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## Models of Inflammation: Carrageenan Air Pouch

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### Abstract

The subcutaneous air pouch is an *in vivo* model that can be used to study the components of acute and chronic inflammation, the resolution of the inflammatory response, the oxidative stress response, and potential therapeutic targets for treating inflammation. Injection of irritants into an air pouch in rats or mice induces an inflammatory response that can be quantified by the volume of exudate produced, the infiltration of cells, and the release of inflammatory mediators. The model presented in this unit has been extensively used to identify potential anti-inflammatory drugs.

### Keywords

inflammation; edema; carrageenan; air pouch; animal model; inflammatory mediator; PGE<sub>2</sub>; TNF alpha; oxidative stress

## INTRODUCTION

The subcutaneous (s.c.) air pouch is an *in vivo* model used to study acute and chronic inflammation. First described in 1953 by Selye, the air pouch is formed by subcutaneous injection of sterile air into the intra-scapular area of the back of the rat or mouse, which can be later injected with a variety of irritants to produce inflammation. The pouch is composed of a lining of cells that consists primarily of macrophage-like (Type A) and fibroblast-like (Type B) cells, which is similar to the synovial cavity (Edwards et al 1981). Injection of a carrageenan solution into the pouch produces an inflammatory reaction that is characterized by an infiltration of cells, increase in exudate, and a marked production of pro-inflammatory mediators, such as prostaglandins, leukotrienes, and cytokines, as well as components of the oxidative stress response. These endpoints can be quantified and used to determine the degree of inflammation, resolution of inflammation, or anti-inflammatory and antioxidative activity of drugs. This unit describes a method to elicit cellular influx and inflammatory exudate in the six-day-old air pouch after subcutaneous injection of sterile air, followed by

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injection of carrageenan in the rat (Basic Protocol) or mouse (Alternate Protocol), based on methods described by Edwards et al. (1981) and Sedgwick et al. (1981). It should be noted that modifications of the basic methods of air pouch inflammation, such as varying the species of rodents, the number and sequence of air injections and the endpoints measured, have been used extensively. In addition, a number of other agents that evoke an inflammatory or immune response can be injected into the air pouch in lieu of carrageenan. These include, but are not limited to, bovine serum albumin (Sin et al 1984, Yoshino et al 1984); mycobacteria (Castro et al 1991); lipopolysaccharide (Nakamura et al 2001), zymosan (Yoon et al 2005), and lipids (Kadl et al 2009, Zhao et al 2011). For the purpose of this protocol, however, we will focus on a single methodology for rats, with modifications for mice, that uses carrageenan as the inflammatory stimulus.

*NOTE:* All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

## **BASIC PROTOCOL**

### **AIR POUCH MODEL IN THE RAT**

This protocol describes a method to elicit cellular influx and inflammatory exudate in the six-day-old air pouch in the rat (see Edwards (1981) and Sedgwick (1981)).

#### **Materials**

Rats weighing ~150 g or mice weighing ~20 to 25 g

Inhalation anesthetic [e.g., 5% (v/v) isoflurane; IsoFlo, Abbott]

70% (v/v) ethanol

0.9% (w/v) saline, sterile

2% carrageenan solution (see recipe)

Lavage solution (see recipe)

ELISA kit(s) specific for inflammatory mediators (e.g., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, prostaglandins; Thermo Scientific)

Isotonic phosphate-buffered saline (PBS; Life Technologies, cat. no. 10010-031), ice cold

Differential staining kit (e.g., Hemacolor; EMD Millipore, cat. no. 111674)

5-, 10-, and 20-ml syringes, sterile

18-, 20- and 23-G, 1- to 1.5-in. needles, sterile

0.2- $\mu$ m syringe filters, sterile

Anesthetic chamber or chemical fume hood

Animal hair clippers

Hemostat and scissors

15-ml conical centrifuge tubes, sterile

Transfer pipets, sterile

Cell counter *or* hemacytometer

Benchtop centrifuge (for 15-ml conical tubes)

Additional reagents and equipment for euthanizing the animal (see Donovan & Brown 2006)

*NOTE:* Besides carrageenan, other irritants such as zymosan or lipopolysaccharide (LPS) can be used to induce inflammation in this model.

*CAUTION:* Make certain that excess anesthetic does not vent into the room; use an appropriate anesthesia chamber apparatus or work in a chemical fume hood.

### Prepare the animals

1. Acclimate the animals for 1 week under standard lighting and temperature conditions. Provide food and water *ad libitum*.
2. Divide the animals into groups of at least six animals, one group for each experimental condition.

*The study protocol should contain a vehicle control group, test compound groups for different doses, and a positive control group (a known anti-inflammatory drug).*

### Create the air pouch

3. At a time point 6 days prior to induction of inflammation, generate the air pouch as follows:
  - a. Fill a 20-ml syringe with room air and attach a 0.2- $\mu$ m sterile filter to the syringe. Then, attach a 23-G, 1-in. needle to the 0.2- $\mu$ m sterile filter outlet.
  - b. Place the animal in a chamber and anesthetize with the method of choice (some alternatives are discussed below).
  - c. Once the animal is unconscious, remove the animal from the chamber, shave the dorsal cervical/thoracic region of the animal and swab the entire region with 70% ethanol.
  - d. Place the needle into the region between the scapulae of the animal and inject 20 ml sterile air subcutaneously to create the air pouch. Return the animal to its cage.

*Inhalation anesthesia is the method of choice when injecting air to create and/or maintain the air pouch. Isoflurane is the anesthetic of choice since it provides a safe, reliable, and fast way to render the animals unconscious, with a short recovery time from the anesthetic*

*state. Isoflurane also can cause minimal depression of the cardiovascular and respiratory system. Although halothane was used in the past, it is hepatotoxic and, if used, it should be in a chemical fume hood or with a proper anesthetic chamber.*

*To confirm the level of anesthesia, pinch the paw to verify the lack of a nociceptive response. An experienced investigator may be able to process animals quickly, so more than one animal could be anesthetized at once. Note that the anesthesia will wear off quickly, so the injection of air should be rapid, but consistent.*

- 4 At a time point 3 days later, anesthetize the rat again as described in step 3, swab the entire back of the animal with 70% ethanol, and inject 10 ml sterile air into the pouch.

*This second injection of half of the air volume will maintain the integrity of the air pouch without causing further tissue injury.*

#### **Induce inflammation**

- 5 At a time point 6 days after the first injection of air, re-anesthetize the animals and induce inflammation by injecting 2 ml of a 2% carrageenan solution into the pouch using a 5-ml syringe attached to a 20-G, 1-in. needle.

*The time of injection for the test compound or the reference drug in the positive control group (e.g., indomethacin or dexamethasone) should be determined according to the pharmacokinetic properties of the drugs. Typically, indomethacin is administered immediately prior to carrageenan, whereas dexamethasone is administered 3 hr prior to carrageenan treatment.*

- 6 At different time points after carrageenan injection, depending on the study design, the rodents should be sacrificed using an approved method. Two options for euthanasia include CO<sub>2</sub> asphyxiation and decapitation (see Donovan & Brown 2006, Leary 2013).

*The release of pro-inflammatory mediators usually occurs at different time points. Depending on what mediator the investigator chooses to analyze, the animal can be sacrificed 1 to 24 hr after the induction of inflammation. Some discussion of the timing of the experiment is provided in Anticipated Results.*

#### **Harvest the air pouch exudate**

- 7 To remove exudate from the air pouch, inject 5 ml lavage solution into the air pouch using a 10-ml syringe attached to an 18-G, 1.5-in. needle. Mix the contents of the pouch by gently massaging the area.

*EDTA is included in the lavage solution to inhibit cell aggregation.*

- 8 Using scissors, open the air pouch by cutting sagittally across the pouch (~2 cm). Grasp the skin flap with a hemostat and collect the entire exudate volume

into a sterile 15-ml centrifuge tube using a transfer pipet. Record the exudate volume and put the tube of exudate on ice.

**Measure endpoints**—Different parameters can be used to evaluate the extent of inflammation and the effect of a given test compound. These endpoints can include measurement of exudate volume and/or the analysis of inflammatory mediators, reactive oxygen species, reactive nitrogen species, cytokines, and/or chemokines in the exudate. Also, the number of white blood cells in the exudate can be counted using an automatic cell counter (Coulter) or a hemacytometer, and differential staining can be used to identify each cell type present in the exudate.

#### **Collect cells from the exudate**

- 9 Remove cells from the exudate by centrifuging the tubes for 10 min at  $1000 \times g$ ,  $4^{\circ}\text{C}$ . Divide the supernatant into 1-ml aliquots and freeze at  $-20^{\circ}\text{C}$  if the analysis of the inflammatory mediators will not be performed immediately. To evaluate inflammatory mediators in the exudate, use commercially available ELISA kits according to the manufacturer's instructions.
- 10 Resuspend the cell pellet in 0.5 to 2 ml ice-cold PBS and use a cell counter or a hemacytometer to determine the total cell number.
- 11 To differentiate cells present in the exudate, use a differential staining kit according to the manufacturer's instructions or fluorescence-activated cell sorting (FACS) methodology.

## **ALTERNATE PROTOCOL**

### **AIR POUCH MODEL IN THE MOUSE**

The carrageenan air pouch model can also be used in mice (20-30 g), providing an advantage over rats due to the opportunity to use genetically modified (knockout) animals. For mice, the injection volumes need to be adjusted as described by Sin et al. (1986) and Garcia-Ramallo et al. (2002). To create the pouch, inject 5 -6 ml of sterile air, followed by 2 -3 ml after 3 days to maintain the pouch. To induce inflammation, inject 1 ml of a 1% carrageenan solution into the existing pouch 6 days after the first injection of air. The needle for injection should be a 25-G. To recover exudate, inject 1 ml lavage solution into the pouch.

### **REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions.*

**Carrageenan: 0.5% to 2.0% (w/v) carrageenan solution**—Pour 500 ml sterile 0.9% saline into a 1-liter beaker and add a stir bar. Slowly add 2.5 g (0.5%) to 10.0 g (2.0%) carrageenan powder (Type IV Lambda, Sigma) to the beaker. Heat the solution to  $90^{\circ}\text{C}$  with stirring, but do not allow the solution to boil. Heating the mixture helps to dissolve the carrageenan. It should take less than an hour to dissolve all of the powder. Pour the solution into a clean 1-liter glass bottle equipped with a cap and sterilize by autoclaving. Cool the

solution to room temperature and divide into aliquots into sterile, 100-ml capped bottles. This solution is stable for at least a week when stored at 4°C following preparation.

**EDTA 54 mM (to be used in the lavage solution)**—Dissolve 10.04 g disodium EDTA dihydrate (Sigma) in 500 ml sterile 0.9% (w/v) saline. Sterilize by filtration using a 0.22- $\mu$ m filter. Store up to 6 months at room temperature or at 4°C.

**Lavage solution**—Add 540 ml sterile 0.9% saline to a 1-liter glass beaker. While stirring, add 60 ml of 54 mM EDTA (see recipe; final concentration 5.4 mM). Prepare fresh daily.

*The lavage solution could also be prepared with PBS or Hanks' buffered salt solution (HBSS) at physiological pH and include various substances depending on the endpoint to be measured.*

## COMMENTARY

**Background Information**—The air pouch model is a convenient in vivo model to study localized inflammation without systemic effects. Subcutaneous injection of air into the thoracic region of the back causes a morphological change in the cellular lining of the pouch, which occurs over several days. This change results in a lining similar to that found in the synovial cavity (Edwards et al 1981). Injection of irritants into this subcutaneous cavity provides a model of the inflammatory response observed in patients with rheumatoid arthritis and other chronic inflammatory diseases. Consequently, this model can be used to screen potential anti-inflammatory compounds. One of the advantages of the air pouch over the injection of irritants directly into the knee joint of animals is that the volume of exudate produced in the air pouch is much larger, enabling the measurement of multiple parameters from the same animal. Besides acute and chronic inflammation, the air pouch model can also be used to study granulomatous inflammation (Vane et al 1994), angiogenesis within granulomatous tissue (Eteraf-Oskouei et al 2015, Eteraf-Oskouei et al 2014), the resolution of the acute inflammatory response (reviewed by Ariel and Serhan, 2007), and evaluation of oxidative stress (Jain & Parmar 2011).

**Critical Parameters**—Rats must be acclimated to their environment for approximately 1 week prior to the experiments to avoid the effects of stress, which could interfere with the inflammatory response.

The exudate volume can be used to ascertain anti-inflammatory effects of test compounds; however, some slight variability in exudate volumes will exist, even between animals in the same experimental groups. After collection of exudates, it is important to immediately separate the cells by centrifugation since some inflammatory mediators may be produced or consumed by the cells. Furthermore, cells should be processed as quickly as possible on ice to preserve cell integrity and to minimize cell death. After removing cells from the exudate, the exudate must be divided into aliquots and frozen (use liquid nitrogen or dry ice) as soon as possible to avoid degradation of inflammatory mediators. In addition, avoid repeated freezing and thawing of the exudate.

It is important to attempt to stop synthesis and catabolism from occurring in the exudate. For example, if prostanoids are being measured, a cyclooxygenase inhibitor should be included in the exudate lavage solution to prevent ex-vivo synthesis of these eicosanoids.

**Troubleshooting**—When a large variation in soluble mediators is observed, it is possible that the exudates were not processed rapidly enough, were not kept on ice, were frozen and thawed too many times, or did not contain compounds to eliminate synthesis or catabolism.

**Anticipated Results**—The theoretical yield of exudate from the pouch in rats is usually 7 ml: 5 ml from the lavage solution and 2 ml from the carrageenan solution. However, volumes recovered may vary from animal to animal. For mice, the exudate volume is also equivalent to the total injection volume of lavage solution and carrageenan solution. The time between induction of the pouch and injection of carrageenan (or other irritants) also influences the magnitude of inflammatory response. Thus, carrageenan injection into a 6-day-old air pouch will elicit greater inflammation than carrageenan injection into a one- or three-day-old air pouch, using endpoints such as exudate volume and total cell number in the exudate (Sedgwick et al 1983). It also should be noted that results can vary depending on the strain of rodent used in the experiments (Delano et al 2005).

Resident cells in unstimulated pouch exudates are predominantly monocytes/macrophages. Between  $1 \times 10^6$  and  $5 \times 10^6$  cells can be harvested from an unstimulated pouch. A significant cellular infiltrate occurs in response to carrageenan administration (Fig. 5.6.1). This infiltrate has been well characterized and consists primarily of polymorphonuclear cells (87%) during the first 24 hr, with a gradual increase in mononuclear cells to 25% of the total by 48 hr (Martin et al 1994).

Injection of carrageenan into the air pouch induces a marked production of biochemical mediators, such as prostaglandins, leukotrienes, and cytokines (Fig. 5.6.2). Increases in these mediators are generally observed within an hour of carrageenan injection and peak at various times thereafter, depending upon the mediator studied.

Table 5.6.1 lists the effects of several pharmacological agents which exhibit anti-inflammatory effects in in the rat and mouse versions of the carrageenan air pouch model.

**Time Considerations**—The carrageenan solution is generally prepared in advance and stored. The procedure for generation of the air pouch usually takes no more than 5 min per animal. The inflammation assay generally takes an entire day, depending upon the number of animals used. It is helpful to have two people involved in collecting and processing the material.

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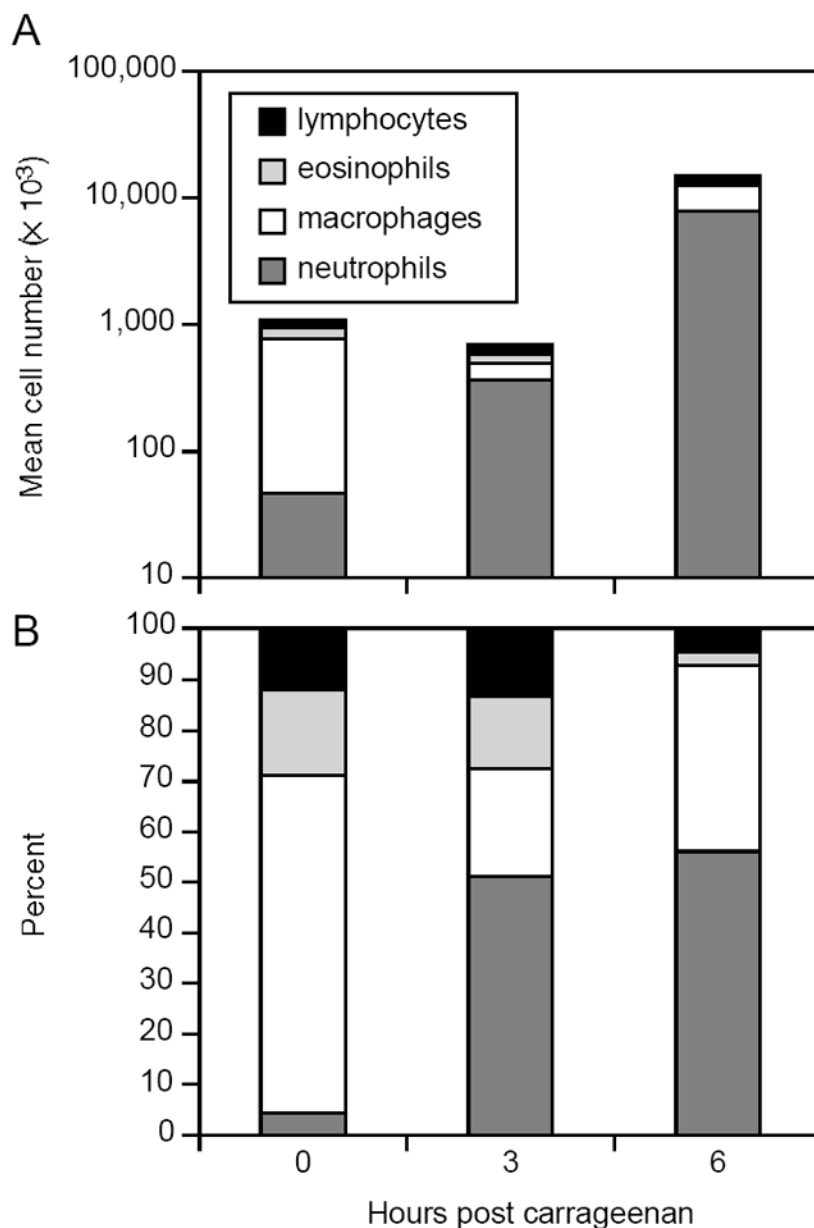
The authors would like to acknowledge the original protocol developed by Whiteley and Dalrymple (2001), which was used as the template for this current protocol.

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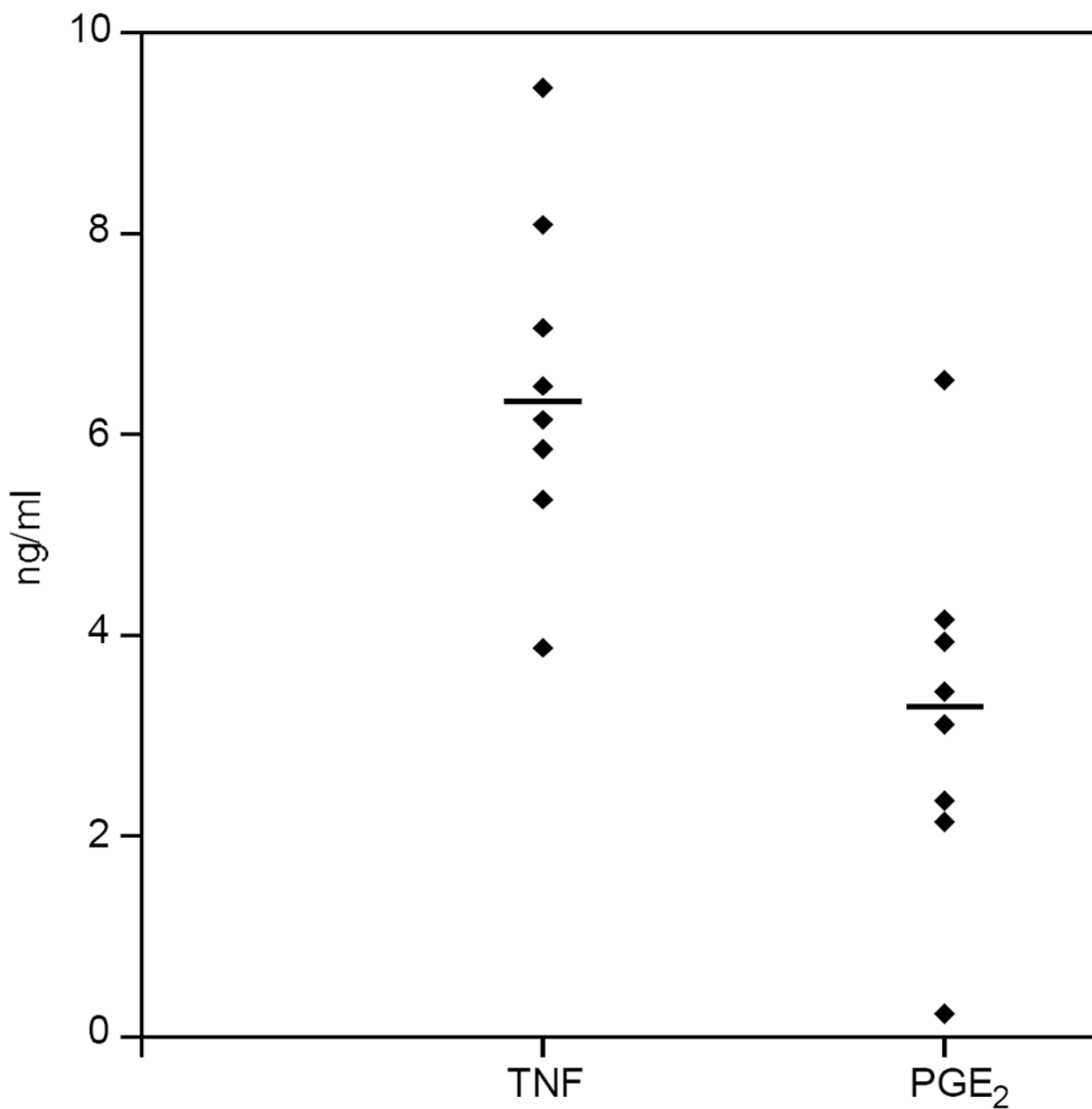
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**Figure 5.6.1.** Analysis of cellular infiltrate in rat air pouch exudates after carrageenan stimulation. Data for each time point represent (A) mean number of cells and (B) percent of total cells for each of four cell types in a group of eight rats for each time point. Data at 0 hr are for untreated animals.



**Figure 5.6.2.**

Amounts of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) present in rat air pouch exudates 3 hr after carrageenan stimulation. Symbols: filled diamond, ng/ml TNF- $\alpha$  or PGE<sub>2</sub> detected for each rat in the experimental group; horizontal line, median level of production for the experimental group. Exudates from unstimulated air pouches contained no detectable levels of TNF- $\alpha$  or PGE<sub>2</sub>. Note the extent of variation among the individuals in both groups.

**Table 5.6.1****Anti-inflammatory Effects of Selected Pharmacological Agents in the Carrageenan Air Pouch Model**

| Species | Drug                                                                                  | Results                                                                                                                                                                               | Reference                                                       |
|---------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
| Rat     | Dexamethasone (80 or 160 µg/kg, i.v.)                                                 | Decreases the total number of leukocytes in the pouch exudate                                                                                                                         | Sedgwick and Lees (1986)                                        |
|         | Indomethacin (30 mg/kg, s.c.)                                                         | Decreases the total number of leukocytes in the pouch exudate                                                                                                                         | Jain and Parmar (2011)                                          |
|         | Dexamethasone (1 mg/kg gavage) or Indomethacin (1 mg/kg gavage)                       | Both drugs block PGE <sub>2</sub> production in the pouch exudate                                                                                                                     | Masferrer et al. (1994)                                         |
|         | Tricyclic Antidepressants (amitriptyline or clomipramine; 90 mg/kg, p.o.)             | Decrease cellular infiltrate of polymorphonuclear cells                                                                                                                               | Gurgel et al. (2013)                                            |
| Mouse   | Dexamethasone (0.5-5 mg/kg, i.p.; ~320 mg/kg, p.o.) or Indomethacin (2-5 mg/kg, i.p.) | Both drugs decrease the number of leukocytes in the pouch exudate; dexamethasone also inhibits TNF levels in the exudate, whereas the effects indomethacin on TNF levels are variable | Romano et al. (1997); Mattei et al. (2015); Vigil et al. (2008) |
|         | Etanercept (5 mg/kg, i.p.)                                                            | Decreases the number of leukocytes and levels of TNF in the pouch exudate                                                                                                             | Mattei et al. (2015)                                            |
|         | Tacrolimus (2-5 mg/kg, p.o.)                                                          | Decreases the number of leukocytes and levels of TNF in the pouch exudate                                                                                                             | Vigil et al. (2008)                                             |