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A Model for Persistent Murine Coronavirus Infection Involving Maintenance via Cytopathically Infected Cell Centres

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SUMMARY

The relatively cell impermeable hygromycin B was found to inhibit viral but not cellular protein synthesis when added to cultures of murine hepatitis virus (MHV)-infected or mock-infected mouse L-2 fibroblasts. Membrane permeability, as judged by influx of sodium ions, has previously been demonstrated to be an MHV E2 glycoprotein-mediated, cytopathic effect of MHV infection in L-2 cells. It is therefore likely that the selective effect of hygromycin B on viral protein synthesis is a reflection of an increased drug penetration into virus-infected cells. Using hygromycin B as a marker for MHV-induced cell membrane cytopathology, the effects of drug treatment on a persistent MHV infection in mouse LM-K fibroblasts were investigated. MHV persistence in LM-K cells, which normally involves a steady state infection of 0·1 to 1% of the cells in culture, was found to be cured by hygromycin B treatment, as measured by the elimination of infectious virus from the supernatant medium. Hygromycin B also resulted in the eradication of MHV-specific RNA from LM-K cells, arguing against the presence of a non-cytopathically or latently infected subpopulation of cells.

Murine coronaviruses, typified by murine hepatitis virus (MHV) are able to produce infections of either an acute or persistent nature. Nevertheless, the mechanisms by which the outcome of such infection is determined remain poorly understood. Studies in cell culture have implicated the involvement of attenuated MHV variants such as heat-sensitive (Holmes & Behnke, 1981), cold-sensitive (Stohlman *et al.*, 1979) and small plaque (Hirano *et al.*, 1981) mutants. Evidence from *in vivo* studies has also been presented suggesting the generation during long-term nervous system infection of virus variants which give rise to altered mRNAs (Jackson *et al.*, 1984; Taguchi *et al.*, 1987). Other studies (Lucas *et al.*, 1977, 1978; Mizzen *et al.*, 1983) showed that persistent infections of MHV could be established in the absence of detectable levels of attenuated virus variants.

Certain strains of MHV produce persistent infections of the nervous system, leading to symptoms reminiscent of certain slowly degenerative neurological conditions in humans. Typically, such persistent MHV infections show a cytopathology indicative of a state of coexistence between isolated pockets of virus-infected cells and normal, uninfected tissue (Bailey *et al.*, 1949; Weiner *et al.*, 1973; Haspel *et al.*, 1978; Nagashima *et al.*, 1979; Sorensen *et al.*, 1980). Alternatively, evidence has been presented which suggests that virus infection of certain brain regions can proceed in a virtually latent manner in the absence of overt c.p.e. (Sorensen *et al.*, 1984). Accordingly, there remains an open question as to whether MHV persistence can be adequately described by a 'carrier culture' mechanism or whether a truly latent infection may be involved.

A major c.p.e. of MHV infection is the production of syncytia, an effect mediated by the viral E2 glycoprotein (Collins *et al.*, 1982). We have noted that expression of MHV E2 protein in infected mouse L-2 fibroblasts also induces cell membrane permeability changes, as determined by leakage of sodium ions (Mizzen *et al.*, 1987). We reasoned that such membrane permeability

changes might be exploited as indicators of MHV-induced cytopathology by the use of certain small compounds not normally able to penetrate uninfected cells. One possible candidate compound is the low molecular weight aminoglycoside, hygromycin B (M_r 527·5), an inhibitor of protein synthesis. Hygromycin B has been used previously in selectively inhibiting translation of cells infected with other viruses known to induce changes in plasma membrane permeability (Benedetto *et al.*, 1980).

We have previously described an *in vitro* model for MHV persistence in which virus propagation appears to be mediated by a small percentage of the total cells in culture (Mizzen *et al.*, 1983). We demonstrate here that the virus-producing cells are cytopathically infected, as indicated by their sensitivity to hygromycin B, while uninfected cells remain resistant. We also show that MHV persistence is 'cured' by hygromycin B treatment, arguing against the involvement of a non-cytopathic, latently infected subpopulation of cells.

We initially examined the effect and specificity of hygromycin B treatment on intracellular protein synthesis in both normal and MHV-infected L-2 cells. The A59 strain of MHV was used for these studies. For the MHV-infected samples, L-2 cells were inoculated at an m.o.i. of 0·1 so that both normal and virus-infected cells would be present in the same culture. By this method any selective effect hygromycin B might have on virus-infected, as compared to uninfected, cells could be observed. Cultures of MHV-infected or mock-infected L-2 cells were incubated in the absence or presence of various concentrations of hygromycin B from 0 to 40 h post-inoculation (p.i.). As shown in Fig. 1, hygromycin B at concentrations of 0·5 mM or higher specifically inhibited MHV protein synthesis without significantly affecting cellular protein synthesis. This result suggests that hygromycin B selectively penetrates into MHV-infected cells due to their increased membrane permeability. Hygromycin B did not inactivate the virus before cell entry. Incubation of MHV with various concentrations of hygromycin B (0 to 1 mM for 1 h at 37 °C) before adsorption did not reduce the number of p.f.u./ml, as determined by plaque assay (results not shown).

MHV normally shuts off host cell protein synthesis very effectively in L-2 cells (Hilton *et al.*, 1986). This is apparent in Fig. 1 (*b*), lane 1, despite the relatively low m.o.i. which was employed in this experiment. Previous studies have documented the high susceptibility of L-2 cells to MHV-mediated cell fusion and the ability of uninfected cells to become rapidly recruited into expanding syncytia (Mizzen *et al.*, 1983). Such recruitment results in the eventual syncytial engulfment of virtually all L-2 cells in culture which, as a consequence, all become translationally subverted to MHV-encoded protein synthesis. In contrast, MHV-infected L-2 cell cultures which were treated with hygromycin B showed greatly reduced development of syncytia, an observation consistent with the idea of selective killing of MHV-infected cell foci. The resultant decrease in MHV-specific protein synthesis and the concomitant reduction in inhibition of host cell protein synthesis are clearly seen in Fig. 1 (*b*).

The persistent infection resulting from MHV infection of mouse LM-K fibroblasts has been previously described (Mizzen *et al.*, 1983). Even when inoculated at high m.o.i., which results in considerable cell death, LM-K cells rapidly equilibrate to a steady state of persistence. This steady state is characterized by the production of virus titres in the order of 10^5 to 10^7 p.f.u./ml; under these conditions approximately 0·1 to 1% of the cells in culture are infected, as measured by infectious centre assay. In order to examine the contribution of cytopathically infected cells to the maintenance of the persistent state, mock- and MHV-infected LM-K cell cultures were incubated in the absence or presence of various concentrations of hygromycin B and monitored over time for virus production and for cell viability.

The number of viable cells (Fig. 2*a*) in MHV-infected LM-K cell cultures grown in the absence of hygromycin B declined between 36 and 72 h p.i. as a result of death of infected cells, but subsequently increased as the remaining cells in culture continued to grow. In all cultures treated with hygromycin B, numbers of viable cells remained fairly constant for the duration of the experiment. (All cultures were initially confluent and would not be expected to increase in cell number beyond their starting levels.)

Virus production (Fig. 2*b*) was found to be suppressed with increasing concentration of hygromycin B. At concentrations of 0·75 mM and 1 mM, production of virus was reduced to non-

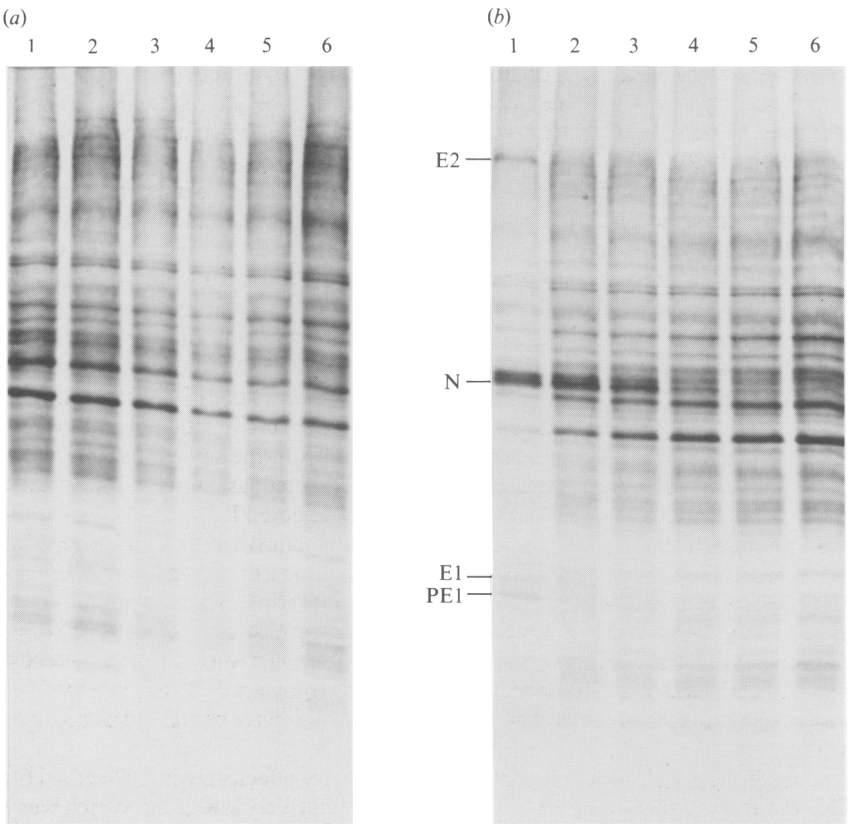

Fig. 1. Selective inhibition of MHV-specific protein synthesis by hygromycin B. Monolayer cultures (24-well plates) of L-2 cells were mock-infected (*a*) or infected with MHV at an m.o.i. of 0·1 (*b*). Virus was adsorbed for 30 min at room temperature. After removal of unbound inoculum, medium (MEM supplemented with 5% foetal calf serum) containing 0, 0·1, 0·25, 0·5, 0·75 or 1·0 mM-hygromycin B (lanes 1 to 6, respectively) was added to the cultures. Cultures were incubated at 37 °C. At 6 h intervals, medium was removed for virus titration and replaced with fresh medium containing the appropriate concentration of hygromycin B. At 40 h p.i., all cultures were radiolabelled by incubation in the presence of [^{35}S]methionine (100 μCi/ml). An SDS–PAGE fluorogram is shown. The positions of structural proteins N, PE1/E1 and E2 are indicated.

detectable levels by 84 or 60 h p.i., respectively. It is evident, by comparison of the cell viability (Fig. 2*a*) and virus production (Fig. 2*b*) data, that only virus-producing cells are affected by the drug treatment.

Immunofluorescence studies, using a polyclonal anti-MHV antibody, also suggest that hygromycin B is cytotoxic for MHV-infected LM-K cells. These investigations indicate that viral antigens cannot be detected in cultures treated with 1 mM-hygromycin B for 108 h p.i. (results not shown).

Despite the absence of detectable virus in hygromycin B-treated MHV-infected LM-K cultures, the possibility remained that MHV persistence might be maintained in a latent manner by non-cytopathically infected cells. Such cells might contain MHV genomic RNA while not actively replicating progeny virions. In order to examine this possibility, RNA was extracted from MHV-infected LM-K cultures maintained for 108 h in the absence or presence of hygromycin B and assayed for the presence of MHV-specific sequences. Dot blot hybridization (Cheley & Anderson, 1984) using a cloned MHV-specific ^{32}P-labelled cDNA (M. Daya, unpublished data), showed the absence of MHV-specific RNA in cultures treated with

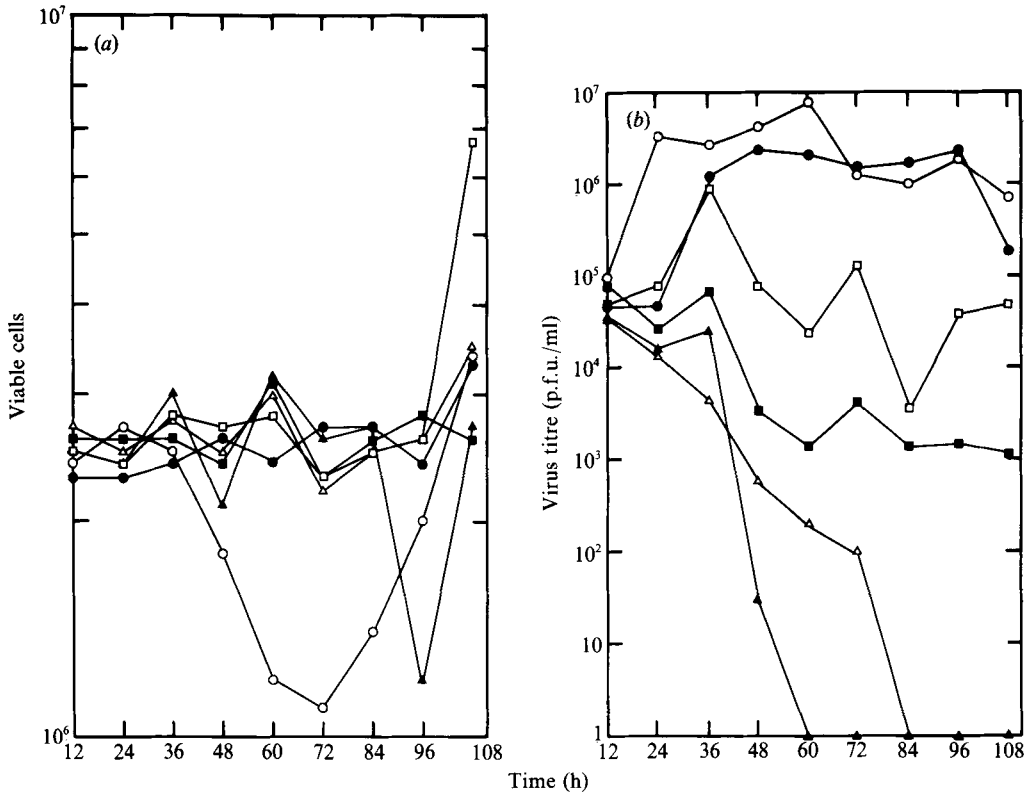

Fig. 2. Curing of persistent MHV infection of LM-K cells. Monolayer cultures (24-well plates) of LM-K cells were mock- or MHV-infected, allowed to adsorb for 30 min at room temperature and then incubated at 37 °C in medium containing hygromycin B at various concentrations (0 mM, ○; 0·1 mM, ●; 0·25 mM, □; 0·5 mM, ■; 0·75 mM, △; 1 mM, ▲). One series of cultures was assayed at 12 h intervals to assess numbers of viable cells, using the trypan blue exclusion method (*a*). A duplicate series of cultures was assayed at 12 h intervals for virus titres of the supernatant medium (*b*).

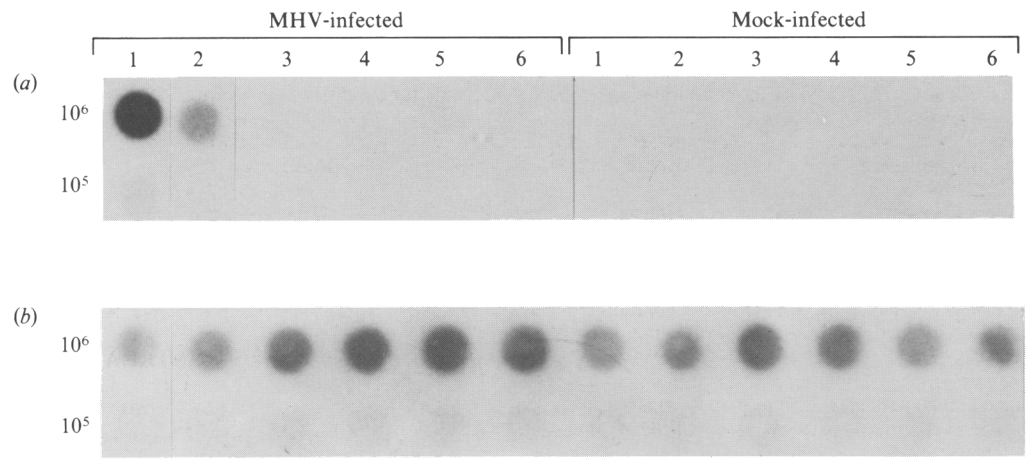

Fig. 3. Absence of non-cytopathically infected cells in persistent MHV infection. Monolayer cultures (10^5 or 10^6 cells, as indicated) of LM-K cells were mock- or MHV-infected and incubated as described in the legend to Fig. 2. Medium was changed every 12 h. At 108 h p.i., RNA was extracted from each culture, in the presence of carrier LM-K cell RNA (70 μg), and subjected to dot blot hybridization using either a cloned MHV-specific ^{32}P-labelled cDNA (M. Daya, unpublished data) (*a*) or β-actin-specific ^{32}P-labelled cDNA (*b*). Hygromycin concentrations were: 1, 0 mM; 2, 0·1 mM; 3, 0·25 mM; 4, 0·5 mM; 5, 0·75 mM; 6, 1 mM.

hygromycin B (Fig. 3). As a control, dot blot hybridization was also performed using a β-actin-specific ^{32}P-labelled cDNA.

MHV-infected LM-K cultures treated for 108 h p.i. with hygromycin B and then grown in medium with no drug showed no increase in c.p.e. or virus production over a further 108 h period (results not shown). This study and the results from immunofluorescence and RNA dot blot hybridization indicate that the majority of infected LM-K cells are eliminated by hygromycin B treatment.

The results presented here show that the mechanism of MHV persistence in mouse LM-K fibroblasts is one by which virus infection is maintained by a small percentage of cells which are cytopathically infected, as indicated by their selective susceptibility to the normally poorly permeable translational inhibitor, hygromycin B.

Effective translation-inhibiting concentrations of hygromycin B are reduced by orders of magnitude when cells are rendered membrane-permeable (Cameron *et al.*, 1986). We have previously directly demonstrated that MHV infection of L-2 cells results in increased cell permeability to sodium ions (Mizzen *et al.*, 1987). Thus while other mechanisms are possible, we feel that the most likely explanation for the hygromycin B sensitivity of MHV-infected cells involves an increased cell membrane permeability. If this is the case, the results from the present study indicate that not only sodium ions but also larger molecules, at least of the molecular dimensions of hygromycin B, are able to penetrate the membranes of MHV-infected cells. Moreover, the results presented here suggest that increased membrane permeability occurs in MHV infection of at least two cell lines (L-2 and LM-K) and indicate that cell leakiness may be a general c.p.e. of MHV.

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