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Fusion Resistance and Decreased Infectability as Major Host Cell Determinants of Coronavirus Persistence

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Mouse hepatitis virus persists in cultures of a subline (designated LM-K) of mouse LM cells but produces a lytic infection in L-2 cells. Persistence in the LM-K cells was not accompanied by production of ts mutants or of soluble anti-MHV factors. Infectious center assay demonstrated an approximately 500-fold lower level of infectibility by MHV of the LM-K cells as compared to L-2 cells. On an infected cell basis, production levels of infectious progeny and viral RNA were comparable between the two cell lines. The extent of virus-induced cell-cell fusion, however, was markedly reduced in the LM-K cells. Cell-mixing experiments showed that both infected L-2 and LM-K cells have the capacity of fusing with neighboring uninfected L-2 cells but not with uninfected LM-K cells. This suggests that the decreased level of fusion observed in the LM-K infection is due not to absence of viral fusion protein at the cell surface, but rather to an inherent resistance of the LM-K cell membrane to MHV-induced fusion. It is believed that such fusion resistance in LM-K cells moderates virus dissemination throughout the culture, thereby contributing to a state of virus persistence.

INTRODUCTION

Within the past few years considerable progress has been made towards characterizing, in molecular terms, some of the events involved in the coronavirus replication cycle. Murine coronaviruses, exemplified by mouse hepatitis virus (MHV), are now known to contain three major structural proteins (Cheley and Anderson, 1981; Sturman, 1977), of which the intravirion localizations (Sturman *et al.*, 1980) as well as some intracellular activities (Collins *et al.*, 1982; Holmes *et al.*, 1981) have been documented. In addition, significant advances have been made concerning the steps involved in RNA transcription, including the characterization of genomic and messenger RNA species (Cheley *et al.*, 1981a, b; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981; Wege *et al.*, 1981) as well as the assignments of virus-specified structural and nonstructural polypeptides to individual messenger RNAs (Leibowitz *et al.*, 1982; Rottier *et al.*, 1981; Siddell *et al.*, 1981). Complementary DNA (cDNA) probes are now available (Cheley *et al.*, 1981a, b; Leibowitz and Weiss, 1981) which may be used for the detection and analysis of MHV RNA.

With these newly gained insights into coronavirus replication, it is now possible to investigate individual parameters in the infectious cycle to determine which factors play a key role in determining the outcome of infection. A number of strains of MHV have received much interest as a result of their demonstrated ability to become persistent under certain conditions, both *in vivo* and *in vitro* (Lucas *et al.*, 1977, 1978; Robb and Bond, 1979; Sorensen *et al.*, 1980). A variety of mechanisms implicated in *in vitro* MHV persistence have been reported in the literature, involving the generation of temperature-sensitive (Holmes and Behnke, 1981), cold-sensitive (Stohlman *et al.*, 1979), and small plaque (Hirano *et al.*, 1981) virus mutants. Studies by Lucas *et al.* (1977, 1978), however, demonstrated that MHV persistence in a number of cul-

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tured cell lines did not entail the production of detectable virus mutants, suggesting that these were not mandatory for establishment of the persistent state.

The present report describes some characteristics of a persistent MHV infection in a subline of mouse LM fibroblasts, in which persistence is host cell-regulated and does not involve any apparent change in the input wild type virus. The results strongly suggest that, in this system, persistence is due not to suppression of virus replication but rather to an ability of the host cell to resist infection and to withstand cytopathic effects, i.e., cell fusion.

MATERIALS AND METHODS

Cells and virus. The A59 strain (Manaker *et al.*, 1961) of MHV was used for these studies. Cells used were the L-2 line (Rothfels *et al.*, 1959), here designated L, and the thymidine kinase-deficient LM line (Kit *et al.*, 1963), here designated LM-K. This latter cell line is not the same as the LM cells (Merchant and Hellman, 1962) used in previous studies (Anderson and Bilan, 1981; Cheley *et al.*, 1981b). For comparison, LM-K cells were found to be more infectible by MHV and gave rise to higher virus titers than LM cells (unpublished).

Infection and culture conditions. Cells were propagated in Eagle's minimal essential medium (MEM), supplemented with 5% fetal calf serum (FCS). Monolayers of either L or LM-K cells were inoculated with pretitered stocks of MHV, adsorbed 30 min at 32.5°, washed three times with MEM, and then incubated at 37° in MEM containing 5% FCS. For long-term culture studies, medium was changed on a daily basis. Aliquots were removed from culture media at various time intervals for analysis of virus yields by plaque assay on L cells (Lucas *et al.*, 1977).

Infectious center assay. To determine the number of infected cells in MHV-inoculated cultures, cells were harvested at 2 hr postinoculation (p.i.) by trypsinization after thrice washing with citrate saline. Harvested cells were freed from trypsin by centrifugation (2 min at 600 *g*) and resuspended in MEM supplemented with 5% FCS. Aliquots of the resuspended cells and dilutions therefrom were plated on monolayers of L cells, allowed to attach for 2 hr at 32.5°, then overlaid with MEM containing 5% FCS and 0.5% methylcellulose (4000 cps). Plaques were read after 24 hr at 37°.

Assay for cell surface fusion activity. Sparse cultures of L and LM-K cells (10^5 cells/35 mm plate) were inoculated with MHV at a multiplicity of inoculation (m.o.i.) of 0.01 and 10, respectively (to produce roughly equal infectious centers). After adsorption and washing as above, cultures were incubated 5 hr at 37°. At this time 10^6 uninfected L or LM-K cells were added to the cultures to fill in the spaces left unoccupied by the original, sparsely seeded cells. Cultures were photographed and scored either positive or negative for syncytial formation after 3 hr incubation at 37°.

RNA extraction and semiquantitation by dot blotting. Cell monolayers (10^6 cells in 35 mm plates) were harvested with a modified guanidine hydrochloride (Strohman, 1977) procedure. Cells were solubilized with 1 ml of 7.6 *M* guanidine-HCl in 0.1 *M* potassium acetate buffer, pH 5.0, and the viscous mixture was homogenized by aspiration five times through a 1-ml sterile plastic syringe (Yale) fitted with a 21-gauge needle. Samples were transferred to tissue culture tubes (Falcon), mixed with 0.6 vol of 95% ethanol, cooled for 12 hr at −20°, and centrifuged at 5000 *g* for 20 min to pellet RNA. RNA pellets were dissolved in 250 μl 7.6 *M* guanidine-HCl in 0.1 *M* potassium acetate, pH 5.0, to which was added 250 μl 20 × SSC (1 × SSC (standard citrate saline) is 0.15 *M* sodium chloride, 0.015 *M* trisodium citrate). Samples were subsequently heated for 15 min at 50°. For dot-blot hybridization, 50-μl aliquots of the samples or of serial 10-fold dilutions therefrom were applied in a Schleicher and Schuell "Minifold" filtration apparatus to nitrocellulose sheets (0.45 μm, Millipore) supported on three layers of slab dryer filter paper (Bio-Rad). Both nitrocellulose sheets and filter paper had been previously equilibrated with 10 × SSC. After sample application, sheets were dried at 80° for 1

hr. Sheets were preannealed, annealed with [^{32}P]cDNA (prepared as described (Cheley *et al.*, 1981a) against MHV nucleocapsid protein mRNA), and washed essentially according to the procedure of Thomas (1980). The ability of this cDNA to detect all positive-sense MHV-specific RNAs identified to date has been documented (Cheley *et al.*, 1981a, b). The annealed and washed sheets were autoradiographed to yield the dot blots. Densitometric scanning analysis was performed on selected sample dots for which autoradiographic intensities were less than saturated.

[^{35}S]*Methionine labeling of MHV polypeptides.* Cultures of L and LM-K cells in 35 mm petri dishes were inoculated at an m.o.i. of 10 and labeled at 8 hr p.i. for 1 hr with [^{35}S]methionine (100 μCi/ml) in 0.5 ml methionine-free MEM. Cells were harvested by scraping and immunoprecipitated (Nusse *et al.*, 1978) using anti-MHV(A59) antiserum. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography as previously described (Cheley and Anderson, 1981).

RESULTS

Comparative Infectability of L and LM-K Cells with MHV

To perform the comparisons documented in subsequent sections of this report, it was initially necessary to standardize results on an infected cell basis. Toward this end, cultures of L or LM-K cells were inoculated at m.o.i.'s ranging from 0.01 to 10 and, following a 2-hr incubation period, numbers of infected cells determined by infectious center assay. Under nonsaturating conditions (i.e., when the m.o.i. is less than 1), LM-K cells were found to be approximately 500-fold less infectable than correspondingly inoculated L cells. Also evident from Table 1 is the observation that comparable numbers of infected cells are produced when L cells are inoculated at an m.o.i. of 0.01 and LM-K cells are inoculated at an m.o.i. of 10. The basis for the differential infectibility of the two cell types is currently under investigation but appears to involve an early event, within the adsorption-penetration phase of the viral replication cycle (unpublished).

TABLE 1

COMPARATIVE INFECTABILITY OF L AND LM-K CELLS WITH MHV

m.o.i.[a]	Number of cells infected[b]		Ratio
	L cells	LM-K cells	
10	1.1×10^6	1.4×10^4	78
5	9.3×10^5	6.4×10^3	145
1	6.0×10^5	1.8×10^3	333
0.5	3.4×10^5	8.1×10^2	420
0.1	9.0×10^4	1.9×10^2	474
0.01	1.1×10^4	2.1×10^1	524

[a] As determined by plaque assay on L cells.

[b] Cultures of 10^6 cells were inoculated with MHV at the m.o.i. indicated, washed, and numbers of infected cells determined by infectious center assay.

Virus Yields from MHV-Infected L and LM-K Cells are Comparable

Since infectious center assays showed that a much higher inoculum was necessary to produce comparable numbers of infected LM-K as L cells, virus yields were determined on cultures inoculated with a range of m.o.i.'s. If L and LM-K cells were equally efficient virus producers, virus outputs from the two cell types would be comparable, once cultures were standardized on the basis of equal numbers of infectious centers. On this basis, virus production from LM-K cells (m.o.i. = 10) would be expected to most closely parallel virus production from L cells inoculated at an m.o.i. of 0.01. In fact, in terms of both virus yields and chronological similarity, the LM virus titration curve appeared most comparable to that of L cells inoculated at an m.o.i. of 0.1 (Fig. 1A). Hence, on an infected cell basis, LM-K cells are at least as efficient as L cells in producing infectious progeny MHV.

In contrast to MHV-infected L cells, cultures of MHV-infected LM-K cells continued to produce progeny virus in an undulating fashion over the 7-day period of study (Fig. 1B). Longer-term studies (data

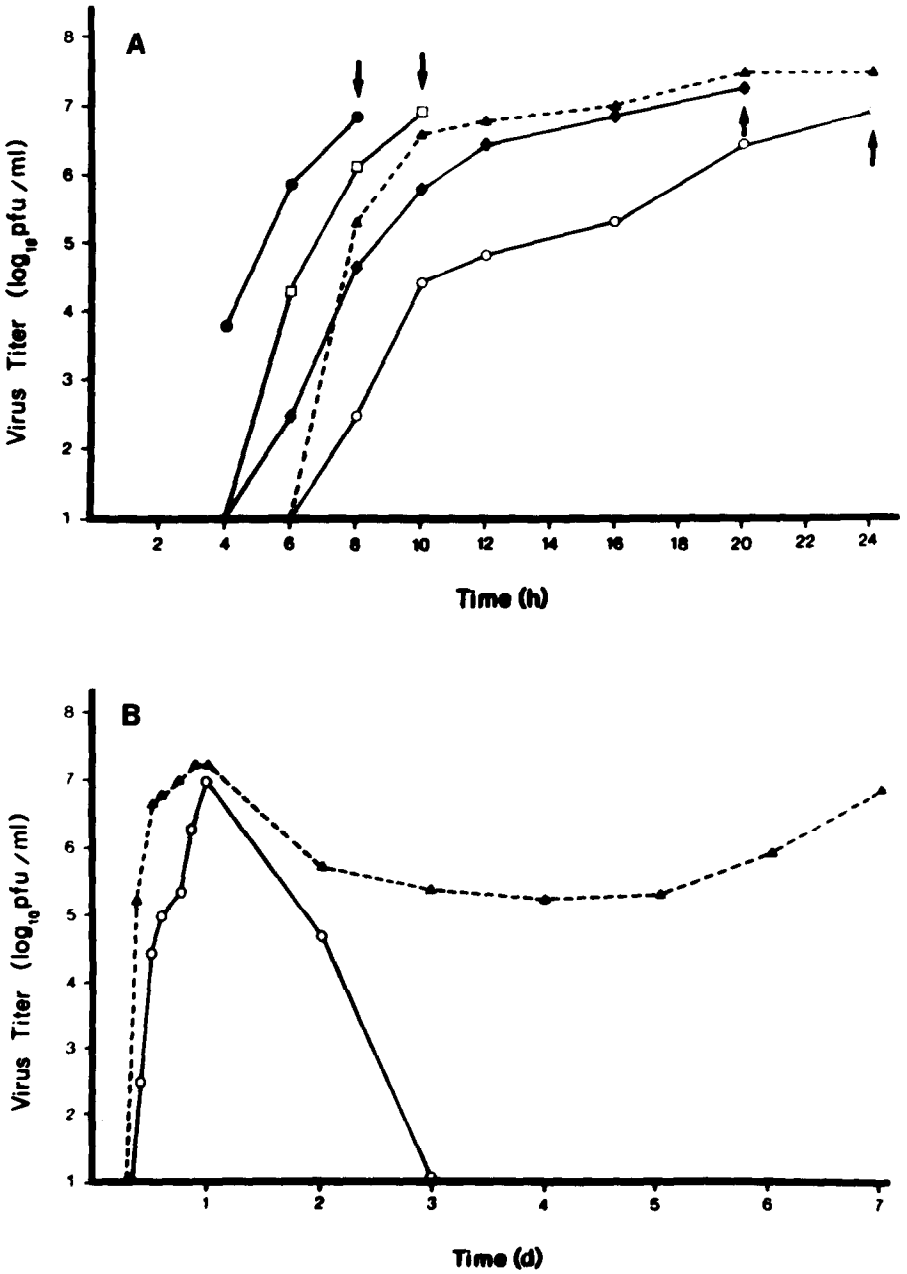

FIG. 1. Virus production from L (solid lines) and LM-K (dashed lines) cells inoculated at various m.o.i.'s. Cultures of L cells were inoculated at m.o.i.'s of 10 (●), 1 (□), 0.1 (♦), and 0.01 (○). LM-K cells were inoculated at an m.o.i. of 10 (▲). Panel (A) short-term (1 day); Panel (B) long-term (7 days). Arrows refer to times of complete fusion of the monolayers.

not shown) have shown that infected LM-K cell cultures continue to produce progeny virus over a period of several weeks, in a cyclical fashion similar to that previously described for a number of persistently infected cell lines (Lucas *et al.*, 1978). Periodic infectious center assays of long-term cultures showed that between 1 and 10% of the cells were infected at any given time-point, suggesting that MHV persistence in

LM-K cells is mediated by a carrier-culture mechanism, involving virus propagation and transmission among a relatively small fraction of the total cell population.

Virus isolated from infected LM-K cells showed no appreciable temperature sensitivity when assayed for growth in L cells at 32.5, 37, or 39.5°. Plaque morphology on L cells was also unaltered from that seen with stock MHV. As with previously described persistent MHV infections (Lucas *et al.*, 1978), it therefore seems unlikely that MHV persistence in LM-K cells necessitates the production of ts virus mutants.

Supernatant MHV-infected LM-K culture fluids, which had been freed from virus by centrifugation at 100,000 *g* for 1 hr had no inhibitory effect on MHV replication when incubated with MHV-inoculated L cells. Therefore, no evidence was found for the production of soluble antiviral factors which might suppress virus production and contribute to persistence.

Levels of Viral RNA Synthesis in MHV-Infected L and LM-K Cells are Comparable

Using an MHV-specific ^{32}P-labeled cDNA probe (Cheley *et al.*, 1981a) the relative amounts of positive-sense viral RNA were determined in MHV-infected L and LM-K cells by dot blotting. For each timepoint assayed, aliquots were taken from three serial 10-fold dilutions of the RNA extracted from 10^6 cells. In this way non-saturating autoradiographic images of the viral RNA in at least one dilution of each sample could be obtained by the dot-blotting technique. In all cultures examined, background levels of viral RNA, i.e., up to 2 hr p.i., were extremely low (Fig. 2), attesting to the high specificity of the probe for MHV-RNA. L cells inoculated at an m.o.i. of 10 showed significant viral RNA at 4 hr p.i.; this increased considerably by 6 hr p.i. before dropping off somewhat by

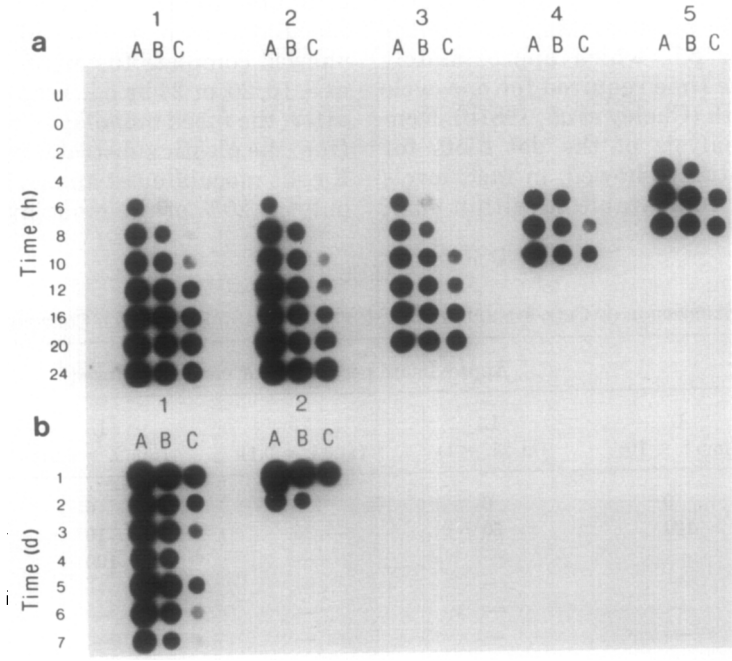

FIG. 2. Dot-blot hydridization analysis of MHV-specific RNA in infected L and LM-K cells, using MHV-[^{32}P]cDNA as the probe. Column numbers 1-5: 1, LM-K (m.o.i. of 10); 2, L (m.o.i. of 0.01); 3, L (m.o.i. of 0.1); 4, L (m.o.i. of 1); 5, L (m.o.i. of 10). Column letters A, B, C refer to serial 10-fold dilutions of RNA samples, corresponding to RNA from 10^5, 10^4, and 10^3 cells, respectively. Panel (a) short-term (1 day) time course; u:uninfected. Panel (b), long-term (7 days) time course.

8 hr p.i. At this time the monolayer was completely fused and starting to detach from the plastic substrate. L cell cultures inoculated with m.o.i.'s of 1, 0.1, and 0.01 showed a progressively more gradual increase of viral RNA, the onsets of which were first detected at 6 hr p.i. Generally, for any given time point after 4 hr p.i. there is an obvious increase in detectable viral RNA in response to the increase in m.o.i. from 0.01 to 10 (Fig. 2, lanes 2–5). LM-K cultures inoculated with an m.o.i. of 10 showed a pattern of viral RNA synthesis most closely resembling that seen in L cells inoculated with an m.o.i. of 0.01, i.e., a 1000-fold lesser inoculum than that used for the LM-K cells. This value is in reasonably good agreement with the difference in infectability of the two cell lines as determined by infectious center assay (Table 1). There would, therefore, appear to be no great difference in the levels of viral RNA synthesis, on an infected-cell basis, between the two cell lines.

The similarity in viral RNA levels between LM-K (m.o.i. = 10) and L (m.o.i. = 0.01) cells is most striking during the first 16–20 hr p.i., which approximately represents the time required for one cycle of virus growth (Cheley *et al.*, 1981b). Densitometric analysis of the dot blots for these two cultures showed, in fact, levels of viral RNA comparable to within 40%, for each of the time points assayed between 6 and 20 hr p.i.

An extended term analysis of viral RNA in LM-K cells (Fig. 2b) demonstrated a somewhat cyclical variation in RNA levels over a period of 7 days. While viral RNA persisted in the LM-K cultures for the duration of the 7-day experiment, the most long-lived L cell infection (i.e., that inoculated with an m.o.i. of 0.01) showed viral RNA only for 2 days, after which time cell lysis and lifting of the fused monolayer terminated the culture. Moreover, the level of viral RNA was seen to drop dramatically between Day 1 and Day 2 in the L cell culture (by 99% by densitometric analysis) consistent with rapid decline of integrity of the culture.

Differential Expression of MHV-Induced Fusion in L and LM-K Cultures

Both size and spread of virus-induced syncytia were found to be much reduced in cultures of MHV-infected LM-K cells as compared to MHV-infected L cells. L cells inoculated at m.o.i.'s of 10, 1, 0.1, or 0.01 showed complete fusion of the monolayer at 8, 10, 20, or 24 hr p.i., respectively; thereafter, the fused monolayer began to detach from the plastic substrate. In contrast, LM-K cells inoculated at an m.o.i. of 10 showed only ca. 20% of the monolayer fused after

TABLE 2

EXPRESSION OF CELL FUSION IN MHV-INFECTED L AND LM-K CELL CULTURES

Time p.i. (hr)	Approximate percentage of monolayer fused[a]				
	L (m.o.i. = 10)	L (m.o.i. = 1)	L (m.o.i. = 0.1)	L (m.o.i. = 0.01)	LM-K (m.o.i. = 10)
2	0	0	0	0	0
9	100	60	20	10	10
24	—	—	—	100	20
48	—	—	—	—	20
72	—	—	—	—	20
96	—	—	—	—	10
120	—	—	—	—	20
144	—	—	—	—	20
168	—	—	—	—	20

[a] Expressed as area percent of total monolayer. Dashes indicate detachment of the fused monolayer from the plastic petri plates.

24 hr p.i. (Table 2). Moreover, in the infected LM-K culture, there was no increase in this level of fusion with time; rather, cell fusion declined slowly (to ca. 10% of the monolayer by Day 4), followed by an increase (to ca. 20% by Day 7). Cyclical variations of cell fusion over periods of several weeks have been a common feature of MHV-infected LM-K cultures maintained in our laboratory.

Syncytial morphology is also indicative of depressed fusion levels in infected LM-K as opposed to infected L cells (Fig. 3). Individual syncytial foci in infected L cell cultures (m.o.i.'s of 0.01 or 0.1) were roughly circular and rapidly expanding, suggesting

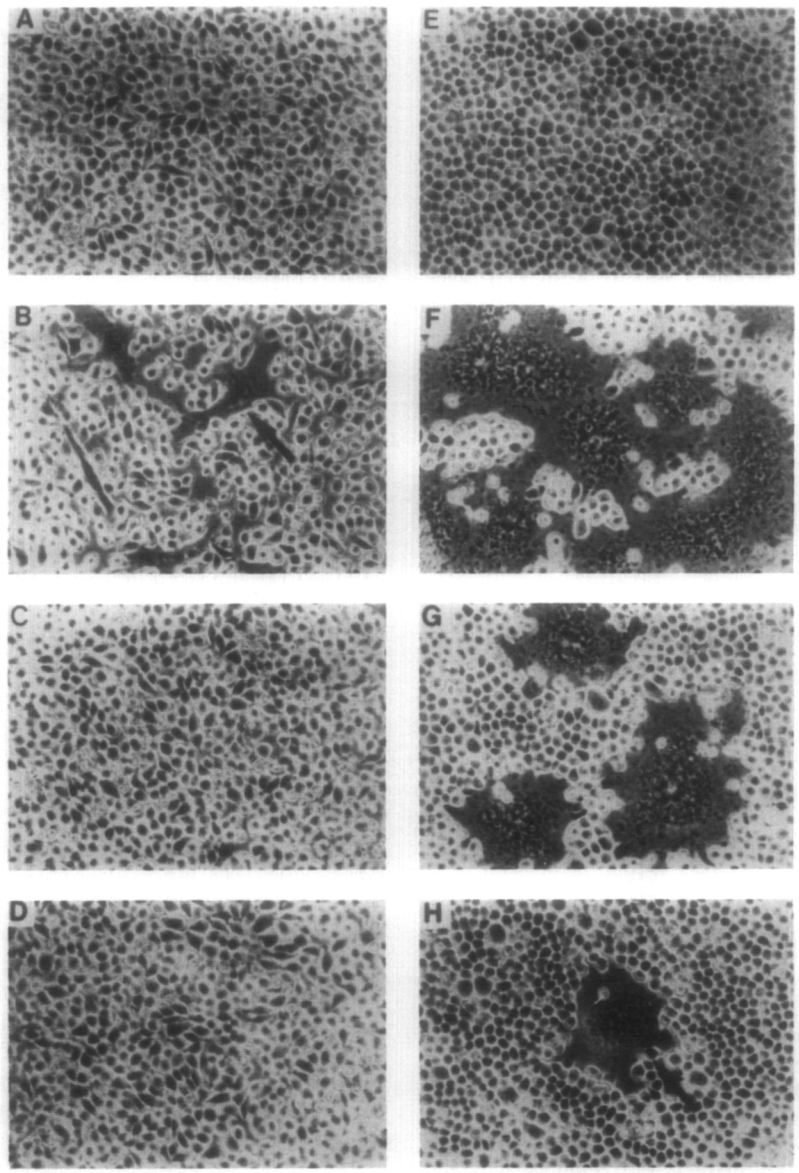

FIG. 3. Light micrographic comparison of MHV-induced cell fusion in L and LM-K cells. Cultures of LM-K (A–D) and L (E–H) were inoculated at m.o.i.'s of 0 (A and E), 10 (B), 1 (C and F), 0.1 (D and G), and 0.01 (H). Photographs of representative fields were taken at 9 hr p.i. Arrow indicates syncytium.

facile recruitment of neighboring uninfected cells. In contrast to those observed in infected L cells, LM-K cell syncytia showed little radial expansion. Rather, they tended to remain narrow and anastomotic in appearance, thus preserving relatively large areas of uninfected cells. This observation is highly suggestive of a dynamic balance between infected and uninfected cells in MHV-infected LM-K cultures, which would contribute to a persistent state.

To test for the presence of viral structural proteins, including the protein responsible for cell fusion (Collins *et al.*, 1982), in MHV-infected L and LM-K cells, immunoprecipitation was performed on extracts prepared from cultures labeled with [^{35}S]methionine at 8 hr p.i. As shown in Fig. 4, all three major viral polypeptides,

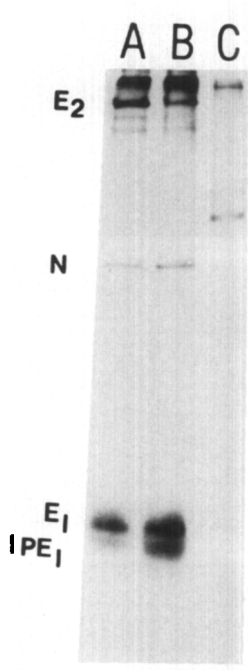

FIG. 4. Demonstration of MHV structural polypeptides in MHV-infected L and LM-K cells. Culture dishes (35 mm diameter) of LM-K (lane A) or L (lanes B and C), inoculated at an m.o.i. of 10, were labeled with [^{35}S]methionine for 1 hr at 0 (lane C) or 8 (lanes A and B) hr p.i. Cell extracts were immunoprecipitated with anti-MHV antiserum, and the immunoprecipitates subjected to autoradiographic SDS-PAGE. Polypeptide designations are as previously given (Cheley *et al.*, 1981a).

E1 (and precursor PE1), E2, and N, were present in both L and LM-K cells, in roughly similar proportions. Thus, the observed lack of fusion in MHV-infected LM-K cells is not likely due to defective synthesis of viral fusion protein. This viral fusion protein has been shown by Collins *et al.* (1982) to be the E2 polypeptide.

Depressed LM-K Syncytiogenesis Is Due to Membrane Resistance to MHV-Induced Fusion

To determine whether the reduced levels of cell fusion observed in MHV-infected LM-K vs L cultures were a consequence of inherent membrane fusion resistance or a lack of expression of viral fusion protein at the cell surface, cell mixing experiments were performed. The ability of infected L or LM-K cells to induce fusion with neighboring, uninfected "indicator" L or LM-K cells was examined. Fusion was assayed within 3 hr after addition of the indicator cells, to monitor contact fusion with the infected cells and to avoid fusion expression as a result of possible infection of the indicator cells (even at an m.o.i. of 10, L cells do not show fusion before 4 hr p.i.).

The addition of indicator L cells to sparse cultures of either infected L or LM-K cells resulted in rapid spread of fusion throughout the indicator cells. However, when LM-K cells were used as indicators, no fusion was observed (Fig. 5). This experiment shows that there is no lack of viral fusion protein at the LM-K cell surface. We, therefore, conclude that the difference in syncytial formation between infected L and LM-K cultures is due to resistance of uninfected LM-K cells to fusion with infected neighboring cells. This resistance must be due to an inherent property of the LM-K cell membrane. Such a conclusion is also inferred by observations made earlier (Fig. 3), namely that syncytia in infected LM-K cultures remain narrow and show little radial expansion when compared to infected L cultures.

DISCUSSION

LM-K cells possess two characteristics which allow cultures to support a persis-

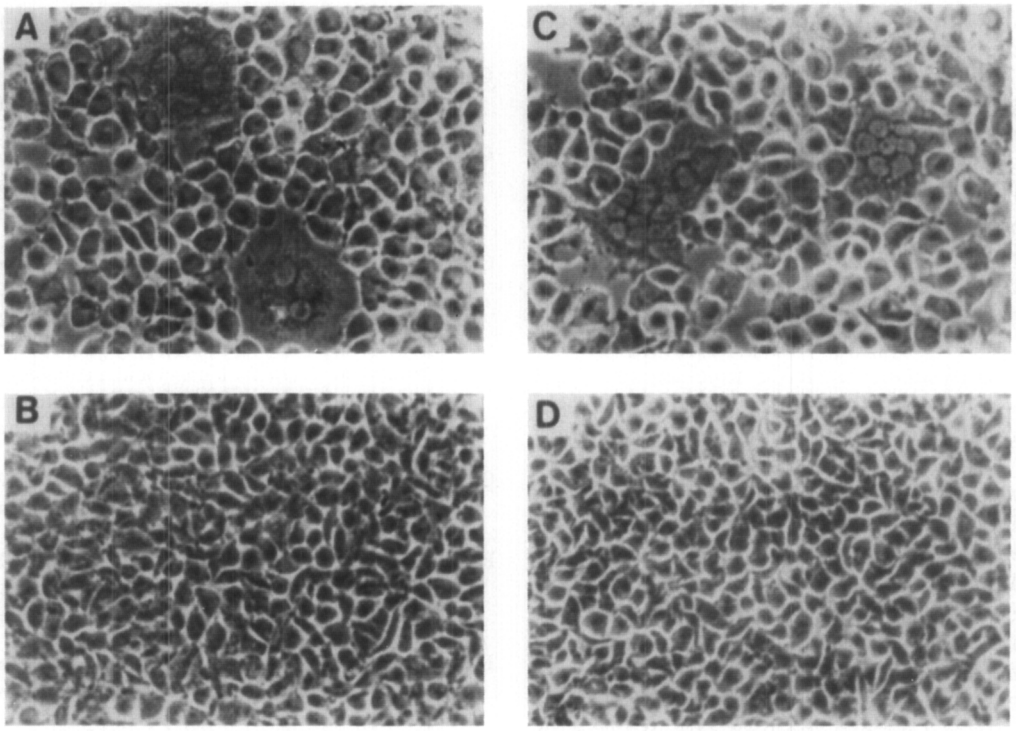

FIG. 5. Demonstration of cell surface fusion activity in infected L and LM-K cells. To washed, sparse cultures of 10^5 infected L (Panels C and D) and LM-K (Panels A and B) cells were added 10^6 uninfected L (Panels A and C) or LM-K (Panels B and D) cells. Photographs of representative fields were taken 3 hr after addition of the uninfected cells.

tent infection of MHV: a reduced level of infectability and a resistance to undergoing syncytial formation, when in contact with MHV-infected cell neighbors. It is apparent that MHV persistence in LM-K cells involves a dynamic balance between infected and noninfected cells. Persistence is, therefore, a feature of the entire culture and not of individual cells. The observed reduced levels of cell fusion in MHV-infected LM-K (as contrasted with L) cell cultures would act as a dissemination-limiting factor, thus contributing to the continued survival of remaining uninfected cells. Survival of uninfected cells is also assured as a result of the relatively higher resistance of LM-K cells to MHV infection. Results presented in this study show that MHV, even at an m.o.i. of 10 (determined on L cells) can only infect about 1% of inoculated LM-K cells. Levels of circulating virus (of the order of 10^7 PFU/ml) would, therefore, be inadequate to ensure infection of a substantial proportion of all LM-K cells in culture (ca. 10^6 cells/culture in the present study).

Despite the documented differences in infectability and manifestation of fusion between L and LM-K cells, it is clear that the two cell lines do not differ much in synthesis of viral RNA or in the production of infectious progeny. Although viral protein was not quantitated in the present study, it is apparent that sufficient quantities are produced in LM-K cells to yield normal virus outputs (compared to L cells). In addition, all three MHV structural proteins are present in infected LM-K cells as well as L cells, as demonstrated by immunoprecipitation. It is also apparent that adequate amounts of viral fusion protein are synthesized and expressed on the surface of infected LM-K cells to induce cell fusion with a "fusion-permissive" cell line, i.e., the L cell. Thus, the reduced incidence of cell fusion in the LM-K culture is likely

due to inherent resistance of the LM-K cell membrane to MHV-induced fusion.

Features displayed by the persistent MHV infection of LM-K cells, such as cycling virus titers, low levels of infectious centers, little cytopathic effect, and nonalteration of input virus are similar to those previously described for MHV infections of a variety of other cell lines (Lucas *et al.*, 1978). It is possible, therefore, that MHV persistence in these cultures (Lucas *et al.*, 1978) may similarly involve dissemination-limiting mechanisms such as reduced infectability and fusion resistance, as described here for LM-K cells.

Although it is conceivable that some genetically altered form of MHV is generated in LM-K cells which accounts for persistence, it is unlikely for the following reasons. First, virus recovered from MHV-infected LM-K cells is indistinguishable from input MHV in terms of non-ts replication and plaque morphology in L cells. Second, persistence in LM-K cells is already established within the first day of virus inoculation (c.f., complete lysis of L cells within this time), arguing against appreciable genetic selection of a "persisting" virus variant. Thus, while virus mutants may indeed arise as a result of prolonged persistence, as has been demonstrated for vesicular stomatitis virus (Holland *et al.*, 1979), it appears from the present study that the establishment of viral persistence need not involve genetic alteration of the infecting virus.

In connection with the present study suggesting MHV persistence may be at least in part determined by cellular resistance to fusion, it is worth noting the studies of Graves *et al.* (1978). These authors were able to prolong survival of measles-infected cell cultures approximately fivefold by using a synthetic inhibitor of cell fusion, SV4814. Cultures so treated continued to produce virus progeny but were protected against normally severe cytopathic effects.

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