

# RECEPTOR-INDEPENDENT INFECTION OF MOUSE HEPATITIS VIRUS: ANALYSIS BY SPINOCULATION

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## 1. INTRODUCTION

Cell entry of mouse hepatitis virus (MHV) is mediated by the interaction of its spike (S) protein and cellular receptor carcinoembryonic antigen adhesion molecule 1 (CEACAM1, MHVR). However, a highly neurotropic MHV, wild-type (wt) JHMV is known to spread to receptor-negative BHK cells from firstly infected receptor-positive DBT cells (MHVR-independent infection).<sup>1</sup> Although the mechanism of this infection is still unclear, it is hypothesized that the S protein attached on cell surface is activated for fusion by natural dissociation of S1 from S2, which was revealed in wt JHMV but not mutants derived from it,<sup>2</sup> with or without MHVR-independent infection activity, respectively.<sup>3,4</sup> Wild-type JHMV fails to infect BHK cells by a standard infection procedure, because the virus is not able to attach cells without MHVR. However, the S protein expressed on cell surface infected with wt can attach onto MHVR-negative cells by overlaying these cells, which induces fusion/infection of MHVR deficient cells (infected-cell overlay test). If MHVR-independent infection occurs as expected above, then we will be able to make wt JHMV infect cells by attaching virions onto cells without MHVR. To test this possibility, we employed the spinoculation method, which has been shown to facilitate the binding of viruses onto cells<sup>5</sup>.

Two strains of JHMV, cl-2 and its soluble receptor resistant mutant, srr7,<sup>6</sup> were used for spinoculation. There is only one single amino-acid substitution in the S2 subunit of srr7 as compared with cl-2. They were spinoculated onto BHK cells, which facilitated the attachment of these viruses to the same extent. It also facilitated the infection of cl-2 virus but not that of srr7, being in good agreement with the result obtained by infected-cell overlay test. Furthermore, dissociation of S1 from S2 was confirmed in cells expressing cl-2 S protein but not srr7 S. These results clearly support the proposed hypothesis for the mechanism of MHVR-independent infection.

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## 2. MATERIALS AND METHODS

### 2.1. Cells, Viruses, and Spinoculation

MHVR-positive DBT and MHVR-negative BHK cells were used as target cells. Wild-type JHMV cl-2 and its mutant *srr7* were used.<sup>6</sup> Spinoculation was performed as previously described<sup>5</sup> with a slight modification. Cells in 24-well plates were infected with viruses in 300  $\mu$ l of medium containing 1  $\mu$ g/ml of concanavalin A and were centrifuged at  $1750 \times g$  for 2 hours at 4°C. After 14 hours incubation at 37°C, cells were fixed, stained with crystal violet to count the number of syncytium.

### 2.2. Expression of S Proteins

Cells infected with vTF7.3<sup>7</sup> were transfected with S protein expression plasmids by electroporation. Twelve hours after transfection, the culture supernatants and cells were separately harvested. The S protein in culture supernatants was collected using anti-S monoclonal antibodies (MAbs).<sup>8</sup> S proteins in the supernatants and in cell lysates were analyzed by Western blot with MAbs.<sup>9</sup>

## 3. RESULTS AND DISCUSSION

### 3.1. Centrifugation Mediates the Infection of cl-2 to Receptor-Deficient Cells

Cl-2 and *srr7* were spinoculated onto MHVR-positive DBT or negative BHK cells, and their infections were monitored by syncytium formation (Table 1). The centrifugation had small effects on virus infection (23-fold increase compared with no-centrifuged plate), when DBT cells were infected with cl-2. In contrast, cl-2 infection of receptor-deficient BHK cells was extensively (ca. 800-fold) increased by centrifugation, while virus hardly infected BHK cells without centrifugation (2.75 syncytia per well). On the other hand, no syncytium formation was observed in BHK cells spinoculated with JHMV *srr7*, while an increase in infection similar to cl-2 was observed in DBT cells. We then examined whether spinoculation increased the attachment of the viruses or not. Real-time PCR showed that attachment of both cl-2 and *srr7* was increased significantly by spinoculation in both DBT and BHK cells (data not shown). These data suggest that spinoculation increased attachment to MHVR-negative cells regardless of viruses used, however, only cl-2 infected those cells as shown in Table 1. This result is in good agreement with the MHVR-independent infection observed by the infected-cell overlay test, suggesting that localization of cl-2 S protein in close proximity is an important condition for MHVR-independent infection.

**Table 1.** Infection of cl-2 and srr7 on DBT and BHK cells by spinoculation.

Viruses	Number of syncytium formed / well <sup>a)</sup>			
	DBT		BHK	
	spin(+)	spin(-)	spin(+)	spin(-)
JHMV cl-2	145000	6120	2200	2.75
JHMV srr7	47800	12800	ND <sup>b)</sup>	ND <sup>b)</sup>

<sup>a)</sup>  $10^5$  pfu/well of viruses were inoculated onto target cells and infection was monitored by syncytium formation. Three independent experiments were done, and mean values are shown.

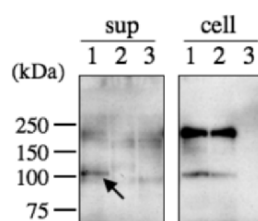
<sup>b)</sup> ND, not detected.

### 3.2. S1 Subunit of cl-2, but Not srr7 S1, Is Dissociated Easily from S2

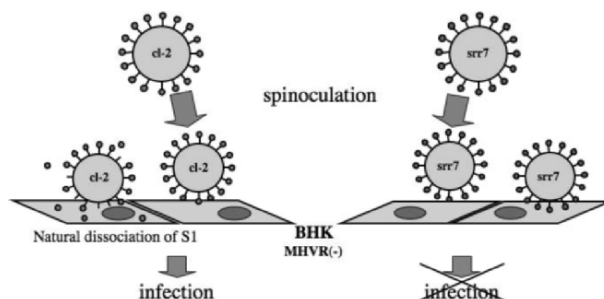
S proteins of both viruses were expressed on BHK cells and analyzed by Western blotting for their S1 dissociation from S2 (Fig. 1). Cl-2 S1 was released into culture supernatants of cells expressing S protein (lane 1) compared with the cells expressing srr7 S protein (lane 2). Because there was no significant difference on expression and cleavage of S protein between two strains, these results suggest that S1 of cl-2 is more releasable than srr7 S1. This result indicates the correlation between S1 dissociation and MHVR-independent infection activity.

## 4. CONCLUSIONS

To verify the proposed mechanism of MHVR-independent infection, we forced viruses attachment onto MHVR-negative BHK cells by spinning cells together with inoculated viruses (spinoculation). Cl-2 with MHVR-independent infection activity successfully infected BHK cells, whereas srr7 without this activity failed to infect. Furthermore, the S1 of cl-2 was removed from S2 in a naturally occurring event, but not S1 of srr7. These findings support a mechanism of MHVR-independent infection proposed from infected cell overlay test that the S protein of cl-2, in which S1 is easily



**Figure 1.** Dissociation of S1 subunit of cl-2 S protein. S protein of cl-2 (lane 1) and srr7 (lane 2) were expressed by T7 vacciniavirus expression system in BHK cells. Released (left panel) and intracellular (right panel) S were detected using S1-specific MAbs 11F. Lane 3 shows samples derived from mock-transfected cells.



**Figure 2.** Schematic diagram for mechanism of MHVR-independent infection of cl-2 mediated by spinoculation.

removed from S2 without MHVR binding, mediates viral-cell membrane fusion, if it is placed onto or close to target cell membrane.

## 5. ACKNOWLEDGMENTS

We thank Shutoku Matsuyama and Keiko Nakagaki for valuable discussions and comments.

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