Virus Research 215 (2016) 114–120

Contents lists available at ScienceDirect

Virus Research

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

Pattern of circulation of MCMV mimicking natural infection upon oronasal inoculation

Shunchuan Zhang[1], Jun Xiang[1], Lowiese M.B. Desmarets, Hans J. Nauwynck*

Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

ARTICLE INFO

Article history:
Received 12 October 2015
Received in revised form
17 December 2015
Accepted 21 December 2015
Available online 28 December 2015

Keywords:
MCMV
Pathogenesis
Oronasal inoculation
Virus dissemination
Non-productive infection

ABSTRACT

Cytomegaloviruses may infect mammals via oronasal route. However, up till now it remains unclear how this exposure leads to a general infection and shedding. To address this issue, BALB/c female mice were oronasally inoculated with either the highly passaged murine cytomegalovirus (MCMV) Smith or the low passaged MCMV HaNa1. Virus titration showed a productive virus replication of both strains in the nasal mucosa from 1 dpi until the end of the experiment (14 dpi), in lungs from 5 until 14 dpi, and in submandibular glands from 7 until 14 dpi. In contrast to MCMV HaNa1, MCMV Smith also established a low level productive infection in abdominal organs (spleen, liver and kidneys) from 5 dpi (spleen), 7 dpi (liver), and 10 dpi (kidneys) until the end of the experiment. Co-culture showed that for both strains, cell-associated virus was detected in a non-infectious form in nasopharynx-associated lymphoid tissues (NALT) from 1 until 14 dpi, in submandibular lymph nodes from 3 until 5 dpi, in deep cervical lymph nodes from 3 until 14 dpi, in mediastinal lymph nodes from 7 until 14 dpi, in spleen from 5 until at least 10 dpi and in the peripheral blood mononuclear cells (PBMC) at 7 and 10 dpi. The present study shows that upon oronasal exposure, MCMV first enters the nasal mucosa and NALT, from where the virus disseminates to the spleen possibly via the draining lymphatic system and blood; a subsequent cell-associated viremia transports MCMV to submandibular glands and for MCMV Smith also to liver and kidneys, where a second productive replication starts.

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1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that establishes life-long, mainly asymptomatic infections. However, these infections may be life-threatening in immunocompromised individuals, such as preterm neonates (Fowler et al., 1992; Lazzarotto et al., 2014), AIDS patients (Moskowitz et al., 1985) and transplant recipients (Neiman et al., 1973; Rubin, 1990). Due to the strict species-specificity of HCMV, it is not possible to study its replication in experimental models. Because of high similarities in CMV's biology and disease spectrum, murine cytomegalovirus (MCMV) infections in mice have been most commonly and widely used as a model to understand the pathogenesis of HCMV infections in humans (Hudson, 1979; Krmpotic et al., 2003a).

Humans can be infected with HCMV by oral exposure, such as consuming HCMV-contaminated breast milk (Asanuma et al., 1996; Hamprecht et al., 2001; Kurath et al., 2010; Stagno and Cloud, 1994; Vochem et al., 1998) and oral uptake of HCMV-contaminated saliva (Cannon et al., 2012; Mocarski et al., 2007). HCMV was also recently detected in the nasal mucosa of healthy individuals (Chan et al., 2002), infants (Wejse et al., 2001), transplant recipients (Kulkarni et al., 2003), and AIDS patients (Jutte et al., 2000; Marks et al., 1996; Yoskovitch and Cantrell, 1998). This oronasal transmission may also be the main route for MCMV infections in mice, since mice have been successfully infected with MCMV by oral exposure to MCMV-contaminated breast milk (Wu et al., 2011), by oral inoculation (Doom and Hill, 2008; Krmpotic et al., 2003b), and by nasal exposure (Cardin et al., 2009; Stahl et al., 2013). Therefore, the oral and nasal cavities are likely to be major primary replication sites of CMV and shedding (Chan et al., 2002; Jutte et al., 2000; Marks et al., 1996; Mocarski et al., 2007; Wejse et al., 2001). In our previous work, an MCMV infection model has been established that mimics natural infection using a recent Belgian MCMV isolate HaNa1 (Zhang et al., 2015). However, it remains unclear how the virus enters and reaches other organs such as salivary glands after natural oronasal exposure.

* Corresponding author at: Laboratory of Virology, Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium. Fax: +32 9 264 74 95.
E-mail address: hans.nauwynck@ugent.be (H.J. Nauwynck).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.virusres.2015.12.016
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As known for a long time, CMV spreads in a strict cell-associated way in mice and humans (Gilbert et al., 1989; Roback et al., 2006; Yeager et al., 1981). Different peripheral blood leukocyte (PBL) subsets have been involved in viremia and latency in both immune-compromised and immune-competent subjects (Reeves and Sinclair, 2008; Revello et al., 1998; Slobedman and Mocarski, 1999). Nonpermissive monocytes likely serve as latent reservoirs that support dissemination by maturing into permissive macrophages or dendritic cells that reactivate virus (Hertel et al., 2003; Reeves et al., 2005; Sinclair, 2008). Besides blood circulation, the lymph drainage may be another important pathway for CMV dissemination as well, since MCMV could be detected in draining lymph nodes (Cardin et al., 2009).

In the oral and nasal cavities, mucosal surfaces, mucosa-associated lymphoid tissues and mucosa-draining lymph nodes (LN) constitute the inductive sites for mucosal immunity against exogenous pathogens (Brandtzaeg et al., 2008; Iwasaki, 2007; Kuper et al., 1992). Since mice have no tonsils, the nasopharynx-associated lymphoid tissues (NALT) are the most important tissues for the generation of mucosal immunity after inhalation of antigens (Bienenstock and McDermott, 2005; Harkema et al., 2012; Kiyono and Fukuyama, 2004; Kuper et al., 1992; Nacer et al., 2014). The NALT is found at the base of the nasal cavity and consists of bilateral strips of lymphoid tissue. In human, the functional homologue of rodent NALT is the Waldeyer's ring (Koornstra et al., 1991; Ogasawara et al., 2011). As previous researchers have reported the detection of HCMV DNA in the Waldeyer's ring (Berger et al., 2007; David et al., 1987), the exact role of these mucosa-associated lymphoid tissues in the dissemination of CMV requires further investigation.

In the present study, BALB/c mice were experimentally inoculated with two MCMV strains (the highly passaged MCMV Smith strain and the low passaged MCMV HaNa1 strain) to elucidate the kinetics of virus dissemination upon oronasal exposure. Blood, lymph nodes and other relevant organs were collected at different time points and used to detect infectious virus by virus titration and cell-associated virus by co-cultivation.

2. Materials and methods

2.1. Cells and viruses

Primary BALB/c mouse embryonic fibroblasts (MEFs) were cultivated at 37 °C with 5% CO_2, in minimum essential medium (MEM) with 10% fetal calf serum (FCS), 2% lactalbumin and a mixture of antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 mg/ml gentamicin), and were used at the second passage. Viruses used in the present experiments were the second passage of clone1 of the MCMV HaNa1 strain, which was isolated from a Belgian domestic mouse in the laboratory of the authors (Zhang et al., 2015), and the MCMV Smith strain from ATCC was at a high passage in the continuous murine bone marrow stromal cell line (M2-10B4). Seven genes from MCMV HaNa1 have been sequenced and submitted to GenBank: m06 gene (accession No.: KR184668), m033 gene (accession No.: KR184669), mck-2 including exon1 (m131 gene) and exon2 (m129 gene) (accession No.: KR184670), m138 gene (accession No.: KR184671), m144 gene (accession No.: KR184672), m152 gene (accession No.: KR184673) and m157 gene (accession No.: KT289520). The nucleic acid/deduced amino acid sequence alignments between the known HaNa1 genes and the corresponding Smith genes were done by MEGA5.21. The results are shown in Table 1.

Table 1
Nucleic acid/deduced amino acid sequence identity of genes/gene products of MCMV strains.

Genes	Sequence identity MCMV HaNa1 & MCMV Smith	
	Nucleic acids	Deduced amino acids
m06	99.8%	100%
m33	99.7%	99.7%
m129	100%	100%
m131	100%	100%
m138	99.5%	99.1%
m144	95%	93.5%
m152	99.2%	98.4%
m157	95.7%	91.5%

2.2. Animals and virus inoculation

Specific pathogen-free 6 to 8-week-old BALB/c female mice were used in the present study. Eighteen mice were inoculated with 10^6 $TCID_{50}$ MCMV HaNa1 in 100 μl PBS via intranasal (25 μl) and peroral (75 μl) route without anesthesia. Since mice are obligate nose breathers (Harkema et al., 2012) and intranasal administration of large volumes (>30 μl) per time can result in direct delivery into lungs (Tan et al., 2014; Visweswaraiah et al., 2002), a small amount of inoculum (5 μl) was repeatedly instilled in each nostril. Each application was done with several minutes interval to avoid direct flow of the inoculum into the lungs. For the oral inoculation, 25 μl inoculum was given three times with a few minutes interval between each inoculation. Another 18 mice were inoculated with MCMV Smith using the same methodology. Mice were kept in isolation and fed ad libitum. For each MCMV strain, three infected mice were euthanized at 1, 3, 5, 7, 10 and 14 dpi. Another 3 mice were mock inoculated with PBS and euthanized at the end of the experiment.

2.3. Collection of blood and tissues

For blood collection, mice were anesthetized with 130 μl of 10 mg/ml sodium pentobarbital (KELA, Belgium). Afterwards, approximately 0.6 ml blood per mouse was taken from the orbital sinus with a heparinized Pasteur pipet and kept in an eppendorf with 0.5 ml PBS containing 5 U/ml heparin (Leo Pharma, Zaventem, Belgium). Plasma was collected by centrifugation (200 g for 10 min) and stored at −70 °C for virus titration. PBMC were isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare). After blood collection, mice were euthanized with another 200 μl of 10 mg/ml sodium pentobarbital (KELA, Belgium), and different tissues were collected under aseptic conditions. Tissues obtained from the respiratory system were nasal mucosa, pharynx, trachea, lungs, thymus, nasopharynx-associated lymphoid tissues (NALT) (collected as described in references (Brandtzaeg et al., 2008; Cisney et al., 2012)), deep cervical LN and mediastinal LN. Tissues collected from the alimentary system were oral mucosa, submandibular glands, esophagus, small intestines, submandibular LN, superficial parotid LN, Peyer's patches and mesenteric LN. Other abdominal organs that were collected were spleen, liver and kidneys. The lymph nodes, thymus, PBMC and half of the spleen were used for co-culture (see below). From the other half of the spleen and the other tissues, 10% homogenates were made in PBS and stored at −70 °C until virus titration was performed.

2.4. Virus titration

MEFs were inoculated with 50 μl of 10-fold serial dilutions of the 10% tissue homogenates. After 1 h incubation at 37 °C, 100 μl of medium were added and the cells were further incubated for 7 days. After 7 days, the presence of a cytopathic effect (CPE) was assessed

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