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A study of the virulence in mice of high copying fidelity variants of human enterovirus 71

Sara Sadeghipour, Peter C. McMinn*

Infectious Diseases and Immunology, Sydney Medical School, Blackburn Building D06, The University of Sydney, NSW 2006, Australia

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ABSTRACT

Polioviruses with a G64S mutation in the 3D polymerase have enhanced replication fidelity and are attenuated in animal models. Here we describe the mouse virulence properties of high replication fidelity 3D polymerase variants of human enterovirus 71 (HEV71), with mutations at positions 3D-S264L, 3D-G64R or at 3D-S264L plus 3D-G64R. Mouse-adapted strains (MP-G64R, MP-S264L and MP-S264L-G64R) were constructed in order to compare the virulence of the 3D polymerase variants with that of mouse-adapted parental virus (MP-26M). MP-S264L and MP-S264L-G64R were attenuated in mice (mean survival time 7.0 and 7.5 days p.i., respectively) compared to MP-G64R and MP-26M (mean survival time 6.5 and 6.0 days p.i., respectively). MP-26M and MP-G64R infection induced early onset, severe generalised necrotising myositis, whereas MP-S264L and MP-S264L-G64R infection induced a later onset, mild and focal skeletal muscle myositis. Our findings demonstrate that only the 3D-S264L mutation attenuates HEV71 in mice, suggesting that the high replication fidelity phenotype is not essential for virulence attenuation in this model.

1. Introduction

Human enterovirus 71 (HEV71) belongs to the genus *Enterovirus* within the family *Picornaviridae* (Knowles et al., 2012). HEV71 was first discovered in 1969 and has recently emerged as an important cause of hand-foot-and-mouse disease (HFMD) associated with a high frequency of acute neurological disease (McMinn, 2002).

The poliovirus 3D polymerase ($3D^{pol}$) has recently gained attention as a promising virulence attenuation target (Pfeiffer and Kirkegaard, 2003; Vignuzzi et al., 2008). A poliovirus variant with a G64S mutation in the $3D^{pol}$ coding region has been shown to express high replication fidelity and to be attenuated in poliovirus receptor-expressing transgenic mice (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006, 2008). Furthermore, G64A, G64T, G64L and G64V mutations in the poliovirus $3D^{pol}$ have been shown to greatly reduce viral fitness and to attenuate virulence (Vignuzzi et al., 2008). Attenuating mutations that increase the replication fidelity of the poliovirus $3D^{pol}$ have also been shown to prevent reversion to virulence of the parental virus phenotype (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2008). Furthermore, we have recently identified two mutations in HEV71 $3D^{pol}$, at positions G64R and S264L, that increase replication fidelity during growth in cell culture (Sadeghipour et al., 2013).

In this study, we examine the relationship between $3D^{pol}$ mutations (G64R, S264L) that confer a high replication fidelity phenotype upon HEV71 and their virulence phenotype in mice. We investigate the virulence of HEV71 strains containing $3D^{pol}$ mutations at positions G64R, S264L and at S264L plus G64R (double mutant) in a newborn BALB/c mouse model of HEV71 infection (Chua et al., 2008). 50% humane endpoints, mean lengths of survival, viral distribution in mouse tissues and histological studies were undertaken in order to compare the virulence of the HEV71 $3D^{pol}$ variants with that of virulent parental virus.

2. Materials and methods

2.1. Virus, cell lines and media

HEV71 strain 26M/AUS/4/99, sub-genotype B3, was isolated in Western Australia in 1999 (Chua et al., 2008; McMinn et al., 2001) during an outbreak of hand, foot and mouth disease. HEV71-26M has been fully sequenced and used to generate an infectious cDNA clone (Chua et al., 2008). MP-26M is a mouse-adapted strain (VP1-G145E) of HEV71-26M (Chua et al., 2008). Human rhabdomyosarcoma (RD; ATCC number CCL-136), African green monkey kidney (Vero; ATCC number CCL-81) and simian virus 40-transfected African green monkey kidney (COS-7; CRL-1651) cell lines were maintained at 37 °C in 5% CO_2. Maintenance medium

* Corresponding author. Tel.: +61 2 9351 2900; fax: +61 2 9351 4731.
E-mail address: peter.mcminn@sydney.edu.au (P.C. McMinn).

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was Dulbecco's Modified Eagle Medium (DMEM) (HyClone) supplemented with 2% foetal bovine serum (FBS) (Bovogen Biologicals) and 2 mM L-glutamine (Sigma). Growth medium was DMEM supplemented with 5% FBS and 2 mM L-glutamine.

2.2. Cloning

In order to select HEV71 strains with high replication fidelity, the 3D-G64R (Sadeghipour et al., 2013), 3D-S264L (Sadeghipour et al., 2013) and 3D-S264L-G64R mutations were introduced into pcDNA3-3D by site-directed mutagenesis using the Lightning Quickchange mutagenesis kit (Stratagene). The 3D coding region fragments carrying the G64R, S264L or S264L-G64R mutations were cloned into the HEV71-26M infectious cDNA clone by digestion with *Mlu*I and *Eco*RV to produce G64R (Sadeghipour et al., 2013), S264L (Sadeghipour et al., 2013) and S264L-G64R, respectively. The 3D gene fragments carrying the 3D-G64R, 3D-S264L or 3D-S264L-G64R mutations were also cloned into the mouse-adapted MP-26M infectious cDNA clone by digestion with *Mlu*I and *Eco*RV to generate MP-G64R, MP-S264L and MP-S264L-G64R, respectively.

2.3. Transfection

Recombinant full-length HEV71 plasmids were transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen) to rescue clone-derived virus (CDV). Briefly, 1.6 μg of either G64R, S264L or S264L-G64R and 1.6 μg pCMVT7-Pol expressing the T7 RNA polymerase were diluted into a final volume of 100 μL containing 4 μL Lipofectamine 2000 in 96 μL OptiMEM (Invitrogen). The two solutions were incubated for 5 min, then combined and incubated for a further 20 min at room temperature. COS-7 cells in 12-well tissue culture plates (CellStar) were covered with 400 μL of the OptiMEM solution. After 4 h incubation at 37 °C, 600 μL of growth medium was added and the transfected cells were incubated for 72 h. Transfected cells were then subjected to three cycles of freeze-thawing and the supernatants clarified by centrifugation at 6500 × *g* for 5 min; 200 μL of transfected COS-7 cell lysate was used to infect RD cells in six-well plates. CDVs were passaged (×1) on RD cells to obtain working stocks. CDV-infected cell culture supernatants were subjected to viral RNA extraction using the QIAamp viral RNA minikit (Qiagen) and viral cDNA synthesis was performed using Superscript III (Invitrogen) and the 3′-UTR-R primer. Viral cDNA was amplified by polymerase chain reaction (PCR) followed by nucleotide sequencing of the 3D coding region to confirm the presence of the 3D-G64R, 3D-S264L or 3D-S264L-G64R mutations or the VP1 coding region to confirm the presence of the VP1-G145E mouse adaptation marker.

2.4. Characterisation of the S264L-G64R double mutant

2.4.1. Investigation of the ribavirin resistance phenotype

The growth of S264L-G64R was compared to parental 26M in RD cell culture for 24 h in the presence of 0 μM, 800 μM or 1600 μM ribavirin. Briefly, RD cells in 12-well plates (Cellstar) were covered with DMEM containing 0 μM, 800 μM or 1600 μM ribavirin for 1 h at 37 °C. The ribavirin-pre-treated RD cells were washed once with phosphate-buffered saline, pH 7.4 (PBS). Cells were infected (MOI = 0.1) with parental 26M or S264L-G64R for 1 h at 37 °C, washed with PBS, followed by the addition of maintenance medium containing either 0 μM, 800 μM or 1600 μM ribavirin. The infected cell cultures were then incubated at 37 °C in 5% CO_2 for 24 h. At the conclusion of the experiment, virus was released from cells by freeze-thawing (×3) and the lysate was clarified by centrifugation at 6500 × *g* for 5 min. The titre of the lysate was determined by $TCID_{50}$ assay on Vero cells, following Reed and Muench (Reed and Muench, 1938).

2.4.2. Plaque purification of S264L-G64R

Twelve-well tissue culture trays (Cellstar) were seeded with Vero cells at a density of 2.2×10^5 cells/well and grown overnight at 37 °C. Ten-fold serial dilutions of virus were inoculated onto Vero cell monolayers at a volume of 200 μL per well. After incubation for 30 min at 37 °C, virus was removed and cells were washed with PBS. Cells were then overlaid with 1 mL of maintenance medium containing 0.5% immunodiffusion-grade agarose (ICN) and incubated at 37 °C in 5% CO_2. After four days incubation, an additional 0.5 mL of maintenance medium containing 0.5% immunodiffusion-grade agarose was added to each well and the cultures incubated for a further three days. Isolated plaques were selected using a pipette tip to draw up the cells from within the plaque. This protocol was repeated three times in order to select plaque-purified virus populations.

2.4.3. Characterisation of the guanidine sensitivity of plaque-purified populations of S264L-G64R during passage in RD cells in the presence of ribavirin

The guanidine sensitivities of plaque-purified populations of S264L-G64R were compared to plaque-purified populations of parental 26M by growth in the presence of either 0 mM or 0.5 mM guanidine for 48 h. Briefly, RD cells were infected (MOI = 0.1) in six-well plates (Cellstar) for 1 h at 37 °C. Cells were washed with PBS and maintenance medium containing either 0 mM or 0.5 mM guanidine was added. Cells were incubated at 37 °C in 5% CO_2 for 48 h. Virus was released from cells by freeze-thawing (×3) and the lysate was clarified by centrifugation at 6500 × *g* for 5 min. The titre of the lysate was determined by $TCID_{50}$ assay, as described above (Reed and Muench, 1938).

2.4.4. RNA extraction, cDNA synthesis and nucleotide sequence analysis

Viral RNA was extracted from parental 26M and S264L-G64R after one or thirteen passages in RD cells in the presence of ribavirin (400 μM), using the QIAamp Viral RNA Mini Kit (Qiagen); 8 μL of viral RNA was reverse transcribed using Superscript III (Invitrogen) and the 3′-UTR-R primer. Viral cDNA was amplified by PCR and gel-purified using a MinElute gel extraction kit (Qiagen). Nucleotide sequencing of coding regions 2C and 3D was performed by the Australian Genome Research Facility. Analysis of DNA chromatograms were performed using the program Chromas™ version 2.33 (Technelysium Pty. Ltd. Australia) and Sequencher version 4.7 (Gen Codes Corporation). Statistical analyses were performed using two-tailed Student's *t*-test; *P* values of <0.05 are considered statistically significant.

2.4.5. Single-step growth kinetics

RD cell monolayers (1×10^5 cells per well) grown overnight in 48-well tissue culture plates (Cellstar) were infected (MOI = 10) for 1 h at 37 °C. Cells were then washed with PBS (×3) and overlaid with 300 μL of maintenance medium per well. Virus was collected at four hourly intervals for 24 h by freeze-thawing (×3) and clarified by centrifugation at 6500 × *g* for 5 min. The first time point (0 h) was collected immediately after the addition of the maintenance medium. Yields were quantified by $TCID_{50}$ assay, as described above (Reed and Muench, 1938).

2.5. Determination of HD_{50} values

In order to minimise distress to the experimental animals, 50% humane endpoints (HD_{50}) were used instead of 50% lethal endpoints (LD_{50}); humane endpoint assays have been shown to be