Chapter 7

Investigating an Immune Response to Bacterial Infection

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Contents

Introduction

The ability of organisms to defend themselves from parasites and pathogens is a fundamental aspect of biology. Because of the risk of infection from blood-born pathogens, and the elaborate protocols required to handle and keep mammals and other vertebrate organisms, it is becoming more difficult and expensive to conduct laboratory exercises that illustrate fundamental immune reactions. Alternative models are needed to teach this important biological process. Invertebrates, namely insects, are excellent models of cellular immune reactions to bacterial infections. We present activities that are appropriate for high school, college, or university biology teaching laboratories.

Insects display two broad categories of defense responses to bacterial infections: humoral and hemocytic (Gupta, 1986, 1991; Bowman and Hultmark, 1987). Humoral responses involve the induced synthesis of anti-bacterial proteins such as cecropins (4 kDa), attacins (12 to 23 kDa), diptericins (8 kDa) and defensins (4 kDa) (Bowman and Hultmark, 1987). The detergent properties of these anti-bacterial proteins disrupt bacterial cell membranes. Insects also synthesize lysozymes, enzymes that directly attack bacteria by hydrolyzing their peptidoglycan cell wall (Dunn, 1986; Russell and Dunn, 1991). Hemocytic responses involve direct interactions between circulating hemocytes and bacteria. Specific cellular defense mechanisms include phagocytosis, nodulation, and encapsulation (Gupta, 1986, 1991).

Nodule formation is the earliest, and quantitatively, the predominant cellular reaction to bacterial infections in insects (Horohov and Dunn, 1983). Nodulation is thought to begin with the formation of hemocyte microaggregations, mainly involving granulocytes and plasmatocytes, which entrap bacterial cells. The process continues with the recruitment of additional hemocytes while entrapping more bacterial cells. Mature nodules are completed with an outer layer of plamatocytes. The overall process ends with melanization into darkened nodules, usually about 0.1 to 0.2 μm in diameter. Nodule sizes can vary. Hemocyte microaggregations remain in circulation, while nodules are usually found attached to the body wall and various internal organs. The nodulation process can clear thousands of bacterial cells from circulating hemolymph within minutes post-infection.

Objectives

By completing this laboratory exercise, students will:

1. Develop a basic understanding of insect hemocyte types, and their relative abundance in tobacco hornworms.
2. Assess the cellular aggregation response to an artificially induced bacterial infection by microscopic observation of hemolymph collected from tobacco hornworms (Optional Activity, refer to Appendix B).
3. Become familiar with the various internal structures of the tobacco hornworm, and assess their normal appearance by dissection and microscopic observation.
4. Assess the nodulation response to an artificially induced bacterial infection of the tobacco hornworm by dissection and microscopic observation.

Materials

Organisms and Equipment

Students will work in groups of two.

1. Insects, tobacco hornworms, *Manduca sexta*, 5th instar, day 1, 2, or 3.
2. Bacterial cultures, grown to stationary phase and titered to 10^7 bacterial cells/ml. (Heat-killed cells may be used for convenience. Refer to "Notes for the Instructor.")
3. 26-gauge, 0.5 inch needles fitted to 1.0 ml syringes (Becton Dickinson, Refer to Appendix C.)
4. Teflon-lined (siliconized may be substituted) needles, 21-gauge, 1.5 inch (Becton Dickinson).
5. Hemacytometer with glass cover slips.
6. *Manduca Saline Buffer* medium for insect cells. (Refer to Appendix A for formula.)
7. Light microscope (Phase contrast optics are recommended but not required).
8. Dissecting microscope (with lamp).
9. Dissecting tray.
10. Dissecting instruments (scissors, forceps, and eight pins).

Notes for the Instructor

Proper handling of bacterial cultures and insect larvae is essential to the success of these laboratory activities. Both organisms can be purchased from a number of biological supply houses with instructions for their proper care (Refer to Appendix C). Insects can be reared on an artificial diet, also available from a number of sources.

An emphasis on safety is important when handling bacteria and hypodermic needles. Although many types of bacteria do not pose a significant threat to human health, they should be treated as though they do. A review of sterile techniques used in microbiology is a good practice when working with microbes. We recommend Koch (1981) as an excellent resource for general methods of bacteriology. Common non-pathogenic strains of *Escherichia coli* may be substituted for *Serratia marcescens* and bacterial cultures may be heat-killed, titered, and stored in the refrigerator for convenience. Heat-killed bacterial cultures are prepared by autoclaving bacterial suspensions at 230°C to 250°C, for 20 to 30 minutes. Heat-killed cultures should not be stored for more than two weeks. Additionally, caution should be exercised when working with hypodermic needles. If you allow students to do their own pericardial punctures and/or injections of bacteria, be sure to go over the proper use and disposal of hypodermic needles.

For convenience and economy of time, it is recommended that the injection of bacteria be done well in advance (2 hours or more) of the scheduled laboratory activity. Consequently, this may call for the instructor(s) to perform the injections. Proper training and practice will help insure a successful laboratory set-up. To demonstrate the immune reaction to an artificial bacterial infection, one-hour incubations will work; however, longer post-infection incubation times will produce more dramatic results.

Generally, this laboratory exercise can be completed in about 3 hours. Depending on the number of activities one intends to accomplish, the completion time may vary. It is recommended that the instructor pre-run the activities to get a better idea of the time required to complete the objectives.

Student Outline

Activity 1: Hemocyte Counting

Background

There are five distinct hemocyte types in tobacco hornworm hemolymph. Granulocytes are round cells with diameters of about 5.4 μm. The nucleus of a granulocyte is round and small, with a diameter of about 2.8 μm. The nucleocytoplasm ratio (diameter of nucleus to diameter of the whole cell) is about 0.5. Cytoplasmic granules, which range in size from about 0.5 to 1.5 μm in diameter, can be seen by focusing through the cells. Sometimes, after several minutes on the slide, very fine filopodia can be seen extending from the surface of the cell.

Plasmatocytes are recognized by their spreading behavior. Even before spreading however, plasmatocytes can be distinguished by their relatively large size (about 8.0 μm) and by their relatively high nucleocytoplasm ratio (0.7). Most plasmatocytes will appear round if examined immediately after being drawn from the insect. However, extensions from the cell surface can be seen within a very short time (3 to 5 minutes), which subsequently develop into lamellipoda that spread on the microscope slide. The overall shape of plasmatocytes becomes fusiform.

Spherulocytes are small cells (about 5.0 μm) with large (about 3.0 μm) cytoplasmic inclusions. The nucleus is relatively small (about 2.5 μm) and is often obscured by cytoplasmic spherules. The nucleocytoplasm ratio of this cell type is about 0.4. Spherulocytes usually do not adhere to glass, but instead stay suspended between the slide and the coverslip.

Prohemocytes are round or oval cells with a diameter of about 5.9 μm. The nucleus is relatively large with a diameter of about 4.5 μm. The nucleocytoplasm ratio is the highest (0.8) among all the hornworm hemocyte types.

Oenocytoids are quite large (10-12 μm in diameter), round cells. They are generally highly refractile, and occasionally an eccentric nucleus can be seen. The nucleocytoplasm ratio is very low (about 0.3) for this cell type. Some evidence suggests that oenocytoid cells contain large amounts of phenoloxidase, indicating a possible role in the recognition of a foreign invader (i.e., bacteria, fungi, protozoans, etc.).

Total Hemocyte Counting Technique

Procedure

1. Obtain a tobacco hornworm larvae and anesthetize it by chilling the larvae on ice for 15 minutes. Surface sterilize the larvae by swabbing its anterior dorsal surface with 95% ethanol.
2. Collection of hemolymph is done by pericardial puncture as describe by Horohov and Dunn (1983). A sterile 21-gauge, Teflon-lined or siliconized, 1.5 inch needle is inserted anteriorly at the thoracic abdominal junction such that the needle penetrates into the pericardial sinus. Hemolymph that drips freely from the needle is collected into a chilled, sterile polypropylene 1.5 ml centrifuge tube. Dilute the hemolymph 1:1 with chilled buffer. Mix gently by inverting the tube several times. See Figure 7.1 below.

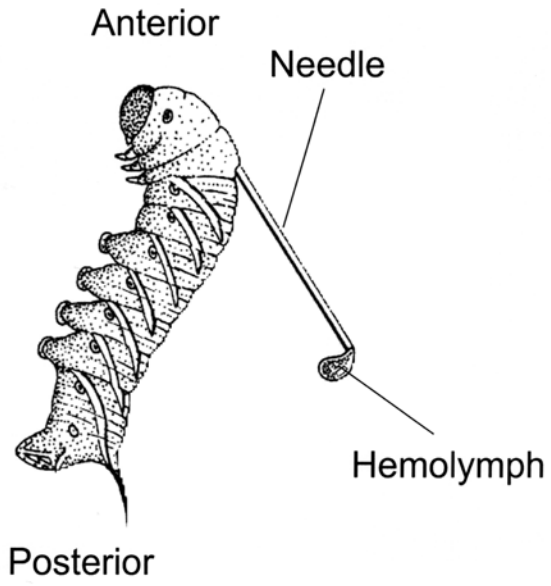

Figure 7.1 Collection of hemolymph.

3. Apply 20 μl of diluted hemolymph to a hemacytometer (Figure 7.2).

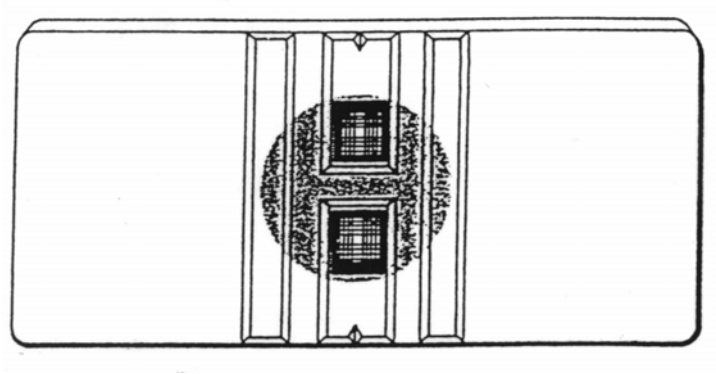

Figure 7.2. Diagram of a hemacytometer. The dark circle indicates areas where hemolymph suspension is to be applied.

4. Using a compound light microscope, count the cells that appear within the counting zones (corner grids) (Figure 7.3). When counting the cells, only consider those that lie within each of the sixteen individual squares and those touching the upper and right hand lines of each square. Do not count those touching the left hand and lower lines. Refer to the pattern followed in Figure 7.4 where there are twelve cells in one of the four counting zones. Record the number of cells counted in each counting zone in Data Table 7.1.

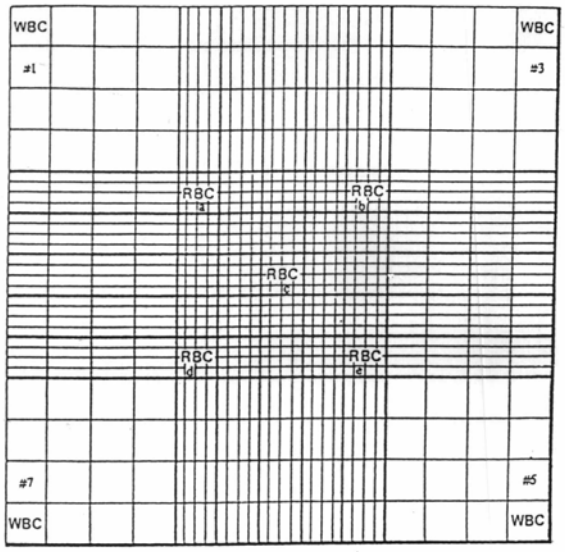

Figure 7.3. Detail of the lined area on the hemacytometer.

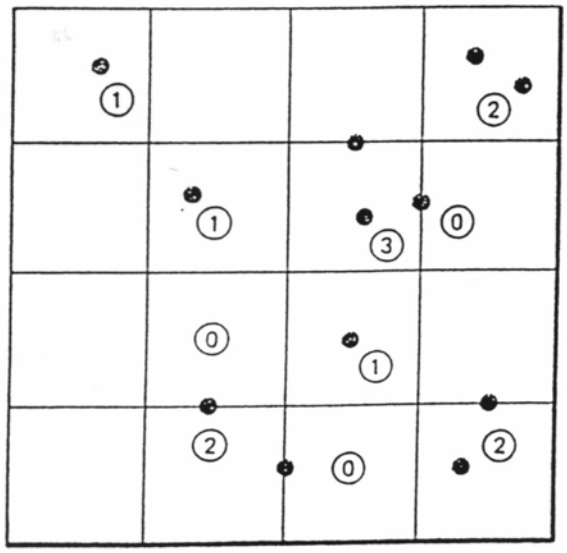

Figure 7.4. Count the cells that are touching the upper and right hand lines of each square in the counting zone. Do not count those touching the left hand and lower lines. In this example there are twelve cells in this section of the hemacytometer.

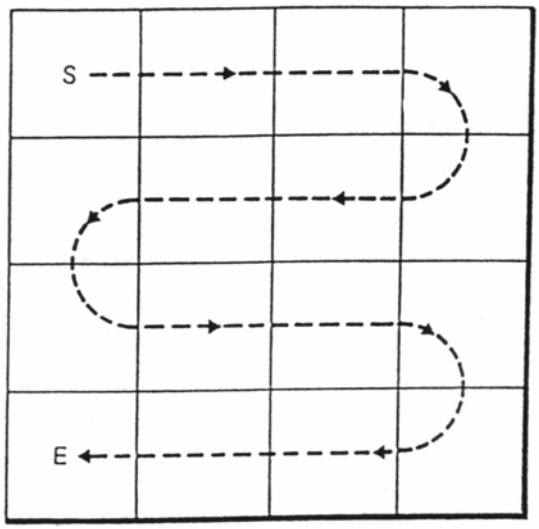

Figure 7.5. Counting pattern used when counting the number of cells with a hemacytometer.

Data Table 7.1. Determination of the number of hemocytes per ml of hemolymph. Use the space to the right of Data Table 7.1 to draw diagrams of cells, record observations, or for calculations.

COUNTING ZONES	NO. OF CELLS
Chamber 1	
1	
2	
3	
4	
Chamber 2	
1	
2	
3	
4	
Total Cells	

5. After you have the total counts for each of the four counting zones, determine the number of cells for a given volume of hemolymph. Use the following formula to determine the number of cells per ml of hemolymph:

$$\text{No. of Hemocytes/ml} = \frac{\text{No. of hemocytes X dilution factor X } 10^5}{\text{No. of counting zones counted}}$$

Activity 2: Assessment of the Nodulation Response to Bacterial Infection

Background

As previously described in the introduction, insects respond to large bacterial infections by nodule formation or nodulation. This process is quantitatively the most important response to bacterial infections (Horohov and Dunn, 1983). Nodulation begins with the formation of hemocyte microaggregations, mainly involving granulocytes and plasmatocytes that entrap bacterial cells. The process continues with recruitment of additional hemocytes, while entrapping more bacterial cells. Mature nodules are completed by attachment of layers of flattened plasmatocytes (phagocytic cells) to the mass of hemocytes and bacterial cells. The last stage is a melanization process, leaving darkened, easily visible nodules attached to the inner sides of the body wall or various organs. As a result, invading bacterial cells are topologically removed from circulation by effectively forming an impermeable wall between the bacterial mass and the remainder of the organism. The nodulation process can clear thousands of bacterial cells from circulating hemolymph within 60 minutes post-infection.

Injection of Bacteria

Procedure

1. Obtain two tobacco hornworm larvae. Anesthetize both larvae by chilling them on ice for 10 to 15 minutes. Select one specimen to serve as a healthy example for comparison with an infected specimen. Leave it one on ice until you are ready to dissect it. The other specimen, to be artificially infected, should be surface sterilized by swabbing the injection site area with 95% ethanol.

2. Obtain a 0.5 inch needle with a 1 ml syringe. Draw 0.3 to 0.4 ml of *Serratia marcescens* bacterial suspension from the stock culture into the syringe. The concentration of bacteria will be approximately 5.5 X 10^7 colony forming units per ml. Remove any air that remains in the chamber by holding the needle and syringe in a vertical position (needle pointing upward) and gently tapping the side of the syringe. When all the bubbles inside the syringe have risen to the top, carefully depress the plunger until bacterial suspension is visible at the tip of the needle. Adjust the volume to the nearest 0.1 ml.
3. Artificially infect one of the larvae by injecting 100 μl of bacterial suspension into it. Injection of the bacterial suspension is done by carefully inserting the needle dorsolaterally above the last two spiracles then moving into the hemocoel parallel to the body wall to avoid injuring the alimentary canal. Move the needle forward into the immediate anterior segment. Depress the plunger to inject 0.1 ml of bacterial suspension. Place any unused portion of the bacteria suspension in the designated waste container.

4. Place the infected specimen back into a small container and allow it to incubate in a warm environment (i.e. on the lab bench) for 1 hour or more. Prepare to dissect the healthy specimen.

Part 1: Dissection of the Healthy Specimen

Procedure

1. Obtain the necessary dissecting instruments and a dissecting pan. Place the specimen (that has been chilling on ice as previously described) in the dissection pan with the dorsal surface up. Place a dissecting pin through the anterior aspect of the body just posterior to the head. Place a second pin just posterior to the dorsal caudal horn at the tail end of the animal.

2. Using small scissors, make an incision through the cuticle posterior to the caudal horn and anterior to the dissecting pin at the tail end. Insert the tip of the scissors just inside the incision and carefully cut in the direction of posterior to anterior along the mid line of the dorsal surface to the head end of the animal.

3. Make two lateral cuts with the scissors at the posterior end of the incision and two more lateral cuts at the anterior end of the incision. Using small forceps, gently pull the flaps of cuticle back away from the mid-line and pin them down. Three dissecting pins per side should be enough.

4. With the aid of a dissecting microscope, examine the exposed hemocoel and identify the various structures. You should be able to find Malpighian tubules, gut, fat body, silk gland, trachae, muscle along the internal body wall, and ventral nerve cord and ganglion.

5. Draw a diagram and label the various identifiable structures. This will be used for comparison with the infected specimen.

Part 2: Dissection of the infected specimen

Procedure

1. Obtain and anesthetize the infected larvae by chilling it on ice for 10 to 15 minutes.

2. Follow the dissecting protocol described above.

3. With the aid of a dissecting microscope, examine the exposed hemocoel for the presence of nodules. Nodules will appear as dark black round bodies that are affixed to the internal tissues. Some may appear to have filipoda-like structures connecting several nodules in a cluster.

4. Count the number of nodules along the dorsal surface of the gut. Additionally, with the forceps, probe the fat body and determine the number of nodules throughout and around the trachae as well.

5. Carefully remove the gut and examine the previously unexposed areas of the hemocoel beginning with the ventral surface of the gut. Further examine the area of the hemocoel ventral to the gut.

6. Determine the total number of nodules. Draw a second diagram for comparison with your previous diagram of the healthy specimen.

7. Clean up your instruments and laboratory station when you are finished.

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Acknowledgments

We thank Jon C. Bedick for creating the drawing in Figure 7.1. His generous gift is greatly appreciated.

Appendices

APPENDIX A: Recipe for the Preparation of Manduca Saline Buffer

The buffer is made of 0.58 g/L Pipes (1,4-piperazinediethanesulfonic acid), suspended with 0.23 g/L NaCl; 2.98 g/L KCl; 3.66 g/L $MgCl_2$; 83.0 g/L sucrose; 1.0 g/L polyvinylpyrrolidone; 30 mg/L penicillin G; 15.0 mg/L phenylthiourea, adjusted to pH 6.5 with 0.1 M HC1 or 0.1 M NaOH. The buffer can be stored in the refrigerator for three to four weeks. Grace's Insect Medium may be substituted.

APPENDIX B: Assessment of the cellular aggregation response to a bacterial infection

By first artificially infecting tobacco hornworms with bacteria as described in Activity 2 (injection of bacteria) and then using the technique described in Activity 1 (counting hemocytes), students will be able to assess the pre-nodule cellular immune response (microaggregation of bacterial cells and hemocytes). Students will look for cell masses containing 7 or more cells in a cluster. These cellular aggregations are described as an early stage of the nodulation process and

can been observed in hemolymph samples taken between 15 to 60 minutes post-infection. By counting the number of cellular aggregations, student will gain insight into the cellular events that take place immediately after infection.

APPENDIX C: Sources for obtaining biological organisms, equipment, and other supplies

Bio-Serv Entomology Division
One 8th Street
Frenchtown, NJ 08825
Phone: 908-996-2155
Fax: 908-996-4123
http://www.bio-serv.com

Carolina Biological Supply Company
P. O. Box 6010
Burlington, NC 27216-6010
Phone: 800-334-5551
Fax: 800-222-7112
http://www.carolina.com

Fisher Scientific
600 Business Center Drive
Pittsburgh, PA 15205-991
Phone: 800-766-7000
Fax: 800-926-1166
http://www.fishersci.com