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Video Article

Use of *In vivo* Imaging to Monitor the Progression of Experimental Mouse Cytomegalovirus Infection in Neonates

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Abstract

Human Cytomegalovirus (HCMV or HHV-5) is a life-threatening pathogen in immune-compromised individuals. Upon congenital or neonatal infection, the virus can infect and replicate in the developing brain, which may induce severe neurological damage, including deafness and mental retardation. Despite the potential severity of the symptoms, the therapeutic options are limited by the unavailability of a vaccine and the absence of a specific antiviral therapy. Furthermore, a precise description of the molecular events occurring during infection of the central nervous system (CNS) is still lacking since observations mostly derive from the autopsy of infected children. Several animal models, such as rhesus macaque CMV, have been developed and provided important insights into CMV pathogenesis in the CNS. However, despite its evolutionary proximity with humans, this model was limited by the intracranial inoculation procedure used to infect the animals and consistently induce CNS infection. Furthermore, ethical considerations have promoted the development of alternative models, among which neonatal infection of newborn mice with mouse cytomegalovirus (MCMV) has recently led to significant advances. For instance, it was reported that intraperitoneal injection of MCMV to Balb/c neonates leads to infection of neurons and glial cells in specific areas of the brain. These findings suggested that experimental inoculation of mice might recapitulate the deficits induced by HCMV infection in children. Nevertheless, a dynamic analysis of MCMV infection of neonates is difficult to perform because classical methodology requires the sacrifice of a significant number of animals at different time points to analyze the viral burden and/or immune-related parameters. To circumvent this bottleneck and to enable future investigations of rare mutant animals, we applied *in vivo* imaging technology to perform a time-course analysis of the viral dissemination in the brain upon peripheral injection of a recombinant MCMV expressing luciferase to C57Bl/6 neonates.

Video Link

The video component of this article can be found at http://www.jove.com/video/50409/

Introduction

Human Cytomegalovirus (HCMV/HHV-5) is a member of the β-herpesvirus family. HCMV is a highly prevalent, opportunistic pathogen which is usually acquired during early life as an asymptomatic infection [1]. Like all herpesviruses, HCMV persists throughout the entire life of the host whose immune system tightly controls viral replication. Episodes of viral reactivation mostly occur in immunocompromised individuals such as transplant patients receiving drugs to prevent graft rejection [2]. In adults, HCMV has also been linked to glioblastomas [3]. In addition, HCMV is a prominent pathogen for newborns with immature immunity [4-6]. Primary infection in the developing fetus or neonate can have severe consequences. HCMV infection is the most common infectious cause of congenital birth defects and childhood disorders in developed countries. It is estimated that the incidence of neonatal HCMV infection affects 0.5-1% of all live births among which 5-10% will suffer from severe symptoms such as microcephaly or cerebellar hypoplasia. In addition, 10% of the infected infants with subclinical viral infection will later develop sequellae leading to mental retardation, hearing loss, visual defects or seizure and epilepsy [7,8].

As opposed to other human herpesviruses such as Herpes Simplex 1 (HSV-1/HHV-1) which can be inoculated to mice *via* different routes of injection [9], cytomegalovirus replication is species specific. This feature has severely hampered investigations of HCMV pathogenesis which are performed in different animal models (mouse, rat, guinea pig, rhesus monkey) and their respective genuine host-specific CMVs. All CMVs exhibit significant similarities in genome size and organization, tissue tropism and regulation of gene expression. They also induce similar pathologies in their respective host. Despite genomic diversity between HCMV and mouse cytomegalovirus (MCMV) (50% of the ORFs present in the human virus are identified in the murine CMV), the mouse model has recently proved to be advantageous, mostly because mutant strains can be tested for their ability to control viral replication *in vivo*. This has led to a genetic screen which enabled an estimation of the number of mouse genes expressed at the adult stage which compose the "resistome" to this virus [10]. Altogether, this indicates that MCMV-infected mice represent an attractive model for the study of host-virus interactions in adults. The exploration of congenital CMV infection is more complex because differences in placental layer organization between human and mice impair the mother-to-fetus transmission of the viral infection in mice. Recently, direct injection of MCMV in the placenta on day 12.5 of gestation has enabled brain infection of mice neonates which led

to hearing impairment [11]. However, most investigations now use intraperitoneal injection of 4-20 hr-old neonates to provide systemic viral dissemination potentially leading to hematogenous brain infection, a model which is more relevant than that of an intracranial injection. This protocol provided important insights into CMV pathogenesis and more particularly, it was demonstrated that MCMV infection of newborns results in viral replication in neuronal and glial cells located in inflammatory foci which are infiltrated with mononuclear cells like macrophages [12]. This report also described altered morphogenesis of the cerebellum accompanied with diminished granular neuron proliferation and migration and the induction of multiple interferon-stimulated genes. The essential role of CD8+T cells for the control of MCMV in the central nervous system was also reported by the same group [13]. An important aspect to consider when analyzing the pathological effect of a microbe is the dynamics of the infection. In the case of MCMV, it is particularly crucial to explore and quantify the progression of viral dissemination into the developing brain in order to understand and anticipate the magnitude of the future neurobiological injuries. Traditionally, the quantification of the progression of an infection requires the regular sacrifice of infected animals to titer the pathogen in tissues, such as the brain, which are otherwise inaccessible. This type of protocol is now challenged by necessary improvement of animal welfare and the 3Rs (Reduce, Refine, Replace) principles [14]. Using *in vivo* imaging technologies may allow a drastic reduction of the number of animals which are necessary in *in vivo* infection experiments. Here, we report and describe a time-course analysis of viral dissemination into the brain upon intraperitoneal MCMV-Luc injection to mouse neonates. Using the same animals, we tracked and monitored *in vivo* the sites of intense viral replication during a 2-week period.

Protocol

1. Preparation of Viral Suspension

1. Obtain the Smith strain of MCMV expressing Luciferase (MCMV-Luc [15]) from Ulrich Koszninowski's laboratory. In this recombinant, the Luciferase gene is inserted in the *ie2* locus of the MCMV genome.
2. To amplify MCMV-Luc, infect a murine bone marrow stromal cell line (M2-10B4, ATCC #CRL-1972) with MCMV at different multiplicity of infection (MOI, 0.001 to 1) [16]. For this, add virus to cultured cells in serum free medium at 37 °C. After one hour, remove the supernatant and replace it with DMEM supplemented with 10% fetal calf serum (FCS). Five days later, harvest the culture medium and store aliquots at -80 °C until further use.
3. Titration of the viral suspension by plaque assay.
    1. Split 3T3 cells (ATCC #CRL-1658) in a 24 well plate at 40,000 cells/well in 1 ml of DMEM supplemented with 10% FCS containing penicillin (200 IU/ml) and steptomycin (200 mg/ml) and incubate 24 hr at 37 °C.
    2. Prepare serial dilutions (10^{-1} to 10^{-8}) of an aliquot of MCMV suspension in DMEM and infect target cells (3T3) with 200 µl of dilution after removing cell culture medium. Incubate 1 hr at 37 °C. Add 1 ml of Top Medium (DMEM 3% FCS, 2% (v/v) gentamycin, 200 IU/ml penicillin, 200 mg/ml streptomycin, 8 g/L Carboxymethyl cellulose) and incubate 5 days at 37 °C.
    3. Fix the cells by adding 200 µl 10% formaldehyde (dilution of formaldehyde with water should be done in a chemical hood to avoid toxic inhalations) to each well, and incubating 6 hr at room temperature. Remove culture medium + fixator by pipetting out and add 200 µl cell staining solution (1% crystal violet (w/v) in 20% ethanol). Before use, dilute 1 part with 9 parts milli-Q water. Incubate 2 min at room temperature, wash the plate 3-4x by soaking it in water, let dry and count the number of plaques with a binocular.
4. Dilute MCMV-Luc in DMEM to obtain 50 PFUs in 50 µl and leave on ice until further use. Using higher viral inoculums induces lethality before the neonates reach 2-3 weeks of age. In our experience, 500 PFUs induce lethality in every neonate 5 to 7 days after injection.

2. Injection of Neonates

1. When neonates (4 to 20 hr-old mice) are available, manipulate the litter and the feces of the cage with gloves before carefully handling the pups to avoid contamination with "foreign" odors that would stress the mother and induce the rejection of the pups.
2. Perform an intraperitoneal injection using an insulin syringe (29 G) as shown in **Figure 1A**. Hold the neonate with the dorsal skin. With ventral side up, introduce the needle under the foreleg and gently push it subcutaneously to attain the peritoneal cavity (a little under the umbilicus). We currently inject 1% Methylene blue diluted in DPBS and monitor survival to practice the technique before performing viral infections. Since Methylene blue is clearly visible during the injection process, we recommend performing this "training" injection first to ensure proper delivery of the viral suspension in the intraperitoneal cavity.
3. Eliminate the tiny blood drops that may arise and place the neonate back into the cage.

3. *In vivo* Imaging

1. On day 7, handle the neonates with the same cautions as previously mentioned. Inject intraperitoneally a mixture of anesthetics (4 mg/ml ketamine, 0.8 mg/ml xylazine) and luciferin (0.15 mg/g body weight) in 50 µl in each animal. Average body weight of the animals is 1-2 g and the dose of anesthetics per animal is: ketamine 40 µg, xylazine 8 µg Wait 12-15 min until luciferin reaches its maximum of emission. This has to be determined beforehand and depends on the luciferin provider and the imaging system used.
2. While the pups are anesthetized, tag them for future identification and harvest a small piece of tissue that can then be used for future genotyping if mutant animals are used.
3. Place the pups in the imaging system and perform acquisition of the light emitted by the whole body. Seven days after infection, the virus has replicated into numerous copies in target organs such as liver, kidneys, lungs and salivary glands; therefore, the duration of exposure should be short. In our case, we perform the acquisition for 10 to 20 sec (**Figure 1B**). The platform in the acquisition chamber of the imaging device needs to be heated to avoid excessive cooling of the body temperature of anesthetized animals. In addition, it is helpful to obtain low resolution images of several animals within one round of acquisition and then, move up the platform closer to the camera lens in order to focus on one part of a particular animal or an isolated organ following dissection. The software must enable rapid changes of the parameters (exposure time, distance sample/lens, sensitivity), quantification of defined regions of interest and future export of snapshot images.

4. To acquire light signal from the head only, cover the body of the neonates lying laterally with a thick, dark paper that will mask the photons emitted at the level of the organs where high viral replication occurs (liver, lungs, salivary glands) and perform acquisition for 5 to 10 min (**Figure 1C**). Perform acquisition of the opposed side of the animal in the same conditions.
5. Reintroduce the pups into the cage and place a heating lamp on top to avoid excessive cooling of the anesthetized neonates. Regularly monitor post-anesthetic recovery. With the indicated dose, anesthesia currently lasts 30 min.
6. Repeat the same procedure on days 9, 11 and 14. On days 11 and 14, change the dose of anesthetics (6 mg/ml ketamine, 1.2 mg/ml xylazine).

Representative Results

A representative experiment is illustrated in **Figure 1**. Upon intraperitoneal injection of 50 PFUs of MCMV-Luc (Panel A shows a similar injection performed with Methylene blue to visualize the subcutaneous route of the needle), neonates were anesthetized and received simultaneously 0.3 mg of the luciferase substrate (Luciferin, Caliper). Fifteen minutes later, animals were placed ventral side up in the acquisition chamber of the IVIS 50 (In Vivo Imaging System, Caliper) and the light emitted by the whole animal was captured during a 10 sec exposure. Panel B shows snapshot images taken at days 7, 9, 11 and 14 with the software (Living Image 3.2, Caliper) dedicated for the analysis. The images were calibrated with the same settings: Max emission is set at 10^7 photons per second (p/sec) and Min at 10^6 p/sec. From these pictures, it appears that the overall viral titer in the entire animal decreases over time. Quantification of the luminescence performed with the same software confirms this observation (not shown). Next, we covered the entire body, except the head, of the animal with a thick, dark paper which prevents the photons emitted by organs such as lungs, liver, kidneys and salivary glands to be detected by the digital camera. This enables longer exposure (5 min in our case) and reveals a discrete spot at the level of the left ear whose intensity increases between day 7 and day 14 (Panel C). A signal also appears at the lower jaw and nose of the animal. Images were set with a Max at 2,000 p/sec and Min at 100 p/sec. This experiment, performed on the same animal between day 0 and day 14, indicates that the evolution of the viral infection can be recorded and quantified with this technology and that the dissemination of MCMV to the brain can be dynamically observed *in vivo*.

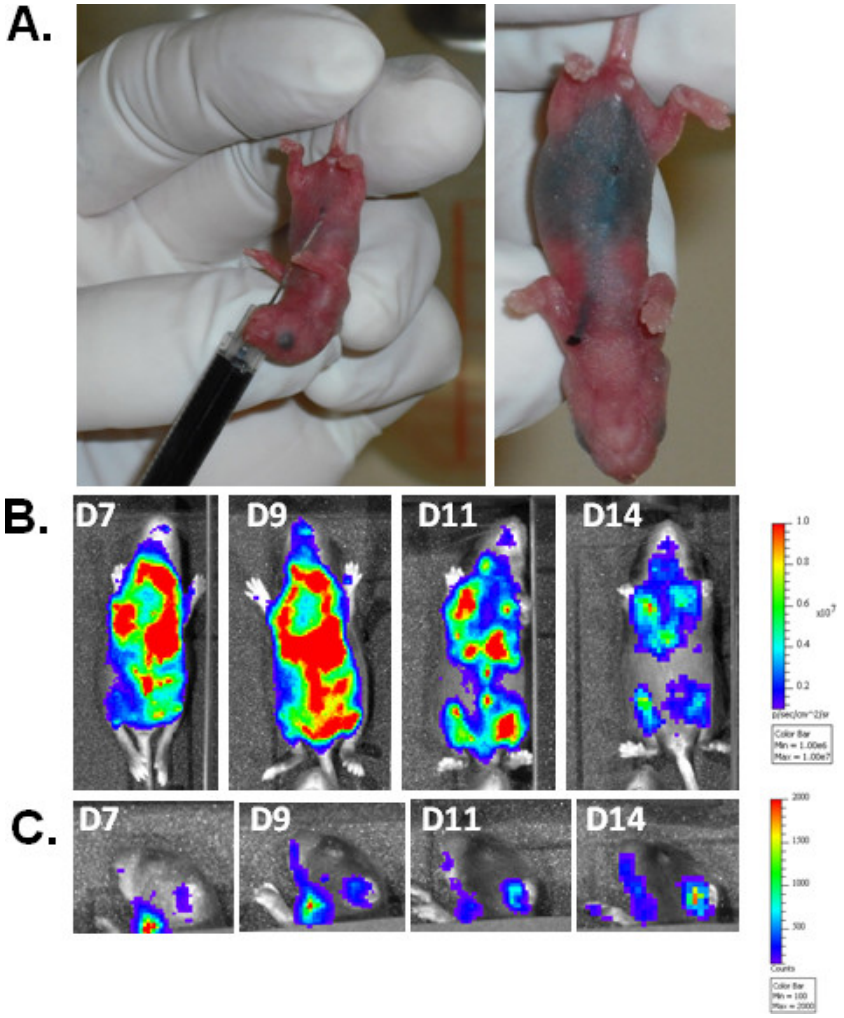

Figure 1. Time-course analysis of a representative mouse neonate experimentally infected with a luminescent cytomegalovirus. A. Intraperitoneal methylene blue injection in neonates. The magnified picture (right) shows the subcutaneous path of the injection on the thorax. **B.** *In vivo* imaging of the same neonate from day 7 to day 14 after MCMV-Luc injection. Luminescence from the whole anesthetized animal is visualized upon luciferin injection and 10 sec exposure. **C.** Luminescence emitted by the head (left side) of the same animal at days 7 to 14. Quenching the photons from the rest of the body with a dark paper enables longer exposure (5 min). Click here to view larger figure.

Discussion

Using *in vivo* imaging technology to monitor MCMV-Luc dissemination in mice neonates, we were able to observe viral spread to the brain of mutant animals, as opposed to wild-type. Further dissection of the animal and *ex vivo* imaging of the brain confirmed the presence of luminescent virus in the central nervous system. In addition, we also performed immunohistochemistry (not shown) on brain thin sections with an antibody specific for MCMV E1 early protein and observed that indeed, MCMV is present in the same areas as those in which luminescence was detected, thus indicating that macroscopic visualization of the light emitted by whole, live anesthetized animals actually reflects the characteristics of viral infection that occurs *in vivo*.

Such analysis has been widely used for adult animals, for instance to monitor tumor growth upon implantation of luminescent cells. In our case, the challenge was to use neonates for which the gaseous anesthesia with isoflurane delivered to the animal inside the acquisition chamber was not possible for technical reasons. Therefore, we were forced to use ketamine/xylazine injection and we adapted the dose to the small size of the neonates. We also handled the pups carefully to avoid the risk of rejection by their mother. By following the recommendations and doses described here, and which are critical for safe manipulation and outcome of the pups, anesthesia of neonates becomes an alternative viable option which enables long periods of immobility required to perform *in vivo* imaging.

Furthermore, because the luminescence level can be quantified with the appropriate software, we demonstrated that the progression of the infection during a 2-week period is characterized by a decreased viral load in the peritoneal organs (liver, spleen, kidneys) and the lungs, while virus spread in the brain can progressively be evidenced. This observation, which could be noticed in mutant animals and not in controls, will be documented with more details and a statistical analysis in a manuscript in preparation where the nature of the mutated gene will also be discussed. Nevertheless, our imaging method constitutes a significant improvement compared to the classical techniques which requires euthanasia of several animals for each time point, followed by time- and resource-consuming methods (plaque assay or quantitative PCR) to

measure viral titers in homogenates prepared from different organs upon dissection. This is also in line with the ethics principles governing animal experimentation which require constant effort to minimize the numbers sufficient to reach statistical significance. Finally, another advantage of following the same animal (tagged at day 7) during the entire duration of the experiment is to reduce inter-individual variations. As a result, quantification made at each time point (days 7, 9, 11 and 14) performed on a group of 6 neonates greatly improves the statistical significance of the results (data not shown).

We currently use this methodology to analyze the impact of mutations in mice on the dissemination of MCMV and a manuscript currently in preparation reports augmented viral spread in the brain of mutant animals. Our experience indicates that comparing a group of 5-6 mutants to an equivalent group of control neonates provides high-quality and significant data. We do not, however, consider this methodology suitable for the screening of phenodeviants generated by a random mutagenesis program.

Disclosures

The authors declare no competing financial interest.

Ethics statement

Animals were maintained under pathogen-free conditions in the animal care facility of the Institut d'Immunologie et d'Hématologie. Handling of mice and experimental procedures were conducted in accordance with the French Law for the Protection of Laboratory Animals. The procedure was approved by the service véterinaire de la Préfecture du Bas-Rhin (France) under the authorization number A-67-345.

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