THE ROLE OF PROTEASE-DEPENDENT CELL MEMBRANE FUSION IN PERSISTENT AND LYTIC INFECTIONS OF MURINE HEPATITIS VIRUS

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SUMMARY

We have defined three categories of cultured cell lines on the basis of their permissiveness (susceptibility to initial infection) to mouse hepatitis virus (MHV). Fully permissive L-2 cells gave rise to 100-1000-fold higher numbers of infectious centers than did semi-permissive LM, LM-K or C-1300 cells, whereas non-permissive Vero or C-6 cells were refractory to MHV infection. On an infected cell basis, there was no deficiency on the part of semi-permissive cell lines to replicate total viral RNA, viral polypeptides or progeny virions. Two of the semi-permissive cell lines (LM and LM-K) supported persistent MHV infection, while a third (C-1300) succumbed to lytic infection. LM and LM-K cells, but not C-1300 cells showed resistance to MHV-induced membrane fusion, even when placed in contact with fusion-active MHV-infected L-2 cells. The ability of a given cell to undergo fusion did not correlate with membrane lipid characteristics (unsaturated fatty acid and sterol content) which contribute to membrane "fluidity". In order to more closely study the parameters of MHV-induced cell fusion, membranes were prepared from MHV-infected L-2 cells and monitored for their fusogenic potential with permissive L-2 cells, semi-permissive LM cells and non-permissive vero cells. Fusion was only observed with the permissive L-2 cells, and only when exogenous protease (trypsin or chymotrypsin) was added. When the membranes were prepared from ^{35}S-methionine-labeled MHV-infected L-2 cells and subjected to protease treatment, the radiolabeled 180,000 dalton form of the E_2-glycoprotein underwent proteolytic cleavage to yield a major product of approximately 90,000 daltons. Both trypsin and chymotrypsin were effective in this proteolytic cleavage and in activating membrane fusion. In a normally permissive, fusogenic infection of MHV in L-2 cells, the protease inhibitors TPCK and ZPCK, but not TLCK, were found to inhibit cell fusion. In MHV-infected L-2 cells, E_2 was found almost exclusively as the 180,000 dalton form but turned over rapidly as shown by pulse-chase studies. TPCK and ZPCK but not TLCK inhibited turnover. The results suggest that L-2 cells contain a protease which cleaves at aromatic amino acids such as phenylalanine, and that this protease cleaves the 180,000 dalton form of the E_2 to peptide fragments, one or more of which may activate cell fusion.

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INTRODUCTION

The mechanisms underlying coronavirus persistence remain poorly understood. One attractive animal model for virus-mediated chronic neurological disease involves infection of rats or mice with the JHM strain of mouse hepatitis virus (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 undergo infections of a chronic nature in rodents.

In addition, however, to producing infections of a slowly progressive type, many MHV strains are also capable of inducing dramatic, acute disease (reviewed by Wege et al., 1982). Elucidating the conditions which favor persistent over acute infections is, accordingly, important to the understanding of MHV-induced disease. In the 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). Specifically, 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 MHV dissemination.

We have previously described an in vitro model for MHV persistence (Mizzen et al., 1983) in which a steady-state balance between virus replication and cell survival is maintained by host-imposed restriction of cell-cell fusion. We describe here studies emphasizing the importance of cell fusion in modulating the course of MHV infection, and further present biochemical evidence as to the mechanism of MHV-induced membrane fusion.

RESULTS

Characteristics of MHV replication in selected cell lines

As shown in Table 1, three cell lines (C-1300, Augusti-Tocco and Sato, 1969; LM-K, Kit et al., 1963; and LM, Merchant and Hellman, 1962) gave rise to much fewer numbers of infected cells (assayed according to Lucas et al., 1977) compared to the fully permissive L-2 cell line, when exposed to the same virus inoculum (MHV-A59; Manaker et al., 1961). For purposes of subsequent analyses it was convenient to define a semi-permissive category of cells with respect to MHV infection. We define semi-permissive cells, in contrast to fully permissive L-2 cells, as giving rise to a substantially lower (100-1000 fold) number of infectious centers. Shown in Table 1 are the results of infectious center assays for fully permissive (L-2), semi-permissive (LM-K, LM and C-1300) and non-permissive (C-6 and Vero) cells.

Two of the semi-permissive cell lines (LM-K and LM) became persistently infected while the C-1300 cell line was destroyed by cytocidal infection within 16 h in a manner approaching that observed in the L-2 cell infection. A major difference between the C-1300 cell line, and the LM-K or LM cell lines, is the expression of virus-induced cell fusion (Table 1). It would therefore appear that, while LM-K and LM cells can support continued MHV infection by restricting virus spread to discrete pockets of infected cells, the C-1300 cells soon succumb to cytocidal infection through unrestricted virus dissemination via cell fusion.

Using L-2 cells as a reference, different moi's of MHV inoculum were used and the resultant numbers of infectious centers were determined. As shown in Table 1, there are fewer infectious centers with decreasing moi, approximating those expected from the Poisson distribution (Dulbecco and Vogt, 1954). Infection of the semi-permissive cell lines with an moi of 10 (as determined on L-2 cells) gives rise to much smaller numbers of infectious centers. Using these data it is possible to assess parameters of virus replication, on an infected cell basis, for the various cell lines. Per infected cell, all three semi-permissive cell lines (LM, LM-K and C-1300), were found to replicate yields of progeny MHV comparable to or higher than those produced by fully permissive L-2 cells (Table 1).

Table 1. Characteristics of MHV Infection of Cultured Cell Lines

Cell	moi[a]	Infectious[b] Centers (%)	Virus output per Infected Cell[c]	Type of Infection
L-2	10	99	61.2	acute, fusogenic
L-2	1.0	59	57.6	acute, fusogenic
L-2	0.1	9	72.1	acute, fusogenic
L-2	0.01	0.9	53.0	acute, fusogenic
C-1300	10	1.5	200.0	acute, fusogenic
LM-K	10	1.5	615.0	persistent, weakly fusogenic
LM	10	0.2	48.2	persistent, weakly fusogenic
Vero	10	0.0	0.0	refractory
C-6	10	0.0	0.0	refractory

a Based on MHV titered on L-2 cells by plaque assay.
b Percentage of cells infected by MHV as determined by infectious center assay.
c Ratio of total plaque-assayable virus in 12 h culture fluid to the total number of infectious centers.

In order to rule out the possibility that any of the semi-permissive cell lines were deficient in permitting viral RNA synthesis, a dot-blot analysis was employed using a radiolabeled virus-specific cDNA probe. For comparison, as we have shown previously (Mizzen et al., 1983), dot-blot analysis of viral RNA from L-2 cells inoculated with decreasing moi's gives rise to the pattern shown in Fig. 1A, rows A-D. At high moi (row A) viral RNA synthesis peaks rapidly and is terminated by 8h PI or shortly thereafter by complete destruction of the cell monolayer. With decreasing moi's (rows B-D), viral RNA synthesis is reduced and is detectable at later times, due to increased survival of the monolayer.

Densitometric quantitation of viral RNA, using dot-blot data, as illustrated in Fig. 1A, permits reasonable comparisons as to the levels of MHV-RNA synthesized in the various cell lines under study. When expressed

in terms of MHV-infected cells, the results shown in Fig. 1B show that viral RNA synthesis in two of the semi-permissive cell lines, LM-K and LM, proceeds even more rapidly than that seen in the fully permissive L-2 cells. Viral RNA synthesis in C-1300 cells parallels very closely that seen in L-2 cells, once standardized on an infected cell basis. It is therefore apparent, that none of the three semi-permissive cell lines examined (LM-K, LM, C-1300) are deficient in synthesis of MHV-RNA.

In the non-permissive cell lines examined, no viral RNA was detected in either MHV-inoculated Vero or C-6 cells (Fig. 1A, rows H, I) indicating that the block in virus replication is one which affects a very early stage in infection.

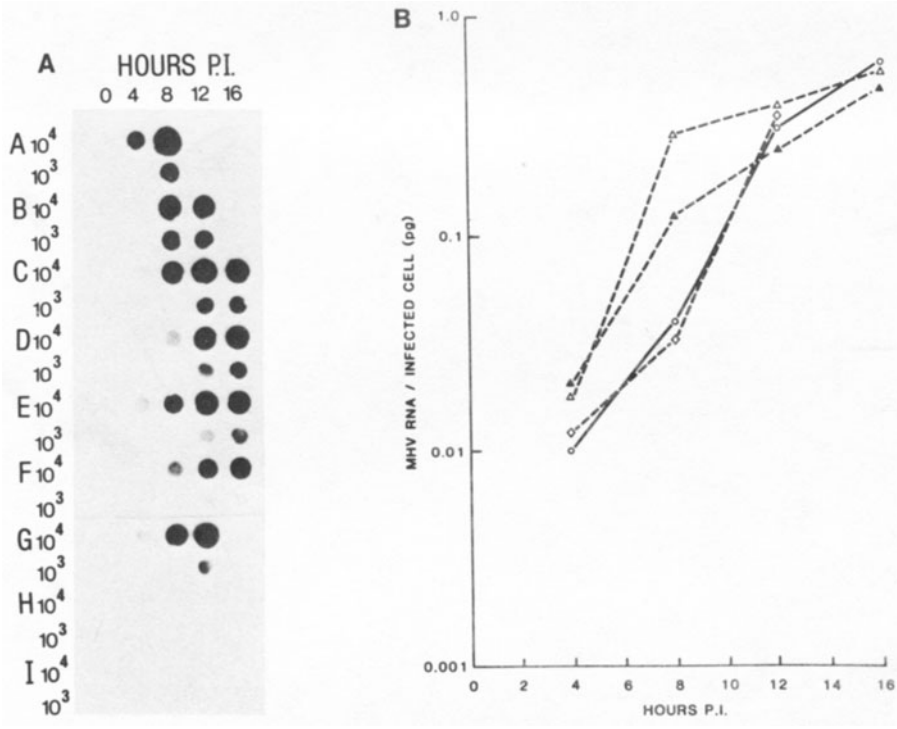

Fig. 1. Quantitation of MHV RNA. A: Dot-blot hybridization (Cheley and Anderson, 1984), using radiolabeled MHV-cDNA probe (Cheley et al., 1981), of virus-specific RNA synthesized in MHV-inoculated L-2 (A-D), LM-K (E), LM (F), C-1300 (G), vero (H) or C-6 (I) cells. Cells were inoculated with MHV-A59 at moi's (titered on L-2 cells) of 10 (A, E-I), 1 (B), 0.1 (C) or 0.01 (D). RNA was extracted at 0, 4, 8, 12 or 16h PI and aliquots corresponding to that extracted from 10^4 or 10^3 cells were applied to the dot-blot. B: Amounts of MHV-RNA detected per infected cell. Representative dot-blot data, as shown in Fig. 1A, were scanned densitometrically in conjunction with parallel determinations on known amounts of MHV nucleocapsid mRNA. (○) L-2, (▲) LM-K, (△) LM, (◇) C-1300 cells.

In order to examine MHV-directed protein synthesis, cultures of MHV-inoculated L-2, LM-K, LM, C-1300 and Vero cells were radiolabeled with ^{35}S-methionine (from 5-7h PI), and cell extracts were immunoprecipitated using anti-MHV serum (Mizzen et al., 1983). Aliquots of the immunoprecipitates

were analyzed by SDS-PAGE and fluorography (Fig. 2A). The three major size classes of MHV structural polypeptides were evident in each of the cell lines examined, with the exception of the non-permissive Vero cells. Of particular interest in the present study is the synthesis and expression of the viral E_2 glycoprotein, which has been previously implicated in mediating cell fusion (Collins et al., 1982). There were no appreciable cell-dependent differences with respect to its apparent molecular weight (MW), and by inference the extent of glycosylation (Fig. 2A).

Quantitation of MHV-directed protein synthesis was performed by ELISA and the results were expressed on an infected cell basis. It is evident from these results (Fig. 2B) that none of the semi-permissive cells studied (LM-K, LM, C-1300) are deficient in permitting levels of viral protein synthesis similar to those seen in the fully permissive L-2 cells.

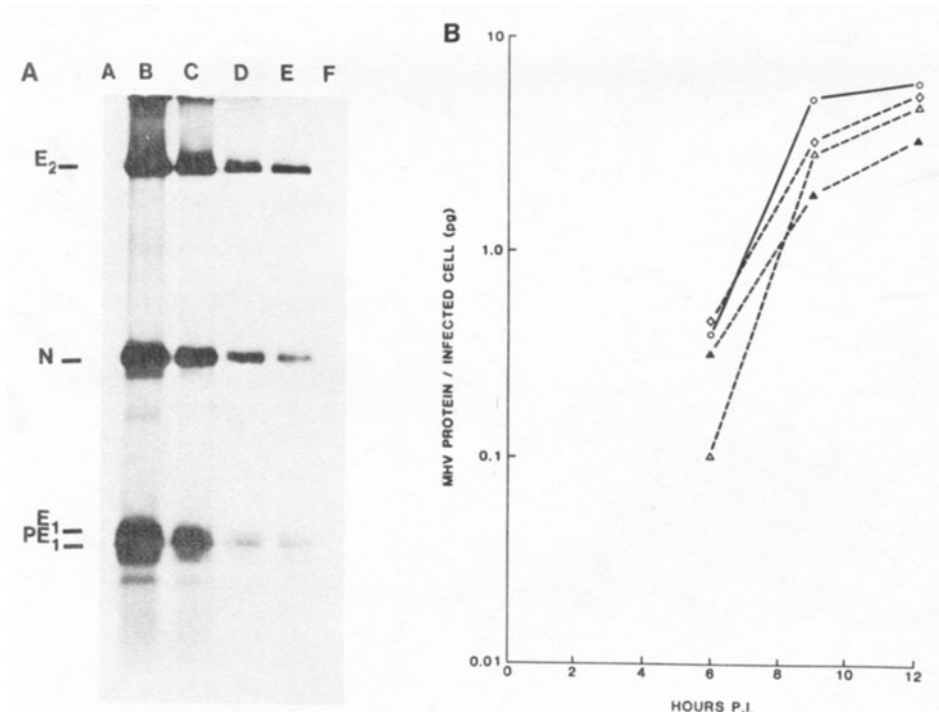

Fig. 2. MHV Protein synthesis. A: SDS-PAGE fluorogram of ^{35}S-methionine labeled proteins from mock-infected (A) or MHV-inoculated (B-F) cultures immunoprecipitated with anti-MHV serum. Cell cultures are L-2 (A and B), C-1300 (B), LM-K (D), LM (E), and Vero (F). B: Amounts of MHV protein detected per infected cell, as quantitated by ELISA. (○) L-2, (◇) C-1300, (▲) LM-K, (△) LM cells.

Assessment of membrane fusion in selected cell lines

Despite showing normal patterns of viral RNA, viral polypeptide and virion production, two of the semi-permissive cell lines, LM and LM-K, support a persistent infection in which there is a marked reduction in cell fusion (Table 1). A contact fusion assay (Mizzen et al., 1983) was performed in which uninfected LM, LM-K or C-1300 cells were seeded in

excess with MHV-infected L-2 cells (Table 2). Contact fusion was observed only with C-1300 cells. However, in the reciprocal assay (infected LM, LM-K or C-1300 cells seeded with uninfected L-2 cells) fusion was evident in each case, demonstrating the presence of fusion-active protein at the surface of the infected semi-permissive cells.

Table 2. Demonstration of Cell Surface Fusion Activity in MHV-Infected Permissive and Semi-Permissive Cells by Contact Fusion Assay

		Infected cell type[a]			
	moi[c]	0.01	1	10	10
		L-2	C-1300	LM-K	LM
Uninfected "Indicator" cell type[b]	L-2	+	+	+	+
	C-1300	+	ND	ND	ND
	LM-K	-	ND	ND	ND
	LM	-	ND	ND	ND

a 10^5 cells seeded in a 35 mm dish were MHV infected at the moi's indicated to produce comparable numbers of infected cells (Table 1).

b 10^6 uninfected cells were added to the sparsely-seeded infected cells to complete the monolayers and after 3 h incubation at 37°, syncytia formation scored as + or -. (ND; not determined).

c As determined by plaque assay on L-2 cells.

Table 3. Lipid Characteristics Related to Membrane Fluidity

Cell		L-2	LM-K	LM	C-1300
Sterol/FA (w/w)		0.142	0.128	0.118	0.088
%FA (w/w)	14:0	0.8	3.1	1.0	2.3
	16:0	15.6	28.0	20.5	27.2
	16:1	1.6	1.7	1.6	1.8
	18:0	25.9	21.0	22.6	18.9
	18:1	7.5	6.4	7.5	8.7
	18:2	26.0	18.6	24.0	18.7
	20:4	12.5	10.2	11.9	8.2
	22:6	10.1	11.0	10.9	14.2
Double bonds/ mol FA		1.61	1.46	1.65	1.51

In order to examine the possibility that cellular differences in susceptibility to MHV-induced fusion were perhaps related to differences in membrane fluidity, determinations of unsaturated fatty acid and sterol content were performed on membrane preparations (Atkinson, 1973) from L-2, LM-K, LM and C-1300 cells. Under normal physiological conditions, membrane fluidity is increased with increasing lipid unsaturation and decreasing sterol content. As shown in Table 3, L-2 and C-1300 cells which both show high sensitivity to MHV-induced fusion were found to have strikingly different sterol contents. The less fusion-susceptible LM and LM-K cells had sterol contents which were intermediate between these two values. It would therefore seem unlikely that sterol-determined membrane fluidity is responsible for the differences in MHV-induced fusion observed among the four cell lines examined. Similarly, L-2 and LM cells, which differ greatly in their susceptibility to MHV-mediated fusion, were found to have similar unsaturated fatty acid contents when expressed as the number of double bonds/mol fatty acids. There is thus no correlation between membrane lipid composition (and consequent membrane fluidity) and the extent of MHV-induced fusion among the various cell lines studied.

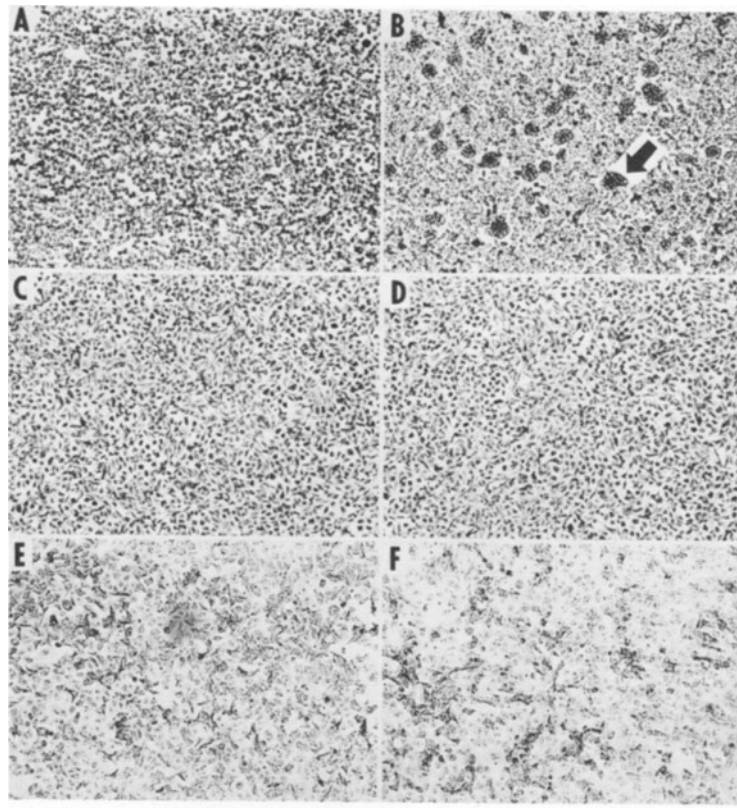

Fig. 3. Light micrographs of uninfected L-2 (A, B); LM (C,D) and Vero (E,F) cells, showing differential fusion induced within 1h at 37° by a plasma membrane fraction prepared from MHV-infected L-2 cells (method of Atkinson, 1973). Samples B, D and F were supplemented with trypsin at a concentration of 10 μg/ml. Arrow indicates a syncytium.

Preliminary experiments indicated that crude lysed cell preparations from MHV-infected L-2 cells rapidly (within 1 h) induce fusion when added

to uninfected L-2 cell monolayers. However, purified membranes (freed of cytosoluble components) prepared from such lysates failed to induce fusion. These findings suggested that a soluble factor was required for the fusion process. Since Sturman et al. (1984) had reported that trypsin-treated MHV virions became fusion-active ("fusion from without"), we investigated the possibility that an intracellular protease was involved in MHV-induced L-2 cell fusion. To better characterize the fusion process, we prepared purified membranes from infected L-2 cells and monitored their ability to induce fusion in fully permissive (L-2), semi-permissive (LM) and non-permissive (vero) cells. As shown in Fig. 3A,C,E, none of these combinations resulted in the manifestation of fusion. However, in the presence of trypsin (or, in other experiments chymotrypsin), there was rapid fusion with L-2 cells (Fig. 3B) but not with LM (Fig. 3D) or Vero (Fig. 3F) cells. In control experiments with protease-treated uninfected L-2 cell membranes, no fusion activity could be demonstrated. This suggests that fusion expression is a consequence of proteolytic activation of viral, rather than cellular polypeptide(s).

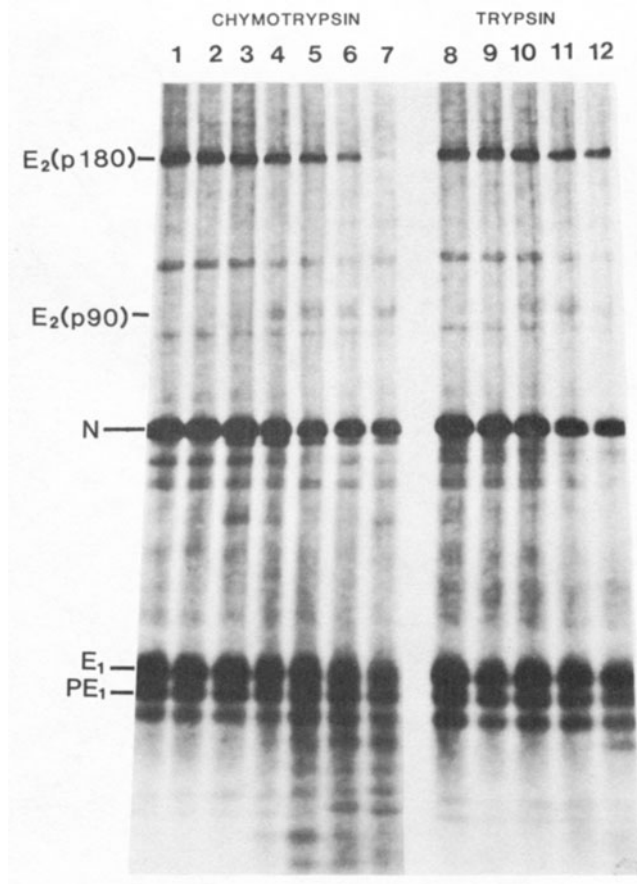

Fig. 4. Protease treatment of membranes prepared from ^{35}S-methionine-labeled MHV-infected L-2 cells. Protease concentrations were 0 μg/ml (1), 0.5 μg/ml (2), 1 μg/ml (3,8), 5 μg/ml (4,9), 10 μg/ml (5, 10), 50 μg/ml (6,11) and 100 μg/ml (7,12) in PBS. Membranes were incubated with protease for 1h at 37°, then lyophilized and subjected to SDS-PAGE and fluorography.

To identify biochemical changes which correlated with the protease-dependent activities of the membranes described above, parallel preparations were obtained from MHV-infected L-2 cells which had been labeled with ^{35}S-methionine from 5-6h PI. The resultant radiolabeled membranes were subjected to protease digestion with either trypsin or chymotrypsin at a range of concentrations including that used above in the fusion assay. Following protease treatment, the distribution of radiolabel among the membrane polypeptides was examined by SDS-PAGE and fluorography. Of interest is the observed reduction in radiolabeled E_2(p180) with increasing concentrations of either trypsin or chymotrypsin (Fig. 4). In addition, beginning at a chymotrypsin concentration of 5 μg/ml or a trypsin concentration of 10 μg/ml, there was detected a polypeptide of apparent molecular weight 90,000 dalton (by comparison with molecular weight protein standards). This polypeptide was related to E_2(p180), as indicated by peptide mapping (data not shown) and is likely related to the 90,000 dalton form of the E_2 which is found in varying amounts in MHV virions (Sturman and Holmes, 1977; Sturman and Holmes, 1984). The extent of proteolysis of E_2(p180) and appearance of the 90,000 dalton form, E_2(p90), do not correlate quantitatively, suggesting that additional proteolytic events may be taking place.

The nature of the E_2 glycoprotein in MHV-infected L-2 cells

In MHV virions, the glycoprotein E_2 may exist in either a 180,000 or 90,000 dalton form (Sturman and Holmes, 1977) and the latter may be responsible for "fusion from without" induced by trypsin-treated virions (Sturman and Holmes, 1984). Since L-2 cells are extremely susceptible to MHV-induced "fusion from within" (Lucas et al., 1977; Mizzen et al., 1983) it is of interest to note that the 180,000 dalton form of E_2 is by far the predominating species labeled in infected L-2 cells (Cheley and Anderson, 1981; Fig. 4, lane 1). Nevertheless, we have consistently observed a rapid decline in pulse-labeled E_2(p180) (eg. Anderson et al., 1979), not all of which can be explained on the basis of incorporation into progeny virions. To examine the possibility that E_2(p180) may be susceptible to intracellular proteolysis, a pulse-chase experiment was performed in the presence or absence of specific protease inhibitors. Cultures of L-2 cells, inoculated with MHV, were maintained in medium containing either no inhibitor, or the inhibitors TLCK, TPCK or ZPCK (at 10^{-4}M). At 5h PI, the cultures were pulsed for 15 min with ^{35}S-methionine and subsequently chased for 2h. As shown in Fig. 5, pulse-labeled E_2(p180) disappeared rapidly during the chase period in normal medium, as well as in medium containing TLCK. However, the protease inhibitors TPCK and ZPCK markedly prolonged the survival time of E_2(p180), suggesting inhibition of proteolysis. The pulse-chase relationships for the remaining radiolabeled intracellular polypeptides, not shown in Fig. 5, did not seem to be affected by the presence of any of the three protease inhibitors.

The protease inhibitors, TPCK and ZPCK, but not TLCK, were also effective in blocking MHV-induced cell fusion. At 7h PI, at which time MHV-infected L-2 cells were completely fused into a syncytial sheet, parallel cultures treated with TPCK or ZPCK showed only the occasional syncytial focus. Given the demonstrated activities of TLCK against trypsin (Shaw et al., 1965), and TPCK, ZPCK against chymotrypsin (Schoellmann and Shaw, 1963; Segal et al., 1971) the above results implicate a serine protease with substrate specificity for a phenylalanine (or other aromatic amino acid)-containing cleavage site in the intracellular processing of E_2(p180) and in activating MHV-induced cell fusion in L-2 cells.

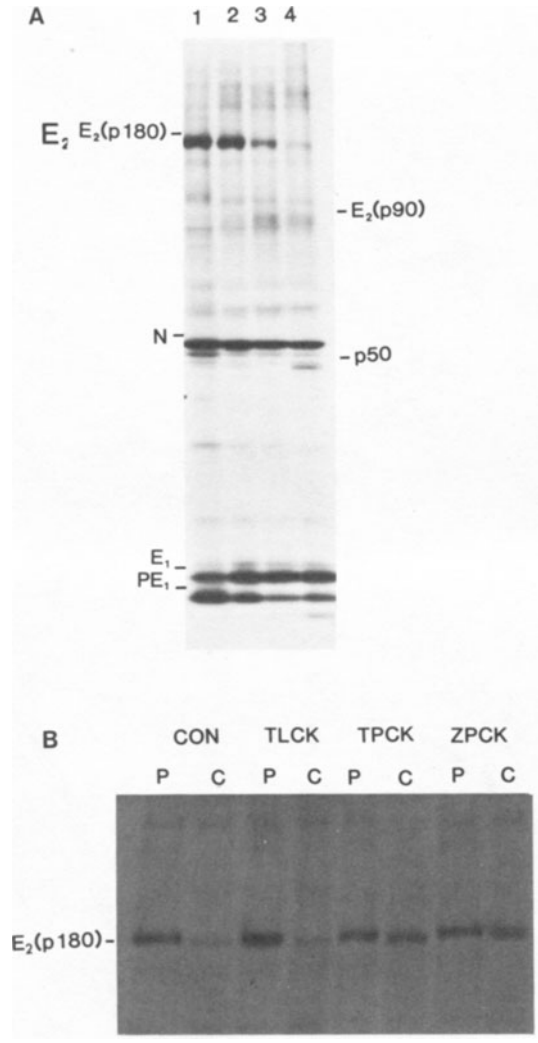

Fig. 5 Behaviour of E_2(p180) in the absence or presence of protease inhibitors. A. SDS-PAGE fluorogram of extracts from MHV-infected L-2 cells (moi=10) pulse labeled with ^{35}S-methionine for 15 min at 5h PI and chased for 0 (lane 1), 45 (lane 2), 90 (lane 3) or 120 (lane 4) min. B. SDS-PAGE fluorogram of extracts from MHV-infected L-2 cells pulse labeled (P) as above and chased 120 min (C) in the absence (Con) or presence of protease inhibitors, TLCK, TPCK or ZPCK at 10^{-4}M.

DISCUSSION

The results presented here suggest that resistance to MHV-induced cell fusion may be a general mechanism of MHV persistence. Our studies, conducted *in vitro*, may well have applicability to chronic infections of MHV *in vivo*. The importance of cell-cell fusion as a virus dissemination mechanism is indicated by *in vivo* studies showing virus persistence in the face of continued neutralizing antibody response (Stohlman and Weiner, 1981; Sorensen et al., 1984) which would inhibit the extracellular mode of spread. Two different modes of spread were indicated by Buchmeier et al.

(1984) in studies which showed that administration of neutralizing monoclonal antibodies blocked MHV-JHM replication in neurons but not in oligodendrocytes. Since JHM-infected neurons, in contrast to nonneuronal cells, do not undergo fusion (Dubois-Dalcq et al., 1982), neurons may transmit virus extracellularly whereas nonneuronal cells appear more susceptible to the fusion mode of spread.

A role for protease has been implicated in enhancing infectivity (Sturman and Holmes, 1977), plaque production (Storz et al., 1981; Otsuki and Tsubokora, 1981) and fusion activity (Storz et al., 1981; Toth, 1982; Yoshikura and Tejima, 1981; Sturman et al., 1984) of coronaviruses. In the case of MHV, trypsin-activated whole virions caused rapid fusion (so-called "fusion from without") with cell monolayers (Sturman et al., 1984); such trypsin-activation was associated with a cleavage reaction in which the 180,000 dalton form of E_2 was converted to two 90,000 dalton forms. Of interest in the present work is our finding that protease activation of MHV-induced fusion occurs in the infected L-2 cell, and that the protease involved appears chymotrypsin-like on the basis of inhibition with TPCK and ZPCK. Although in the infected L-2 cells a chymotrypsin-like protease is implicated, our experiments with exogenously added membranes prepared from MHV-infected L-2 cells, indicate that both trypsin and chymotrypsin are capable of activating cell fusion. It would appear that, extracellularly both enzymes are capable of exposing an otherwise hidden fusogenic determinant, presumably in the E_2. Analogous to the experiments of Sturman et al. (1984) with trypsin-treated virions, we find that protease-treated membranes exhibit cleavage of E_2 (p180) to E_2(p90). Although E_2(p180) undergoes rapid clearance, presumably through extensive proteolysis, we rarely detect significant amounts of E_2(p90) in MHV-infected L-2 cells. We therefore conclude that either catalytic amounts of E_2(p90) are sufficient to induce cell fusion, or that additional products of E_2(p180/p90) proteolysis may have fusogenic activity.

Our results with the permissive (L-2) and semi-permissive (LM) cell lines strongly implicate differences in cell fusion as factors contributing to reduced virus dissemination and a tendency to chronic infection. Despite the demonstration that proteolysis is necessary for cell fusion, it is apparent from the present report and from our previous work (Mizzen et al., 1983) that differences in fusion expression are not due to lack of appropriate protease activity in semi-permissive cell lines. In particular, even in the presence of exogenous protease, membranes prepared from MHV-infected L-2 cells fail to induce fusion with semi-permissive LM cells. It therefore appears that membrane characteristics inherent to permissive and semi-permissive cells play an important role in determining whether the outcome of MHV infection is acute or persistent.

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