Pathogenesis of Fusion Deficient Recombinant Mouse Hepatitis Viruses

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1. INTRODUCTION

It has been shown that MHV-4 (JHM) can utilize both endosomal and nonendosomal pathways for entry into cells, depending on the strain of the virus and the nature of the cell being infected (Nash and Buchmeier, 1997). Wildtype MHV-4 fuses at the cell surface. However, persistent MHV-4 infection of a neuronal cell line (OBL21a) gave rise to the acid-dependent fusion variant OBLV60. Previous studies demonstrated that there were 8 amino acid differences between the S genes of MHV-4 and OBLV60. Elimination of neutral pH fusion was dependent only on amino acid alterations at positions 1067 (Q to H), 1094 (Q to H), and 1114 (L to R) within the first heptad repeat of the S2 subunit (Gallagher *et al.*, 1991). Additionally, it was shown that intranasal inoculation with MHV-4 resulted in fatal encephalitis as a consequence of widespread distribution of infection in the brain. In contrast, the OBLV60 variant grew preferentially in the glomerular and mitral layers of the olfactory bulb and no fatal encephalitis was observed (Pearce *et al.*, 1994).

To test the hypothesis that altered entry into cells, caused by the inability to induce fusion at neutral pH, affects virulence and pathogenesis, we used targeted RNA recombination to select recombinant viruses containing the MHV-4 spike protein (S₄), carrying the three point mutations. We describe here our initial characterization of these recombinant viruses.

2. RESULTS AND DISCUSSION

We used the technique of targeted RNA recombination (Masters *et al.*, 1994), to create recombinant MHV-A59 viruses that carried either the wildtype S₄ gene or the S₄ gene encoding the Q1067H, Q1094H and L1114R mutations (Figure 1).

2.1 *In vitro* replication of recombinant viruses

In cell culture, the R120 and R121 recombinants showed a more efficient replication than the wildtype MHV-4 S recombinant (S₄), S₄R29. At 24 hours post-infection, R120 and R121 reached viral titers of 3.9×10^7 and 1.3×10^7 PFU/ml respectively, while S₄R29 titers were at 2.4×10^3 PFU/ml. Because the R120/R121 recombinants carry the 3 amino acid alterations (Q1067H, Q1094H, and L1114R) that are associated with a fusion negative phenotype at neutral pH, it is possible that the cells infected with these recombinants remain viable longer, due to decreased cytopathicity.

2.2 *In vitro* fusion assays

To demonstrate that these three amino acid mutations indeed confer a fusion negative phenotype to R120/R121, the ability of our recombinants to induce cell-to-cell fusion was assayed *in vitro* on L2 and DBT cells. For comparison, wildtype MHV-4 virus and the OBLV60 variant were also assayed for their ability to induce cell-to-cell fusion *in vitro*. Syncytia were visible 6 hours postinfection in cells infected with either wildtype MHV-4 virus or the S₄R29 recombinant. In contrast, in cells infected with viruses carrying the Q1067H, Q1094H, and L1114R mutations (R120/R121 and OBLV60), no fusion was observed even 20 hours postinfection (data not

shown). These results confirm previous data that these 3 amino acid alterations are associated with a fusion negative phenotype at neutral pH (Gallagher *et al.*, 1991).

2.3 Effect of ammonium chloride on viral replication

We tested the effect of ammonium chloride (NH₄Cl), a lysomotropic agent, on the replication of our recombinants *in vitro*. Vesicular stomatitis virus (VSV), which is known to enter the cells via the endosomal pathway, was used as a control in our assays. The presence of NH₄Cl inhibited the replication of OBLV60 and R120 to a similar extent as VSV, and to a much greater degree than wildtype MHV-4 or the S₄R29 recombinant. At an ammonium chloride concentration of 60 mM (the highest concentration used in this study), VSV, OBLV60 and R120 viral replication was completely inhibited. At this concentration, the replication of MHV-4 and S₄R29 was only 50% inhibited. These results suggest that R120, like OBLV60 and VSV, enters cells via the endosomal pathway.

2.4 Virulence of recombinant viruses

Mice inoculated either intracranially (IC) or intranasally (IN), with serial dilutions of S₄R29 or R120/R121, were observed for disease and death and an LD₅₀ value was calculated for each virus. The results are shown in Table 1. Recombinant viruses R120/R121, carrying the 3 amino acid alterations, displayed a higher LD₅₀, or a less virulent phenotype, than S₄R29, which carries the wildtype S₄ gene.

Table 1. Virulence and replication in brain of recombinant viruses

	Virus	LD ₅₀ (Log)	Viral titers in brain (Log)	
			3 d.p.i.	5 d.p.i.
intracranial	S ₄ R29	0.45	4.39	5.69
	R120/R121	> 3.70	3.01	5.37
intranasal	S ₄ R29/R22 ^a	2.74	4.49	4.68
	R120	> 5.00	3.91	6.36

^a S4R22 (Phillips *et al.*, 1999) is an MHV-4 spike recombinant in the background of A59 independently isolated from S4R29. It has the same sequence and phenotype as S4R29 and was used to determine the LD₅₀ by intranasal inoculation.

2.5 Replication in brain

To determine whether the observed attenuation with R120/R121 corresponded with a decreased viral replication *in vivo*, we inoculated mice either IC (10 PFU) or IN (5000 PFU). The amount of virus in the brain was determined at various times postinfection by plaque assay on L2 cell

monolayers. S₄R29 and R120/R121 replicated to equivalent levels in the brain following IC inoculation (Table 1). Following IN inoculation, our initial results suggested that R120/R121 replicated to a higher level than S₄R29. These results suggest that the attenuation observed with the R120/R121 viruses is not a result of lower levels of replication *in vivo*.

3. SUMMARY

In this study we have demonstrated that recombinant viruses carrying the amino acid mutations Q1067H, Q1094H, and L1114R were unable to induce fusion at neutral pH, replicated more efficiently in L2 cells, and that infection was delayed by ammonium chloride. These results suggest that the R120/R121 recombinants most likely use the endosomal pathway to enter cells. In this sense they are similar to the pH-dependent MHV-4 variant OBLV60.

We were able to observe an attenuated virulence *in vivo*, despite the fact that our R120/R121 recombinants replicated to comparable (IC) or higher (IN) titers than the S₄R29 recombinant in the brain. Preliminary results showed that the level of inflammation observed in infected mice is consistent with the attenuated virulence, but they cannot be explained by the high titers of replication.

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