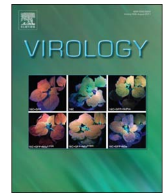
Virology 509 (2017) 195–204

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Differential replication of Foot-and-mouth disease viruses in mice determine lethality

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ARTICLE INFO

Keywords:
Pathogenesis
Lethality
FMDV
Adult mice
2C protein
Capsid

ABSTRACT

Adult C57BL/6J mice have been used to study *Foot-and-mouth disease virus* (FMDV) biology. In this work, two variants of an FMDV A/Arg/01 strain exhibiting differential pathogenicity in adult mice were identified and characterized: a non-lethal virus (A01NL) caused mild signs of disease, whereas a lethal virus (A01L) caused death within 24–48 h independently of the dose used. Both viruses caused a systemic infection with pathological changes in the exocrine pancreas. Virus A01L reached higher viral loads in plasma and organs of inoculated mice as well as increased replication in an ovine kidney cell line. Complete consensus sequences revealed 6 non-synonymous changes between A01L and A10NL genomes that might be linked to replication differences, as suggested by *in silico* prediction studies. Our results highlight the biological significance of discrete genomic variations and reinforce the usefulness of this animal model to study viral determinants of lethality.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of wild and domestic cloven-hoofed animals. The disease has major economic impact due to severe productivity losses and to the restrictions imposed to the trade of animals and animal products from FMD-affected regions (Sobrino, 2004). Prevention and control of FMD is achieved by sanitary profilaxis as well as vaccination of susceptible animals in endemic areas.

The etiological agent of FMD is *Foot-and-mouth disease virus* (FMDV), the prototype member of the genus *Aphthovirus* within the *Picornaviridae* family. The viral particle is an icosahedron which encloses a single-stranded positive-sense RNA of approximately 8200 nucleotides that is linked covalently to the viral protein VPg at its 5′ end. The viral genome is flanked by untranslated regions at both its 5′ and 3′ termini and encodes a polyprotein that is subjected to co-translational cleavage to produce the 4 capsid proteins (VP4, VP2, VP3 and VP1) and 10 non-structural proteins (Lpro, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3Cpro and 3Dpol) (Grubman and Baxt, 2004). Like other RNA viruses, FMDV replication is catalyzed by an error-prone viral RNA polymerase (3Dpol) and consequently the virus appears as a population of different but phylogenetically-related variants known as the viral quasispecies (Haydon et al., 2001; Domingo et al., 2003, 1980; Sobrino et al., 1983). The virus exists as 7 immunologically different serotypes (A, O, C, Asia, SAT1, SAT2, SAT3) and multiple subtypes that elicit effective neutralizing antibodies that do not confer cross-protection among serotypes.

Natural FMDV infection in cattle occurs mainly *via* the respiratory route by aerosolized virus and subsequent primary infection of nasopharynx and lung, followed by a viremic phase and dissemination to secondary replication sites (reviewed by Arzt et al., 2011). Although many aspects of FMD in natural hosts have been studied extensively, viral and host factors related to FMDV virulence and pathogenesis are not completely understood. It is well known that FMDV leader proteinase (Lpro) plays a role as a virulence factor, since viruses of serotype A lacking this region or carrying mutations within Lpro

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http://dx.doi.org/10.1016/j.virol.2017.06.012
Received 1 February 2017; Received in revised form 1 June 2017; Accepted 12 June 2017
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display an attenuated phenotype in cell culture and show no replication in cattle and pigs (Chinsangaram et al., 1998; Mason et al., 1997; Díaz-San Segundo et al., 2012). Other viral proteins such as 3A or structural proteins have also been proposed as virulence determinants both in cell culture and in natural hosts. Indeed, Pacheco et al. demonstrated that 3A protein of type O1Campos is relevant for replication in cattle and in primary cell cultures of bovine origin (Pacheco et al., 2013), and this viral protein has also been associated with FMDV adaptation to the guinea pig (Núñez et al., 2001). Other authors have shown that VP1, VP2 and VP3 proteins are determinants of FMDV pathogenesis in cattle and swine (Lohse et al., 2012; Bøtner et al., 2011), and deletions of the RGD motif in VP1 (Rieder et al., 1996) or acquisition of positive charge have been related to attenuation in cattle and swine (Borca et al., 2012; Sa-Carvalho et al., 1997; Zhao et al., 2003; Lawrence et al., 2016a, 2016b).

The study of FMDV virulence factors in natural hosts has a major limitation related to the logistics and cost of experimentation with large animals. To overcome this difficulty, several laboratory animal models have been developed that may be useful to study particular aspects of FMD pathogenesis and FMDV biology (Habiela et al., 2014; Skinner, 1951; Waldman and Pape, 1920). In particular, after footpad subcutaneous (sc) injection or intraperitoneal (ip) inoculation the virus causes an acute infection that can be lethal in adult C57BL/6 mice. Subcutaneous injection produces a systemic infection with viral dissemination to different organs (heart, lung, brain, kidney, liver, spleen, pancreas, and thymus) that leads to death of animals at 48–72 hpi. Finally, the disease is associated with a pronounced lymphopenia and depletion of splenic $CD4^+$ and $CD8^+$ T-lymphocytes (Salguero et al., 2005).

Interestingly, C57BL/6 mice proved to be adequate to detect virulence differences among FMDV variants of the same serotype and thus this animal model appears as a powerful tool to study viral factors related to virulence and/or pathogenesis (Salguero et al., 2005). In fact, in a study on the virulence of field strains that circulated in Argentina during the 2000–2002 epizootic, we demonstrated previously that FMDV A/Arg/00, as opposed to FMDV A/Arg/01, does not cause death of C57BL/6 mice when inoculated ip even at doses as high as 10^7 pfu per animal; these observations mirrored the pathogenic behavior of field strains (García Núñez et al., 2010; Mattion et al., 2004).

In this work, we further characterize two FMDV A/Arg/01 variants in terms of pathogenicity in adult mice and replication in cell culture.

2. Material and methods

2.1. Viruses and cell lines

A01NL and A01L viruses were obtained from the National Institute for Animal Health (SENASA, Argentina). Viruses were isolated during the FMDV outbreak that occurred in Argentina during years 2000–2001 and belong to serotype A/Arg/01. All experiments were conducted using fourth cell passages of each FMDV in baby hamster kidney cells (BHK-21 clone 13; ATCC CCL10). Quantification of viral particles present in various samples were determined in BHK-21 cells by plaque assay (pfu/ml) or alternatively the 50% tissue culture infective dose ($TCID_{50}$) was calculated by the end point dilution method using the formula of Reed and Muench (Reed and Muench, 1938).

Cell lines used in this study were BHK-21, PK15-C1 (ATCC, PTA-8244), IBRS-2, MDBK (ATCC CCL22), fetal bovine kidney cells (FBK) and ovine kidney cells (OK) (Zabal and Fondevila, 2013). Cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle´s medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco-BRL/Invitrogen, Carlsbad, CA, USA).

2.2. Mice

Eight to 10-week-old female C57BL/6J/LAE mice were purchased from University of La Plata, Argentina. Mice were maintained under specific pathogen-free conditions and allowed to acclimatize to the biosafety level 4 OIE (BSL-4 OIE) animal facility at the Institute of Virology, INTA, for 1 week prior to experiments.

Experiments with mice were performed in accordance with the Institutional Committee for the Use and Care of Experimentation Animals (CICUAE-INTA protocols Nos. 46/2013 and 49/2015).

2.3. Animal infection and processing of samples

Groups of mice were inoculated with A01L or A01NL by ip injection with 100 microliters of a viral suspension containing the indicated amount of each virus. Mice were examined for clinical symptoms twice daily. Animals were euthanized at a humane endpoint when showing irreversible signs of pain or disease (hypothermia, hunched posture, lethargy).

At different times post inoculation, mice (n=4 per experimental group) were euthanized and organs (liver, spleen, pancreas, thymus, lung, heart and brain) were harvested and weighed. Half of the tissues was used for histological analysis. The other half was mechanically disrupted and resuspended in DMEM supplemented with HEPES 25 mM pH 7.4; cell suspensions were frozen at −80 °C. Lung and heart samples were used exclusively for histological analysis. Whole blood samples were collected in heparinized tubes at different times post-inoculation. Plasma was separated by centrifugation, aliquoted and stored at −80 °C.

For cross-protection experiments, groups of mice (n=5) were inoculated ip with A01NL (10^5 PFU per animal) or DMEM. Fourteen days after first inoculation, mice were bled to determine neutralizing antibody titers and immediately inoculated ip with a lethal dose of A01L virus (10^4 PFU per animal) (Molinari et al., 2010). Mice were bled 24 h post-A01L infection and heparin-anticoagulated plasma samples were stored at −80 °C for quantification of viremia. Animals showing irreversible signs of disease at 24 h post-A01L inoculation were sacrificed to avoid suffering. Surviving mice were examined for clinical symptoms daily for 7 additional days.

2.4. Histopathology

Samples from different organs were fixed in 10% buffered formalin (pH 7.2) for histopathological studies. After fixation, samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin. Three micrometer-thick sections were cut and stained with hematoxylin and eosin (H & E). Histological grading was made blindly by an experimented pathologist.

2.5. Quantification of neutralizing antibodies

Anti-FMDV neutralizing antibodies were measured by the variable serum-constant virus method as described by Quattrocchi et al. (2011). Briefly, heat-inactivated sera were serially diluted and dilutions were incubated with 100 $TCID_{50}$/well of infectious FMDV for 40 min at 37 °C. The virus-serum mixtures were transferred onto BHK-21 cell monolayers and cytopathic effect was recorded after 48 h incubation at 37 °C in a 5% CO_2 containing atmosphere. Titer of neutralizing antibodies was calculated as log (1/last serum dilution that neutralizes 50% of wells).

2.6. Quantification of IFN-α

Interferon alpha was quantitated (pg/ml) in plasma samples with Mouse IFN alpha Platinum ELISA kit (eBioscience, Vienna, Austria).

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