

Immunotoxicity takes two general forms. One is an immunosuppression, resulting in an inability of the immune system to adequately defend against invaders such as cancer cells or pathogens. However, another type of immunotoxicity is an enhancement of the immune response or hyperreactivity of the immune system. In this situation, the offending substance or allergen is immunogenic and mobilizes the humoral and/or cellular arms of the immune system. Autoimmunity or hypersensitivity is the result. Asthma is one possible consequence of an increased immunoreactivity or an allergic response in the lung, either to environmental antigens or to antigens encountered in the workplace, i.e., occupational asthma.

The asthmatic response is divided into an induction phase (sensitization) and an effector (elicitation or challenge) phase. The induction phase involves the first exposure to a substance and a specific immune response is evoked. Re-exposure or challenge with the allergen then causes the effector phase—the asthmatic symptoms. Occupational asthma is categorized as immunological and non-immunological, depending on whether an induction phase or latent period is necessary to evoke the asthmatic symptoms. In addition, within the category of immunological occupational asthma, latitude is given for IgE, non-IgE, or unknown immune mechanisms that can lead to the asthma symptoms.

Regardless of cause, asthma is characterized by reversible airway obstruction, airway hyperresponsiveness to a variety of agonists including methacholine, mucus production, airway remodeling, and cell infiltration into the lung, particularly eosinophil infiltration. Various trigger factors for asthma, such as exercise, ozone, pollutants, viral infection, emotion, and passive smoke can have profound effects on the asthmatic response to allergens and exacerbate an ongoing inflammatory situation in the lung. In addition, high exposures to noxious substances have been reported to initiate asthmatic symptoms in the lung, without a latent period.

In toxicology, two primary areas of investigation prompt the measurement of the asthmatic response in an animal: (1) identification of chemicals or proteins that cause asthma, i.e., respiratory allergens, and (2) identification of exposures that will exacerbate existing asthma. Ovalbumin-induced asthma in the mouse is a model system that can readily be used to identify substances that could exacerbate existing asthma. The effect of exposure to a toxic substance on endpoints of the ovalbumin-induced asthmatic response is determined. With regard to identification of respiratory allergens, much effort has been expended to develop reliable methods. The LLNA (UNIT 18.2) can identify substances that have the potential to cause allergy after topical exposure, i.e., hazard identification. By determining if exposure to a substance results in proliferation of lymph node cells, the LLNA assay identifies substances that induce an immune response. Whether continued exposure to these substances, or exposure via the respiratory route, will result in asthma requires measurement of the asthma symptoms in an animal model after re-exposure or elicitation of the response.

With these potential uses in mind, the Basic Protocol describes a method to sensitize and challenge a mouse using the protein allergen ovalbumin to reliably elicit asthma symptoms. Ovalbumin is widely used in experimental systems in immunology and has been extensively used to explore the effector mechanisms in rodent models of asthma. In addition, ovalbumin is a known cause of occupational asthma in egg processing facilities. The assessment of characteristic asthma symptoms in the mouse are described by: airway

hyperresponsiveness to the agonist methacholine in a conscious unrestrained mouse (see Support Protocol 1), cellular infiltration into the airspace (see Support Protocol 2), and cellular infiltration into the lung (see Support Protocol 3). As described here, the Support Protocols are used with mice sensitized and challenged with ovalbumin as in the Basic Protocol, however, they could also be used in non-sensitized animals to determine if inflammatory stimuli such as endotoxin, diesel exhaust, and other substances can cause airway hyperresponsiveness and cellular infiltration into the lung of the mouse.

MOUSE SENSITIZATION AND INTRANASAL CHALLENGE

Numerous methods are available for sensitization and challenge of a mouse using ovalbumin to elicit the symptoms of asthma. One such protocol that consistently results in an asthmatic response is described here using a Balb/c mouse. The mouse is injected by the intraperitoneal route with ovalbumin and alum as an adjuvant. After a selected waiting period for induction of the specific immune response, mice are then challenged intranasally on 3 successive days to elicit airway hyperresponsiveness and cellular infiltration into the lung. Depending on the purpose of the experiment, control mice are either sensitized with alum solution without ovalbumin, or challenged with water intranasally rather than ovalbumin. In some instances, both types of controls are indicated as well as animals sensitized with alum alone and challenged with water. After the time period of interest, the asthma symptoms are evaluated as outlined in Support Protocols 1, 2, and 3.

This protocol will be concerned with using ovalbumin as an antigen to sensitize and challenge the mouse. To determine if exposure to other chemicals or proteins results in asthma symptoms, the properties and normal exposure routes of the substance need to be taken into account. Convenient experimental systems are not always available, since many substances are not water soluble and the primary exposure is inhalation of dust. Some animal models of asthma caused by the occupational allergen trimellitic anhydride (TMA) have used sensitization of the mouse or guinea pig intradermally with TMA suspended in oil, followed by intratracheal challenge with TMA conjugated to albumin (Hayes et al., 1992b; Fraser et al., 1995; Regal et al., 2001). This method of sensitization and challenge with TMA resulted in eosinophil infiltration into the lung. Other methods of exposure to low-molecular-weight occupational allergens such as toluene diisocyanate and TMA have been tried, including inhalation of the dust (Hayes et al., 1992a), topical exposure to the dust (Zhang et al., 2002) and intratracheal exposure of oil suspensions of the molecule (Ebino et al., 2000). Various asthma endpoints have been examined after sensitization and challenge. In many of the studies, the primary concern was induction of the immune response as evidenced by IgE production or cytokine elaboration in local lymph nodes, i.e., hazard identification. Work still needs to be done to effectively and efficiently use rodent models of asthma to identify chemical or protein exposures that will cause asthma.

Materials

- Ovalbumin for sensitization (see recipe)
- Inject Alum aluminum hydroxide solution (Pierce Chemical)
- Normal saline solution (endotoxin-free)
- Mice of selected strain, age, and sex (e.g., Balb/c, 6 to 8 weeks old)
- Ovalbumin for challenge (see recipe)
- Ketamine/xylazine (see recipe)
- Water (endotoxin-free)
- 2-ml polypropylene screw-cap vials
- Micro stir bar
- Animal balance (accurate to 0.1 g)
- 1-ml syringes and 27-G × 1/2-in. needles
- 100- μ l pipet and tips

Sensitize with ovalbumin (day 1)

1. Thaw 1.2 ml ovalbumin for sensitization and begin stirring in a 2-ml screw-cap plastic vial with a micro stir bar. Add 0.4 ml Imject Alum dropwise to 1.2 ml solution of ovalbumin for sensitization or normal saline solution (endotoxin-free).

This is enough ovalbumin for sensitization of 5 to 7 mice. Stir each vial continuously for 30 min at room temperature to allow adsorption of the antigen.

Reagents and containers for sensitization and challenge should be endotoxin free. Use endotoxin-free reagents and sterile plastic ware. Alternatively, treat any glassware with E-Toxa clean concentrate (Sigma) or similar reagent to remove endotoxin contamination. Treating clean glassware at 175°C for a minimum of 3 hr will also destroy endotoxin. Ovalbumin can have endotoxin contamination. The amount of endotoxin contamination in a particular lot of ovalbumin can be determined using a Limulus Amebocyte Lysate assay supplied by Sigma (E-Toxate Kit) or BioWhittaker.

2. Weigh mouse and calculate volume for intraperitoneal injection at 0.2 ml/20 g mouse (0.5 mg ovalbumin/kg). Swirl the ovalbumin/aluminum hydroxide suspension prior to loading the calculated volume into a 1-ml syringe with attached 27-G × 1/2-in. needle.
3. Pick up mouse by the tail and allow the mouse to grab the cage top with its front paws. Pinch the skin at the nape of the neck firmly with thumb and forefinger of the other hand, holding the tail between the palm and other fingers.
4. Inject calculated volume intraperitoneally by tilting the animal slightly head down so the intestines fall forward, insert needle bevel up in the lower right quadrant of the abdomen, slightly off the midline, and then pull back slightly on the plunger before injecting to ensure that no fluid is withdrawn.

The needle should be inserted high enough to avoid the bladder and the cecum and not deep enough to penetrate kidneys or spinal column.

If yellow or greenish-brown fluid is withdrawn, the bladder or the cecum may have been penetrated. If this is the case, discard the syringe and its contents and start again.

5. Return the mouse to the cage. Repeat steps 2 through 5 if necessary, for other mice.

Intranasally challenge with ovalbumin (days 14 to 16)

6. Thaw ovalbumin solution for challenge and mix gently by inversion.
7. Weigh mouse and calculate volume of ketamine/xylazine solution for intramuscular injection (0.1 ml/20 g mouse). Load 1-ml syringe with 27-G × 1/2-in. needle with ketamine/xylazine.

Ketamine is a Schedule III controlled substance and requires proper licensing and storage in an authorized locked cabinet.

8. Calculate volume of ovalbumin for challenge or water (endotoxin-free) for intranasal instillation (80 µl/20 g mouse; 5 mg/kg) and load 100-µl pipet with proper volume.

Alternatively, a mouse serum albumin solution in water can be used as a control challenge.

9. Pick up mouse by the tail and allow it to grab the cage top with its front paws. Pinch the skin at the nape of the neck firmly with thumb and forefinger of the other hand, holding the tail and hind limb between the palm and other fingers.
10. Inject calculated volume of ketamine/xylazine (from step 7) into muscle of hind limb. Return mouse to cage until anesthesia takes effect in ~3 to 5 min.
11. Remove anesthetized mouse from cage and hold gently with nostrils pointing straight up. Using the 100-µl pipet, slowly drip calculated volume of ovalbumin for intranasal

instillation (from step 8) over nostrils, alternating nostrils until entire volume disappears from view. Return mouse to cage.

12. Repeat daily for 3 days at approximately the same time of day for each intranasal instillation.

A convenient challenge time is first thing in the morning so that the evaluation of airway hyperresponsiveness (see Support Protocol 1) and cellular infiltration (see Support Protocols 2 and 3) can be measured at 24 and/or 48 hr post-challenge.

SUPPORT PROTOCOL 1

MEASURING AIRWAY HYPERRESPONSIVENESS IN A CONSCIOUS UNRESTRAINED MOUSE

A hallmark of asthma in the human is increased reactivity of the airways to numerous different agonists, i.e., airway hyperresponsiveness. As the severity of the asthma increases, the reactivity of the airways to methacholine increases. A positive methacholine challenge test is used clinically in the differential diagnosis of asthma. In the mouse, various methods have been developed to measure pulmonary function. Whole-body plethysmography in conscious unrestrained animals (Hamelmann et al., 1997) uses changes in a plethysmograph box pressure to follow breathing patterns and calculate the dimensionless value-enhanced pause (PenH). The inspiratory time, the decay of the expiratory time, and the maximum changes in box pressure during inspiration and expiration are used to calculate PenH. Values of PenH have been shown to correlate with measures of pulmonary resistance and compliance obtained by invasive methods in anesthetized mice (Hamelmann et al., 1997). Various studies including a recent paper by Lundblad et al. (2002) have questioned the validity of PenH as a reliable indicator of bronchoconstriction in the mouse. Though the value of the method continues to be debated, it has the distinct advantage of being a high throughput, non-invasive method that allows measurement of individual animals over time, in the absence of confounding effects of anesthesia.

Materials

Methacholine (see recipe)

Mice sensitized and challenged with ovalbumin and appropriate control animals
(see Basic Protocol)

Endotoxin-free saline

Buxco whole-body plethysmograph with Softmax XA software for whole-body
flow-derived parameters (Buxco Electronics); four chambers monitored
simultaneously

Balance (accurate to 0.1 g)

1. Remove methacholine stock solution from freezer.
2. Calibrate Buxco plethysmograph system per manufacturer's instructions.
3. Weigh mice.
4. For 4 animals, mix the following proportions of methacholine stock + endotoxin-free saline:

6 mg/ml	0.0625 ml + 3.9375 ml
12 mg/ml	0.125 ml + 3.875 ml
24 mg/ml	0.25 ml + 3.75 ml
48 mg/ml	0.5 ml + 3.5 ml
96 mg/ml	1 ml + 3 ml.

Place solutions on ice until loaded in the nebulizer cup.

5. Place mouse in whole-body plethysmograph box.

Depending on the available equipment, many mice can be monitored and exposed to methacholine simultaneously. Randomly assign treatments to each of the whole-body plethysmographs so as not to bias the results.

6. Place 4 ml endotoxin-free saline in the nebulizer cup. Nebulize for 2 min and then monitor respiratory variables for an additional 6 min during the drying phase.
7. Remove nebulizer cup and discard any remaining saline. Place 4 ml of 6 mg/ml methacholine in the nebulizer cup and repeat nebulization for 2 min plus a 6-min monitoring cycle.

The changing of solutions in the nebulizer should be accomplished within 1 min or use a consistent time period between doses.

8. Repeat step 7 with 12, 24, 48 and 96 mg/ml methacholine, using the same 2-min nebulization period and 6-min monitoring cycle.
9. Remove the mice from the chambers and return them to their cages.
10. Export the data and save in Excel spreadsheet.
11. Refill nebulizer cup with 4 ml endotoxin-free saline and run another sequence to flush through the tubing.
12. Shut down the air flows, disassemble, and wipe clean all chambers before running a second set of animals.

The system should also be recalibrated before running another set of animals.

MEASUREMENT OF CELLULAR INFILTRATION INTO THE AIRSPACE

A characteristic of the asthmatic response is eosinophil infiltration into the lung and airspace. Depending on the strain of mouse and the ovalbumin doses chosen for sensitization and challenge, cellular infiltration will be evident in the airspace and lung tissue of the animal anywhere from 6 hr after the last challenge to many days later. A common time point chosen is 48 hr after the last intranasal challenge, when both the measurement of airway hyperresponsiveness, as outlined in Support Protocol 1, and eosinophil infiltration are apparent. Cellular infiltration into the airspace is assessed using bronchoalveolar lavage (BAL). In this technique, the animal is euthanized, the trachea is cannulated, and PBS or other appropriate solution is introduced via the tracheal cannula to collect cells from the airway and alveolar space (also see UNIT 18.4). If airway hyperresponsiveness to methacholine is determined, wait at least 30 min before BAL to allow the methacholine-induced airway constriction to subside. This allows better recovery of BAL fluid and cells.

Materials

Mice sensitized and challenged with ovalbumin and appropriate control animals
(see Basic Protocol)
Phosphate-buffered saline (PBS; APPENDIX 2A; or from GIBCO)
50 mg/ml pentobarbital sodium (Abbott)
Unopette Microcollection System solution (Becton Dickinson)
Normal guinea pig serum (Sigma)
Methanol
Modified Wrights' stain (Diff Quik, VWR)

Balance for weighing mice (accurate to 0.1 g)
10-ml beaker
3-ml syringes

SUPPORT PROTOCOL 2

Immunotoxicology

18.3.5

Thread (4-0 silk)
 Forceps (blunt, curved tip, serrated micro-dissecting forceps, Roboz Surgical Instruments)
 Tracheal cannula (20-G needle, bevel filed off)
 Dissecting scissors (5 1/2-in. blunt, Roboz Surgical Instruments)
 Iris scissors (3-in., Roboz Surgical Instruments)
 1.5-ml siliconized eppendorf tubes (low adhesion, Dot Scientific)
 Refrigerated, low-speed centrifuge
 Analytical balance (accurate to 0.001 g)
 1.5-ml microcentrifuge tube
 Neubauer hemacytometer (or automated cell counter such as a Coulter counter)
 96-well U-bottomed microtiter plates (Sarstedt)
 Shandon Cytospin 3 centrifuge (Shandon/Lipshaw)

Perform bronchoalveolar lavage (BAL)

1. Weigh the mouse sensitized and challenged with ovalbumin and appropriate control animals (see Basic Protocol).
2. Measure 1.8 ml endotoxin-free PBS for lavage into a 10-ml beaker. Prefill a 3-ml syringe with ~0.9 ml PBS and leave the syringe in the beaker.
3. Inject the mouse with 50 mg/ml pentobarbital (0.12 ml/20 g mouse) intraperitoneally.

Once the animal does not respond to a toe pinch of the hind foot, it is ready for insertion of the tracheal cannula.

If bleeding the mouse prior to BAL, be sure to monitor mouse continuously so that bleeding by retro-orbital or intracardiac method can be done before the heart stops beating or the blood pressure rapidly decreases. This dose of pentobarbital is intended to euthanize the animal.

Pentobarbital is a Schedule II controlled substance and requires proper licensing and storage.

4. Immobilize the animal on its back. Make a midline incision over the trachea using dissecting scissors. Cut through muscle layers over the trachea and using the forceps, isolate the trachea and put two threads around it, each ~12 cm long. Make a loop in thread 1 over the trachea in preparation for tying a knot around the tracheal cannula (see Fig. 18.3.1). Tie a knot in thread 2, leaving a 4- to 5-cm diameter loop.

When making an incision, stick to the midline so as to avoid severing arteries and veins. Be careful not to sever the trachea. When isolating the trachea, insert forceps with points together under the trachea and then spread the points apart underneath the trachea to spread the tissue. Grab both threads with the forceps and pull under the trachea to then form the loops of threads 1 and 2 as depicted in Figure 18.3.1.

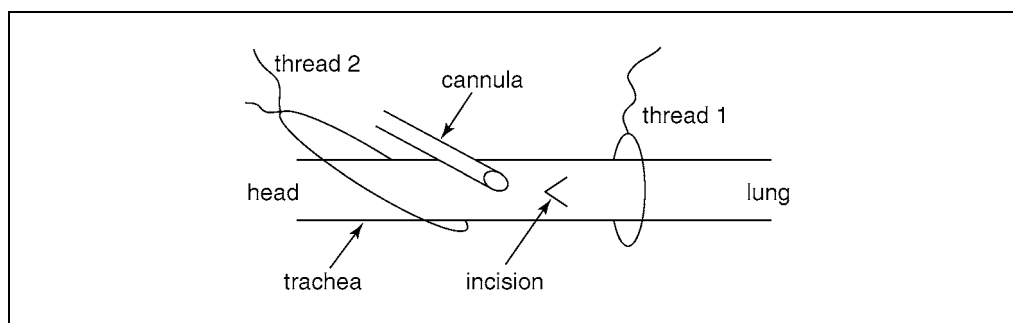


Figure 18.3.1 Schematic of tracheal cannulation of mouse.

5. Gently lift the loop of thread 1, nearest the lungs, to lift the trachea up. With iris scissors, make a V-shaped incision half way through the trachea. Insert the blunt needle cannula into the opening, advancing it ~3 to 5 mm into the trachea towards the lungs. Tie thread 1 securely with a single knot.

Thread 2 can be used to put a small amount of tension on the trachea while making the incision and inserting the cannula.

6. Attach the 3-ml syringe containing room temperature PBS to the cannula. Gently deliver the volume of PBS into the lungs. Withdraw the fluid slowly until bubbles begin to appear, disengage the syringe from the cannula, and put the BAL fluid into a 1.5-ml siliconized eppendorf tube on ice. Repeat with the same volume of PBS and add to the BAL fluid already collected. Continue using the syringe to withdraw as much lavage fluid from the animal as possible.

Throughout the procedure it is best to continue holding the hub of the cannula with one hand while manipulating the 3-ml syringe with the other hand until all of the lavage fluid is returned.

7. If the animal is to be used for assessment of cellular infiltration into the lung as well, proceed immediately to Support Protocol 3.

Count cells and determine differentials

8. Centrifuge the BAL fluid 12 min at $\sim 600 \times g$, 4°C .
9. On an analytical balance, tare a 1.5-ml microcentrifuge tube. Using a 1-ml pipet, remove the supernatant from the cell pellet, being careful not to disturb the pellet. Add this BAL fluid to the tared 1.5-ml microcentrifuge tube. Record the weight of the BAL supernatant and freeze the supernatant (assume $1\text{ g} = 1\text{ ml}$).

Depending on the purpose of the experiment, antibodies, mediators, or other substances may be measured in the BAL supernatant.

10. Resuspend the cell pellet gently in 0.125 ml PBS and keep on ice.
11. Add $5\text{ }\mu\text{l}$ of cell suspension to $45\text{ }\mu\text{l}$ Unopette solution in a 1.5-ml microcentrifuge tube. Allow to sit for 10 min to ensure that any red blood cells are lysed.

The Unopette Microcollection System solution is an ammonium oxalate diluent to lyse any red blood cells.

12. Load a standard Neubauer hemacytometer with the diluted cell suspension, in duplicate, and count the cells in each of the nine large squares, including those that lie on the top and left-hand perimeters. Do not count cells lying on the bottom and right-hand perimeters. Average the cell counts and calculate the number of white blood cells/ml of the original solution using the following formula:

$(\text{Average no. of cells}/9) \times 10^5 = \text{cells/ml in original cell suspension resuspended to } 0.125\text{ ml.}$

Before loading the hemacytometer, make sure the cells are uniformly suspended in the liquid. In a control mouse, expect $1\text{--}10 \times 10^4$ cells total. In ovalbumin-sensitized and challenged animals, the number of white blood cells may be increased two to ten times.

13. Remove aliquots of 2×10^4 cells in 10- to 40- μl volume for cytopins. Dilute cells if necessary.
14. For cytopins, mix 2×10^4 cells, $130\text{ }\mu\text{l}$ PBS, $10\text{ }\mu\text{l}$ normal guinea pig serum, and $15\text{ }\mu\text{l}$ methanol in the well of a 96-well U-bottomed microtiter plate.

Table 18.3.1 Effect of Ovalbumin Challenge on Bronchoalveolar Lavage Cell Numbers^a

Treatment	<i>N</i>	White blood cells ($\times 10^4$)	Macrophages ($\times 10^4$)	Eosinophils ($\times 10^4$)	Neutrophils ($\times 10^4$)
Water	5	6.5 $\pm$ 2.4	5.9 $\pm$ 2.3	0.04 $\pm$ 0.01	0.47 $\pm$ 0.20
Ovalbumin	8	7.8 $\pm$ 1.3	6.7 $\pm$ 1.1	0.29 $\pm$ 0.11 ^b	0.79 $\pm$ 0.24

^aThirteen Balb/c mice were sensitized with ovalbumin and challenged with either ovalbumin or water as described in the Basic Protocol. At 48 hr after the last intranasal challenge, bronchoalveolar lavage was conducted as described in Support Protocol 2. Values represent mean $\pm$ SEM.

^b $p < 0.05$ compared to water treatment.

15. Add entire cell mixture to double cytospin funnel and cytospin 3 min at 1000 rpm with low acceleration, using double cytoslides for duplicate samples.
16. Air dry slides and stain with Diff Quik solution as recommended by the manufacturer.
17. Count 200 cells per spot (400 total), differentiating them as lymphocytes, macrophages, eosinophils, and neutrophils.
18. Express data as % cell type. Use total white blood cells from the mouse to calculate the total number of each cell type recovered from the mouse (typical results are seen in Table 18.3.1).

MEASURING CELLULAR INFILTRATION INTO THE LUNG

After sensitization and challenge of mice with ovalbumin (see Basic Protocol), depending on the experimental goals, a common scenario involves evaluation of airway hyperresponsiveness at 24 and/or 48 hr after the last intranasal challenge (see Support Protocol 1), followed by euthanasia of the mouse 30 to 60 min later for evaluation of cellular infiltration into the airspace and lung. The BAL as outlined in Support Protocol 2 is conducted, and then the lung lobes are removed as outlined in this protocol for the determination of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) as indicators of the numbers of eosinophils and neutrophils, respectively, in the remaining lung tissue. Alternatively, the mouse can be euthanized and the lung lobes removed as outlined in this protocol without doing BAL.

Processing of lung lobes differs depending on whether EPO or MPO will be measured. For EPO, lung homogenates are centrifuged and the supernatant discarded. The resulting pellet is resuspended in the cetrimide buffer and sonicated prior to 2 cycles of freeze/thaw and sonication. EPO is tightly bound to membranes or contained within specific granules and is not liberated until the sonication and freeze/thaw cycles (Dimayuga et al., 1991). After freeze/thaw cycles and sonication, EPO activity is associated with both soluble and insoluble fractions, therefore, the assay of EPO uses both. For MPO, the enzyme is located within the primary granules of neutrophils. Extraction of MPO depends on disruption of the granules as well as the presence of cetrimide, which renders MPO soluble in aqueous solution (Bradley et al., 1982). The solubilized MPO is then separated from tissue debris by centrifugation. Aminotriazole is used in the MPO assay to inhibit any EPO contamination of the MPO sample.

Materials

- Mice sensitized and challenged with ovalbumin and appropriate control animals (see Basic Protocol)
- 50 mg/ml pentobarbital sodium (Abbott)
- Cetrimide (hexadecyltrimethylammonium bromide; see recipe)

Balance (accurate to 0.1 g)
 2-ml glass vials (12 × 32-mm; Fisher)
 Analytical balance (accurate to 0.0001 g)
 Small hemostat (4 1/2-in.)
 Dissecting scissors (5 1/2-in. blunt, Roboz Surgical Instruments)
 Kimwipes in petri dish
 Luxo magnifier (Daigger)
 12 × 75-mm polystyrene round-bottomed tubes (Falcon)
 Drying oven (Carbolite)
 Homogenizer (Janke Kunkel Ultra-Turrax T-25, 24,000 rpm) with 8-mm generator probe for aqueous and organic solutions
 250- and 50-ml beakers
 Ear plugs
 Forceps
 1.0-ml pipet
 Refrigerated, low-speed centrifuge
 Sonicator (e.g., Daigger VC-130 ultrasonic processor) with microtip
 Refrigerated, high-speed centrifuge and 12 × 75-mm high-speed centrifuge tubes
 1.5-ml microcentrifuge tubes

Remove lung lobes

1. Weigh the mouse sensitized and challenged with ovalbumin and appropriate control animals (see Basic Protocol).
2. Preweigh a 2-ml glass vial on an analytical balance to 0.0001 g and record the weight.
3. Inject the mouse with 50 mg/ml pentobarbital (0.12 ml/20 g mouse) intraperitoneally.

Once the animal does not respond to a toe pinch of the hind foot, it is ready for removal of the lungs.

If bleeding the mouse prior to surgery, be sure to monitor mouse continuously so that bleeding by retro-orbital or intracardiac method can be done before the heart stops beating or the blood pressure rapidly decreases. This dose of pentobarbital is intended to euthanize the animal.

Once the animal has been euthanized, the lung lobes should be removed as quickly as possible and placed on ice in cetrimide.

Pentobarbital is a Schedule II controlled substance and requires proper licensing and storage.

4. Immobilize the animal on its back. Make a midline incision through the chest wall, being careful not to cut through the lungs. Clamp the trachea as high up as possible with a small hemostat, and use dissecting scissors to remove the heart and lungs intact, still attached to the trachea.
5. Invert the heart and lungs on a Kimwipe in a petri dish so the heart is underneath the lung lobes, as if observing by the dorsal view of the heart and lungs in situ.

If the trachea is facing away, then the large left lobe is on the left side (see Fig. 18.3.2). Work quickly so tissue does not dry out.

6. Remove the inferior lobe of the right lung (see Fig. 18.3.2) for determination of MPO and the left lung lobe for determination of EPO. Be sure to trim pieces of the heart, esophagus, and fat tissue, using the Luxo magnifier for easy visualization.

The remainder of the lung without the trachea is used to determine the wet/dry weight of the lung.

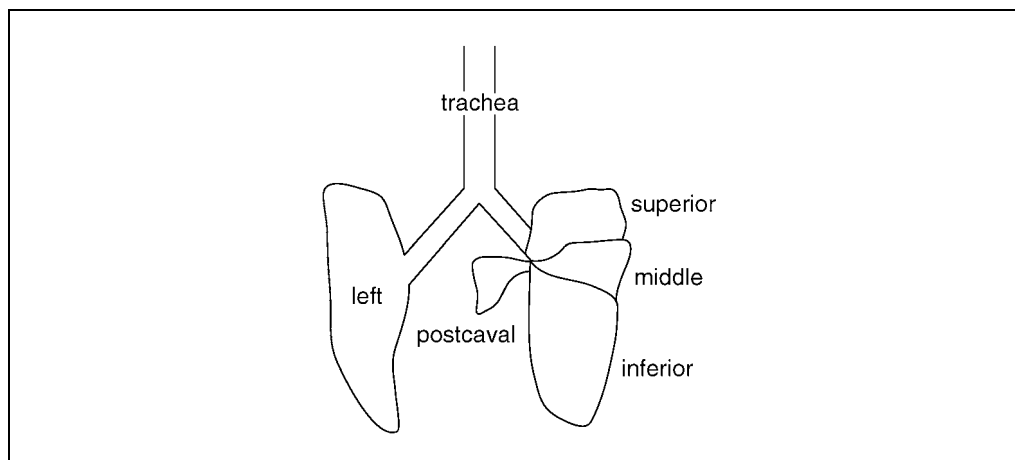


Figure 18.3.2 Schematic of dorsal view of mouse lung lobes. Lobes are named as described by Cook et al. (1983). The pattern of lobation may vary with the strain of mouse.

7. Tare a plastic 12 × 75-mm tube on the analytical balance and place the inferior lobe in the tube. Record the wet weight. Add 0.5 ml cetrimide to the tube and immediately place on ice.
8. Tare a second plastic 12 × 75-mm tube and place the left lobe in the tube. Record the wet weight. Add 0.5 ml cetrimide to the tube and immediately place on ice.
9. Place the wet/dry piece of lung in the preweighed 2-ml glass vial and record the weight of the vial plus the wet lung. Dry in a drying oven for 48 to 72 hr at 80°C to constant weight. Record weight of dried lung plus vial.

Homogenize left lung lobe and inferior lobe for enzyme assays

10. Rinse homogenizer by placing tip in 250-ml beaker of distilled water and turning it on for ~15 sec.

Use ear protection, e.g., ear plugs, when operating the homogenizer.

11. Wipe down tip thoroughly with Kimwipes and remove any debris remaining on tip with forceps.
12. Place the 12 × 75-mm tube containing lung lobe into a 50-ml beaker of wet ice.
13. Remove cap, and homogenize sample on ice ~20 sec at 24,000 rpm.

Take care to keep the sample on ice, and rotate the tube to homogenize the sample evenly.

14. Using a 1.0-ml pipet, rinse homogenizer with 0.5 ml of cetrimide, being sure to allow all liquid to drain into homogenized sample tube, giving a total volume of 1 ml plus the lung lobe.
15. Replace homogenized sample back on ice.

Prepare left lobe for eosinophil peroxidase (EPO) assay

16. Centrifuge the tube containing homogenized left lobe for 30 min at 400 × g, 4°C.
17. Discard supernatant and resuspend pellet in 0.5 ml cetrimide.
18. Sonicate for 15 sec, output 3, with tube on ice. Freeze sample at –20°C.

Use ear protection when operating the homogenizer and sonicator.

19. Thaw, sonicate, and freeze (–20°C) the sample two additional times prior to determination of EPO (see Support Protocol 4). Store samples frozen at –20°C until assayed.

Place sample on ice immediately after thawing at room temperature. If samples are to be stored longer than 2 to 3 months, place at -70°C .

Prepare inferior lobe for MPO assay

20. Sonicate the sample containing the homogenized inferior lobe for 15 sec, output 3 with tube on ice. Freeze sample at -20°C .

Use ear protection when operating the sonicator.

21. Thaw, sonicate, and freeze (-20°C) the sample two additional times prior to centrifugation.

Place sample on ice immediately after thawing at room temperature.

22. Thaw sample, transfer to a 12×75 -mm high-speed centrifuge tube, and centrifuge 30 min at $12,100 \times g$, 4°C .

23. Transfer supernatant to 1.5-ml microcentrifuge tube and store frozen at -20°C until assayed (see Support Protocol 5).

If samples will be stored longer than 2 to 3 months, place at -70°C .

EOSINOPHIL PEROXIDASE (EPO) ASSAY

Eosinophils contain EPO and neutrophils contain MPO. Eosinophil peroxidase from tissues is measured as described by Strath et al. (1985) and Cheng et al. (1993). OD changes at 490 nm are monitored using the substrate solution *o*-phenylenediamine dihydrochloride (OPD) in a pH 8.0 buffer with 1 mM hydrogen peroxide. Assessment of EPO and MPO activity in a mixed cell population that contains both eosinophils and neutrophils is possible because of differences in the pH optimum for EPO and MPO (Worthington Enzyme Manual, 1972), as well as the ability of aminotriazole to inhibit EPO with minimal effects on MPO (Cramer et al., 1984). Thus, to achieve relative specificity for each enzyme, the EPO assay is conducted at pH 8.0, whereas the MPO assay is conducted at pH 6.0 in the presence of aminotriazole.

Materials

Homogenized left lung (see Support Protocol 3)
0.1% and 1% (v/v) Triton/Tris buffers (see recipes)
o-Phenylenediamine dihydrochloride (OPD; see recipe)
30% H_2O_2 (Sigma)

Spectrophotometer with visible light and kinetics software
Sonicator (Daigger) with microtip
Vortex
96-well U-bottomed microtiter plates (Sarstedt)
Glass cuvettes
Small squares of Parafilm ($\sim 2 \times 2$ -cm)

CAUTION: Wear gloves throughout the procedure because OPD is a potential carcinogen. Hazardous waste containing OPD should be collected for proper disposal. Cover the work area with plastic-backed absorbent paper and wipe down all surfaces at the end with water.

1. Set up spectrophotometer for kinetic assays with a recommended starting point of determining OD every 5 sec for 2 min at a wavelength of 490 nm.
2. Thaw frozen homogenized left lung lobe sample (see Support Protocol 3, step 19). Keep tube on ice during assay.

Place sample on ice immediately after thawing at room temperature.

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**SUPPORT
PROTOCOL 5**

3. Sonicate homogenized lung samples 15 sec on ice. Vortex before withdrawing 150 μ l for dilution.
4. For a 1:2 dilution in a 96-well U-bottomed microtiter plate, mix 150 μ l sample, 30 μ l of 1% Triton/Tris buffer, and 120 μ l of 0.1% Triton/Tris buffer. Serially dilute 70 μ l of the 1:2 dilution back in 70 μ l of 0.1% Triton/Tris buffer to a 1:4 dilution.
5. Place the following into cuvette, in order: 860 μ l of 0.1% Triton/Tris buffer, 50 μ l sample dilution, and 8.5 μ l OPD. Top cuvette with Parafilm and invert to mix. Wipe sides of cuvette clean. Insert into the spectrophotometer and take a baseline reading at 490 nm. Remove cuvette from spectrophotometer.
6. Add 1 μ l of 30% H_2O_2 , place Parafilm on top, invert two times, and immediately place cuvette into spectrophotometer and begin to take kinetic readings.
7. When the reaction is complete, usually within 2 min, remove cuvette, pour contents into a hazardous waste container, rinse cuvette once with water, and pour into waste container. Finally, rinse cuvette eight to ten times with water and discard the waste down the drain.
8. If it is necessary to run an undiluted sample because the signal from the 1:2 dilution is very low, add 100 μ l of 1:2 dilution to 810 μ l of 0.1% Triton/Tris buffer, and 8.5 μ l OPD. Take a baseline reading and then start the reaction as step 6 using 1 μ l of 30% H_2O_2 .

MYELOPEROXIDASE (MPO) ASSAY

Myeloperoxidase (MPO) is a constituent of neutrophil granules. For the assay of MPO, the hydrolysis of H_2O_2 is monitored by the change at 460 nm of *o*-dianisidine dihydrochloride (ODA) in a pH 6 phosphate buffer in the presence of the EPO inhibitor aminotriazole (AMT). Units of MPO activity are calculated as described in the Worthington Enzyme Manual (1972) and by Ormrod et al. (1987).

Materials

Frozen homogenized inferior lung lobe sample (see Support Protocol 3)
0.05 M potassium phosphate buffer, pH 6.0 (see recipe)
o-Dianisidine dihydrochloride (ODA; see recipe)
Aminotriazole (AMT; see recipe)
0.5 mM H_2O_2 (see recipe)

Spectrophotometer with visible light and kinetics software
Glass cuvettes
Small squares of parafilm ($\sim 2 \times 2$ -cm)

CAUTION: Wear gloves throughout the procedure because ODA and AMT are potential carcinogens. Hazardous waste should be collected for proper disposal. Cover the work area with plastic-backed absorbent paper and wipe down all surfaces at the end with water.

1. Set up spectrophotometer for kinetic assays with a recommended starting point of determining OD every 5 sec for 3 min at a wavelength of 460 nm.
2. Thaw frozen homogenized inferior lung lobe sample (see Support Protocol 3, step 23). Keep sample tubes on ice during the assay.

Place sample on ice immediately after thawing at room temperature.

3. Place the following into cuvette, in order: 873 μl of 0.05 M potassium phosphate buffer, pH 6.0, 30 μl undiluted sample, 100 μl ODA, and 10 μl AMT. Top cuvette with Parafilm and invert two times to mix. Wipe sides of cuvette clean.

If AMT solution yellows during the assay, discard aliquot and thaw a new one.

4. Insert cuvette into spectrophotometer. After 3 min, take baseline reading at a wavelength of 460 nm.

This 3-min waiting period ensures that AMT has time to inhibit any EPO activity present.

5. Remove cuvette, add 10 μl of 0.5 mM H_2O_2 , place Parafilm on top, invert two times, and immediately place cuvette into spectrophotometer and begin to take kinetic readings.
6. When the reaction is complete, usually within 3 min, remove cuvette, pour contents into hazardous waste container, rinse cuvette once with water, and pour into waste container. Finally, rinse cuvette eight to ten times with water and dispose of the waste down the drain.
7. If MPO activity is very low, double the sample size by adding 843 μl of 0.05 M potassium phosphate buffer, pH 6.0, 60 μl undiluted sample, 100 μl ODA, and 10 μl AMT. Wait 3 min, take a baseline reading, and then start the reaction as above using 10 μl of 0.5 mM H_2O_2 .

If ODA crystallizes in cuvette during the assay, discard the ODA aliquot and thaw a new one.

The background reading is a good check to determine that the sample was added and that the ODA is not crystallizing (the background will be higher than normal).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Aminotriazole (AMT)

3-Amino-1,2,4 triazole (Sigma)

Dissolve at 42 mg/ml water (final concentration of 0.5 M)

Store in 0.2-ml aliquots up to 1 year at -20°C

Aminotriazole is a potential carcinogen, care should be taken when handling.

Cetrimide

Add 0.5 g cetrimide (Hexadecyltrimethylammonium bromide; Sigma) to 100 ml of 0.05 M potassium phosphate buffer, pH 6.0 (see recipe). Store up to 4 weeks at 4°C . Warm to room temperature before use.

H_2O_2 , 0.5 mM

Add 8.5 μl of 30% H_2O_2 (Sigma) to 4.99 ml 0.05 M potassium phosphate buffer, pH 6.0 (see recipe). Prepare on the day of the assay, keep on ice, and discard any remaining solution at the end of the assays.

Ketamine/xylazine

1 ml 100 mg/ml ketamine

1 ml 20 mg/ml xylazine

8 ml normal saline solution

Mix and store up to 1 month at room temperature

Ketamine is a controlled substance (Schedule III) and requires proper licensing and storage in an authorized locked cabinet.

Methacholine

To 19.2 g acetyl- β -methylcholine chloride (Sigma), add endotoxin-free saline up to 50 ml in a graduated cylinder. Dissolve at room temperature, transfer solution to ice bath, and dispense into 1-ml aliquots. Final solution concentration is 0.384 g/ml. Freeze 1-ml aliquots up to 6 months at -80°C .

ODA

Dissolve 1.67 mg/ml *o*-dianisidine dihydrochloride (Sigma) in water. Store in 1-ml aliquots up to 1 year at -20°C .

o-Dianisidine dihydrochloride is a potential carcinogen, care should be taken when handling.

OPD

Dissolve 90 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) in water. Store 50- μl aliquots up to 1 year at -20°C .

Ovalbumin for challenge

Dissolve 1.25 mg/ml ovalbumin (Grade V; Sigma) in endotoxin-free water. Allow to dissolve for 2 to 3 hr or overnight at 4°C . Stir gently and dispense into convenient aliquots (1-ml) for freezing at -20° or -80°C up to 2 years.

Ovalbumin can have endotoxin contamination. The amount of endotoxin contamination in a particular lot of ovalbumin can be determined using a Limulus Amebocyte Lysate assay. Reagents and kits for this assay are commercially available from, e.g., Sigma (E-Toxate Kit) or BioWhittaker.

Be sure to use endotoxin-free water and sterile plastic ware. Alternatively, treat any glassware with E-Toxa clean concentrate (Sigma) to remove endotoxin contamination. Treating clean glassware at 175°C for a minimum of 3 hr will also destroy endotoxin.

Ovalbumin for sensitization

Dissolve 0.067 mg/ml ovalbumin (Grade V; Sigma) in endotoxin-free saline. Allow to dissolve for 2 to 3 hr or overnight at 4°C . Stir gently and dispense into 1.2-ml aliquots in 2-ml polypropylene screw-cap vials for freezing at -20° or -80°C up to 2 years.

Ovalbumin can have endotoxin contamination. The amount of endotoxin contamination in a particular lot of ovalbumin can be determined using a Limulus Amebocyte Lysate assay. Reagents and kits for this assay are commercially available from, e.g., Sigma (E-Toxate Kit) or BioWhittaker.

Be sure to use endotoxin-free saline and sterile plastic ware. Alternatively, treat any glassware with E-Toxa clean concentrate (Sigma) to remove endotoxin contamination. Treating clean glassware at 175°C for a minimum of 3 hr will also destroy endotoxin.

Potassium phosphate buffer, 0.05 M (pH 6.0)

1.361 g KH_2PO_4 dissolved in ~ 150 ml water
pH to 6.0 with 0.1 M K_2HPO_4
Add water to 200 ml
Store up to 6 months at 4°C
Warm to room temperature before use

Triton/Tris buffer, 0.1% (v/w)

0.657 g Tris (50 mM) dissolved in ~ 60 ml water
Add 100 μl of Triton X-100

Mix well with stir bar
Adjust pH to 8.0 with 2 N HCl
Add water to 100 ml
Store up to 1 month at 4°C
Warm to room temperature before use

Triton/Tris buffer, 1% (v/w)

0.657 g Tris (50 mM) dissolved in ~60 ml water
Add 1 ml of Triton X-100
Mix well with stir bar
Adjust pH to 8.0 with 2 N HCl
Add water to 100 ml
Store up to 1 month at 4°C
Warm to room temperature before use

COMMENTARY

Background Information

Numerous studies have investigated the mechanism of ovalbumin-induced asthma in the mouse. Recent reviews summarize particular aspects of the mechanism in murine asthma models (Wills-Karp, 2000; Leong and Huston, 2001; Abraham, 2003). Bernstein et al. (1999) provides an overview of the studies done in occupational asthma in humans and in animal models. It is apparent that a wide variety of mouse model systems are used in investigating the asthmatic response. Asthma is a chronic inflammatory immune disease with a prominent role for CD4+ T cells producing a Th2 pattern of cytokines. When appropriately sensitized and challenged, the characteristic components of the asthmatic response can be demonstrated in murine asthma models: immediate bronchoconstriction to antigen, increased airway microvascular permeability, airway hyperresponsiveness to an agonist such as methacholine, and inflammatory cell infiltration into the lung, particularly eosinophils. Mice readily make IgE antibody to parallel the human situation. The mouse model of asthma has the advantage that extensive information is available regarding the mouse genome and numerous inbred strains and transgenic mice are available to examine specific hypotheses. A disadvantage of the mouse model is that extensive exposure to the antigen along with the use of an adjuvant may be required to reproducibly elicit the asthmatic response in a short period of time. This is in contrast to a species such as the guinea pig where the lung is the target organ and bronchoconstriction and eosinophil infiltration readily occur after antigen exposure.

In mouse models, the expression of different components of the effector phase of the

asthmatic response, and the magnitude and time course of those responses, may differ depending on the routes of sensitization and challenge with the allergen, the dose of antigen, as well as the inbred strain of animal used. When examining 12 different strains of mice, Brewer et al. (1999) found a wide range of responses in both airway hyperresponsiveness to methacholine (zero- to three-fold change) and eosinophilia in the BAL (2% to 60%). Karp et al. (2000), using different strains of mice, found that complement component C5 is a susceptibility locus for airway hyperresponsiveness in ovalbumin-induced asthma. Thus, C5 deficient strains of mice such as A/J mice very readily develop airway hyperresponsiveness to ovalbumin sensitization and challenge.

Zhang et al. (1997) directly compared different routes of ovalbumin administration (intranasally versus intraperitoneally) in Balb/c mice and concluded that without local allergen exposure intranasally the mice did not develop airway hyperresponsiveness. The dose of allergen is also important in determining the asthmatic response. Mice sensitized with low doses of ovalbumin and alum intraperitoneally presented with both eosinophilia in the lung and airway hyperresponsiveness, whereas higher sensitizing doses by the same route resulted in a reduced eosinophilia and the absence of airway hyperresponsiveness (Sakai et al., 1999).

For sensitization, numerous studies have employed ovalbumin plus alum sensitization intraperitoneally. Alternatively, repeated aerosol administration of ovalbumin has also been used for sensitization. With regard to the ovalbumin challenge, repeated aerosol administration or multiple intranasal challenges help ensure a strong reproducible asthmatic response. Intratracheal challenge can also be

used to elicit the asthmatic symptoms and avoids exposure of the upper airways, but it is somewhat more invasive. Aerosolizing the ovalbumin requires a nebulizer and exposure chamber, as well as greater quantities of ovalbumin. The intranasal route has the advantage of simplicity, convenience, and a known dose of ovalbumin is delivered to the animal. Southam et al. (2002) systematically examined the effects of anesthesia, instillation volume, and position of the animal on the delivery of ovalbumin to the lung by intranasal instillation. The Basic Protocol utilizes three successive intranasal challenges to elicit the asthmatic response in the mouse. However, fewer challenges may also be sufficient. Tomkinson et al. (2001) successfully used a single intranasal challenge with ovalbumin to determine the time course of the airway hyperresponsiveness and eosinophil infiltration into the lung of the mouse.

In an asthmatic response, antigen challenge itself initiates an airway obstruction. After the response to antigen, the asthmatic airway becomes hyperresponsive to numerous agonists, including acetylcholine. The airways are more responsive to a lower dose of acetylcholine and the maximal response is also increased. Methacholine is a muscarinic agonist that is not as susceptible as acetylcholine to spontaneous hydrolysis and thus is useful in experimental situations to stimulate the muscarinic receptor. Many different methods are available to assess airway hyperresponsiveness. The one presented in this unit is the measurement of PenH using whole-body plethysmography in a conscious unrestrained mouse. Besides being minimally invasive, this technique allows high throughput and is not confounded by anesthesia. Airway reactivity can be assessed in a single animal at multiple time points to evaluate the time course of the event. Studies have been done to validate the technique as a measure of airway obstruction (Hamelmann et al., 1997). However, the method has been criticized as an indirect and imprecise measure of pulmonary function that is subject to changes in temperature and humidity (Lundblad et al., 2002). One experimental strategy is the use of PenH as an initial measure of airway hyperresponsiveness, followed by selected studies using more traditional measures of pulmonary mechanics in an anesthetized animal. Such methods in anesthetized ventilated mice may include measure of the airway pressure time index (Ewart et al., 1995), measurement of input impedance using the forced oscillation tech-

nique (Gomes et al., 2000), or a plethysmographic method of measuring pulmonary resistance and compliance (Martin et al., 1988). Isolated tracheal strips from ovalbumin-sensitized and -challenged mice have also been used to evaluate airway hyperresponsiveness, using either methacholine (De Bie et al., 1996) or electrical field stimulation (Hamelmann et al., 1999) as the constricting stimulus.

Many studies have utilized eosinophil peroxidase (EPO) and myeloperoxidase (MPO) as indicators of eosinophils and neutrophils, respectively, in lung tissue. The assays are quantitative and representative of the tissue as a whole. Measurements of EPO and MPO do not require histological interpretation and many measurements can be done in a short period of time. However, no information regarding the site of localization of the eosinophils and neutrophils—i.e., perivascular, interstitial, peribronchial, parenchymal—is obtained by measuring EPO and MPO in homogenized lung tissue.

The literature regarding the assay of EPO activity from cells and tissues is quite varied as to the tissue fractions analyzed. Some studies utilize the supernatant fraction after homogenization, freeze thawing and sonication in a Triton/Tris or cetrimide buffer (Collins et al., 1995; Hessel et al., 1997; Humbles et al., 1997). Other studies use both the soluble and insoluble fractions after a similar tissue preparation (Cheng et al., 1993; Dimayuga et al., 1991; Pettipher et al., 1994; Kung et al., 1995; Turner et al., 1996). Therefore, since methods vary considerably, especially homogenization and sonication techniques and buffers, it is very important in each laboratory to check soluble and insoluble fractions for EPO activity and confirm that the activity is inhibited by aminotriazole.

Besides measurement of cellular infiltration into the lung and airway hyperresponsiveness, mouse models of asthma lend themselves to the measurement of numerous other parameters not covered in this unit. Serum samples can be obtained and used for the measurement of antibodies. Antibody, protein, cytokines, and chemokines can be measured in the BAL fluid. Evidence of cell secretion (EPO) or cell injury (lactate dehydrogenase) may be evident in the BAL fluid. With proper precautions, the lung lobes can be used for isolation of RNA, cytokine measurements, histology, or immunocytochemistry. Collagenase digestion of lung lobes can be used to isolate lymphocyte populations for characterization.

Critical Parameters and Troubleshooting

As with any experiment, appropriate control groups of animals are essential and the protocol for sensitization and challenge will depend on the experimental question. Endotoxin contamination of solutions needs to be minimized because endotoxin will lead to neutrophilia itself, independent of an allergic reaction. Tissues should be removed from the animal as quickly as possible, keeping cells and tissues on ice in between manipulations. All samples should be frozen within 8 hr of removal from the animal.

Standard mouse feed is generally free of ovalbumin. However, if difficulties arise in seeing airway hyperresponsiveness or cellular infiltration, the possibility of ovalbumin exposure in the feed should be considered. Excessive exposure to ovalbumin could lead to tolerance and lack of reactivity to the allergen. As always, good animal husbandry is key to healthy animals and successful experiments.

When measuring EPO and MPO, it is important to use low amounts of enzyme so that the reaction rate is linear. If too much sample is used, the linear portion of the reaction will occur before the OD readings have begun. In the EPO assay, sonication prior to using the sample for assay is essential. Large pieces of tissue debris can interfere with the kinetic measurements. Low amounts of EPO in the sam-

ple will sometimes be difficult to distinguish from background. It may be useful to run EPO samples with and without the EPO inhibitor AMT so that noise can be distinguished from signal.

Anticipated Results

PenH for each mouse is averaged over the entire 8-min monitoring period to obtain an average PenH for each saline or methacholine dose. Data can then be expressed as the average for each dose, or as the ratio of the methacholine response over the control saline response for each animal. A change in control PenH values over time may indicate that environmental conditions are affecting baseline airway function. For example, excessive mold in the bedding or dust exposure may affect baseline PenH for the animals, and thus affect the airway responsiveness to methacholine after ovalbumin sensitization and challenge. Typical PenH results for the sensitization and challenge described in the Basic Protocol are shown in Figure 18.3.3. The concentration response curve to methacholine in the control animals tends to plateau. In contrast, PenH continues to increase over the methacholine concentration range in ovalbumin sensitized and challenged animals. Airway hyperresponsiveness is more apparent at 24 hr after the last intranasal challenge compared to the 48-hr time-point. The time course of the response

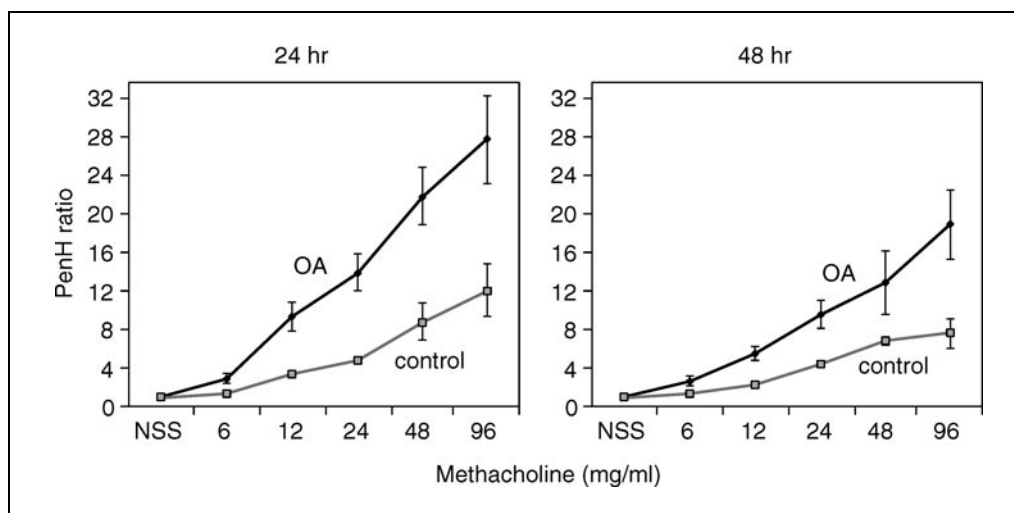


Figure 18.3.3 Six Balb/c mice were ovalbumin (OA)-sensitized and challenged as described in the Basic Protocol. At 24 and 48 hr after the last intranasal challenge, airway hyperresponsiveness to inhaled methacholine was determined as described in Support Protocol 1. PenH was determined and expressed as the PenH ratio (average PenH over the 8-min time interval with methacholine aerosol divided by the average PenH over the 8-min saline (NSS) aerosol exposure time). At 24 hr, the average PenH for NSS exposure was 0.58 ± 0.03 and 0.60 ± 0.09 for the control (water-challenged) and OA-challenged animals, respectively. At 48 hr, the average PenH for NSS exposure was 0.93 ± 0.06 and 0.80 ± 0.11 for the control (water-challenged) and OA-challenged animals, respectively. Values represent the mean $\pm$ SEM for 3 animals.

Table 18.3.2 Effect of Ovalbumin Challenge on EPO and MPO Measurements in the Lung^a

Mouse	Challenge	Lobe for wet dry weight ^b		Left lobe ^c		EPO			Inferior lobe ^d		MPO			
		Wet weight (g)	Dry weight (g)	Wet weight (g)	Dry weight calculated (g)	OD/min ^e	Total OD/min ^f	EPO/g dry weight ^g	Wet weight (g)	Dry weight calculated (g)	OD/min ^h	Total OD/min ⁱ	Total units ^j	MPO/g dry weight ^k
1	OA	0.0118	0.0027	0.0486	0.0111	0.137	1.37	123.4	0.0412	0.0094	0.032	1.100	0.0974	10.362
2	OA	0.0130	0.0030	0.0525	0.0121	0.693	6.93	572.7	0.0325	0.0075	0.015	0.512	0.0454	6.047
3	OA	0.0143	0.0030	0.0556	0.0117	0.342	3.42	292.3	0.0427	0.0090	0.034	1.171	0.1037	11.519
4	Water	0.0106	0.0027	0.0404	0.0103	0.029	0.29	28.2	0.0275	0.0070	0.017	0.578	0.0512	7.311
5	Water	0.0109	0.0025	0.0473	0.0108	0.042	0.42	38.9	0.0312	0.0072	0.010	0.341	0.0302	4.195
6	Water	0.0123	0.0029	0.0417	0.0098	0.031	0.31	31.6	0.0288	0.0068	0.015	0.511	0.0452	6.650

^aSix Balb/c mice were sensitized with ovalbumin (OA) and challenged 3 successive days with either ovalbumin or water as described in the Basic Protocol. The left lung lobe was removed for EPO measurements and the inferior lobe for MPO measurements as described in Support Protocol 3 at 48 hr after the last intranasal ovalbumin challenge. In this particular experiment, the postcaval lobe was used for wet/dry weight because the superior and middle lobes were used for RNA isolation. EPO, eosinophil peroxidase; MPO, myeloperoxidase.

^bLobe for wet/dry: Wet weight of postcaval lobe; Dry weight of postcaval lobe determined after 3 days at 80°C.

^cLeft lobe: Wet weight of left lobe; Dry weight calculated = (left lobe wet weight) × [(dry weight of lobe for wet dry)/(wet weight of lobe for wet dry)].

^dInferior lobe: Wet weight of inferior lobe; Dry weight calculated = (inferior lobe wet weight) × [(dry weight of lobe for wet dry)/(wet weight of lobe for wet dry)].

^eEPO OD/min = OD/min in EPO assay for 50 µl undiluted sample.

^fEPO total OD/min = EPO OD/min × 10. This is the total EPO for the 0.5-ml sample containing the whole left lobe.

^gEPO/g dry weight = (EPO total OD/min)/(Left lobe dry weight calculated).

^hMPO OD/min = OD/min in MPO assay for 30 µl undiluted sample.

ⁱMPO total OD/min = (MPO OD/min) × (1 + Wet weight of inferior lobe – Dry weight calculated of inferior lobe)/0.030. This is the total MPO from the inferior lobe, accounting for the additional sample volume added by the water in the lung lobe.

^jMPO total units = (MPO total OD/min)/11.3. MPO is expressed in units, where one unit is defined as that degrading 1 µmol of peroxide/min at 25°C, using the absorbance index of H₂O₂ of 11.3 (µmol/ml)⁻¹·cm⁻¹.

^kMPO/g dry weight = (MPO total units)/(Inferior lobe dry weight calculated).

will differ depending on the sensitization and challenge regimen used.

Since Support Protocol 4 is a kinetic assay, the intent is to determine the initial reaction rate. Thus, the slope of the OD change over time is determined before it maximizes, and OD/min changes do not need to be large. The software for the kinetics program being used should be set to determine the OD/min from only the linear portion of the 2-min data-collection period. This is often no more than the first 30 to 60 sec. The number of sample dilutions assayed depends on the consistency of the OD/min readings obtained after they are corrected for the dilution. For ovalbumin-sensitized and -challenged mice, undiluted samples to 1:4 dilutions are usually within range. In samples with very few eosinophils, the kinetic readings show a great deal of noise due to the tissue debris. Background can be determined by conducting the assay in the presence of aminotriazole to inhibit EPO activity. Typical results are calculated as shown in Table 18.3.2 with OD/min values ranging from 0.031 to 0.693 for 50 μ l of undiluted sample. In ovalbumin-challenged animals with higher EPO, the OD/min was determined on diluted samples and corrected for the dilution to arrive at the OD/min for a 50 μ l undiluted sample. Final data is expressed as EPO/g dry weight lung, which is significantly greater for the ovalbumin-challenged animals compared to their water-challenged controls.

Since Support Protocol 5 is a kinetic assay, the intent is to determine the initial reaction rate. Thus, the slope of the OD change over time is determined before it maximizes, and OD/min changes do not need to be large. The software for the kinetics program being used should be set to determine the OD/min from only the linear portion of the 3-min data-collection period. This is often no more than the first 60 to 90 sec. The MPO activity should be completely inhibited by 0.1 mM sodium azide but not by 5 mM aminotriazole. In the absence of hydrogen peroxide, no change in OD should be evident. Typical results are shown in Table 18.3.2. OD/min for 30 μ l undiluted sample range from 0.01 to 0.034. Final data is expressed as total MPO units/g dry weight of lung. As seen in Table 18.3.2, challenge with ovalbumin using the Basic Protocol does not result in a large increase in MPO in the lungs of sensitized mice as compared to water-challenged controls.

Time Considerations

The time from sensitization of the mouse to measurement of airway hyperresponsiveness and cellular infiltration is a 16-day period. Typically, 8 animals per day is a manageable number. This allows the intranasal challenge of 8 animals on 3 consecutive days first thing in the morning. Airway hyperresponsiveness measurements can then begin on the morning of the fourth or fifth day, depending on the experiment. Conducting methacholine dose response curves for the pulmonary function measurements requires $\sim$ 2 hr for each group of mice. Thus, for 8 mice, measure airway hyperresponsiveness in 4 mice as Group 1 from 8 to 10 a.m. and 4 mice as Group 2 from 10 a.m. to 12 p.m. Then begin BAL and harvesting lung tissue in Group 1. For lavaging and removing lungs, allow $\sim$ 10 to 15 min/mouse (2 hr total), with one person lavaging the lungs and another person removing the lung pieces. BAL cells can then be counted, cytopins made, lung tissue homogenized, and frozen by the end of the day. On the following day, further freeze/thaw and sonicate the lung homogenates, along with differential count of the cytopins can be completed. Later, EPO and MPO assays can be conducted. The wet/dry weight of the lung is available after 2 to 3 days of drying, and the final results may then be calculated.

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