

Chapter 12

Induction of Allergic Rhinitis in Mice

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Abstract

We describe a method for allergic rhinitis (AR) induction in mice. Methodology involves nasal infusions of small volumes of ovalbumin for both initial sensitization and challenges. The latter are frequent and carried out over several weeks. This methodology more closely resembles natural AR induction than does the common use of systemic sensitization, often with adjuvants, followed by nasal challenges with relatively large allergen volumes. Also described are methodologies for collection of cardiac blood and perfusion for preparation of histological samples, both essential in verifying AR induction in individual animals.

Key words Allergic rhinitis induction, Murine allergic rhinitis model, Nasal tissues, Cardiac blood collection, Mouse perfusion, Nasal sinuses, Ovalbumin, Olfactory epithelium, Respiratory epithelium, Eosinophils

1 Introduction

Allergic rhinitis (AR) is the most common atopic disease [1] and can often cause seriously compromised olfactory function [2, 3]. It is induced by repeated nasal exposure to low levels of allergenic material. Mice provide convenient models in which to study the onset, progression, termination, and amelioration of AR. However, to date, in efforts to guarantee robust immune responses in relatively short experimental periods, most murine AR studies have used models that involve subcutaneous or intraperitoneal systemic initial sensitization, with only the subsequent challenges being delivered nasally. Moreover, adjuvant is often used in conjunction with sensitization; and the nasal challenges often involve relatively large, rather than small, infusate volumes and high allergen concentrations (e.g., [4–8]).

In contrast, McCusker and colleagues, in investigations of murine upper and lower airway allergic diseases [1], recognized the unnaturalness of such induction methods. McCusker et al. noted that the use of the nasal route for both sensitization and challenge and the use of both low allergen doses and numerous

small exposures over a period of several weeks more closely reflect natural allergic induction than do the other models. They also used the non-microbially derived protein ovalbumin (OVA) as their allergen. This avoids induction of additional innate, non-AR immune responses to microbe-associated molecular patterns of microbial antigens (e.g., [9–11]). Using their consequently developed protocol [1], McCusker et al. were able to show induction of pronounced allergic reactions as measured by the standard indicators of OVA-specific IgE and IgG serum levels, pronounced upper and lower airway eosinophil infiltration, and increased IL-5 and polymorphonuclear leukocyte presence in postchallenge bronchoalveolar lavage fluid.

Our own interests concerned AR effects on olfactory capabilities in affected animals. To investigate these capabilities, it was first necessary to demonstrate the induction of allergic responses in the nasal cavity itself, using slight modifications of the McCusker protocol [12]. Our protocol is detailed below. Induction of AR was verified by high OVA-specific serum IgE levels in ELISA blots and by pronounced nasal cavity eosinophil infiltration. These two AR indicators were highly correlated in all study animals.

Our study further examined the effects of extended allergen exposure, such as would occur with chronic or perennial seasonal allergen exposure. This extended OVA exposure was found to cause noticeably more pronounced nasal histological changes [12]. Interestingly, nasal responses appeared complicated, with olfactory epithelial histological changes being secondary to respiratory epithelial responses. That intriguing issue, along with analysis of numerous other histological and molecular components of the observed responses, still awaits examination, as do the effects on olfactory function itself. Distinct nasal sinus responses were also noted, but these were not further investigated.

2 Materials

2.1 Animals

1. Virus-free 7–11-week-old BALB/c mice (*see Note 1*), housed under conventional conditions in the institutional animal facility and treated strictly according to the NIH and institutional animal care protocol requirements throughout the duration of the entire experiment.

2.2 Reagents (*See Note 2*)

1. Phosphate-buffered saline (PBS), pH 7.4, prepared from a commercial concentrate solution by dilution with H₂O (*see Note 3*).
2. Allergen: 1.0 % (wt/vol) OVA in PBS (*see Note 4*). Make this in 10 ml batches: Add 0.1 g OVA to 10 ml PBS; vortex to dissolve. Filter sterilize (0.02 µm pore size), and aliquot into sterile plastic vials, 40–50 µl/vial. Store at –20 °C. Thaw as needed on the day of use.

3. PBS controls: Dilute from the 10× concentrate above and then filter sterilize, aliquot, and store in the same manner as the OVA.
4. Anesthetics for blood collection and perfusion: Ketamine and xylazine (0.65 and 0.035 mg, respectively, *per g* b.wt. for each mouse; *see* **Note 5**).
5. Fixative for histological preparation: Paraformaldehyde (PFA) in PBS (*see* **Note 6**), 1 N sodium hydroxide (NaOH) for titration to solubilize the PFA. PFA is made and stored as 16 % in H₂O and on the day of perfusion is then diluted to 4 % with PBS and H₂O (*see* Subheading 3).

2.3 Additional Materials

1. For blood collection and perfusion: ½–1 in. 22 G disposable needles and thin polyethylene tubing, cut into 1.5–2.5 cm and 20–25 cm lengths. Internal tubing diameter should be just wide enough to tightly fit over the needles.
2. Metal file and wire/metal cutters for cutting disposable needle tips.
3. Nail polish.
4. Perfusion pump.
5. Various sized syringes for anesthetization; blood collection; and perfusion, if fluid delivery by syringe is preferred to a perfusion pump.
6. Various surgical scissors and tweezers for tissue isolation.
7. Small centrifuge tubes for blood; jars for collected specimens; and Pasteur pipettes.

3 Methods

3.1 Nasal Infusions

1. Nasal infusions are carried out in a procedure hood in the animal facilities following institutional guidelines.
2. Wipe the hood and work spaces with disinfectant.
3. Mice are housed at a maximum of five per cage. Animals receiving allergen (OVA) or control buffered saline (PBS) should be housed separately. Before beginning infusions prepare a fresh cage for each cage of animals to be infused. Infuse all animals from the same cage sequentially, and transfer each to the same clean cage immediately after infusion (*see* **Note 7**).
4. Before removing an animal from its cage for infusion, have ready for use two pipettors, one for each naris, fitted with sterile 10 µl tips and each filled with 7.5 µl (*see* **Note 8**) of the appropriate solution (allergen or buffer). This avoids having to change and fill pipette tips between the infusions into each side while still holding a mouse. Do not allow the pipette tips to touch any surfaces.

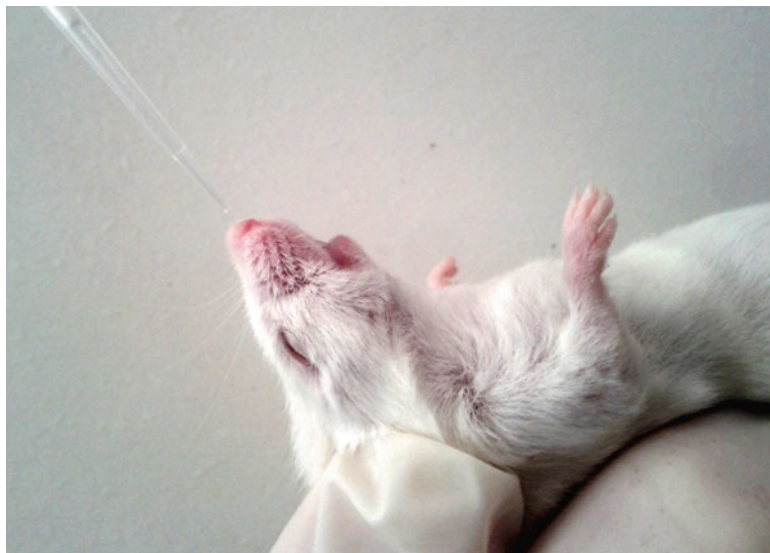


Fig. 1 Image of mouse being held for nasal fluid infusion. The head and neck are held firmly between the thumb and forefinger of the nondominant hand. The trunk and hind legs are held firmly but gently with the remaining free fingers of that hand (not pictured). The pipettor is held in the dominant hand, with the pipette tip placed just above and slightly to the outer side of the naris opening so that the infusion fluid is dispensed right over the naris

5. Remove an animal to be infused from its cage (*see Note 9*). With the thumb and first finger of the nondominant hand grasp the animal's neck and neck skin quite firmly behind the ears and along the neck. Firmly but gently anchor the hind legs and trunk with the remaining fingers of that hand. Tip the mouse back so that its nose points upwards, and bring the tip of the first pipette to one of the nares. Holding the pipette tip over the naris from the side, infuse the solution onto the naris opening (Fig. 1). To maximize the amount of fluid reaching the posterior-most nasal regions, hold the mouse on its back for several seconds until it stops struggling. Mice are obligate nose breathers, so the fluid will be inhaled. Repeat for the second naris using the second prepared pipette. Place the infused mouse into the fresh cage, and then prepare the set of pipettes for the next mouse. When finished infusing all mice from a given cage, remove the old and fresh cages from the hood and wipe down the hood surface with disinfectant before starting the next cage of mice (*see Notes 10–14*).
6. The infusion schedule is given in Fig. 2. Chronically exposed mice are treated for either 6 or 11 weeks with either the sterile 1 % OVA or PBS (PBS chronic controls) solutions above. For the 6-week exposures, mice receive daily infusions for 5 days

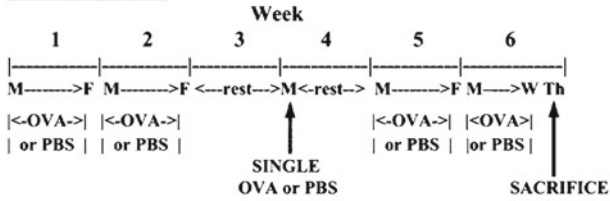
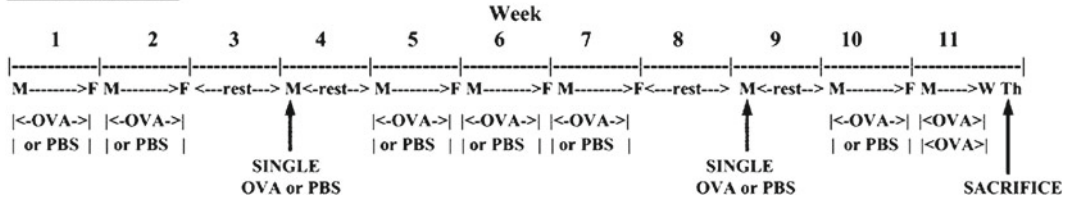
6-week exposures**11-week exposures**

Fig. 2 Nasal infusion protocols for 6- and 11-week chronic exposure regimens for murine allergic rhinitis induction. Ovalbumin (OVA) is the allergenic infusate and phosphate-buffered saline (PBS) the control. Infusions occur on weekdays (*see Note 15*). For the 6-week chronic exposures, infusions occur Monday (M)–Friday (F) of weeks 1–2. A 2-week rest period follows in weeks 3–4 with single infusions on Monday of week 4 (*see Note 16*). Daily infusions are then resumed for the 5 days of week 5 and the first 3 days of week 6. Animals are sacrificed on the Thursday (Th) of week 6. For 11-week chronic exposures, this pattern is modified so that daily nasal infusions occur on the 5 weekdays in weeks 1–2, 5–7, and 10 and on the first 3 days of week 11. There are also two break periods, in weeks 3–4 and 8–9, with single infusions occurring on the Mondays of weeks 4 and 9. The specific days of the week on which infusion is performed can be altered to suit researchers' needs as long as the temporal pattern is maintained

for weeks 1 and 2 (*see Note 15*). Week 3 is a rest week, with no infusions. This is followed by single bilateral infusions on the first day of week 4 (*see Note 16*). The daily infusion pattern is then resumed for the 5 days of week 5 and the first 3 days of week 6. The mice are then sacrificed on the fourth day of week 6, 1 day after their final infusion. For the 11-week exposure animals, daily infusions occur in weeks 1–2 and 5–7. Rest periods similar to that of weeks 3–4 of the 6-week exposure schedule occur in weeks 3–4 and 8–9 (*see Note 16*), with single bilateral infusions occurring on the first day of both weeks 4 and 9. Daily nasal infusions then resume for the 5 days of week 10 and the first 3 days of week 11. The mice are sacrificed on the fourth day of week 11. Acutely treated animals receive single bilateral 7.5 μ l infusions of OVA or PBS 1 day prior to sacrifice. Untreated controls receive neither OVA nor PBS prior to sacrifice (*see Note 17*).

7. It is essential to verify that AR has indeed been induced in any animal included in the subsequent analyses. We utilize both ELISA of blood serum OVA-specific IgE levels and the Luna stain [13] for histological verification of nasal epithelial

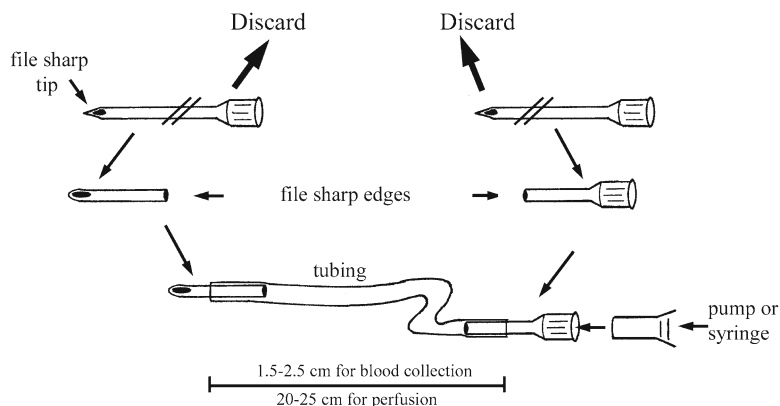


Fig. 3 Preparation of needle/polyethylene tubing apparatus for murine blood collection and perfusion. The dispensing end of the apparatus (*left*) is prepared by cutting a $\frac{1}{2}$ –1 in. 22 G disposable needle close to its plastic adaptor end. The sharp tip and the rough cut edges are blunted using a metal file. The blunted cut end is threaded into thin polyethylene tubing just wide enough to tightly hold the cut needle. The adaptor end of the apparatus (*right*) is prepared from a second disposable needle cut close to its pointed tip. The cut end of this needle is also blunted and fitted into the free end of the polyethylene tubing. Polyethylene tubing should be 1.5–2.5 cm long for blood collection apparatus and 20–25 cm for perfusion apparatus. Additionally, for the perfusion tubing a small drop of nail polish should be added just below the needle opening to prevent the needle from slipping out of the ventricle (not shown)

eosinophil infiltration [12] (*see Note 18*). It is also highly advisable that investigators of AR familiarize themselves with the morphology and tissue distribution through the entire extent of the mouse nasal cavity. Consequently, anesthetization, collection of cardiac blood, and fixation by transcardial perfusion are described below. For molecular and biochemical analysis for which unfixed tissue is required, only the ELISA would be possible.

3.2 Preparation of Cardiac Insertion Needles for Blood Collection and Perfusion (Fig. 3)

1. Prior to the day of perfusion prepare needles for blood collection and perfusion. Slightly blunt the tips of $\frac{1}{2}$ –1 in. 22 G disposable needles with a metal file. Cut off and discard the plastic adaptor ends. Blunt the rough cut edges of the remaining needle tubes (*see Note 19*). Carefully thread one of these blunted top ends into a piece of thin polyethylene tubing just wide enough to hold the needle tightly. For blood collection the polyethylene tubing should be 1.5–2.5 cm long; for perfusion the tubing should be 20–25 cm long. The shorter length for blood collection provides less volume for loss or coagulation of drawn blood while still being long enough to provide some flexibility during blood collection. The longer length for

perfusion allows for maximum flexibility and maneuverability, but is not so long as to add to the clutter of instruments, perfusion fluids, and fluid waste containers that fill the hood during perfusion.

2. Trim off the pointed tips of a second set of needles, leaving most of the needle length (~0.5–1 cm) still attached to the plastic adaptor tops. Blunt the cut edges. Thread these cut ends into the free ends of the polyethylene tubing prepared above. The finished adapted needles are essentially thin polyethylene tubes that can be inserted into a mouse ventricle at their tips and attached to the nipple of a syringe or perfusion pump tubing via their plastic adaptor ends.
3. Additionally, for the needles to be used for perfusion, place a small drop of nail polish just below the opening of the needle tip and allow it to dry. This will serve to anchor the tip of the needle in the ventricle during perfusion.

3.3 Animal Anesthetization

1. After the allergen and PBS exposure period, animals are sacrificed according to institutional protocols and the requirements of subsequent procedures. Anesthetization is required. All procedures that follow should be carried out in a hood using gloves and proper eye cover.
2. On the day animals are to be perfused or otherwise sacrificed, weigh the animals, and calculate the combined total weight.
3. Cover the work space with plastic-backed absorbent paper.
4. Animals are deeply anesthetized by intraperitoneal injection of their individual weight-based calculated volumes of ketamine and xylazine. These are used in a combined “cocktail” solution with final drug concentrations of 9.8 mg of ketamine and 0.49 mg of xylazine/1.0 ml of anesthetic, with PBS as the diluent (*see Note 5*). These ketamine and xylazine concentrations work out to 0.65 ml of the “cocktail” for each 25–30 g mouse (0.022–0.026 ml of “cocktail”/g b.wt.). Allow an additional 0.1 ml/mouse in case extra anesthetic is needed to fully anesthetize any individual mice.
5. Each animal is anesthetized just prior to the start of the blood collection, perfusion, or other procedure being carried out. Deep anesthesia is indicated by the absence of an eye blink and/or tail pinch response, depending on individual institutional regulations. This usually requires 5–10 min. If the animal is not completely anesthetized in this time, administer additional anesthetic in 0.05 ml increments. Once deep anesthetization is achieved, subsequent procedures can be initiated.

3.4 Blood Collection by Cardiac Puncture for ELISA

1. Place the fully anesthetized animal on its back in a container large enough to ultimately hold all of the perfusion buffer and fixative but small enough to allow ready access to the animal.
2. With small scissors and surgical tweezers cut open the abdominal skin, cut through the diaphragm from the abdominal cavity into the thoracic cavity, and gently lift the ribs out of the way or remove them.
3. Rapidly insert a needle apparatus prepared for blood collection, with a 0.5–1.0 ml disposable syringe attached, into the base of the left ventricle. Gently draw as much blood as possible into the syringe from the heart.
4. Quickly but gently remove the needle from the ventricle. Be careful not to enlarge the needle penetration hole if the animal is to be subsequently perfused.
5. Dispense the blood into a small plastic centrifuge tube and immediately place this in the cold and store upright overnight so that the serum collects on the top. The next morning spin down the blood samples ($13,800 \times g$ on a tabletop centrifuge for 5 min), carefully collect the serum from the top layer of each, and store that in individual vials or containers at -80°C until used for allergen-specific ELISA.
6. If the nasal tissues are to be used for molecular or biochemical studies and no perfusion is to be carried out, cut the head from the remainder of the body. Trim off the skin and lower jaw, and carefully remove the palate to expose the nasal cavity. Isolate the nasal septum and store as appropriate for subsequent procedures. The nasal turbinates and nasal sinuses can also be removed as needed. If desired, the olfactory and respiratory epithelia can be further isolated from the septal epithelium using a dissecting microscope.

3.5 Transcardial Perfusion of Animals for Histological Examination

1. Several days before perfusion, prepare a stock solution of 16 % PFA (*see Note 20*). For each 100 ml of 16 % PFA, add 16 g of PFA powder to a beaker containing 100 ml of H_2O and a spinning magnetic stir bar on a stirring/hot plate set at a moderate spin speed. Carefully heat the mixture to 60°C . The fluid will be cloudy. Slowly add 12 drops of 1 N NaOH with a Pasteur pipette and continue stirring until the PFA dissolves and the fluid clears. A small amount of additional NaOH may be necessary to fully clear the solution. Do this slowly, using a lower concentration of NaOH (e.g., 0.1 N). Turn off the heat, and let the stirring continue until the solution cools to room temperature. To hasten cooling move the beaker to an unheated stir plate and continue stirring. If making large volumes of PFA, the beaker can also be placed in an ice bath on this unheated stir plate. Store the 16 % PFA at $4\text{--}8^{\circ}\text{C}$ for up to a month.

2. On the day of perfusion, prior to anesthetization of any mice, prepare a solution of 4 % PFA in PBS. Note that the PBS final concentration should be 1×. Allow 60–80 ml/mouse for perfusion with a perfusion pump. Thus, for each mouse to be perfused, mix 20 ml of 16 % PFA, 8 ml of PBS 10× concentrate, and 52 ml of H₂O.
3. Perfusion can be carried out using a perfusion pump [12] or by manually perfusing using 50 ml syringes. If manually perfusing, fill one syringe with PBS and the other with fixative (*see Note 21*). If a perfusion pump is used, it should be set up according to the manufacturer's directions. Connect two pieces of polyethylene tubing (2–3 mm internal diameter) to the pump input tubing via a 3-way adaptor. Immerse the free end of one piece of this tubing into a container of the 4 % PFA and that of the other into a container of PBS (*see Note 22*). Attach one of the needles prepared previously for perfusion to the pump output tubing. Check to make sure that no air bubbles remain in the tubing prior to starting perfusion.
4. After completion of blood collection (Section 3.4), carefully replace the blood collection needle in the ventricle with one prepared for perfusion and attached to the pump or syringe. Do not enlarge the existing hole or make a new one. That can lead to loss of perfusion fluid through the original hole and reduced perfusion fluid pressure through the body, resulting in a less than optimal perfusion. The dried nail polish drop will help to hold the perfusion needle in place in the ventricle during perfusion.
5. If no cardiac blood collection was carried out, anesthetize the animal, expose the heart, and insert the perfusion apparatus-attached needle into the left ventricle as described above.
6. With the perfusion needle inserted into the ventricle, begin the flow of PBS at a rate of 10–15 ml/min (*see Note 23*). *Immediately* clip the right atrium with sharp scissors so that the perfusate will exit from there after transiting through the entire body. Initial fluid will be pink due to blood carried from the body. Once the exiting fluid is clear in color (*see Note 24*) and the animal thoroughly exsanguinated, quickly switch to the fixative (*see Note 25*). Perfuse the animal with fixative until it or, in the case of a fluid block below the heart, its head, jaws, and neck are completely stiff. This should take 3–5 min, but can sometimes take longer. During both PBS and fixative delivery, the animal should be checked periodically to make sure that the needle remains inserted into the ventricle.
7. Turn off the pump.
8. Cut the head from the body. Since AR involves the nasal cavity and associated tissues, only the head above the palate needs to be kept. Trim off the skin and lower jaws (*see Note 26*).

Immerse the trimmed head in a jar containing sufficient fixative to cover it, and using a Pasteur pipette, force a gentle stream of fixative through the nostrils to flush out any trapped air (*see* **Note 20**). Store the head in fixative overnight at 4–8 °C. The next morning thoroughly wash out the fixative by immersion in running water for 15 min followed by several additional changes of water for up to an hour (*see* **Notes 27 and 28**).

9. Follow all institutional guidelines for disposal of animal carcasses, parts, and tissues as well as fixative wastes.

4 Notes

1. BALB/c mice are preferable because this strain had been found to give more robust AR responses than either C57BL/6 or CBA/J mice in studies using the *Schistosoma mansoni* egg antigen as the allergen [10]. However, given that that allergen may also be inducing innate, non-AR responses to microbe-associated molecular patterns, in a truly thorough investigation other strains should ultimately be examined as well.
2. For all solutions use reagent-grade reagents and either deionized or distilled water (H₂O). Use protective gloves when working with anesthetics, fixatives, and animals. Follow all institutional usage and waste disposal requirements.
3. PBS can also be prepared de novo from powdered ingredients following readily available directions. However, to prevent microbial growth, all preparations should be made and stored as 10× concentrations and then subsequently diluted as needed. Other isotonic physiological buffers could also be used as controls and allergen diluent.
4. Ovalbumin was used as the allergen because it is not an inducer of toll-like receptors and the innate immune response, which could greatly complicate any analysis of AR. Other non-microbially derived allergens could also be used as long as they do not induce innate responses.
5. To minimize injection trauma to the mice, xylazine and ketamine are administered in an anesthetic “cocktail.” Using 100 mg/ml of ketamine and 20 mg/ml of xylazine commercial preparations, the “cocktail” represents a vol/vol ratio of 87.5 % PBS, 9.8 % ketamine, and 2.4 % xylazine. This anesthetic “cocktail” should not be prepared more than a few hours prior to use. Strictly follow all institutional and governmental regulations for drug storage, use, and disposal.
6. We used 4 % PFA. However, investigators should use whatever fixative that best suits their own subsequent histological, molecular, or biochemical needs.

7. Maintaining this pattern for infusion avoids any confusion as to whether a particular animal has been infused. To avoid accidental infusate errors, infuse all animals receiving the same infusate before starting the animals receiving the other infusate solution and have tubes of only the appropriate infusate in the hood. Any exposure to the wrong infusate solution could induce an unintended immune response, invalidating the results from that particular mouse.
8. McCusker et al. [1] used 5 μ l of infusate/naris. We have used 7.5 μ l instead to guarantee that at least 5 μ l gets into each side. Given animal squirming, we found that in our hands 7.5 μ l was a more reliable volume to use. However, to insure that resulting immune responses do indeed induce only AR and not additional immune responses, much larger infusate volumes should be avoided.
9. Mice move very quickly. Thus, rather than completely uncovering the cage, slide the cage top back just enough to grab any mouse in the cage (since all in the cage will be receiving the same infusate).
10. Mice should be infused bilaterally rather than trying to use one side as a control. It is very difficult to completely guarantee that infusate from one side will not spill onto the other naris. Moreover, side-to-side and inter-animal histological differences readily occur [12]. For this reason each nasal cavity also must be analyzed separately for histological investigations.
11. It is helpful to relax one's arms during infusion procedures so that pipettes will be held as steady as possible. This also helps confine the infusate to the side being infused. Practice infusions may be useful.
12. When infusing many mice, it helps to routinely infuse either the right or the left naris first for each mouse. This avoids the possibility of infusing one naris twice and the other not at all. Not discarding pipette tips until both nares have been infused also helps to verify that both nares have indeed been infused.
13. To clearly see the nares it is helpful to wear magnifying reading glasses. If the reading glasses will be worn over regular glasses, purchase a pair large enough to fit over the regular glasses but not so large as to slip down. If additional magnification is still required, a magnifying glass such as used in crafts projects with a flexible stem and clamp holder that allows it to be clamped to the edge of the hood cover is invaluable.
14. To help insure that fluid indeed is reaching the olfactory regions in these experiments, test infusions should be carried out with a vital dye (e.g., Evan's Blue [1]) and the extent of dye dispersal determined histologically.

15. In addition to its convenience, the 5-day-per-week pattern for nasal infusion is reflective of often intermittent AR-inducing allergen exposure (C. McCusker, personal communication).
16. The weeks 3–4 rest period, including the single intranasal challenge on the first day of week 4, was found essential for maximizing the serum IgE responses in the McCusker site-specific nasal sensitization and challenge regimen ([1], and McCusker, personal communication). For the same reason, a similar break from daily infusions was included in weeks 8–9 of the 11-week extended exposure studies [12]. We assume that any further extensions of exposure periods should include additional similar breaks as appropriate, but always including one in the 4th and 3rd weeks prior to animal sacrifice.
17. It is essential to include chronic PBS control animals. Our study [12] found that both chronic and acute PBS, as well as acute OVA exposure, cause *non*allergic swelling of the respiratory epithelia, which must be considered in the overall conclusions.
18. It is highly advisable that investigators of AR familiarize themselves with the morphology, histology, and tissue distribution through the entire extent of the mouse nasal cavity. This includes the turbinates, sinuses, septal organ, vomeronasal organ, and relative distribution of olfactory versus respiratory epithelia. All are nasal cavity spatial landmarks and important in determining the degree of AR-induced epithelial disruption. Thorough familiarity should be gained for both unexposed normal morphology as well as for AR-induced changes. It was our experience that important points found in our AR study [12] had been previously overlooked or dismissed because many earlier investigators lacked a thorough nasal cavity familiarity. This includes our findings of nasal epithelial type-specific effects of AR, with the implication that nasal effects of AR are unexpectedly more complex than previously suspected. Readers are referred to that study [12].
19. Blunting the pointed tips of the needles lessens the chance of piercing through the back side of the heart during blood collection and perfusion; blunting the cut edges lessens the likelihood of tearing the polyethylene tubing.
20. All preparation and use of PFA should be carried out in a ventilated laboratory hood using gloves and goggles. Institutional regulations for its usage and disposal should be strictly followed.
21. The perfusate solutions can be chilled depending on subsequent needs.
22. Mark the tubing for PBS and for the fixative with different colors of lab tape near their insertion into the 3-way adaptor so that the different fluids they contain can be readily identified.

23. This flow rate is based on an optimum fixative delivery of approximately 1 ml/gb.wt./min for perfusion of an entire mouse (Dr. E. Weiler, personal communication). Perfusion can also be carried out using handheld syringes as long as care is taken to maintain an even fluid pressure and flow rate. In our experience a flow rate of 2.5 ml/min is acceptable for mice when using a handheld syringe. In either case, flow must be low enough to avoid damage to the nasal epithelia.
24. This can be tested by holding the corner of a piece of lab tissue wipe to the cut right atrium.
25. Failure to completely exsanguinate an animal can result in formation of blood clots on exposure to fixative within blood vessels and subsequently poor fixation due to limited fixative access to trans-clot regions.
26. If using a perfusion pump, the time during specimen isolation and trimming is a convenient time in which to flush pump tubing with PBS for several minutes to clear the fixative in preparation for exsanguination of the next animal or to fully clear it after the conclusion of all perfusions. Soiled instruments can also be immersed in a beaker of distilled water with soft toweling at the bottom during this time and then wiped clean of coagulated blood so as to have them clean before proceeding to the next animal.
27. In our experience the total time required *per* mouse is about 30 min: 5–10 min for anesthetization, 5–6 min for PBS exsanguination, 5–7 min for fixation, and time for trimming the head. Time can be saved by using the anesthetization time of a mouse to clean up the instruments and work space from the previous perfusion.
28. Alternatively, multiple changes of PBS can be used in place of water depending on the specific experimental requirements. However, it is absolutely essential to completely wash out all fixative from the dissected tissues as it can interfere with subsequent procedures.

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