An endocrinology laboratory exercise demonstrating the effect of confinement stress on the immune system of mice

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Brehe J, Way AL. An endocrinology laboratory exercise demonstrating the effect of confinement stress on the immune system of mice. Adv Physiol Educ 32: 157–160, 2008; doi:10.1152/advan.00023.2007.—This article describes a simple laboratory exercise for examining the effect of stress on the immune system in mice. Mice are subjected to confinement stress for 1 h, after which a sample of blood is collected via the caudal vein. Blood samples are smeared onto microscope slides, air dried, and stained with Wright’s Giemsa stain. When differential white blood cell counts are performed, there are noticeable differences between the neutrophil and lymphocyte counts of stressed versus control mice. The protocol is simple enough for students to perform, and the entire experiment can be completed within 3 h. Examples of ways in which the basic protocol can be modified to accommodate a shorter laboratory class are provided. This hands-on laboratory experiment provides students with experience using the scientific method to investigate the interaction between the endocrine and immune systems in response to stress.

Glucocorticoids; differential white blood cell count

THE INTERPLAY between the nervous, endocrine, and immune systems becomes readily apparent during stress. In general, the stress response occurs in an animal if it perceives an external condition (stressor) that threatens to compromise its well being. There are two phases to the stress response, each involving a different set of hormones.

Catecholamines are released in the first phase and are always associated with sympathetic stimulation. Under the influence of the “fight or flight” response, epinephrine and norepinephrine are secreted from the adrenal medulla, causing a host of physiological responses. Many of the responses associated with the first phase of the stress response occur within seconds of the perceived threat and include rapid heart rate, bronchiolar dilation, increased pupil size, increased fat catabolism, and increased glycogenolysis, among others.

The second phase of the stress response involves glucocorticoids, a group of steroid hormones released from the adrenal cortex. Stress interrupts the normal rhythmic release of glucocorticoids, resulting in higher levels of glucocorticoids that elicit a wide range of physiological responses, enabling the body to withstand stress. Glucocorticoids enhance some of the effects of catecholamines released in the first phase of the stress response and, in addition, promote gluconeogenesis, mobilize fatty acids from adipose, and stimulate catabolism of stored protein. Both glucocorticoids and catecholamines oppose the action of insulin and promote fuel availability for the brain and exercising muscles (4, 6, 7).

Glucocorticoids have profound suppressive effects on the immune system, many of which can be readily observed shortly after exposure to a stressor. Specifically, the percentage and absolute number of neutrophils in blood increase, and the percentage and absolute numbers of eosinophils, monocytes, lymphocytes and basophils decrease within 1 h (4). This effect of glucocorticoids on mouse leucocytes was demonstrated as early as 1951 (11). Later, confinement-induced changes in the immune system were shown to be consistent with adrenal cortical stimulation in mice (5). Catecholamines, on the other hand, have been observed to elicit an increase in the number of lymphocytes and monocytes within 2 h of injection in humans (7). Changes in leukocyte percentages have been ascribed to the release of these cells from bone marrow reserves and redistribution to other tissue compartments (3).

Without being physically harmful to the animal, confining mice has been shown to be effective in creating an environment stressful enough to increase glucocorticoid release and elicit an immune system response (6). This can be used in a college laboratory course to demonstrate how stress can affect the immune system. We developed a physiology laboratory exercise that demonstrates the effect of glucocorticoids on the immune system of mice by measuring differential white blood cell (WBC) counts before and after confinement stress is applied. The lesson is adaptable to different levels of instruction, requires minimal equipment, and does not involve physical harm to the animals. The full protocol can be completed in <3 h and may be modified to fit a laboratory period of <2 h.

The objectives and outcomes for the exercise will depend on the course level and how the exercise is implemented. The following are sample objectives that could be met by students undertaking the exercise:

1. Describe the roles adrenal gland hormones play in the body’s response to stress.
2. Describe the mechanisms by which stress causes the release of glucocorticoids.
3. Describe the negative effects of glucocorticoid release on the immune system.
4. Investigate the effect of confinement stress on the immune system by monitoring WBC distribution.
5. Review the identification and function of leukocytes.
6. Review the protocol of the WBC differential.
7. Apply the appropriate statistical tool in the analysis of data.

MATERIALS AND METHODS

Animal handling and sample collection. Mice can be obtained from established laboratory animal supply houses (e.g., Simonsen Laboratories or Taconic Farms). Protocols involving the use of animals are typically first approved by a school’s Institutional Animal Care and Use Committee or Institution Review Board prior to implementation.
The basic protocol requires at least two mice per student group. The mice are divided into two groups: one group that will be stressed by confinement (experimental group) and one control group that will not be confined. Prior to the treatment period, a blood sample is obtained from each animal by caudal vein collection, in which the tail is wiped with an alcohol swab and one of the lateral tail veins is nicked with a sterile scalpel blade or small (27 gauge) needle approximately two-thirds the way down the length of the tail (12). A drop of blood is placed on a glass microscope slide, and a smear is immediately made and allowed to air dry. It is simplest to place the mouse in a restraint chamber to collect the blood sample. These chambers can be purchased in a variety of sizes to suit the size of the mouse (e.g., no. AH 63-0127, Harvard Apparatus) or made from PVC pipe (Fig. 1). PVC pipe of 1.25-in. diameter can be purchased at a hardware store and cut into lengths of 4-5 in. with a saw. An electric drill with a 3/8-in. bit can be used to drill three rows of air holes equally spaced around the tube with each row having three holes. The ends are secured with no. 6 black rubber stoppers.

After the initial blood samples have been collected, control mice are returned to their holding cages and placed in a quiet space in the laboratory where disturbance is kept to a minimum. Although restraining the control mouse to obtain a blood sample will initiate a stress reaction, the response can be kept to a minimum by returning the animal to its holding cage as soon as possible. Experimental mice are confined in their individual chambers for 1 h. Although movement of the mouse is restricted when in the chamber, the animal is not prevented from turning around or making many normal postural adjustments provided that the chamber is the appropriate size for each mouse. After 1 h, a blood sample is collected from mice in both groups using the previously described procedure, placed onto a microscope slide, smeared, and air dried.

Slide staining and evaluation. All smears are stained with Wright-Giemsa stain (no. WG16-500ML, Sigma Chemical) using the protocol provided by the manufacturer. Briefly, a thoroughly dried blood film is placed feather edge down in ~50 ml of stain for ~30 s. Dipping the slide rapidly for the first 5–10 s reduces artifacts on the microscope slide. The slide is removed from the stain and placed in distilled water for ~10 min to destain. It is not necessary to agitate the slide while it is in the distilled water. Slides are then rinsed briefly in running distilled water and allowed to air dry thoroughly before evaluation.

Lids must be kept on the staining jars to prevent evaporation. Wax pencils rather than indelible markers are recommended for marking the microscope slides since the stain will remove the marker ink. If the blood smears are to be used for more than one class, it is helpful to glue coverslips to the slides using commercially available adhesive (e.g., Permount no. SP15-100, Fisher Scientific).

Fig. 1. The home-made confinement chamber on the right is about the same diameter as the commercially available chamber on the left. A hand drill was used to provide holes for ventilation in a short section of PVC pipe. Rubber stoppers were used at the ends.

A differential WBC count is performed on each slide. Slides are evaluated by light microscopy using an oil-immersion objective. The procedure for preparing blood smears and performing a differential WBC count can be obtained from most physiology and microbiology laboratory manuals (2, 8). To evaluate the stained blood smears, the student systematically moves the slide across the microscope stage and records each type of WBC until 100 cells have been counted. From these counts, the percentage of each type of WBC can be calculated. Since many students will be evaluating blood smears for the first time, it is useful to pool data from the student groups and provide averages to the class.

RESULTS

Figures 2 and 3 show the results of a student exercise in an animal physiology laboratory. Figure 2 shows a substantial decrease in the percentage of lymphocytes in experimental animals (from 73.4% to 58.1%) compared with the control group (from 69.7% to 67.8%) with a P value of 0.0035 using an unpaired t-test. Figure 3 shows the neutrophil results of the same exercise. There was a large increase in the percentage of neutrophils in the experimental mice (from 25.3% to 39.8%) compared with the control group (from 27.8% to 29.3%) with a P value of 0.0016.
Tables 1 and 2 show the student group raw data upon which Figs. 2 and 3 were based. We include these raw data to demonstrate the outcome of a typical laboratory experience. As can be seen, although the percentage changes varied, almost all groups did see a decrease in the percentage of lymphocytes and an increase in the percentage of neutrophils. Pooling the data allows the class to clearly see the results and affords an opportunity to apply a statistical analysis to the data such as an unpaired t-test to compare the two groups. It also provides an opportunity to explore sources of error in collecting data.

**DISCUSSION**

Circulating catecholamines and glucocorticoids both affect immune system function. The complex relationship between these two groups of hormones and the immune system has been reviewed by several authors (7, 9) and is covered to various extents in most physiology texts. When introducing this laboratory exercise to students, it is advisable to give a brief overview of the first and second phases of the stress response delineating the activity of each group of hormones. Students should also be made aware that there is a normal diurnal rhythm to the release of glucocorticoids that is unrelated to stress.

Stress interrupts the normal rhythmic release of glucocorticoids by overriding the negative feedback mechanism. The resulting higher levels of glucocorticoids cause the second phase of the stress response. The actions of these hormones take up to an hour to observe once the endocrine gland is signaled and elicit a wide range of physiological responses that enable the body to withstand stress.

This laboratory exercise demonstrates to students one way in which glucocorticoids affect the immune system through modulation of WBC numbers. The negative actions of glucocorticoids may keep the components of the immune system from overresponding to an injury, causing damage to nearby healthy tissue or precipitating an autoimmune response (1, 9). Although suppression of the immune system may be appropriate in an animal that has been through an acute challenge, it can produce deleterious effects if the stress is chronic.

Neutrophil percentages were reported as part of the white blood cell differentials performed by 10 student groups before and after stress was applied to male mice following the described protocol. Each data point is the average of two counts performed by two students. The final percentages of the experimental group was significantly different from the initial percentages. P = 0.0035 by an unpaired t-test.
and human WBCs, it may be useful to have prepared mouse blood smears available for students to practice counting prior to the exercise.

Figure 4 shows the effect of applying confinement stress on consecutive days. The percentage difference between the initial and final count is reduced each day and is almost eliminated by the fourth day. Although Pitman et al. (10) did not find that rats habituated to mild restraint stress, it appears that the effect of confinement in mice is dramatically reduced when the animal is repeatedly tested. If an instructor teaches multiple laboratory sections, care must be taken that the same mice are not restrained in consecutive sessions.

The instructions to students will vary depending on the manner in which the instructor chooses to utilize the exercise. The basic protocol can be used simply to illustrate the effect glucocorticoids have on the immune system in a demonstration. It can also be used in a hypothesis-driven laboratory in which students begin by formulating a hypothesis as to whether a given parameter will influence how confinement stress affects the differential WBC count in the animals. If young (<30 days) and old (>1 yr) mice are provided to the students, they can develop a hypothesis as to whether aging would result in a reduced response of the immune system to glucocorticoids or whether the release of glucocorticoids in response to stress might be impaired. If male and female mice can be provided, students might hypothesize which gender would give the greater response. Photoperiods can also become a parameter, with students developing a hypothesis as to whether the stress response differs depending when it is applied. The protocol can also be used in student research projects to examine parameters modifying the animal’s reaction to stress.

The full basic protocol takes ~2 h to complete depending on the number of students in the laboratory section. For classes that meet for a shorter time, blood smears can be prepared ahead of time, and, after an introduction to the experimental protocol, the students can read the slides. In fact, one student conducted a research project using the basic protocol and then developed a laboratory module that is used in a freshman level anatomy and physiology course. The student conducted the experiment, prepared, and read the slides and then developed a slide presentation that is used in the laboratory course to introduce students to the protocol. The students are then asked to form their own hypothesis and read the slides. The data collected in class can then be compared with the data collected by the student investigator. In this modification, the students still benefit from the experience of collecting data and evaluating experimental results despite the time constraints of a short laboratory period (<2 h).

In conclusion, this straightforward protocol provides a hands-on experience in which students can investigate the effects of stress on the immune system. The procedures are simple, the resources required for the experiment are inexpensive, and differences between confined and control mice are easily observed after 1 h. This exercise provides the student with insight into the interplay between the endocrine and immune systems, reinforcing the integrative nature of physiology.

REFERENCES