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Infections of neonatal and adult mice with murine CMV HaNa1 strain upon oronasal inoculation: New insights in the pathogenesis of natural primary CMV infections

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ABSTRACT

In healthy individuals, naturally acquired infections of human cytomegalovirus (HCMV) are generally asymptomatic. Animal models mimicking the natural primary HCMV infections in infants and adults are scarce. Here, neonatal and adult BALB/c mice were inoculated oronasally with a Belgian isolate HaNa1 of murine cytomegalovirus (MCMV). None of the mice showed clinical symptoms. In neonatal mice, a typical systemic infection occurred. In adult mice, viral replication was restricted to the nasal mucosa and submandibular glands. Infectious virus was not detected in trachea, oral mucosa, pharynx, esophagus, small intestines of both neonatal and adult mice at all time points. Nose was demonstrated to be the entry site. Double immunofluorescence staining showed that in nose infected cells were olfactory neurons and sustentacular cells in olfactory epithelium and were macrophages and dendritic cells in nasopharynx-associated lymphoid tissues (NALT). Neonatal and adult mice developed similar antibody response pattern, though former magnitude was lower. In summary, we have established intranasal (without anesthesia) infections of neonatal and adult mice with murine CMV HaNa1 strain, which mimic the range and extent of virus replication during natural primary HCMV infections in healthy infants and adults. These findings might bring new insights in the pathogenesis of natural primary CMV infections.

1. Introduction

In general, primary infection of human cytomegalovirus (HCMV) is asymptomatic in healthy infants and adults, therefore little information is currently available on the natural primary infection of HCMV (Britt, 2006). The earliest phase of cytomegalovirus infection has not been well studied in humans or experimental animal models. Following natural community exposure to HCMV, the entry site has been unknown (Arvin et al., 2007). Due to the strict species-specificity of cytomegaloviruses (CMV), HCMV cannot be studied experimentally in animal models.

In vivo studies of HCMV are generally modeled using murine cytomegalovirus (MCMV) in mice (Shellam et al., 2007). The most commonly used MCMV strain is the Smith strain, which has been maintained by either salivary gland passage in laboratory mice or by passage in cultured mouse embryonic fibroblast or fibroblastic cell lines (Shellam et al., 2007). It is possible that the Smith strain has acquired significant genetic and biological differences from early passages of the Smith isolate (Smith et al., 2008). Therefore, there might be doubts on the relevancy of using this strain in infection models for giving extrapolations to HCMV infections in humans. For HCMV it has been demonstrated that extensively passaged laboratory strains show significant biological differences compared to low-passage clinical isolates (Prichard et al., 2001). Therefore, the use of low-passage isolates is necessary and measures should be taken to avoid adaptation.

In addition, the pathogenesis of MCMV has been studied using a variety of routes of inoculation (Shellam et al., 2007), e.g. intraperitoneal, intranasal (with anesthesia), footpad. So far, the question how MCMV replicates in vivo via a natural infection route has remained unanswered. Infections requiring injection or anesthesia cannot be considered natural. Generally, intranasal or oral route without anesthesia is widely accepted to be the natural infection routes.

Recently, we isolated a new MCMV isolate, HaNa1, from *Mus musculus*. Partial gene sequences have been submitted to GenBank (accession number: KR184668 (m06 gene), KR184669 (m033 gene), KR184670 (MCK2 gene), KR184671 (m138 gene), KR184672 (m144 gene), KR184673 (m152 gene), KT289520 (m157 gene)). Our previous study demonstrated that in vitro MCMV HaNa1 isolate grew to a

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~10-fold lower yield in comparison with the MCMV Smith strain in mouse embryonic fibroblast (MEF) and MCMV HaNa1 isolate was more cell-associated than the MCMV Smith strain (Zhang et al., 2015). In vivo MCMV HaNa1 isolate reached higher titers in the nasal mucosa and submandibular glands than MCMV Smith strain. Only the Smith strain established productive infections in the spleen, liver and kidneys of adult mice. In the present study, neonatal and adult mice were experimentally inoculated with MCMV HaNa1 isolate oronasally, and the virological outcome and antibody response were analyzed.

2. Materials and methods

2.1. Cell culture and virus

Primary BALB/c MEF were prepared by trypsin dispersion of 16–17 days old fetus from BALB/c mice and were cultured 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 50 μg/ml gentamicin). The MCMV HaNa1 isolate was originally isolated with secondary MEF from a mouse (*M. musculus*), caught alive in Belgium. Subsequently, it was passaged on secondary MEF to produce viral stocks. The second passage of HaNa1 isolate was used for inoculation.

2.2. Inoculation of mice

Specific pathogen-free (SPF) BALB/c mice were provided by Faculty of Veterinary Medicine of Ghent University. Female BALB/c mice were inoculated at the age of 3 days (neonates) or 8 weeks (adults). For the inoculation of adults, 10^5 $TCID_{50}$ MCMV HaNa1 in 100 μl PBS was pipetted into the nares (25 μl) and mouth (75 μl) without anesthesia. For the inoculation of neonatal mice, 10^5 $TCID_{50}$ MCMV HaNa1 in 10 μl PBS was pipetted into the nares (2.5 μl) and mouth (7.5 μl) without anesthesia. All animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University.

2.3. Virological analysis

At various days post inoculation (dpi), mice were sacrificed for collection of tissues and blood. Tenfold dilutions of 10% homogenates were brought onto monolayers of secondary MEF for 1 h at 37 °C and afterwards cells were washed and followed for the presence of cytopathic effect. After 7 days, the virus titer was calculated as 50% tissue culture infectious dose ($TCID_{50}$), according to the Reed and Muench formula. Peripheral blood mononuclear cells (PBMC) were isolated as previously published (Zhang et al., 2015) and cocultured with MEF monolayer to examine viremia.

2.4. Immunofluorescence staining

The detection of viral antigen positive cells in tissues was done by an immunofluorescence staining. Cryosections of 12 μm were fixed in 4% paraformaldehyde at 4 °C for 10 min, followed by permeabilization in 0.1% Triton X-100. Samples were incubated with biotinylated mouse anti-MCMV polyclonal antibodies (pAb) at 37 °C for 1 h. Then, samples were thoroughly washed and incubated with fluorescein isothiocyanate (FITC) conjugated streptavidin (Invitrogen, 1:200) at 37 °C for 1 h. After 3 further washes in PBS, the nuclei were counterstained with Hoechst. Fluorescence was visualized using a Leica TCS SP2 confocal microscope and analyzed with ImageJ.

MCMV antigen-positive cells were quantified with the Leica TCS SP2 confocal microscope (magnification 200×) according to the previous quantification method (Zhang et al., 2015). Twenty consecutive sections of 12 μm were analyzed per organ. The size of the sections was determined by the number and size of the visual fields at 200× magnification. Finally, the number of MCMV antigen-positive cells was calculated as an average value per 10 mm^2, independent of their localization and distribution within the section.

2.5. Double immunofluorescence staining

Double immunofluorescence staining was used to identify MCMV-infected cells in the nasal mucosa and NALT. Sections were incubated with biotinylated mouse anti-MCMV polyclonal antibodies (pAb) and cell markers (goat anti-olfactory marker protein (OMP) for olfactory neurons, 1:500 (Wako); rabbit polyclonal anti-cytokeratin-18 for epithelia, 1:150 (Abcam); rat anti-mouse CD68 for tissue macrophages, 1:50 (eBioscience) and hamster anti-mouse CD11c for dendritic cells, 1:50 (eBioscience)) at 37 °C for 1 h. The sections were washed three times with PBS and incubated at 37 °C for 1 h with the corresponding secondary antibodies: streptavidin-Texas Red-X or FITC conjugate, 1:200 (Invitrogen); FITC-goat-anti-rabbit IgG, 1:200 (Invitrogen); Alexa Fluor® 594-rabbit-anti-goat IgG, 1:200 (Invitrogen); Alexa Fluor® 488-goat-anti-rat IgG, 1:200 (Invitrogen); Alexa Fluor® 488-goat-anti-hamster IgG, 1:200 (Jackson ImmunoResearch). After 3 further washes in PBS, the nuclei were counterstained with Hoechst. Fluorescence was visualized using a Leica TCS SP2 confocal microscope and analyzed with ImageJ.

2.6. MCMV-specific antibody determined by immunoperoxidase cell monolayer assay (IPMA)

The antibody titer of MCMV-specific antibodies was determined by an in-house developed IPMA as previously published (Zhang et al., 2015). Briefly, twofold dilutions of inactived plasma, starting with an initial dilution of 1:16 in PBS-Tween with 10% normal goat serum, were added to fixed monolayers of MEF infected with MCMV HaNa1 in 96-well plates and incubated at 37 °C for 1 h, followed by a thorough washing. Biotinylated rat anti-mouse IgM (eBioscience, 1:100), biotinylated sheep anti-mouse IgG (GE healthcare, 1:200), biotinylated rat anti-mouse IgG1 (eBioscience, 1:100), biotinylated rat anti-mouse IgG2a (eBioscience, 1:100), biotinylated rat anti-mouse IgG2b (Biolegend, 1:100), biotinylated goat anti-mouse IgG2c (Abcam, 1:100), or biotinylated rat anti-mouse IgG3 (Biolegend, 1:100) were added to the plates and incubated at 37 °C for 1 h. After three washes, streptavidin-biotin horseradish peroxidase complex (GE, 1:200) was added to the wells and incubated at 37 °C for 1 h. Finally, NaAc-AEC-H_2O_2 was added into each well at room temperature (RT) for 30 min and then the reaction was stopped with PBS. The IPMA titer was calculated as the reciprocal value of the highest plasma dilution that gives a visual staining of infected MEF, as determined by light microscopy.

2.7. Virus neutralization assay

Neutralizing antibody titers were determined in a virus neutralization assay. Twofold dilutions of inactivated plasma were mixed with 100 $TCID_{50}$ MCMV HaNa1 and incubated at 37 °C for 1 h. The mixture was then added to MEF monolayers and incubated at 37 °C for 1 h. The cells were then washed, overlaid with fresh medium, and incubated at 37 °C for 7 days. The neutralizing titers can be defined as the reciprocal of the highest dilution of plasma preventing 100% cytopathic effect in MEF cell cultures.