Chapter 3

Modeling Asthma in Mice Using Rhinovirus Infection

J. Kelley Bentley

Abstract

Rhinovirus (RV) infection is linked to early life wheezing and exacerbation of adult asthma. RV infection can be modeled in adult and neonatal mice. This chapter outlines methods for the production of standardized human rhinovirus A1B and mouse infection. The chapter also describes methods to couple infections with allergen (ovalbumin and house dust mite) administrations. The production of the virus involves its amplification, purification, and concentration. In order to standardize the concentrated RV stock, a plaque assay on HeLa cells is outlined as a method of calibrating infectivity. Once the number of plaque-forming units is determined, the standardized virus is used for mouse infection.

Key words Rhinovirus, Mouse, Asthma, Airways, Inflammation

1 Introduction

Human rhinovirus (RV) infection is associated with early-life wheezing and later asthma development. Consistent with this, RV infection exacerbates allergen-induced airway disease in adult mice [1, 2]. More recently, we have shown that RV-A1B infection also causes an asthma-like phenotype in neonatal mice [3–5]. The mechanism by which RV-A1B can cause this in mouse pups involves activation of the lung innate immune response.

RV-A1B is a minor group virus that binds to the low-density lipoprotein (LDL) receptor in humans [6] and mice [7, 8]. Major group A and group B RVs bind ICAM-1. Because of differences in the human and mouse ICAM-1 protein, ICAM-1-binding major group A and group B RV cannot be successfully used to infect mice, although they can be grown in the ICAM-1-expressing human HeLa cell line by the methods described in this chapter. RV-C binds cadherin-related protein 3 (CDHR3). HeLa cells normally do not express CDHR3. However, when transformed with a lentiviral vector expressing human CDHR3, the resulting stable cell line becomes a good host for RV-C and has been used for large-scale

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preparation of this virus [9]. Data from our laboratory [10] show that the laboratory mouse instilled with this RV-C15 can mount an inflammatory response very much like the human response [11]. Therefore, the methods developed here are likely suitable for the production of other RVs and enteroviruses provided the HeLa cells are transformed to express the appropriate viral receptor.

The methods discussed here include obtaining, enriching, and standardizing RVA1B from H1 HeLa cells for infection of adult or neonatal mice. The chapter also describes allergen sensitization and challenge of adult mice and how to couple viral inoculations with allergen administrations.

2 Materials

2.1 Rhinovirus Preparation

1. Sterile plastic 50-mL centrifuge tubes.
2. Sterile plastic culture dishes, 10 cm and 15 cm sizes.
3. Sterile multiwall culture dishes.
4. Pipettors and micropipettors.
5. 10- and 25-mL sterile pipettes.
6. Refrigerated centrifuge capable of holding multiple 50-ml tubes for centrifugation at 1250 × g.

2.1.1 Preparation of H1 HeLa Cell Stocks

1. Complete media: Dulbecco's minimal essential media (DMEM), 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10% fetal bovine serum (FBS).
2. Trypsin–EDTA: 0.25% trypsin, 2.21 mM EDTA in DMEM.
3. Cell storage media: 6 ml of serum-free media, 2 ml of DMSO, and 2 ml of FBS. Serum-free media contain DMEM, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.
4. H1 HeLa cells (ATCC CRL-1958).
5. Sterile laminar flow hood.
6. 37 °C and 33 °C 5% CO_2 incubators.
7. 10-cm sterile cell culture dishes.
8. Hemocytometer and a microscope or an alternative cell counting device.
9. Trypan blue solution: 0.4% trypan blue dye in sterile PBS.
10. Cryotubes for cell storage.
11. Ultralow freezer (−70 °C or lower).
12. Liquid nitrogen dewar for long-term cell storage.

2.1.2 Preparation of Viral Seeding Stocks

1. Complete media: see above.
2. Infection media: DMEM, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.
3. Propagation media: DMEM, 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 3% FBS, 2 mM $MgCl_2$.
4. Rhinovirus RV-A1B (ATCC VR-1645).
5. 33 °C 5% CO_2 incubators.
6. Ultralow freezer (−70 °C or lower).

2.1.3 Large-Scale Preparation of Viral Stocks

1. Complete media (see above).
2. Infection media (see above).
3. Propagation media (see above).
4. H1 Hela Cells in a 25-cm plate, 90% confluent.
5. Rhinovirus (RV-A1B) seeding stocks.
6. 10-cm and 15-cm cell culture plates.
7. Ultralow freezer (−70 °C or lower).

2.1.4 Concentration and Purification of the Virus

1. Viral supernatant prepared as in Subheading 3.1.3.
2. CM-PBS: cell culture grade endotoxin-free PBS with Ca^{2+} and Mg^{2+}.
3. RNase A.
4. DNase 1.
5. 41-mm fiberglass pre-filter.
6. 0.45-μM cellulose acetate low-protein binding filter.
7. Ultrafiltration unit: 8400 Series Millipore Stir Ultrafiltration Cell, 400 ml.
8. 100-kDa Millipore ultrafiltration membrane.
9. Blunt-ended forceps.
10. Nitrogen gas tank with pressure regulator and tubing to attach the ultrafiltration unit to the nitrogen tank.
11. A magnetic stirring plate.
12. A refrigerated cold room (4 °C) to hold the nitrogen tank, ultrafiltration unit, and magnetic stirrer for the RV concentration.
13. Ultralow freezer (−70 °C or lower).

2.1.5 Determination of the Viral Titer by Plaque Assay

1. HeLa-H1 culture.
2. Polystyrene 6-well cell culture plates.
3. Dilution medium: CM-PBS with 0.2% FBS.

4. Top agarose: dissolve 2.5 g of low melting point SeaPlaque GTG agarose [FMC] in 50 ml of CM-PBS and sterilize. Aliquot into 6 mL volumes in sterile 50-ml conical tubes (*see* **Note 1**).
5. Supplemented medium: DMEM, 40 mM $MgCl_2$, 0.1% cell culture grade endotoxin-free bovine serum albumin (BSA). To prepare the supplemented medium, add 2 ml of sterile-filtered 1 M $MgCl_2$ and 0.5 ml of sterile-filtered 10% endotoxin-free BSA to 47.5 ml of DMEM.
6. Sterile 5-, 15-, and 50-ml polypropylene tubes with caps.
7. 500-ml glass beakers.
8. Microwave oven and/ or a stirring hot plate.
9. Side to side rocking platform set at low frequency (30 cycles per minute or less).
10. Water bath set to 44 °C.
11. Formalin solution: 3.4% formaldehyde in PBS.
12. Crystal violet solution: 1% crystal violet, 20% ethanol in water.

2.2 Sensitization and Challenge of Adult Mice with an Allergen

1. 8–12-week-old adult mice (*see* **Note 2**).
2. Ovalbumin (OVA) solution 1: 20 mg/ml of endotoxin-free OVA in PBS (prepared on the day of mouse injection). To prepare this solution, we use Imject Ovalbumin (Pierce, Rockford, Ill). Imject Ovalbumin is a sterile lyophilized OVA protein in buffered salts (PBS). Prior to injection into mice, hydrate a sterile 20 mg vial of Imject Ovalbumin with 1 mL of sterile deionized water by injecting the water directly into the sterile vial.
3. Alum adjuvant: We use Imject Alum (Pierce). Imject Alum is a sterile aqueous solution of aluminum hydroxide (40 mg/mL) and magnesium hydroxide (40 mg/mL).
4. OVA/alum mixture: Prepare fresh right before injecting into mice. Combine 0.5 ml of the OVA solution 1 with 0.5 ml of Imject Alum. Mix well by either vortexing or pipetting up and down until a stable emulsion is achieved. This will produce a 1 mg/0.1 ml OVA solution in Alum emulsion.
5. Sterile 1–3-ml syringe.
6. Sterile needles.
7. Sterile PBS.
8. Isoflurane/Forane U.S.P.
9. Anesthesia machine allowing controlled vaporization of isoflurane.
10. Pipettors and tips.

11. OVA solution 2: per single intranasal instillation, dissolve 100 μg of endotoxin-free OVA in 50 μl of PBS.
12. HDM solution: per single intranasal instillation, dissolve 100 μg of *Dermatophagoides pteronyssinus* (house dust mite/HDM) extract in 50 μl of PBS.

2.3 Instillation of Rhinovirus into Mice

1. Adult (8 weeks of age or older), neonatal (6 days of life), or juvenile (21 days of life) mice.
2. Rhinovirus of a titer at least 2×10^7 plaque-forming units per ml.
3. Sterile PBS.
4. Isoflurane/Forane U.S.P.
5. Anesthesia machine allowing controlled vaporization of isoflurane.
6. Pipettors and tips.

3 Methods

3.1 Rhinovirus Preparation

3.1.1 Preparation of H1 HeLa Cell Stocks

1. Perform all steps under the hood using a sterile technique.
2. Seed cells into a 10-cm dish with 10 ml of complete medium. To do this, rapidly thaw the cells at 37 °C if frozen. Dilute the cells (about 1 ml) into 10 ml of DMEM in a sterile centrifuge tube, sediment them for 5 min at 1000 × g, and resuspend them in complete media.
3. Pipette cells into a sterile 10-cm dish in 10 ml of complete medium and place the dish in the incubator.
4. Split cells when they achieve confluency of 70–80%. This is accomplished by aspirating the spent media off the plate, adding 2 ml of trypsin–EDTA, and dislodging the cells from the plate surface with pipette trituration.
5. Add the cells in trypsin–EDTA solution to 9 ml of serum-containing complete medium, mix, and sediment them by centrifugation for 5 min at 1000 × g.
6. Resuspend the cell pellet in a small (for example, 3 ml) volume of complete media and perform cell count.
7. Plate cells in three 10-cm dishes (5×10^5 cells/dish). These cultures will be used to prepare a viral seeding stock.
8. Excess cells should be frozen down for future use. Mix cell suspension 1–1 with cell storage media and freeze in 1 ml aliquots.

3.1.2 Preparation of Viral Seeding Stocks

1. Culture H1 HeLa cells in 10-cm plates. Grow to 90% confluence.
2. For each plate, dilute 20 μl of the ATCC RV-A1B stock with 3 ml of infection media. Because there are three plates, dilute 60 μl of virus stock to 9 ml.
3. Remove media from the plate and add 3 ml of the diluted viral suspension.
4. Gently rock the plate so that the viral suspension covers the cell monolayer.
5. Incubate for 90 min at 33 °C (*see* **Note 3**).
6. Add 7 ml of complete media to each plate. (Do not remove the viral suspension from the plate.)
7. Incubate for 24 h at 33 °C.
8. Remove media with the virus, add 10 ml of propagation medium, and incubate for 48–72 h at 33 °C. Most cells will be cytopathic, and many will float. Scrape the plate and collect the supernatant along with the floating cells.
9. Aliquot 0.5 ml into sterile microcentrifuge or cryogenic storage tubes and store at −80 °C.

3.1.3 Large-Scale Preparation of Viral Stocks

1. Day 1: seed H1 HeLa cells in twenty 15-cm dishes. See the general instructions above in Subheading 3.1.1. about seeding cells.
2. Grow the cells until they are 90% confluent.
3. Infect 10 plates with the rhinovirus seeding stock, and sham-infect 10 plates with infection media without the virus. The infection protocol is provided below (*see* **steps 4–10**).
4. Suction the spent media from H1 HeLa plates.
5. For each plate, dilute 25 μl of the rhinovirus seeding stock in 5 ml of infection media to prepare the working suspension of the virus.
6. Add 5 ml of the working suspension of the virus into each plate (add the infection medium with no virus or cell extract to the sham plates).
7. Incubate at 33 °C for 60 min.
8. Add 20 ml of the complete media to each plate. Do not remove infection media from the plate.
9. Incubate for 4–5 days at 33 °C.
10. Check plates under the microscope to look for cytopathic effect (Fig. 1). This should be visible in 2 days. When cells are completely cytopathic (generally 4–5 days, *see* **Note 4**), scrape the plate, collecting these cells in the media with the floating cells. Store in 50-ml test tubes. Freeze at −70 °C or colder (*see*

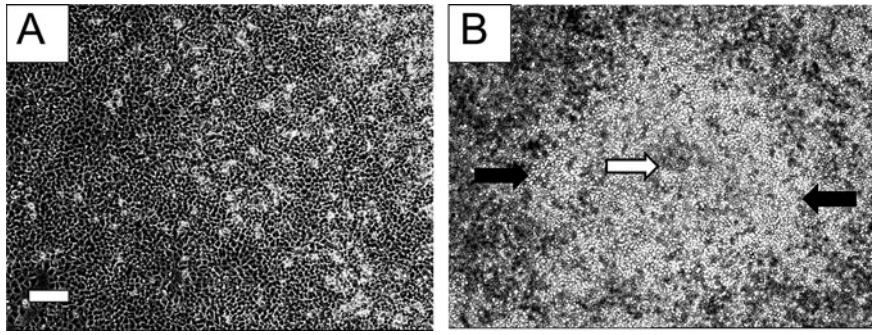

Fig. 1 Plaque formation under agarose at 96th hour of incubation. (**a**) HeLa cells treated with sham. (**b**) A single plaque at a 10^{-5} dilution of RV-A1B. HeLa cells were plated at 500,000 per well in a 6-well plate and treated on the next day with either sham cell lysate (**a**) or RV (**b**) at a variety of dilutions as described in Subheading 3.1.5. At 96 h post-treatment, cells were photographed through the agarose prior to processing for staining. (**b**) Shown is a single large plaque, with its center at the white arrow and approximate edges indicated with black arrows. Plaques are much better defined with crystal violet staining (Fig. 2). The white bar in (**a**) is 200 microns; both (**a**) and (**b**) were photographed through a 5× objective

Note 5). Sham-infected plates should be treated the same way but separately through the prep to give a comparable sham for experimental use.

3.1.4 Concentration and Purification of the Virus

1. Thaw the 50-ml tubes containing the viral supernatant or the sham culture supernatant (*see* Subheading 3.1.3, **step 10**), then freeze-thaw them twice more.
2. Centrifuge for 10 min at 1250 × g to remove cell debris.
3. Put supernatant in −80 °C freezer until purification and concentration.
4. Thaw the viral supernatant (or the sham supernatant).
5. Upon thawing, add RNAse A and DNAse 1 to a final concentration of 10 μg of RNase A per ml and 10 U (about 1 μg) of DNase1 per ml, mix well, and hold on ice until ultrafiltration.
6. In a sterile hood, open the filtration device from its package.
7. Place the pre-filter on top of the filter disc.
8. Apply vacuum and add thawed supernatant containing the virus (or the sham supernatant) on top of the pre-filter.
9. Collect the virus-containing filtrate (or the sham filtrate) for purification and concentration of virus (or sham).
10. Autoclave the ultrafiltration unit or bathe it in 70% ethanol for 10 min to sterilize it. Rinse the ethanol off with sterile PBS.
11. Immerse the ultrafiltration membrane in a plate of 70% alcohol for 15 min to wet it.
12. Transfer the membrane to a new plate containing sterile PBS. Use sterile blunt end forceps to transfer the membrane.

13. Rinse the membrane with sterile distilled water twice.
14. Transfer the membrane to the bottom plate of the ultrafiltration unit, glossy side up, and assemble the ultrafiltration unit. Attach the ultrafiltration unit to the pressure regulator tubing of the nitrogen tank. The whole apparatus should be assembled in a cold (4 °C) room.
15. Wash the membrane in the unit with 300 ml of PBS three times at very low pressure (5 pounds per square inch, PSI) using the nitrogen tank and regulator in the cold room.
16. Pour up to 300 ml of the viral supernatant (or the sham supernatant) into the ultrafiltration device in the cold room and apply pressure to ~40 PSI (should never exceed 75 PSI). Note that 300 ml is the maximum capacity of the device that we use; other devices may have different capacities. Wait until the volume decreases to 10 ml.
17. Add 300 ml of CM-PBS and wash the membrane by applying pressure.
18. Repeat this step two more times. Finally, use CM-PBS for the last wash.
19. Collect the final concentrate (10 ml) and store it in 0.5 ml aliquots at −70 °C or lower. After the determination of titer, the virus may be concentrated further if needed. A final titer of at least 1×10^8 plaque-forming units (PFU) per ml is required.

3.1.5 Determination of the Viral Titer by Plaque Assay

1. At 24 h before infection, plate HeLa-H1 cells at 0.5×10^6 cells in 2 ml of complete medium per well in a 6-well plate.
2. Seed 2 (or more) wells for every dilution or condition to be tested.
3. Immediately before virus addition, check the culture plates. Wells should contain an even monolayer of ~80% confluency. Small gaps are fine. A confluent monolayer will not allow enough time for the plaques to fully develop.
4. Prepare 1:10 serial dilutions of the virus in a sterile dilution medium. Keep in mind that each well should receive 500 µl of the diluted virus. For an assay with duplicate replica wells, prepare 7 tubes, each containing 1350 µl of the diluent and one tube containing 2997 µl of the diluent. Add 3.0 µl of the concentrated viral stock to the tube containing 2997 µl of the diluent (10^{-3}), vortex thoroughly, and transfer 150 µl down the line in series (1:10 dilutions). The appropriate testing range is usually 10^{-5}–10^{-10}.
5. Remove the medium from the cell cultures and add 500 µl of the appropriate virus dilution to each well. Using less than 500 µl in six-well plates tends to result in dry spots.

6. Cover the plates and place them on a rocker for 30 min to allow even adherence of virus to the cells.
7. Next, place the plates in a humidified CO_2 (5%) incubator at 33 °C for another 30 min.
8. Prepare the agarose overlay medium. Supplemented medium should be at 44 °C. Put one of the prepared 50-ml tubes with 6 ml of top-agarose into a glass beaker with water not enough to float the tube. Remove the cap or lay it loosely on top and microwave briefly several times until agarose is liquid. It will be viscous. Keep in hot water prior to dilution. Add 44 ml of supplemented medium to the agarose tube. Mix *well* and put the tube in the 44 °C water bath. Allow sufficient time to equilibrate at 44 °C to avoid killing the cells when added to the wells. This makes a 0.6% agarose solution for an overlay of the infected cell monolayer (*see* **Notes 6** and **7**). Agarose is used to prevent virus progeny from spreading from an infected cell to adjacent cells. The infected cells will die, leaving the adjacent cells intact, leading to the formation of plaques (*see* **Note 8**).
9. Remove the virus inoculum from the plate by tilting the plate and aspirating from the edge. Wash cells once with PBS. Carefully add 2 ml of the 0.6% agarose solution to each well, not disturbing the cells. Tilt the plate and add agarose at the lower edge of the wells, and then level the plate to let it flow over the surfaces. One tube of 50 ml is sufficient for 4 six-well plates at 2 ml/well.
10. When the agarose has hardened, transfer plates to the humidified CO_2 (5%) incubator at 33 °C. Cytopathic effect is usually visible by microscope in about 1.5 days, visible by eye and ready for staining on the second day after inoculation.
11. Remove the overlay medium and the agarose layer gently. To accomplish this, overlay the agarose with 2 ml of formalin and transfer the plate to 4 °C refrigeration for 1 h. Then, loosen up the top agarose by nicking the edge of the agarose so that the formalin gets under the agarose layer. Taking a spatula, rim the agarose plug and remove it carefully while still cold (*see* **Note 9**).
12. Fix the cells with 2 ml of fresh formalin solution for 30 min.
13. Stain the cells with the freshly prepared crystal violet solution for 10 min.
14. Aspirate the stain and arrange plates sideways (vertical) for a short time to allow the excess of the crystal violet solution to run off the monolayer, and then invert (upside-down) to finish draining. See Fig. 2.
15. Calculate viral titer. Titer is expressed in plaque-forming units (PFU) per ml. Count the number of plaques and use the

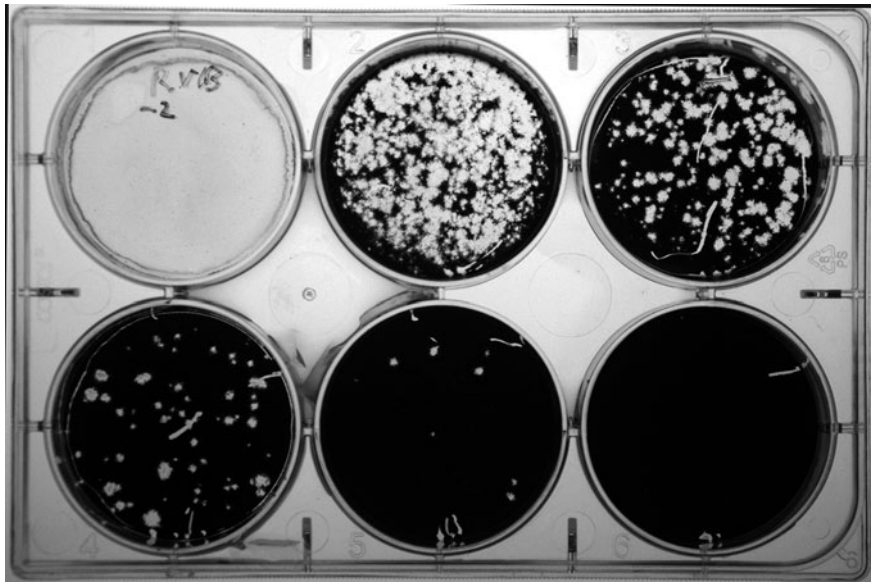

Fig. 2 Plaques after crystal violet staining. As described in Subheading 3.1.5 and in the legend to Fig. 1, HeLa cells were infected with RV at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} dilutions. At 96 h at 33 °C, cells were fixed, the agarose was removed with a spatula, and the fixed cells were stained with crystal violet. Plaques at the appropriate dilution appear as punctate dots. When plaques become confluent, the well clears out entirely, as is evident in the upper left well at 10^{-2} dilution. Streaked wells are indicative of touching the monolayer with a spatula

following formula to determine the titer in PFU/ml of the viral stock: average number of plaques in well × tube dilution factor = PFU/ml.

Sample calculation: You counted 13 and 17 plaques in duplicate 10^{-7} wells with 0 and 2 plaques in the 10^{-8} wells. For calculations, you should use the 10^{-7} count because it is more accurate. The average count from 10^{-7} wells is 15. This gives a titer of 1.5×10^8 PFU/ml.

3.2 Sensitization and Challenge of Adult Mice with an Allergen

3.2.1 Ovalbumin Allergen Sensitization and Challenge

1. On day 0, anesthetize an 8–12-week-old mouse with isoflurane. After the mouse is fully anesthetized, perform intraperitoneal (IP) injection with 0.1 ml of the OVA/alum mixture.
2. On day 7, repeat IP injection (*see* **step 1**). In addition, perform an intranasal (IN) inoculation with 50 μl of the OVA solution 2. Use 50 μl of PBS for the control group.
3. On days 14 through 19, perform IN inoculation with the OVA solution 2 or PBS.
4. Study mice on day 21. Alternatively, if a viral challenge is to be given, administer the virus or sham as described in Subheading 3.3.1 on day 21 and study the mice on days 22 or 23.

3.2.2 House Dust Mite Allergen Sensitization and Challenge

1. On days 0, 7, 14, 15, and 16, anesthetize an 8–12-week-old mouse with isoflurane. After the mouse is fully anesthetized, perform IN instillation using 50 μl of the HDM solution. Use PBS for the control group.
2. Study mice on day 18. Alternatively, if a viral challenge is to be given, administer the virus or sham as described in Subheading 3.3.1 on day 18 and study the mice on days 19 or 20.

3.3 Instillation of Rhinovirus into Mice

3.3.1 Rhinovirus Treatment of Adult Mice

1. Anesthetize an adult mouse as discussed in Subheading 3.1.1 and **Note 10**.
2. Once anesthetized, intranasally administer 50 μl of the concentrated virus to the mouse. The mouse should be given at least 5×10^6 PFU (virus suspension should be at 1×10^8 PFU per ml). This should produce an inflammatory response within 24 h. The appropriate sham to use is a sham lysate concentrated and prepared in exactly the same way as virus. *See* **Note 11**.
3. When coupled with an allergen sensitization model, the virus is usually given after the last challenge step in the time course, as described above in Subheadings 3.1.1 and 3.1.2.
4. Study mice 24–48 h post-infection. *See* **Note 12** for a short discussion of the pathological changes you might expect.

3.3.2 Rhinovirus Treatment of Neonatal and Juvenile Mice

1. Anesthetize a neonatal mouse or a juvenile mouse as discussed in Subheading 3.1.1 and **Note 10**.
2. Once anesthetized, intranasally administer 20 μl of RV-A1B (2×10^6 PFU) or an equal volume of sham (prepared as described above from HeLa cell lysates) to the neonatal mouse. Administer 30 μl of RV-A1B (3×10^6 PFU) or sham to the juvenile mouse.
3. Analyze mice afterward per experimental need. As with adult mice, an initial mixed monocytic–neutrophilic response is to be expected by 24–48 h post-infection. In neonates, a late response develops, peaking at 6 days post-infection. This response is characterized by expansion of group 2 innate lymphoid cells (ILC2s) (*see* **Note 13**).

4 Notes

1. A very high grade endotoxin-free low melting point agarose is required to get unequivocal plaque formation. Endotoxin-contaminated agarose causes cytopathic effect and cell death all on its own, interfering with the production of distinct plaques.
2. Better results have been seen with females over male mice and BALB/c over C57BL6 mice [11].

3. RV replicates better at the temperature of the nasal airway epithelium (33–35 °C) than at 37 °C [12].
4. It is not advisable to wait until all cells are completely detached from the plate, as by that time, the virus is no longer propagating but starting to decrease in infectivity.
5. After the initial amplification, do not purify the virus before its primary storing as a seeding stock. At this step, the idea is to get the virus as concentrated as possible without physical concentration. This allows an initial amplification of the RV.
6. Low-melting point agarose is almost impossible to dissolve unless mixed thoroughly with ice-cold PBS to get a uniform suspension before heating. In hot water, clumps spontaneously form. These clumps are wet on the outside but remain dry on the inside. Suspend the agarose uniformly in the ice-cold buffer before trying to dissolve it with heating.
7. Autoclave in a 250-ml bottle to avoid boil-over and dispense 6 ml into 50 ml capacity sterile disposable centrifuge tubes. Using a 25-ml pipette for four tubes at a time (24 ml) while agarose is still hot (microwave briefly after removing from autoclave) works well. Try to dispense hot agarose into the tips of the tubes so that it will be easier to reheat. Figure at least one 6-ml tube per four six-well plates.
8. If the agarose medium turns yellow and the cells take on a crystalline appearance under the microscope, plaques will not develop beyond this point and monolayer will start to fall apart if the incubation is continued. The plaques formed by RV infection of HeLa cells under agarose never completely clear out with cell lysis. Instead, there are areas of localized cell stasis and cytopathic effect (see Fig. 1). They do detach easily from the plate once the agarose is removed and allow the macroscopic determination of infection loci as plaques when stained (see Fig. 2).
9. Be careful to avoid touching the underlying cell monolayer. Agarose should pull off without disturbing the cells. Touching the cell layer will lead to streaks (Fig. 2). Small fragments of agarose will come off in the following washes, and attempting to remove the extra fragments by picking them off will only damage the cell monolayer. It may be helpful to refrigerate the agarose-covered wells with a fixative for a half hour to harden the agarose further if the fragmentation of the agarose on removal is a problem.
10. Mice must be fully anesthetized in order to inhale an allergen or virus solution through the nose. Mice do not typically breathe through their mouth, but a conscious or unanesthetized mouse will swallow the solution instead of inhaling it through the nose. Mice are anesthetized with 4–5% isoflurane

for induction, and then kept at 1–2% for maintenance until treated intranasally with allergen or virus. Mice should rapidly recover post-treatment as they are returned immediately to their cage.

11. Problems with the administration of the virus might be due to simply not injecting the virus into the nose or airways because the mouse was insufficiently anesthetized and blew out the virus or swallowed instead of inhaling it.
12. At 24–48 h post-infection, the adult mouse response to RV instillation is typically a diffuse inflammation detected by histochemical staining of the lung as a cellular infiltration into the peribronchiolar region. This may be highly localized or more general, depending on how well the instillation was performed. Flow cytometry on whole lung suspensions or bronchoalveolar lavage will reveal more CD45(+) cells (mostly macrophages and neutrophils) at this time, peaking at 48 h. Chemokines like CXCL1 and 2 will be increased at both the RNA and protein levels. Inflammasome activation can be detected by western blot. Other parameters of pulmonary response to RV include increased airway resistance at 48 h as measured by plethysmography. Overall the response to rhinovirus alone should resemble "type 1" inflammation with a nonlethal resolution by 6 days post-infection [2].

    When coupled with an allergen, expect more mucous metaplasia, increased hypertrophy and hyperplasia of the airway smooth muscle, and increased airway resistance. The immune cytometric profile will resemble more of a "type 2" immune response with more eosinophils than to RV alone [2].
13. Baby mice typically show an initial response to RV similar to that of the adult mice, but by day 6 post-infection, type 2 responses may be seen [4, 5].

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