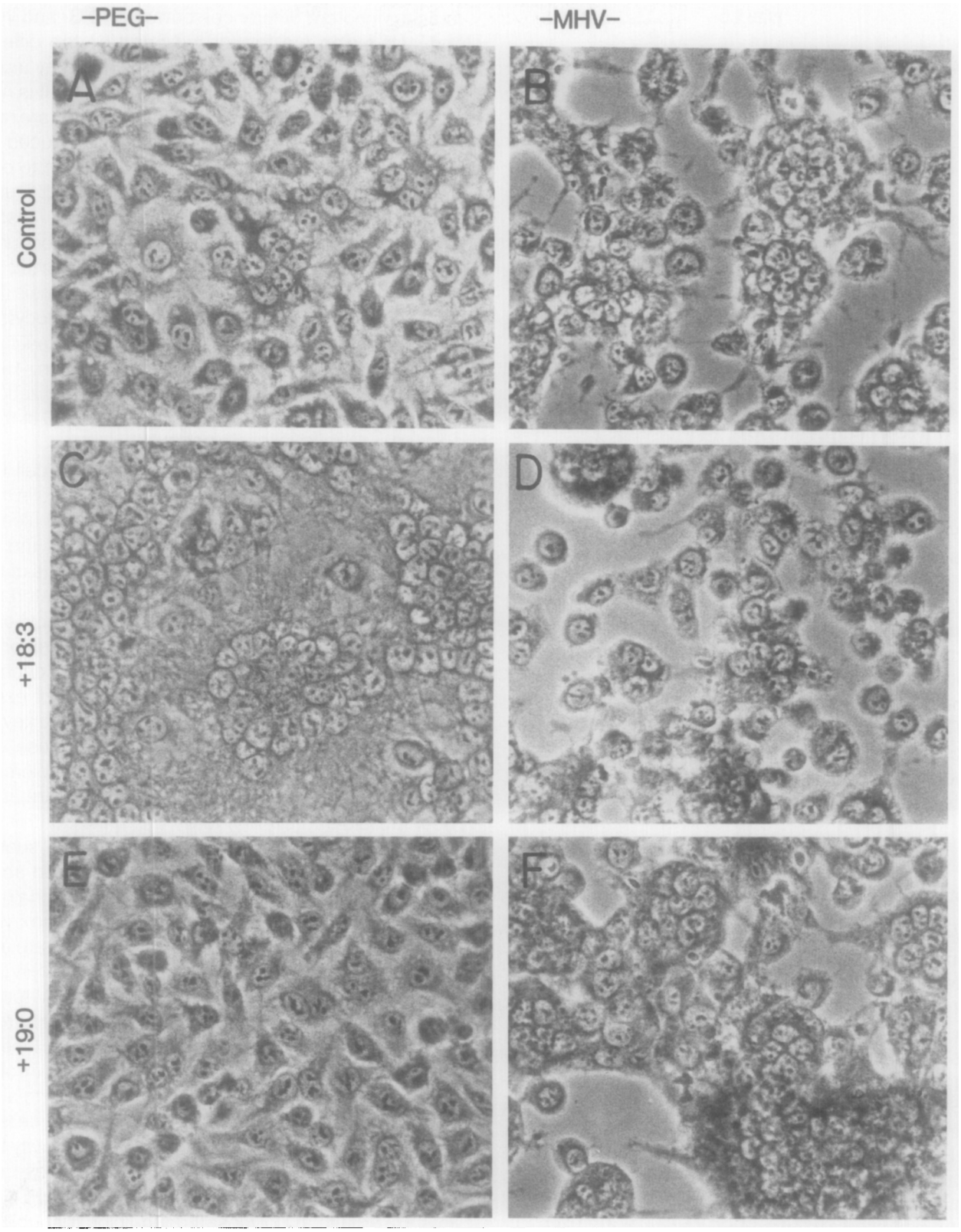

FIG. 5. Fusion of F_{16} cells by viral and chemical agents. Parallel cultures of F_{16} cells were treated with polyethylene glycol (A, C, E) or MHV (B, D, F) as described under Materials and Methods. Control cultures of this cell line exhibit moderate sensitivity to both fusogens (A and B). As is shown in (C) and (D), however, cells grown in 10^{-4} *M* polyunsaturated fatty acid (18:3) exhibit a PEG-sensitive/virus-resistant phenotype reminiscent of the parental cell line Clone 1D. By contrast, growth in saturated fatty acid (19:0; (E) and (F)) produced cells which were resistant to PEG fusion but virus sensitive, as normally seen only in F_{40}.

TABLE 3

TITERS OF VIRUS GROWN IN CLONE 1D AND F_{40} CELLS

	VSV (PFU/ml × 10^{-7})				MHV (PFU/ml × 10^{-5})		
Cell line	+19:0[a]	None	+18:2c[a]	+18:2t[a]	+19:0[a]	None	+18:3[a]
Clone 1D	17	7	9	7	6	<0.1	<0.1
F_{40}	60	69	50	46	244	188	242

[a] 10^{-4} molar lipid supplement; "c" indicates *cis* configuration; "*t*", *trans*.

Murine hepatitis virus grew well in F_{40} cells, producing ~2×10^7 PFU/ml, but Clone 1D cells reproducibly produced at least 1000-fold less virus. Supplementation of Clone 1D cells with saturated fatty acids produced a low, but detectable, titer of infectious virus. Under these conditions, MHV induced considerable fusion (cf. Fig. 4E). Although the modification of F_{40} cells with unsaturated fatty acids dramatically suppressed MHV-induced fusion (Fig. 4D), lipid supplements did not appear to affect virus production in these cultures.

The extent of virus infection was also monitored by indirect immunolabeling of MHV-treated cells. Infected cultures of F_{40} cells confirmed that most cells (including many of those which remained unfused) were producing viral protein. In contrast, infected cultures of Clone 1D showed viral antigen only in fused cells (Fig. 6). The pattern of labeling in Clone 1D suggests that infection is spread only by fusion with adjacent cells, sometimes over long distances through attachment processes from giant multinucleates.

DISCUSSION

Control of virus-induced fusion by lipid composition

We have used a series of mutant cell lines, resistant to chemical fusogens (such as PEG) by virtue of their altered lipid content, to probe the relationship between host cell lipid composition and virus-induced cell fusion. For a wide variety of fusogenic enveloped viruses, virus-induced fusion is *inversely* correlated with PEG-induced fusion; i.e., PEG-sensitive cells are virus resistant, and vice versa. The inverse relationship between virus and PEG fusion extends to other cell lines as well: the BHK-21 cell line, for example, frequently studied because of its high susceptibility to virus-induced fusion (Holmes and Choppin, 1966; Helenius *et al.*, 1980), is highly resistant to PEG (Wang *et al.*, 1982).

It is interesting that PEG-resistant cells are unusually susceptible to virus-induced fusion. Presumably, this finding reflects fundamental differences in the mechanisms by which different fusogens destabilize membranes. Inherent differences between PEG- and virus-induced fusion are further indicated by the different spectrum of polykaryocytosis in cells fused by murine hepatitis virus, as seen in Table 1 and Fig. 3. It is notable, however, that the selection of cells which are resistant to the fusogenic effects of PEG has produced mutants which are also altered in their response to other membrane-active agents, such as fusogenic viruses. Other membrane-associated phenomena are also altered in these cells, including spontaneously occurring fusion (Roos and Choppin, 1985b), nonspecific adsorptive endocytosis and fluid-phase pinocytosis (Li *et al.*, 1986) (but not receptor-mediated endocytosis; Ryser *et al.*, 1988), and tumorigenicity and capacity for metastatic spread *in vivo* (Roos and Choppin, 1984). Like virus-induced fusion, spontaneous hybridization is inversely correlated with susceptibility to PEG in these cells (Roos and Choppin, 1985b).

Comparisons between the fusion-altered cell lines under study and the parental Clone 1D cells from which they were derived have previously revealed unusual differences in the lipid composition of the mutants (Roos and Choppin, 1985a). Two aspects of their lipid composition are particularly well correlated with the PEG-resistant phenotype: increased acyl-chain saturation and the presence of high concentrations of ether-linked neutral lipid. Although these two phenomena may be metabolically linked, a variety of experiments indicate that acyl-chain saturation is the key factor in regulating the fusion response of these cells (Roos, 1988). Through the use of lipid supplements added to the growth medium of cells in culture, it has been possible to exert virtually complete control over the response of cells to chemical fusogens such as PEG (Roos and Choppin, 1985b). As reported above, we have now found that the alteration of acyl-chain saturation can be successfully exploited to control virus-induced fusion as well. Thus F_{16} cells, which are intermediate in their response to both PEG and MHV, can be pushed in either direction: toward the MHV-fusible/PEG-resistant phenotype of F_{40} cells or the MHV-resistant/PEG-fusible phenotype characteristic of Clone 1D, solely by manipulation of the fatty acid content of the medium (Fig. 5). These findings apply equally to a variety of other cell lines (Roos *et al.*, 1987). BHK cells are often studied because of their extreme sensitivity to virus-induced fusion (as noted above), but these cells can be rendered virus-fusion-resistant (and PEG-fusible) by growth in polyunsaturated lipids (unpublished observations). The ability to control both viral and chemical fusion in parallel (although in opposite directions) may reflect similarities in the site (albeit not the mechanism) of action at the cell membrane. This new-

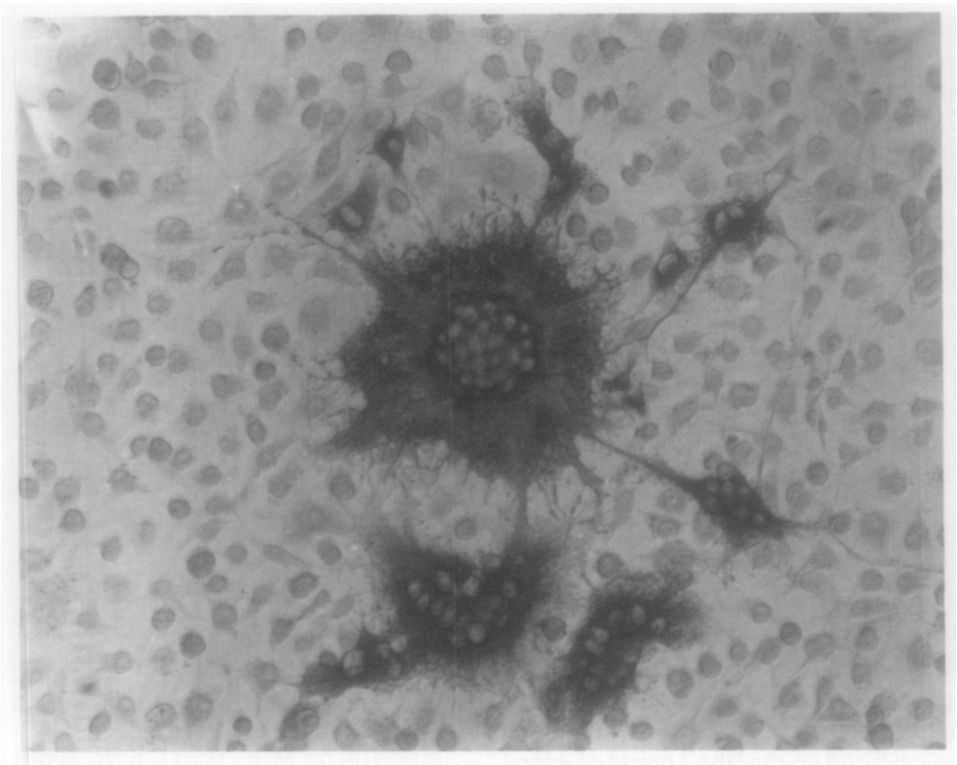

FIG. 6. Immunostaining of viral proteins in infected cells. Clone 1D cells infected with murine hepatitis virus were fixed and processed for microscopy as described under Materials and Methods, using a polyclonal mouse antiserum directed against MHV structural proteins. Viral antigen was found only in multinucleated giant cells, one of which is shown here. Thin extensions can be seen connecting the central syncytium with those outlying cells which stained positively for viral antigen. Neither fusion nor viral protein production was observed in uninfected cultures (not shown).

found control over cell fusion *in vitro* may also prove useful in technological applications (Roos, 1989).

Mizzen *et al.* (1983) studied MHV-induced fusion in an LM TK^- mouse cell line closely related to our Clone 1D cells. These investigators also found LM TK^- cells to be resistant to virus-induced fusion (by comparison with the MHV-sensitive cell line L2; Rothfels *et al.*, 1959) due to some "inherent property of the LM TK^- cell membrane." Our finding that acyl-chain composition serves to regulate MHV-induced fusion provides a possible biochemical basis for these authors' observations.

Membranes, viral pathogenicity, and host range

Several investigators have studied correlations between acyl-chain composition and various aspects of virus virulence. Increased content of unsaturated fatty acids appears to be associated with a decreased hemolysis in Newcastle disease virus (Blenkharn and Apostolov, 1980) and Sendai virus (MacDonald *et al.*, 1984). Both reports attribute this phenomenon to decreased ability to fuse with the target membrane, consistent with our observations for Clone 1D cells, which are naturally unsaturated relative to F_{40}. Increased fatty acid saturation is associated with increased virulence (Blenkharn and Apostolov, 1981; Nozawa and Apostolov, 1982), as seen in our highly saturated mutant cell line F_{40}, which is highly susceptible to virus-induced fusion.

The susceptibility of Clone 1D and F_{40} cells to virus infection did not always parallel susceptibility to virus-induced fusion. VSV fused F_{40} cultures considerably better than Clone 1D cells, but formed plaques with equal efficiency and grew to high titers in both cell lines. In MHV-infected cultures, however, F_{40} produced >1000-fold more virus than did Clone 1D. Of particular interest is the observation that control cultures of Clone 1D cells supplemented with saturated fatty acids (a treatment which strongly stimulates fusion; cf. Fig. 4E) exhibited a distinct increase in the production of infectious virus (Table 2). The ability of lipid alteration to stimulate the production of infectious virus from a nonpermissive cell line demonstrates that lipid composition can play a functional role in the control of viral host range.

Many viruses are characterized by species and tissue tropisms, and coronaviruses typically exhibit a very limited host range (Lucas *et al.*, 1977; Wege *et al.*,

1982). Host permissiveness for MHV varies considerably in different mouse strains and different tissues or cell lines (Bang and Warwick, 1960; Sturman and Takemoto, 1972; Dubois-Dalcq *et al.*, 1982; Tardieu *et al.*, 1986). Genetic resistance can result from absence of the MHV receptor from target tissues (Boyle *et al.*, 1987), or from defects in later steps in the viral replicative cycle such as penetration, RNA synthesis, or virus assembly and release. MHV-mediated fusion is secondary to binding of the peplomeric glycoprotein E2 to the 110-kDa receptor in the membranes of susceptible cells (Holmes *et al.*, 1984, and unpublished observations) and requires cleavage of the E2 molecule into two 90-kDa fragments (Holmes *et al.*, 1984; Frana *et al.*, 1985; Sturman *et al.*, 1985; Van Dinter and Flintoff, 1987; Kooi *et al.*, 1988).

We have not yet determined at what stage in the replicative cycle MHV is inhibited in Clone 1D cells, but the block appears to be early, before the synthesis of viral antigens. The patterns of fusion, antigen distribution, and virus yield observed in MHV-infected Clone 1D cultures suggest that once inside, the virions readily replicate in these cells, but that release of infectious virus may be inhibited so that the spread of infection is limited to fusion with adjacent cells (Fig. 6). This pattern of infection is characteristic of cases where the maturation of virus particles is blocked at the cell membrane, by external experimental manipulations (Graves *et al.*, 1978; Merz *et al.*, 1981), because of a viral defect (Hall and Choppin, 1981), or through a viral genetic lesion introduced through recombinant techniques (McCune *et al.*, 1988). Particularly for enveloped viruses, assembly of new virions appears to be a difficult step, requiring complex membrane interactions (Dubois-Dalcq *et al.*, 1984). In attempting to determine the basis for the inhibition of virus production in Clone 1D cells it may be instructive to examine not only virus-permissive mutants (such as F_{40} cells) but also precursors in the isolation of the LM TK^- cell line (Kit *et al.*, 1963), as mouse fibroblast cell lines are generally susceptible to MHV infection (Mizzen *et al.*, 1983; Sturman *et al.*, 1985; Frana *et al.*, 1985; Kooi *et al.*, 1988).

The observation that at least one aspect of viral pathogenesis—cell fusion—can be regulated in this system by cellular acyl-chain saturation suggests that differences in host and tissue lipid composition may also affect viral host range and tissue specificity in nature. The system described above, where a series of well-characterized, closely related cell lines vary dramatically in their response to virus, should prove useful in determining the functions of lipids in the course of virus infection.

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