ON THE MEMBRANE CYTOPATHOLOGY OF MOUSE HEPATITIS VIRUS INFECTION AS PROBED BY A SEMI-PERMEABLE TRANSLATION-INHIBITING DRUG

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ABSTRACT

Previous studies of the membrane fusion process have permitted the characterization of membrane permeability changes concomitant with MHV-induced cytopathology. One indication of membrane permeability in MHV-infected cells is their sensitivity to translational inhibition by the normally impermeable amino-glycoside, hygromycin B (Macintyre, G., Wong, F. and Anderson, R. (1989) J. Gen. Virol. 70, 763-768). In the present study, we examine the hygromycin B sensitivity of acutely infected mouse fibroblast L-2 cell and macrophage cultures as well as persistently infected mouse fibroblast LM-K cell cultures. The results suggest that membrane permeability alterations (as indicated by hygromycin B sensitivity) are a common feature of these MHV infections. Hygromycin B "cured" persistently infected LM-K cells as indicated by the absence of detectable virus antigen by immunofluorescence and by the absence of infectious virus even after removal of the drug or co-cultivation with untreated L-2 cells. The results argue against the maintenance of MHV infection by a mechanism involving latently or non-cytolytically infected cells. We conclude therefore that at least one mechanism for MHV persistence depends on virus propagation by cytolytic infection of a small, dynamically changing, fraction of the total cells present in culture.

INTRODUCTION

Murine coronaviruses, typified by murine hepatitis virus (MHV) are able to produce infections of either an acute or persistent nature. Some evidence from in vitro (1,2) and in vivo (3,4) studies suggests that virus variants are produced which may account for the persistence of infection, while other studies (5-7) showed that persistent infections of MHV could be established in the absence of detectable levels of virus variants.

Some strains of MHV can produce persistent infections of the nervous system, which are characterized by symptoms similar to those of certain slowly degenerative neurological conditions in humans. MHV infection may persist due to a state of co-existence between isolated pockets of virus infected cells and normal, uninfected tissue (8-12). However, other evidence (13) suggests that virus infection of certain areas of the brain can proceed in a virtually latent manner in the absence of overt cytopathic effect (c.p.e.).

We have previously noted membrane permeability changes during acute MHV infection of mouse L-2 fibroblasts (14). Mouse LM-K fibroblasts which

Coronaviruses and Their Diseases
Edited by D. Cavanagh and T.D.K. Brown
Plenum Press, New York, 1990

support persistent infection of MHV also show membrane permeability alterations, as determined by sensitivity to the normally impermeable translation-inhibiting drug, hygromycin B (15). MHV persistence of LM-K cells, which normally involves a steady state infection of 0.1 - 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 media. Hygromycin B also resulted in the eradication of MHV-specific RNA from LM-K cells (15).

In the present paper, we investigate further the mechanism of MHV persistence in LM-K cells and extend our studies using hygromycin B to mouse macrophages, which are an important target of MHV infection in the animal.

MATERIALS AND METHODS

Cells and Virus. L-2 cells (16) and LM-K cells (17) were grown as monolayers in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). After inoculation with MHV-A59 (18), virus was allowed to absorb for 30 min at room temperature. The cells were then incubated at $37^{O}C$ in MEM containing 5% FCS and the relevant concentration of hygromycin B. The amount of infectious virus present in culture medium after drug treatment was measured by titration on L-2 cells by plaque assay (5).

Co-cultivation rescue study. MHV-inoculated LM-K cells, maintained in various concentrations of hygromycin B for 108 h, were trypsinized, centrifuged and mixed with an equal number of untreated L-2 cells. Following plating and incubation at $37^{O}C$, aliquots were taken from the supernatant media for assay of infectious virus by plaque assay (5).

Isolation of Peritoneal Macrophages. Four month old Balb/c mice were injected (i.p.) with 1.5ml 2% starch in 0.9% NaCl two days prior to the harvesting of peritoneal macrophages. Mice were sacrificed by cervical dislocation and the peritoneal cavity was washed twice with MEM plus 20% FCS. The total peritoneal wash was centrifuged at 1000g for 1 min and the pellet washed with MEM plus 20% FCS. The final pellet was resuspended in MEM plus 10% FCS, plated out and incubated overnight to allow the macrophages to adhere to the wells. Erythrocytes were removed by gently rinsing the monolayers with MEM plus 10% FCS. The monolayers were then infected with MHV as described for L-2 cells.

Immunofluorescence. At 42h PI cultures incubated in the absence or presence of hygromycin B were fixed with 5% acetic acid/95% ethanol for 3 min and washed. After blocking with 30% goat serum, samples were incubated overnight with polyclonal anti-MHV antiserum, washed and treated for 1h with goat anti-mouse FITC. After washing and mounting, cells were examined by immunofluorescent microscopy and scored for the presence of viral antigen.

RESULTS

Curing of Persistent MHV Infection by Hygromycin B

As previously reported, treatment of persistently infected LM-K cells with hygromycin B (0.5mM or higher) reduced virus propagation, viral RNA and viral antigen to undetectable levels (15). As a further verification that complete curing of persistent MHV infection could be achieved by hygromycin B treatment, persistently infected LM-K cultures, maintained for 108 h in the presence of drug, were returned to drug-free medium (indicated by arrow in fig. 1) and monitored for a further 108 h for the appearance of infectious virus. As shown in Fig. 1, cultures which were treated with drug at concentrations of 0.5mM or higher were in fact cured, showing no subsequent release of virus after removal of the drug.

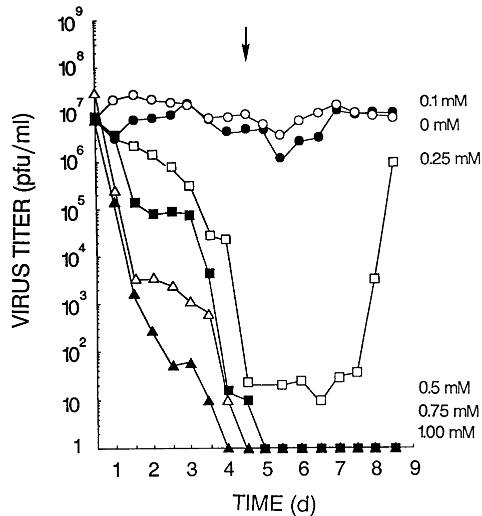

Figure 1

Curing of Persistent MHV Infection by Hygromycin B:
Removal of Drug Does Not Reactivate Infection

As a further check of the efficacy of hygromycin B in curing persistently infected LM-K cells, a co-cultivation rescue experiment was performed. This type of experiment is based on those used with occasional success in other virus systems in which release of infectious virus from a latently infected cell may be achieved by co-cultivation with a permissive cell type.

Accordingly, L-2 cells, known to be highly permissive to MHV (5-7), were added in a 1:1 ratio to persistently infected LM-K cells which had been treated for 108 h with various concentrations of hygromycin B. Following incubation in drug-free medium aliquots were removed from the supernatant media for infectious virus assay. As shown in Table 1, no virus was released by this co-cultivation rescue method from persistently infected LM-K cells which had been treated with hygromycin B at 0.5mM or higher.

In conjunction with our previous results using cDNA and antibody probes (15) the above studies strongly argue against the presence of even a small fraction of persistently infected LM-K cells in which the infection is resistant to hygromycin B and therefore free of virus-induced membrane cytopathology.

Susceptibility of MHV Infection of Macrophages to Hygromycin B

Peritoneal macrophages from Balb/c mice are susceptible to MHV infection and show a similar outcome to the infection as L-2 cells, including virus production and overt c.p.e. characterized by fusion of the cell monolayer (19). Monolayer cultures of L-2 cells and peritoneal macrophages were infected with MHV at an m.o.i. of 0.1. The cultures were incubated in the absence or presence of hygromycin B at various concentrations. At 42h PI the monolayers were examined by immunofluorescent microscopy and scored for the presence of viral antigen. The amount of viral antigen that could be detected in L-2 cells decreased with increasing concentrations of the drug. Hygromycin B had a similar but more severe effect on the MHV infection of peritoneal macrophages. At the lowest concentration of drug, 0.1mM, there was a marked decrease in the amount of viral antigen as compared to the infected, untreated macrophage culture. Treatment with hygromycin B at 0.25mM and above decreased viral antigen to undetectable levels in the peritoneal macrophages (Table 2). Thus, MHV infection of both L-2 cells and peritoneal macrophages can be cured by treatment with hygromycin B.

Table 1

Curing of Persistent MHV Infection by Hygromycin B:
Absence of "Rescuable" Virus by Co-cultivation

	Virus Titer (pfu/ml)	
	6h	24h
[Hygromycin B, mM]		
0	4.6×10^6	3.4×10^6
0.1	5.1×10^6	3.4×10^6
0.25	1.4×10^5	3.2×10^6
0.5	0	0
0.75	0	0
1.0	0	0

MHV-infected LM-K cells treated for 108h with the relevant concentration of hygromycin B were harvested, mixed with an equal number of L-2 cells and plated out. The amount of virus present in media harvested at 6 and 24h post-mixing was quantitated by plaque assay.

Table 2

Susceptibility of MHV Infection of Macrophages to Hygromycin B

	Relative Immunofluorescence	
	L-2	Macrophages
[Hygromycin B, mM]		
0	+ + + +	+ + + +
0.1	+ + + +	+
0.25	+ + +	-
0.5	+ + +	-
0.75	+	-
1.00	-	-

MHV-infected L-2 cells and peritoneal macrophages were treated with various concentrations of hygromycin B for 42h PI. The monolayers were then stained for immunofluoresence as described in Materials and Methods.

DISCUSSION

The results from this and our previous study (15) suggest that hygromycin B sensitivity may be a common feature of MHV infections in various cell types. Hygromycin B sensitivity is also associated with infections of several other viruses (20). While the mechanism of action of hygromycin B in virus-infected cells remains uncertain, evidence has been presented by other groups, suggesting the involvement of virus-induced membrane changes, either at the level of the plasma membrane (20) or the endosome (21). From our results, it is evident that whatever these changes are, they occur in both acute and persistent infections of MHV. Persistent MHV infection of LM-K cells, for example, can be cured by hygromycin B treatment which selectively eliminates MHV-infected cells. The maintenance of MHV persistence, at least in LM-K cells, therefore involves the expression of this aspect of viral cytopathology which predisposes the host cell to hygromycin B sensitivity.

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