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Deficient incorporation of spike protein into virions contributes to the lack of infectivity following establishment of a persistent, non-productive infection in oligodendroglial cell culture by murine coronavirus

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ABSTRACT

Infection of mouse oligodendrocytes with a recombinant mouse hepatitis virus (MHV) expressing a green fluorescence protein facilitated specific selection of virus-infected cells and subsequent establishment of persistence. Interestingly, while viral genomic RNAs persisted in infected cells over 14 subsequent passages with concomitant synthesis of viral subgenomic mRNAs and structural proteins, no infectious virus was isolated beyond passage 2. Further biochemical and electron microscopic analyses revealed that virions, while assembled, contained little spike in the envelope, indicating that lack of infectivity during persistence was likely due to deficiency in spike incorporation. This type of non-lytic, non-productive persistence in oligodendrocytes is unique among animal viruses and resembles MHV persistence previously observed in the mouse central nervous system. Thus, establishment of such a culture system that can recapitulate the in vivo phenomenon will provide a powerful approach for elucidating the mechanisms of coronavirus persistence in glial cells at the cellular and molecular levels.

Introduction

Murine coronavirus mouse hepatitis virus (MHV) is a member of the *Coronaviridae*. It is an enveloped, positive-strand RNA virus. The viral envelope contains three or four structural proteins, depending on viral strains (Lai and Cavanagh, 1997). The spike (S) protein is a glycoprotein with a molecular weight of approximately 180 kilo Dalton (kDa). For some MHV strains such as JHM and A59, the S protein can be cleaved by a furin-like proteinase into two subunits: the amino terminal S1 and the carboxyl-terminal S2. The S1 subunit is thought to form the globular head of the spike and is responsible for the initial attachment of the virus to the receptor on cell surface. The S2 subunit, which forms the stalk portion of the spike and which anchors the S protein to the viral envelope, facilitates the fusion between viral envelope and cell membrane and cell–cell fusion (Chambers et al., 1990; de Groot et al., 1987; de Haan et al., 2004; Gallagher et al., 1991; Kubo et al., 1994; Luytjes et al., 1987; Nash and Buchmeier, 1997; Stauber et al., 1993; Suzuki and Taguchi, 1996; Zhu et al., 2009). It is therefore an important determinant for viral infectivity, pathogenicity and virulence (Boyle et al., 1987; Collins et al., 1982; Phillips et al., 1999). The small envelope (E) protein and the membrane (M) protein play a key role in virus assembly (Vennema et al., 1996; Yu et al., 1994). The nucleocapsid (N) protein is a phosphoprotein of approximately 50 kDa and is associated with the RNA genome to form the nucleocapsid inside the envelope (Lai and Cavanagh, 1997; Stohlman and Lai, 1979). Upon entry into host cells, the viral genomic RNA serves as an mRNA for translation of the viral polymerase polyprotein from the 5′ most overlapping open reading frames 1a and 1b (Lai and Cavanagh, 1997). The polyprotein is then processed into 16 nonstructural proteins (nsp's), which possibly along with host factors form replication and transcription complexes that generate a nested-set of subgenomic mRNAs (Lai and Cavanagh, 1997; Snijder et al., 2003). Each subgenomic mRNA is translated into a structural or nonstructural protein. The structural proteins are assembled into virions in cytoplasmic vesicles (Vennema et al., 1996), which are then released (exocytosed) from the infected cell.

MHV can infect rodents, causing hepatitis, enteritis, and central nervous system (CNS) diseases. In the CNS, acute encephalitis usually occurs during the first week of infection, and acute demyelination can be detected histologically as early as 6 days post infection (p.i.). By the end of the second week, if the mice survive virus infection, most of the viruses are cleared from the CNS, and demyelination develops. Although infectious virus can no longer be isolated from the CNS during the chronic phase (≈3 weeks p.i.), viral RNAs are continuously detectable by Northern blot or reverse transcription-polymerase chain reaction (RT-PCR). Demyelination continues to peak at around 30 days p.i., and then slowly decreases until over a year p.i.,

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concomitant with viral RNA persistence (Bergmann et al., 2006; Das Sarma et al., 2000; Fleming et al., 1993a; Knobler et al., 1981, 1982; Lavi et al., 1984). Although the mechanisms of MHV-caused CNS demyelination are not known, it is believed that the host immune response plays an important role in the demyelination process (Dandeker and Perlman, 2002; Fleming et al., 1993b; Lai and Cavanagh, 1997; Lane et al., 2000; Matthews et al., 2002; Sorensen et al., 1987; Wang et al., 1990; Wu and Perlman, 1999, Wu et al., 2000). It has been shown that the development of demyelination in mice is associated with viral RNA persistence (Das Sarma et al., 2000; Knobler et al., 1982), but the mechanism of CNS persistence remains largely unknown.

Previously, a number of laboratories have attempted to establish an in vitro cell culture system for MHV persistence (Chen and Baric, 1996; Lavi et al., 1987; Sawicki et al., 1995; Schickli et al., 1997). While these studies have led to the establishment of MHV persistence in fibroblast 17Cl-1 cells, astrocytoma DBT cells and primary oligodendrocytes, infectious viruses continued to be produced, albeit at an extremely low level (Chen and Baric, 1996; Lavi et al., 1987; Sawicki et al., 1995; Schickli et al., 1997). Thus, such persistence does not resemble the phenomenon observed in mouse CNS. Although we have previously reported the establishment of a persistent, non-productive infection in a rat progenitor oligodendrocyte cell line (Liu et al., 2003; Liu and Zhang, 2005), the cells that harbor persistent viruses have not been confirmed, largely due to difficulty in separating infected cells from uninfected cells in a mixed culture. In an effort to establish a robust in vitro system that can faithfully recapitulate the in vivo phenomenon, in the current study we made several modifications in our experimental approach. We used a recombinant MHV that expresses a green fluorescence protein (GFP) such that infected cells can be easily identified and separated from non-infected cells. Using this approach, we were able to identify a new type of mouse oligodendroglial cell line (N20.1) that was persistently infected with MHV. Consistent with the results in rat progenitor oligodendrocytes (Liu and Zhang, 2005), viral RNA persisted for over 14 passages without the production of infectious virus. Significantly and unexpectedly, we discovered that persistently infected oligodendrocytes continued to produce viral particles but that these particles were deficient in incorporation of spike proteins on the virion surface, thus providing a mechanism for the lack of viral infectivity. To our knowledge, this is the first report in virology describing non-productive, persistent infection that continuously produces virus particles without infectivity due to a lack of spike protein incorporation.

Results

Establishment of persistent MHV infection in mouse oligodendroglial cells

We previously reported the establishment of a persistent MHV infection in a rat progenitor oligodendrocytic cell line CG-4 cells (Liu and Zhang, 2005). However, it was not clear in that study whether all cells or only a fraction of the cell population were initially infected with MHV. This question is important because the mechanism of persistence could be different under these conditions. To unequivocally address this question and to identify additional cell lines for such studies, we used the recombinant MHV-A59/GFP, which expresses the GFP, and two additional mouse oligodendrocytic cell lines N20.1 (Verity et al., 1993) and Oligo-neu (Jung et al., 1995). The rationale for using MHV-A59/GFP was to quantitatively assess the susceptibility of the cell population to virus infection and to track the extent of viral persistence following continuous passage of the cells. Thus, CG-4, N20.1 and Oligo-neu cells were infected with MHV-A59/GFP at an m.o.i. of 10, and were observed for GFP expression. We found that less than 0.1% of the CG-4 cells expressed strong GFP at 24 and 48 h p.i. (data not shown) while approximately 25% of N20.1 cells (Fig. 1B, a–b) and <20% of Oligo-neu cells exhibited strong fluorescence for the same time period (data not shown). Because of the highest cell number of GFP expression and relative ease in culture, N20.1 cells were used in all subsequent experiments described in this report.

Infected N20.1 cells were subjected to flow cytometric sorting (Fig. 1). Strong GFP-expressing cells were separated from non-GFP-expressing cells and were re-seeded into dishes (Fig. 1B, c–d), which were designated as VP1 (for passage 1 of virus-infected cells). To our surprise and disappointment, most of the green fluorescence disappeared by days 7 to 10 after seeding (Fig. 1B, e–f). Nevertheless, we continued to replenish the medium every other day and allowed the cells to grow to monolayers for additional 15 to 20 days. The monolayer cells were further passaged approximately every 8 days and were designated as VP2, VP3, and so forth.

To determine whether the infection was aborted after disappearance of the green fluorescence at the end of VP1, monolayer cells (approximately by 30 days after seeding of GFP-positive cells) were collected and the presence of viral genomic RNAs in the cells was determined by RT-PCR using 5 pairs of primers that represent 5 regions throughout the viral genome (Fig. 2A). Indeed, viral genomic RNAs were detected with all 5 pairs of primers (Fig. 2B), confirming the presence of the viral genome. We then isolated intracellular RNAs from various passages of the infected cells (VP1 to VP14) and used RNAs from acutely infected (VP0) and mock-infected N20.1 cells as a positive and negative control, respectively, for RT-PCR with the primer pair 5′N and 3′N. Results showed that viral genomic RNAs were present in all passages tested (Fig. 2C). Thus, real-time qRT-PCR (using primer pair 5′qPCR and 3′qPCR) was used to further quantify the viral genomic RNAs in these passages. It was found that in general the amount of viral genome decreased drastically from VP0 to VP4, but relatively slowly thereafter (Fig. 2D). These data demonstrate that viral genomic RNAs established persistence in N20.1 cells for a period of time long after the disappearance of GFP expression.

Loss of GFP expression is associated with aberrant transcription of subgenomic mRNA4 coding for GFP in persistently infected oligodendrocytes

Since viral genomic RNAs persisted (Fig. 2), we assumed that viral genomic replication must have taken place continuously, and so must have been the expression of the viral polymerase polyprotein, the ORF 1a/1b product. However, the eventual disappearance of GFP-positivity led us to further examine potential defects in viral gene expression in persistently infected N20.1 cells since the GFP ORF is under the control of the consensus intergenic sequence for subgenomic mRNA 4 (Fig. 3A) (Das Sarma et al., 2002). Thus, both the genomic regions encompassing the GFP coding sequence and the subgenomic mRNA4 at VP4 were specifically amplified by RT-PCR using the pair of primers 5′IG4–3′G5a and 5′Leader–3′G5a, respectively, and the PCR products were sequenced directly or following shotgun cloning. Interestingly, we found that the entire GFP ORF was intact within the persistent viral genome (data not shown) but that there were multiple species of subgenomic mRNA4 varying in size (Fig. 3B). These mRNAs contained a leader RNA fused at multiple sites downstream of the AUG translation initiation codon, thus effectively abolishing the expression of the GFP gene, a phenomenon reminiscent of the aberrant transcription described previously for MHV (Fischer et al., 1997; Zhang and Lai, 1994).

Expression of viral genes in persistently infected oligodendrocytes

The finding of aberrant transcription of subgenomic mRNA4 (GFP) led us to further examine whether the other viral subgenomic mRNAs were also transcribed aberrantly. Thus, the expression of viral subgenomic mRNAs coding for structural proteins and their protein products was assessed in various passages of the persistently infected N20.1 cells. As an example shown in Fig. 4, mRNA7 (for N gene) and mRNA3 (for S gene) were detected in various passages of the persistently infected cells but not