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Cytomegalovirus Infection: Mouse Model

Ilija Brizić[1,#], **Berislav Lisnić**[1,#], **Wolfram Brune**[2], **Hartmut Hengel**[3], and **Stipan Jonjić**[1]

[1]Department of histology and embryology and Center for proteomics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

[2]Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

[3]Institute of Virology, Medical Center – University of Freiburg, and Faculty of Medicine, University of Freiburg Freiburg, Germany

Abstract

This unit describes procedures for infecting newborn and adult mice with murine cytomegalovirus (MCMV). Methods are included for propagating MCMV in cell cultures and for preparing a more virulent form of MCMV from salivary glands of infected mice. A plaque assay is provided for determining MCMV titers of infected tissues or virus stocks. Also, a method is described for preparing the murine embryonic fibroblasts used for propagating MCMV and for the plaque assay.

Keywords

mice; cytomegalovirus infections/virology; disease models; animal; virus replication; viral plaque assay/methods; viral load; salivary gland/virology; mouse cytomegalovirus/isolation & purification; herpesviridae infections/virology; fibroblasts/virology; MCMV

Introduction

Cytomegaloviruses (CMVs) are prototypes of the β subfamily of herpesviruses. Their large DNA genomes (~230 kb) contain more than 200 annotated open reading frames (ORFs) which, together with an emerging understanding of highly complex patterns of gene expression (Stern-Ginossar et al., 2012), places CMVs among the mammalian viruses with the largest coding capacity. Although a high degree of species specificity is characteristic for cytomegaloviruses, viruses with a similar genomic structure and pathobiology can be found in virtually all mammalian species analyzed. In vivo, CMVs infect a broad range of cells and tissues including fibroblasts, smooth muscle cells, endothelial and epithelial cells, stromal cells, and myeloid cells. As for herpesviruses in general, these viruses are not eliminated from an organism following primary infection. Instead, the virus persists in its host in a state of latency, where the full genome but no infectious progeny are detectable. Reactivation of latency occurs frequently and results in virus shedding and occasionally recurrent disease

Corresponding author: Prof. Stipan Jonjic, stipan.jonjic@medri.uniri.hr, tel: 00385 51 651 170, fax: 00385 51 651 176.
[#]these authors contributed equally to this work

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Methods for the construction of recombinant human and murine CMV, and for investigation of different aspects of CMV pathogenesis

(Britt and Alford, 1996). The manifestation of the primary infection, as well as frequency and severity of recurrent disease, are controlled by the immune system. Thus, CMVs have proved to be valuable tools for studying virus-host interactions and multiple aspects of immunological control of infection.

The strict species specificity of CMVs precludes experimental infection of animals with human cytomegalovirus (HCMV). Therefore, rodent (e.g., mouse, rat, and guinea pig) and rhesus cytomegaloviruses have been used as model systems to understand HCMV disease and to answer basic questions that cannot easily be addressed in clinical research. Among these, murine cytomegalovirus (MCMV) is by far the best studied. MCMV as a model for HCMV infection has played an immense role in the discovery of numerous immune-evasive genes and elucidation of the pathogenesis of CMV infection.

This unit describes procedures for infecting newborn and adult mice with MCMV (Basic Protocol). Methods are included for propagating MCMV in cell cultures (Support Protocol 1) and for preparing a more virulent form of MCMV from salivary glands of infected mice (Support Protocol 2). Support Protocols 3 and 4 describe plaque assays for measuring MCMV titers of infected tissues or virus stocks. Finally, Support Protocol 5 describes a method for preparing the murine embryonic fibroblasts used for propagating MCMV and for determining MCMV titers. The protocols should serve as a starting point for immunologists and virologists interested in the immune control of CMV infection and other aspects of virus-host interactions. References to more specialized virological and immunological procedures are given.

NOTE: MCMV is not a hazardous agent as it does not infect humans. Growth and purification of MCMV in vitro can be performed in any cell culture laboratory with basic equipment including a laminar flow hood, a microscope, and a cell culture incubator. This is especially true if permissive fibroblast cell lines are used instead of primary murine embryonic fibroblasts (MEFs). Productive infection of fibroblasts results in cell rounding, plaque formation, and finally cell death, which can be followed by microscopic inspection. Analysis of MCMV infection and dissemination in vivo, by contrast, requires at least good basic experience in animal handling.

NOTE: All cell culture procedures and preparation of virus and cells must be performed under sterile conditions in a laminar flow hood. Unless specified otherwise, cells and virus are grown at 37°C in a humidified atmosphere with 5% CO_2.

Basic Protocol

Infection of Mice with Cytomegalovirus

To study CMV infection in mice, newborn or adult animals are infected with either cell culture–or salivary gland-derived MCMV. Different routes of infection are commonly employed: intraperitoneal, subcutaneous (most commonly footpad), or intravenous injection. Although other routes might more closely reflect a natural infection, such as intranasal or oral routes of infection, they are more difficult to control or relatively inefficient (Tan, Frederico, & Stevenson, 2014; Wu et al., 2011).

Materials

Mice: C57BL/6, BALB/c or other strain (newborn or adult)

Purified cell culture-derived murine cytomegalovirus (MCMV; see Support Protocol 1) or salivary gland-derived virus (SGV, see Support Protocol 2)

PBS (APPENDIX 2A)

DMEM (APPENDIX 2A)

Complete medium (see recipe)

Stainless steel wire mesh (0.45-mm grid size)

70-100 μm Cell Strainer

Sterile Syringe Plunger

Petri dish (60 mm × 15 mm)

Metal beads (*optional step*) – *stainless steel, diameter: 5 mm*

Bead homogenizer *(optional step) – e.g. Mill Mix 20 bead beater, speed up to 20 Hz, with 2 adaptors for 2 mL Eppendorf tubes (12×2 mL each)*

Additional reagents and equipment for parenteral injection (*unit 1.6*), euthanasia (*unit 1.8*), removal of lymphoid organs (*unit 1.9*), removal of salivary glands (unit 1.11), and determining viral titer in tissues (see Support Protocol 3)

1a. *Infection of newborn mice*: Inject newborn mice intraperitoneally with 100-500 PFU of purified MCMV in a volume of 50 μL of PBS or DMEM.

Intraperitoneal injection of newborn mice should not be performed directly through the abdominal wall. To prevent the return of injected fluid, the needle should be inserted in the thoracic region and passed under the skin before it enters the peritoneal cavity. Take special care to avoid puncturing liver.

Newborn mice get sick after infection with as little as 100 PFU of cell culture-derived virus. Infection of newborns with 1,000 PFU results in severe disease (runting, hair loss, general failure to thrive) and very high mortality rates. Due to variations in salivary gland virus preparations and its higher virulence, cell culture-derived MCMV is recommended for infection of newborn mice. When infected with lethal dose, newborn mice start to die around day 10 post infection.

Newborn mice of different strains vary in their susceptibility to MCMV. For instance, unlike adult mice, newborn BALB/c mice are more resistant to MCMV than C57BL/6. Therefore, we recommend to use a higher dose of 400 PFU for infection of BALB/c newborn mice, whereas lower dose (100 PFU) should be used for C57BL/6 newborns. The susceptibility of newborns of other mouse strains should experimentally be determined.

1b. *Infection of adult mice*: Inject adult mice intravenously, subcutaneously (hind footpad), or intraperitoneally (*unit 1.6*) with 1-5 × 10^5 PFU of purified MCMV.

Adult immunocompetent mice usually do not get sick after infection with 100,000 PFU of cell culture–derived virus.

SGV is much more virulent, with an LD_{50} for adult BALB/c mice at ~100,000 PFU. Mice infected with this dose will all develop severe disease with prominent hepatitis. For non-lethal experiments dose of ~10,000 PFU is recommended for BALB/c mice. SGV should be injected intraperitoneally.

2. Euthanize animals (*unit 1.8*) on various time points post infection and collect organs of interest (e.g., salivary glands, spleen, liver; *unit 1.9 and UNIT 1.11*). Freeze whole organs individually at -20°C or -80°C in 1.5 mL microcentrifuge tubes containing 1 mL of complete medium (see recipe) for titration.

Organs can be frozen for months at -20°C or -80°C.

If pieces of organs are frozen they should be weighed and titer should be expressed per gram of tissue.

Selection of time points for collection of organs depends on the experimental setup. For example, innate cytokines responses peak at 36 h after infection, NK cell response 2-4 days, and $CD8^+$ T cell response 7-14 days after infection.

3. Thaw organs and homogenize them by passing them through a 0.45-mm stainless steel wire mesh (salivary glands, lungs) or through a 70-100 μm cell strainer (liver, spleen). Use the plunger of a syringe (or a similar sterile device) to squeeze the organ tissue through the mesh, collecting the homogenized material in a small petri dish.

Alternatively, metal beads can be added to the organs in 1.5 mL microcentrifuge tubes and homogenized in bead homogenizer. The settings for organ homogenization should be adjusted according to particular instrument used, as well as the organ and tissue being processed.

4. Rinse the mesh with 2 mL complete medium to recover residual material in the petri dish.

If organs are mashed in bead homogenizer, organs will be in 1 mL complete medium.

5. Immediately determine MCMV titer in organ homogenate dilutions using the plaque- assay (see Support Protocol 3).

NOTE: Organ homogenates can be toxic for cultured cells especially when used at low dilutions. This is most frequently observed for liver, pancreas, and salivary gland homogenates. Therefore, it might be necessary to start titration at a higher dilution (e.g., 1:2000) for these organs.

Virus titers in different organs, most commonly spleen, lungs, liver and salivary glands are determined at different time points after infection, depending on the aspect of CMV biology under study. While titers in spleen, liver, and lungs peak within two weeks after infection, titers in the salivary glands increase with delayed kinetics as compared to visceral organs.

Support Protocol 1

Preparation of MCMV Stocks

One of the most prominent characteristics of MCMV is its ability to infect a wide range of different cell types (Shellam, Redwood, Smith, & Gorman, 2007). Nonetheless, due to their high susceptibility to MCMV infection, growth properties and appearance of plaques, primary MEFs are still the cells of choice for the production of virus stocks, plaque assays and detection of latent virus. As an alternative to primary MEFs, immortalized cell lines, such as NIH 3T3, Balb/c 3T3 or M2-10B4 (Lutarewych et al., 1997) can also be used for propagation of MCMV. However, NIH 3T3 and Balb/c 3T3 cells grow very fast and it can be difficult to discern virus plaques from surrounding cells due to their rapid growth. Therefore, unlike MEFs, cultures of NIH 3T3 cells should be infected with MCMV when they reach 10% to 50% confluence. In addition to immortalized cell lines, adherent mouse newborn cells (MNCs) have recently been suggested as a comparable, if not preferable, substitution for primary MEFs (Le-Trilling & Trilling, 2017). This protocol describes a method suitable for preparation of any wild type or recombinant MCMV stock using primary MEFs, or other suitable cells described above, as targets for infection.

Materials

Murine embryonic fibroblasts (MEFs; see Support Protocol 4) or other suitable cells (e.g., NIH 3T3, ATCC #CRL-1658; BALB/c 3T3, ATCC #CCL-163; M2-10B4, ATCC #CRL-1972; or MNC - mouse newborn cells)

Complete medium (see recipe)

Murine cytomegalovirus (MCMV): Smith (ATCC #VR-194, VR-1399) or any other strain

15% (w/v) sucrose cushion (see recipe)

Six 15-cm cell culture dishes (150 mm × 20 mm, sterile, non-pyrogenic, free of DNAse, RNAse and human DNA)

Disposable cell scraper

Phosphate-buffered saline (PBS; APPENDIX 2A)

Benchtop centrifuge with a rotor capable of holding multiple 50-mL centrifuge tubes and delivering up to 2000 × *g*

50-mL, 30 × 115 mm, blue cap, conical centrifuge tubes

Ultracentrifuge with a swing-out or fixed-angle rotor capable of holding 30-mL ultracentrifuge tubes and delivering up to 70000 × *g* at +4°C

30-mL polycarbonate (PC) Oak Ridge ultracentrifuge tubes, or equivalent capable of withstanding sustained 70000 × *g* at +4°C

Ultrasonic Bath Sonicator

1. Seed each of the six 15-cm cell culture dishes with ~4.8 × 10^6 primary MEF cells per dish in a complete medium and grow cells until the cell monolayer reaches ~80-90% confluence (~1.1 × 10^7 cells/dish). See Support Protocol 4 for preparation of primary MEF.

   Cells should reach target confluence in 2 to 3 days after seeding. To ensure consistent quality and high titers, cells used for the production of viral stocks are usually passaged no more than two-three times before infection.

2. For each dish, prepare the MCMV inoculum by combining ~1.1 × 10^5 PFU of MCMV with 6 mL of ice-cold complete medium in a 50-mL tube on ice and mix well.

   When crude viral preparations of unknown titers, such as unpurified supernatants of infected cells, are used as a starting material for virus production, combine 50-100 μL of such preparation with 6 mL of complete medium to obtain an inoculum for infection.

3. Remove the medium from MEF monolayers and infect cells by adding 6 mL of the MCMV inoculum from the previous step to each dish. Since each dish contains ~1.1 × 10^7 cells and 6 mL of virus inoculum contain ~1.1 × 10^5 PFU of MCMV, cells are infected at an MOI of ~0.01 PFU/cell.

   The layer of virus inoculum over the target cells should be made as thin as possible to completely cover the cell monolayer, without exposing the cells to desiccation.

4. Incubate the dishes at 37°C in a 5% CO_2 incubator for 4-6 hours and tilt the dishes carefully every 30 min to ensure even distribution of virus inoculum and to prevent desiccation of cells.

5. To each plate add 14-24 mL of complete medium prewarmed to 37°C and incubate the dishes for 3-5 days at 37°C in a 5% CO_2 incubator, until all of the cells in a monolayer exhibit a cytopathic effect.

   The highest titers are obtained if the virus is harvested when all cells in a monolayer are completely infected. Therefore, the progress of infection should be monitored daily and can be considered complete when all cells exhibit a visible cytopathic effect (rounding) and start detaching from the plate.

6. Collect all cells and supernatants from each plate into sterile, pre-chilled 50-mL centrifuge tubes on ice. To prevent tryptic digestion of surface virion proteins, use a cell-scraper, and not trypsin, to detach any adherent cells.

7. *Optional*: If the aim of the investigator is to produce only a crude (unpurified) viral stock, prepare 1-5 mL aliquots of the suspension from Step 6, release the cell-associated virus by performing 2-3 freeze/thaw cycles (freeze at -80°C/thaw at 37°C in a water bath) and store the crude virus suspension at -80°C. Otherwise, if the aim is the production of a purified virus, skip this step and proceed directly with step 8 of the protocol.

   Unpurified virus usually has a titer in the range of 2×10^6 pfu/mL. Generally, repeated freezing and thawing at this step should not affect the viral titers significantly, while at the same time it will reduce the percentage of multi-capsid virions.

8. Separate the virion-containing supernatant from cell debris by centrifugation for 30 min at 2000 × *g* and +4°C.

   Supernatant from this step can also be used as a crude (unpurified) virus stock and stored at -80°C.

   Starting from here and in all subsequent steps, the samples should be kept on ice or at +4°C.

9. Transfer the supernatants into sterile, pre-chilled 30 mL ultracentrifugation tubes and pellet the virus by centrifugation for 90 min at 50000 × *g* and +4°C.

   Take special care about fill capacity of the ultracentrifuge tubes used in this step. Do not fill the tubes beyond the rated volumes specified by the manufacturer, since unsupported fill volumes may lead to leakage, deform the tubes and/or damage the centrifuge.

   30 mL PC ultracentrifugation tubes used in our lab have a maximum fill volume of 23 mL. Typically, we divide the 180 mL of supernatant from step 8 into nine ultracentrifugation tubes, filling each tube with 20 mL of virus-containing supernatant.

10. Decant the supernatant and leave the ultracentrifuge tubes with the pellet, and a small amount (~300 μL) of residual medium covering the pellet, on ice in a cold room at +4°C overnight.

11. Using a 1000 μL micropipette, thoroughly resuspend the virus pellet in each tube with the residual medium, pool all resuspended pellets and place the pooled virus suspension on ice.

    Depending on the number of ultracentrifugation tubes and volumes of residual medium left covering the pellet in step 10, pooled virus suspension will regularly have an approximate volume between 2.8 mL and 4.5 mL.

12. To each of the two new sterile ultracentrifuge tubes on ice add 15 - 18 mL of cold 15% sucrose cushion, slowly layer equal amounts of virus suspension from the previous step on top of the cushion in each tube and centrifuge for 90 min at 70000 × *g* and +4°C.

Again, to prevent accidents and damage to equipment, make sure that the total volume of sucrose cushion and layered virus suspension corresponds to fill volumes recommended by the manufacturer of ultracentrifugation tubes.

13. Carefully decant the supernatant and remove any traces of the remaining liquid by aspiration. Then add 300 µL of ice-cold PBS to the viral pellet in each tube and leave the tubes on ice in a cold room at +4°C overnight, while making sure that the PBS completely covers viral pellet.

To facilitate resuspension and prevent aggregation of virus particles, it is imperative to use PBS lacking Mg^{2+} and Ca^{2+} at this step.

14. Thoroughly, but gently, resuspend and pool pellets from each tube into cold Eppendorf tube on ice.

15. To dissociate microscopic virus clumps, sonicate the virus suspension on ice, using a frequency of 20 kHz and three 10 second pulses of 150 watts each.

Ultrasonication has been shown to slightly increase the infectivity of MCMV preparations.

16. Homogenize the virus suspension by pipetting up and down several times on the ice.

17. Divide the viral suspension into 25-µL aliquots and store at -80°C (stable for years).

If properly executed, production of MCMV from six 15-cm cell culture dishes according to this protocol should yield 600 µL of virus suspension with an approximate titer of 1-5 × 10^8 PFU/mL for a wild type MCMV.

18. Thaw one aliquot and determine the virus titer as described in the Support Protocol 3.

Support Protocol 2

Preparation of Salivary Gland Virus

Salivary glands are the most important site for horizontal spread of the MCMV. Virus produced in salivary glands (SGV) is much more virulent than virus produced in other organs or in cultured cells (Osborn & Walker, 1971). The high virulence is due to an uncharacterized epigenetic change in the virus that occurs in salivary glands (Chong & Mims, 1981). Therefore, for lethal dose experiments, the more virulent salivary gland-derived virus is frequently used. However, although useful in certain instances, one should bear in mind that experiments using lethal dose of SGV are not an accurate representation of a natural infection. SGV is prepared by serial passage of salivary gland homogenates in 4- to 6-week-old infected BALB/c mice (or other strain).

Materials (also see Basic Protocol)

BALB/c female mice (4 to 6 weeks old)

PBS (APPENDI 2)

Dounce tissue homogenizer with tight fitting pestle (2 mL to 15 mL size, depending on the number of salivary glands collected)

Prepare initial SGV

1. Initiate the first passage by footpad injection of 2×10^5 PFU of cell culture–derived virus (see Support Protocol 1) into 4- to 6-week-old BALB/c mice.
2. After 2 weeks, euthanize animals (*unit 1.8*) and collect salivary glands (*UNIT 1.11*), pooling the parotid, greater sublingual, and mandibular glands.

   Glands can be stored for months at –80°C.
3. Prepare an organ homogenate (3 mL per mouse) as described (see Basic Protocol, steps 3 and 4), but use PBS instead of complete medium. Keep material cooled (on ice) throughout the entire procedure.
4. Remove the tissue debris by centrifugation for 5 min at 800 × *g* at 4 °C, and freeze aliquots of the supernatant at -80 °C.

Passage virus in mice

5. Determine the virus titer in the salivary gland homogenate (see Support Protocol 3) and infect new 4- to 6-week-old BALB/c mice with 5×10^4 PFU by the intraperitoneal route.

   Salivary gland homogenates can be toxic for cultured cells especially when used at low dilutions. Therefore, it might be necessary to start titration at a higher dilution (e.g., 1/2000).

6. Repeat steps 2 through 4 for a total of at least three passages in mice prior to stock production.

Prepare SGV stock

7. Isolate salivary glands and prepare a homogenate from third-passage (or higher) mice.

   The desired yield is 2 mL per mouse used.

8. Dounce the homogenate on ice 20 times using prechilled Dounce tissue homogenizer.

9. Pellet debris by centrifuging 5 min at 800 × *g*, 4°C.

10. Transfer supernatant to a separate tube, resuspend pellet with a small volume (3 to 5 ml) of PBS, and dounce and centrifuge again.

11. Pool supernatants and freeze as 250- to 500-µl aliquots at −80°C (stable for years).

12. Thaw one small aliquot and determine the virus titer (see Support Protocol 3).

Support Protocol 3

Determination of MCMV Titer Using Plaque Assay

Measurement of virus titers in organ homogenates or stock preparations of MCMV is easily achieved by performing a simple plaque assay. This protocol uses murine embryonic fibroblasts as target cells to quantitate titers of MCMV in samples of virus-containing solutions. However, other suitable cell lines, listed in the *Materials* section below, can also be used as target cells for plaque assay. Virus titers are expressed as PFU/mL or PFU/organ.

Polymerase chain reaction (PCR), as well as quantitative PCR, can be used for detection and quantification of MCMV instead of standard plaque assay, or in cases when the determination of viral titers by plaque assay is deemed cumbersome and/or impractical (Abecassis et al., 1993; Farrell et al., 2017; Kamimura & Lanier, 2014; Lemmermann et al., 2015). Both methods have also been used to investigate sites of latency (Farrell et al., 2017; Griessl et al., 2017; Kamimura & Lanier, 2014; Koffron et al., 1998; Kurz et al., 1997; Pollock & Virgin, 1995; Vliegen et al., 2003)

Materials

Murine embryonic fibroblasts (MEFs; see Support Protocol 4) or other suitable cells (e.g., NIH 3T3, ATCC #CRL-1658; BALB/c 3T3, ATCC #CCL-163; M2-10B4, ATCC #CRL-1972; 10.1 fibroblasts (Harvey & Levine, 1991) or MNC - mouse newborn cells)

Test sample: organ homogenate from MCMV-infected mice (prepared in Basic Protocol), MCMV stock (unpurified or purified; prepared in Support Protocol 1), or SGV (prepared in Support Protocol 2)

Complete medium (see recipe)

Methyl cellulose nutrient overlay (see recipe)

Inverted microscope

48-well cell culture plates

Sterile 1.5 mL microcentrifuge tubes (include size)

1. The day before titration, plate primary MEF cells on 48-well plates in a complete medium at a concentration of 2×10^5 cells/well. The amount of wells that need to be seeded with MEF cells depends on the total number of serial dilutions required for titration of viral stocks or organ homogenates. In general, for titration of a single viral stock solution, 30 wells on a 48-well plate need to be seeded with MEF cells (27 wells for titrating 9 serial dilutions in triplicate + 3 wells containing non-infected cells for negative control). Similarly, 15 wells should be seeded with MEF cells when titrating an organ homogenate. Incubate the plates at 37°C in a 5% CO_2 incubator overnight.

The next day, cell monolayer should be 80%-90% confluent for titration.

2. Thaw virus suspension or mouse organs homogenates on ice just before use.

3. Prepare serial dilutions of virus stock or organ homogenates on ice, as follows:

Serial dilutions of purified or crude virus stocks, ranging from 10^{-2} to 10^{-10}

a. Label nine sterile microcentrifuge tubes 2 to 10 and add 990 μL of complete medium to tube labeled 2 and 900 μL of complete medium to tubes labeled 3-10.

b. Prepare a 10^{-2} dilution of the virus stock by adding 10 μL of virus stock to 990 μL of complete medium in tube 2 and mix thoroughly by pipetting.

c. Using a new pipette tip, prepare a 10^{-3} dilution by transferring 100 μL from tube 2 to 900 μL of complete medium in tube 3 and mix thoroughly by pipetting. Continue making subsequent 10-fold dilutions by changing tips, transferring 100 μL of previous dilution to succeeding microcentrifuge tubes and mixing.

Serial dilutions of organ homogenates, ranging from 10^{-1} to 10^{-4}

a. Label four sterile microcentrifuge tubes 1 to 4 and to each tube add 900 μL of complete medium.

b. Prepare a 10^{-1} dilution of the organ homogenate by adding 100 μL of the organ homogenate to 900 μL of complete medium in tube 1 and mix thoroughly by pipetting. Continue making subsequent 10-fold dilutions by changing tips, transferring 100 μL of previous dilution to complete medium in remaining microcentrifuge tubes and mixing.

The extent to which organ homogenates or virus stocks should be diluted depends on the properties of the particular virus and mice strain being used, as well as on the organ for which the viral titers are being determined. For example, when mice are infected with SGV, up to 10^{-8} dilution of the organ homogenate should be prepared for determination of viral titers. Therefore, other serial dilutions in addition to the ones described above can be prepared when necessitated by the experimental setup.

4. Remove the medium from previously prepared MEF monolayers in 48-well cell culture plates (from step 1) by aspiration. Do not tilt the plates, so that a small amount of medium remains in the wells. Add 100 μL of each serial dilution to three wells on a 48-well plate.

The sensitivity of the assay can be enhanced ~25 fold (range 10 to 50) by centrifuging the plates at 1000 × g for 30 min after addition of virus. Centrifugal enhancement of virus infection was analyzed in detail by Hudson (1988).

5. Incubate the plates 1 hr at 37°C in a CO_2 incubator.

   During this step, the virus attaches to target cells. Double-check that the plates are placed in a horizontal position.

6. Add 500 µL to 1000 µL of the methyl-cellulose nutrient overlay to each well and incubate the plates in a CO_2 incubator at 37°C for 3-4 days.

   The methyl-cellulose nutrient overlay is a highly viscous medium which restricts the spread of a progeny virus only to cells near the initial site of infection. In this way, plaques can be formed. Due to its high viscosity, the methyl-cellulose nutrient overlay cannot be dispensed with a micropipette and should instead be poured out of a sterile graduated cylinder.

   Note that one pfu corresponds to ~500 viral genomes. This is in part due to the fact that virus preparations contain multicapsid virions as well as defective or non-infectious particles (Kurz et al., 1997).

7. Count the number of plaques in each well using an inverted microscope and calculate the number of plaque forming units per milliliter of original virus stock suspension by multiplying the number of counted plaques in each well with the dilution factor of the corresponding serial dilution used to infect cells in the well.

Support Protocol 4

Preparation of Primary Murine Embryonic Fibroblasts

Due to their high productivity, murine embryonic fibroblasts (MEFs) have traditionally been used for propagation of MCMV in cell culture. Although alternatives are available (e.g., immortalized fibroblast cell lines), MEFs still represent the gold standard for virus propagation and titration.

Materials

Pregnant mouse

PBS without calcium and magnesium (APPENDIX 2)

Trypsin solution: 0.25% (w/v) trypsin/1 mM EDTA in PBS

Complete medium (see recipe)

DMEM (Appendix 2A)

Sterile surgical instruments: scissors, scalpel, forceps

2- to 4-mm sterile glass beads

Sterile gauze

Stainless-steel wire mesh (0.45-mm grid size)

15-cm cell culture dishes

6-cm cell culture dishes

50 mL centrifuge tube

Additional reagents and equipment for euthanasia (*unit 1.8*) and counting cells (APPENDIX 3B)

1. Euthanize pregnant mouse at day 15 to 16 of gestation by cervical dislocation (*unit 1.8*). Surgically remove mouse embryos. Wash embryos in sterile PBS and remove placenta and tissues around embryos on sterile gauze. Wash embryos in sterile PBS. Try to remove as much embryonic liver and intestine as possible. Wash embryos in sterile PBS.

   The strain of mouse used is not important.

2. Mince embryos in DMEM in a petri dish using scissors or a scalpel. Rinse tissue fragments with PBS to remove erythrocytes and cell debris.

   Rinsing can also be done on a stainless-steel wire mesh.

3. Transfer tissue fragments to a 100-ml Erlenmeyer flask. For each 5 to 8 embryos (one litter), add 15 mL trypsin/EDTA solution, a magnetic stirrer, and enough 2- to 4-mm sterile glass beads to cover flask bottom with one to two layers. Stir slowly at 37°C for 30 min.

   If stirring is performed at room temperature, allow more time (~1 hr) for tryptic digestion.

4. Add 15 mL trypsin/EDTA solution to the tissue fragments. Stir 30 min at 37°C. Repeat this procedure once more (total three trypsin additions/incubations).

5. Filter cell suspension (45 ml) through a few layers of sterile gauze or a 0.45-mm stainless steel wire mesh into 6-cm cell culture dish and transfer to 50 mL centrifuge tube.

6. Centrifuge suspension 10 min at 250 × *g*, room temperature. Discard supernatant and resuspend cell pellet in 50 mL complete medium.

   Alternatively, filtering and centrifugation (steps 5 and 6) can be done after each stirring step before adding more trypsin to the remaining tissue fragments. This is advisable if more than three rounds of tryptic digestion are performed in order to increase the yield of cells.

7. Determine cell concentration by trypan blue exclusion (APPENDIX 3B) and seed 1–2 × 10^7 cells per 15-cm cell culture dish. Incubate overnight at 37°C in a humidified 5% CO_2 incubator.

   Note that there will be many dead cells and debris in the cell suspension.

8. On day 2, remove medium and cell debris and add fresh medium. Continue incubation for three more days.

9. On day 5, when cells have reached confluence, freeze cells (10^6 cells per vial) or split at a 1:4 ratio.

   For virus production and virus titration, MEFs can be used up to the fourth passage. Cells can be stored frozen for years in liquid nitrogen. See APPENDIX 3g for cryopreservation methods.

Reagents and Solutions

Use distilled, deionized water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.

Complete medium

Minimal essential medium (MEM) with Earle salts, or Dulbecco's modified Eagle medium (DMEM) with:

3% (v/v) FBS, heat inactivated 1 hr at 56°C

2 mM l-glutamine

100 U/ml penicillin

0.1 mg/ml streptomycin

10 mM HEPES

Store up to 4 weeks at 4°C

For titration of organ homogenates of animals that have not been maintained in specific pathogen free conditions (SPF), add 0.2 mg/ml gentamicin and 2.5 μg/ml amphotericin B.

Sucrose cushion, 15% (w/v)

50 mM Tris·Cl, pH 7.8 (APPENDIX 2)

12 mM KCl

5 mM Na_2EDTA

15% (w/v) sucrose

Sterilize by autoclaving or filtration

Store up to 6 months at room temperature

Methyl-cellulose nutrient overlay—In a glass bottle, combine 8.8 g methyl cellulose (4000 centipoise, Sigma) with 320 mL distilled water. Stir with a large magnetic stirrer. Autoclave 30 min at 121°C, leaving the magnetic stirrer inside the bottle. Remove from autoclave while still hot and stir again until methyl cellulose has completely dissolved (usually a few hours). Add 40 mL 10× MEM or 10× DMEM, 40 mL FBS, 40,000 U penicillin, 40 mg streptomycin, and 4 mL of 1 M HEPES. Add l-glutamine and sodium bicarbonate according to the medium supplier's recommendations. Store up to 6 months at −20°C.

When titrating homogenates of mouse organs of animals that have not been maintained in specific pathogen free conditions (SPF)s, add 80 mg gentamicin and 1 mg amphotericin B.

Alternatively, another viscous medium containing a 1:1 (v/v) mixture of gum tragacanth solution (1.6% w/v, Sigma) and 2× MEM supplemented with FBS and antibiotics (as above) can be used.

Commentary

Background Information

Clinical manifestations of CMV infection: Most people contract a CMV infection during early childhood or adolescence. Seroprevalence rates range from 45% to 99% in the adult population. Infection of humans with HCMV usually causes no or only minimal symptoms in immunocompetent individuals. However, in the immunocompromised or immunologically immature individuals, primary as well as recurrent CMV infection can result in severe or even fatal disease.

HCMV is one of the most common opportunistic pathogens in immunocompromised individuals (Mocarski, Shenk, Griffiths, & Pass, 2013), and the virus-induced disease can be a consequence of primary infection, reinfection or reactivation of a latent virus. Usually, the most severe symptoms of infection occur in allogeneic stem cell transplant patients and untreated AIDS patients, while more moderate, but still dangerous, symptoms can develop in solid organ transplant patients and cancer patients who received immunosuppressive therapy. Manifestations of active HCMV infection in these patients can range from mild fever to multi-organ disease accompanied with different complications (Boeckh & Geballe, 2011). For example, the most common manifestation of infection in stem cell transplant patients is HCMV pneumonia, which often causes death, while CMV retinitis, frequently resulting in loss of sight, is a common complication in untreated AIDS patients. The affliction of other organs (e.g., central nervous system) with CMV is also seen, but mostly in the terminal stages of the disease (Britt & Alford, 1996). In addition, HCMV is a leading viral cause of congenital infections. Only 10-15% of infected infants display symptoms of infection, usually involving multiple organs (Boppana, Pass, Britt, Stagno, & Alford, 1992). Congenital HCMV infection may result in severe long-term sensorineural sequelae, most common being hearing loss, mental retardation, and microcephaly.

Early diagnosis and timely therapeutic intervention are critical for the positive outcome of CMV infection in immunocompromised individuals, whereas serologic parameters, as well as techniques for direct virus detection, are used to monitor patients at risk. Currently, the most important drug for treatment of HCMV is a nucleoside analog ganciclovir; valganciclovir (ganciclovir prodrug), foscarnet (CMV polymerase inhibitor), cidofovir (cytosine analog) and leflunomide (virion assembly inhibitor) are also often employed, and recently, letermovir (terminase inhibitor) has been approved for treatment of CMV. There is no approved HCMV vaccine.

In recent years, the HCMV infection has been associated with the development of a variety of conditions in which there is no clear evidence of viral replication. These pathologies

include tumors (most commonly glioblastoma), atherosclerotic cardiovascular diseases, Alzheimer's disease, accelerated immune senescence in older population and others.

MCMV strains: Two laboratory strains of MCMV are commonly used, strain Smith and strain K181 (Hudson, Walker, & Altamirano, 1988; M. G. Smith, 1954). The genomes of both strains have been cloned as Bacterial Artificial Chromosomes (BACs) in *E.coli* (UNIT 10.32) and BAC-derived variants of these strains are nowadays used as widely as their original, cell-propagated counterparts. Historically, the K181 strain was considered to be slightly more virulent *in vivo*. However, a single base-pair deletion in the MCK-2 gene was shown to be responsible for the attenuated salivary gland phenotype of the viruses derived from the Smith strain BAC. Restoration of the full-length MCK-2 allele resulted in an enhanced replication in salivary glands of the virus reconstituted from such a “repaired” BAC (Jordan et al., 2011). In addition, viruses derived from Smith strain BAC were recently shown to harbor a point mutation in m12 gene, which differentially affects the virulence of virus in mice in a strain-dependent manner (Aguilar et al., 2017). In summary, even if the extent to which genomes of different laboratory strains differ from the prototypical wild-type MCMV is still not known, such strains still provide the best opportunity to perform highly controlled studies of MCMV pathogenesis. For example, MCMV lacking the *m157* gene is often used as a surrogate “WT virus” in a C57BL/6 mouse strain, due to strong attenuation of the true WT virus mediated by interaction of activating NK cell receptor Ly49H with the product of the *m157* gene (Bubic et al., 2004). A series of low passage field isolate MCMV strains are also available that provide an opportunity to perform studies using a more ‘natural’ virus (Gorman et al., 2006; L. M. Smith, McWhorter, Masters, Shellam, & Redwood, 2008). In addition, amenability of MCMV to genetic manipulation has led to development of viral strains that were engineered to express various reporters like green fluorescent protein (GFP) or luciferase (Klenovsek et al., 2007; A. J. Redwood et al., 2005). Such viral strains can be used to perform non-invasive, whole-animal monitoring of the viral spread and pathogenesis and, as such, offer an alternative to classical methodology which often requires the sacrifice of experimental animals.

Investigation of MCMV infection in various mouse strains: Different inbred laboratory mouse strains show significant differences in their ability to control MCMV infection. For example, BALB/c, BALB.B, B10.D2, B10.A, and A/J mouse strains were found to be genetically more susceptible to MCMV than the moderately resistant C57BL/10 and highly resistant C57BL/6J, CBA/CaH, BALB.K, B10.BR, and Ma/MyJ strains (Lathbury, Allan, Shellam, & Scalzo, 1996; Lawson, Grundy, & Shellam, 1988). These differences are strongly associated with early immune effector mechanisms providing control of the virus. Whereas BALB/c mice control CMV infection predominantly using adaptive cytotoxic $CD8^+$ T cells (Reddehase, Mutter, Munch, Buhring, & Koszinowski, 1987; Riddell et al., 1992), the more resistant strains exhibit the high activity of natural killer cells as well (Bukowski, Warner, Dennert, & Welsh, 1985; Bukowski, Woda, & Welsh, 1984). Several genetically controlled innate host resistance mechanisms to MCMV have been identified in inbred mice, including *Cmv1*, *Cmv2*, *Cmv3*, and *Cmv4* (Alec J. Redwood, Shellam, & Smith, 2013). All these mechanisms are mediated by enhanced NK cell response. The best-

characterized example is C57BL/6 strain, which encodes the NK cell receptor Ly49H in the genetic locus *Cmv1*.

Various immunodeficient and genetically altered mouse mutants have helped to assess the contribution of different immune effector functions to the control of MCMV infection. Beside NK and $CD8^+$ T cells which play a key role in virus control, multiple layers of innate and adaptive immune response provide significant contribution to the protection against virus. Intrinsic immunity, myeloid cells and professional antigen presenting cells, ILC1, $CD4^+$ T, γδ T and B cells all provide certain level of protection. However, it is important to emphasize that vast majority of immune cell subsets exhibit redundancy during control of MCMV infection. The best example are $CD8^+$ T cells, historically considered to be essential for virus clearance. Yet, in the absence of $CD8^+$ T cells, $CD4^+$ T cells can substitute the role of $CD8^+$ T cells and provide efficient protection (Jonjic, Pavic, Lucin, Rukavina, & Koszinowski, 1990). In immunocompetent mice, systemic reactivation and recurrent disease is prevented by hierarchical and redundant immune mechanisms (Polic et al., 1998).

MCMV infection has been studied in a number of mouse mutants that reflect conditions seen in human hereditary, as well as in mice with acquired immunodeficiencies. Irradiation as well as immunosuppressive chemotherapy markedly increase the severity of different aspects of CMV disease similar to those seen in humans.

Since MCMV, in contrast to HCMV, does not cross the placenta, an experimental system in which newborn mice are infected with MCMV intraperitoneally immediately after birth, is used as a model for studying congenital infection. In addition, central nervous system (CNS) in newborn mice is at a developmental stage which is equivalent to the developmental stage of human fetus at 15 weeks of gestation, a period when HCMV infection in humans is most frequently acquired during pregnancy. Upon intraperitoneal infection of newborn mice, the virus disseminates to various tissues, including the brain where infection results in a widespread, focal, non-necrotizing encephalitis (Koontz et al., 2008). CNS pathology in infected newborn mice closely recapitulates the pathology occurring during the congenital HCMV infection, most evident in smaller cerebellum size and thicker external granular layer. Moreover, the infection causes hearing loss associated with inner ear inflammation and loss of spiral ganglia neurons. Altogether, this model provides an excellent opportunity to study pathogenesis and immunobiology of congenital CMV infection.

In recent years humanized mouse models have been developed and employed to study HCMV infection (UNIT 15.21). Several reports have provided evidence that certain aspects of immune response to HCMV infection and HCMV pathogenesis can be studied in such a model (Crawford, Streblow, Hakki, Nelson, & Caposio, 2015) Both T and B cell response to HCMV infection can be investigated, as well as certain aspects of innate immunity. In addition, by engrafting tissue of interest, pathological processes can be followed as well as HCMV latency and reactivation. Rapid development of humanized mouse model has been seen in the last decade. Therefore, it is expected that this model will become even more important not only in studies of HCMV pathogenesis, but also in increasing the translational potential of CMV research.

Analysis of virus mutants in vitro and in vivo: Initially, MCMV mutants have been generated by homologous recombination in cultured fibroblasts. Today, genomes of both wild-type and mutant MCMV strains can stably be maintained and propagated in BACs in E. coli, and BAC mutagenesis is successfully employed to introduce targeted modifications into the MCMV genome (*UNIT 10.32*). In addition to BAC mutagenesis, recently developed CRISPR/Cas9-mediated genome editing technology can also be used to generate desired CMV mutants (Bierle, Anderholm, Wang, McVoy, & Schleiss, 2016; van Diemen et al., 2016). Unlike the HCMV genome that can accumulate large scale deletions, MCMV genome appears to be more conserved during serial passaging *in vitro*. Nonetheless, point mutations causing loss of function have been identified in MCMV genome following serial *in vitro* passage, and therefore warrant special care when studying non-essential genes.

A set of MCMV mutants with large deletions (10-15 kb) of non-essential genes is readily available, enabling a rapid identification of MCMV genome regions containing genes that are involved in the observed phenotype. By constructing mutants with successively smaller deletions the identity of a specific gene responsible can be revealed. However, in some cases interpretation of the results can be complicated by the fact that certain ORFs encode multiple transcripts and/or proteins (e.g. Fig1A). Nonetheless, such an approach led to the identification of viral genes that are important for virulence and immune evasion. Strikingly, both human and murine CMV have independently evolved a plethora of immune evasion genes and it is considered that CMV's success as persistent pathogen is a direct consequence of its viral immunosubversive mechanisms. For example, CMVs are targeting nearly every aspect and both arms of the immune system (Hanley & Bollard, 2014): from cells and mechanisms of innate immunity such are interferon responses (Trilling, Le, & Hengel, 2012), programmed cell death (Handke, Krause, & Brune, 2012), myeloid cells (Brinkmann et al., 2015) and NK cells (Lisnic, Lisnic, & Jonjic, 2015) to various mechanisms targeting adaptive immune responses (Lemmermann, Bohm, Holtappels, & Reddehase, 2011).

Critical Parameters and Troubleshooting—Preparation of MEF cells is a procedure that requires some practice to obtain optimal results. Even though antibiotics are included in the culture medium, it is important to avoid contamination of embryos during removal from the uterus, mincing, and trypsinization. Excessive trypsinization results in decreased cell viability. Inadequate mincing and trypsinization, by contrast, will lead to low yields of cells.

For many purposes, permissive and permanently growing fibroblast cell lines such as NIH 3T3 (which are available in many labs) can be used instead of MEFs. Permanent cell lines are easy to handle and grow faster. On the other hand, rapid growth can also be a disadvantage of these cells. Once the cells have reached 100% confluence, they lose infectability. Furthermore, individual plaques are more difficult to visualize as growth of uninfected surrounding cells may be faster than the cell-to-cell spread of infection. For limiting dilution and titration experiments it is therefore critical to determine the optimal cell density before seeding cell culture dishes with cells (split cells at a 1:4 to 1:20 ratio on the day before use). The use of newborn calf serum (NCS) instead of FBS slows down the growth of some fibroblast cell lines. Host cell components are present in virus preparations, and therefore, it should be considered to use the virus grown on syngeneic cells for in vivo experiments.

Clearly, MEFs are also the cells of choice when determining organ titers as these cells are most susceptible to MCMV infection (when used at a low passage). Generally, organ titers of test and control animals should be determined in parallel to avoid distortion of results due to inconstant cell culture conditions.

Inadvertent contamination of cell culture dishes with MCMV is usually not a problem. If this occurs, it is easily detected by CPE and plaque formation. When different virus strains or mutants are grown at the same time, care must be taken to avoid cross-contamination of viruses.

Animals should be bred under specific-pathogen-free (SPF) conditions. When infecting mice with MCMV, it is important to use separate cages to avoid horizontal virus transmission from test to control animals.

Anticipated Results—Crude virus preparations grown on MEFs as described usually reach titers in the range of 10^6 PFU/ml. Purified virus will have a higher infectious titer due to volume reduction (~10^7 to 10^9 PFU/ml), but some infectivity is lost during the process of purification. Repeated freezing and thawing also reduces titers and should be avoided.

After infection of mice, the magnitude of organ titers depends on the virus used (strain, mutant versus wild type, cell culture–derived virus versus SGV), the susceptibility of the mouse strain, and immunosuppressive regimens. It is obvious that adequate controls should be included when performing infection experiments (e.g., comparison to wild-type virus when analyzing virus mutants, comparison to normal or wild-type mice when infecting immunosuppressed or mutant mice). An example for an in vivo experiment is depicted in Figure 19.7.1.

Time Considerations—Production and purification of a virus stock will take up to two weeks. The purification procedure itself, however, can be done in two days. Most of the time is required for the expansion of cells and virus growth.

For in vivo experiments, a carefully planned time schedule is critical. Once organs are collected and frozen, synchronous determination of organ titers should be performed at a convenient time point for the reasons outlined above.

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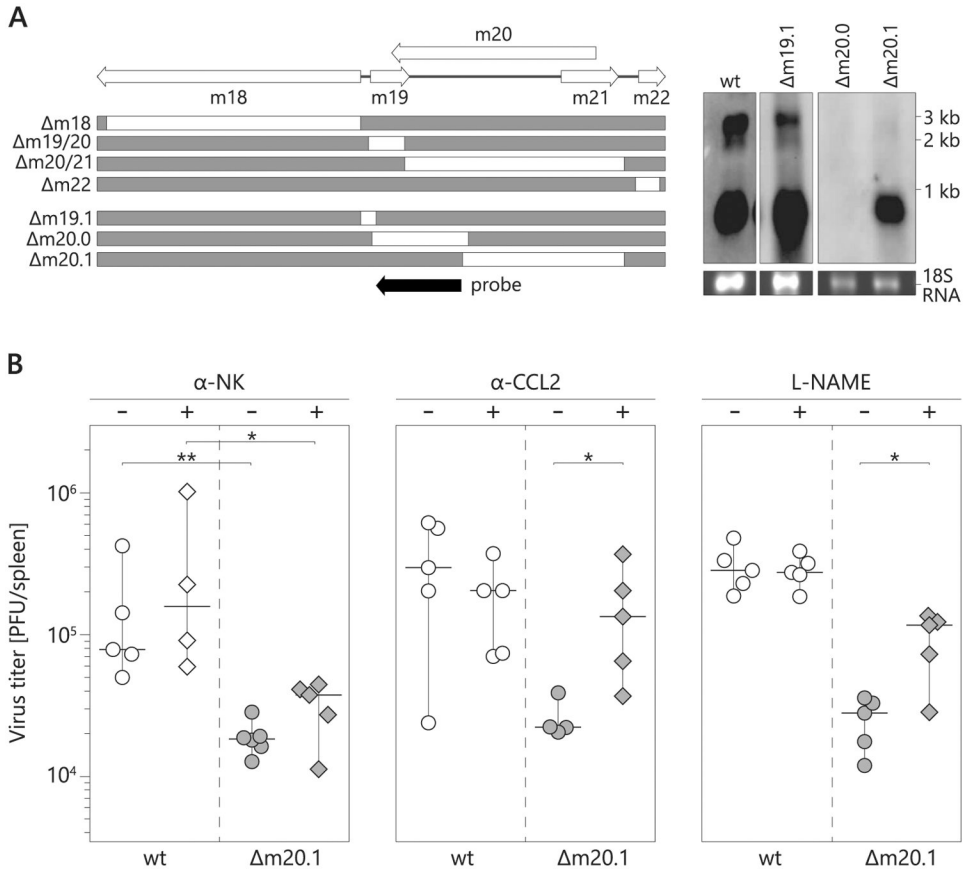

Figure 19.7.1.

A) Identification of viral regulator of PVR. By screening a library of MCMV mutants containing large deletions of non-essential genes we demonstrated that the protein responsible for PVR retention is encoded by the gene located in the m18-m22 gene region. In order to narrow down the genomic region encoding the function of interest, new recombinant viruses with individual deletions in region m18-m22 were generated as shown in the left part of the Figure 1A. White boxes represent deleted gene regions in recombinant viruses. Previously annotated ORFs are shown as white arrows. The black arrow represents the RNA probe used for the Northern blot analysis. Since ORF m20 significantly overlaps with ORFs m19 and m21, it was not feasible to construct individual deletion mutants for m19 and m21 ORFs. Transcripts were identified using the RNA probe (Figure 1A right). B) MCMV lacking ORF m20.1 is controlled in an NK cell, CCL-2 and iNOS dependent manner. BALB/c mice injected with NK depleting antibody, CCL2 blocking antibody, L-NAME or untreated mice were i.p. or i.v. njected with 2×10^5 PFU/mouse of wild-type or Δm20.1 mutant virus. Titers in organs of individual mice 4 d p.i. are shown as circles and diamonds); horizontal bars indicate the median values. Figure adapted from (Lenac Rovis et al., 2016).