Observation of Phagocytosis and Respiratory Burst in Neutrophils

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I. Overview

Goals: students will learn introductory concepts of immunology through 2 laboratory exercises and classroom discussion.

Technical skills: centrifuging micropipetting microscopy (an extended activity could be use of the oil immersion

lens)

These labs could be integrated into various parts of the curriculum, depending on teaching style and the constraints of a particular school system. Because some of the background material may be a bit difficult to master, I would recommend teaching it after the students have a knowledge of basic chemistry and cell function.

II. Science Background

The body has many protective mechanisms to keep things from harming it. The immune system is responsible for defending the body against intruders that are constantly attacking it.

The immune system is usually divided into 2 parts:

1. innate: system of defense that is non-specific and available before exposure to antigens (molecules recognized as foreign)

2. adaptive: system of specific defense that develops when one is exposed to invaders. For example, chemicals called antibodies are made by certain leukocytes (white blood cells) to specifically match and bind to antigens that are presented to them.

In this lesson, we are going to concentrate on the innate system.

The first line of defense is a remarkable barrier called skin. Unbroken skin is dry, salty, and acidic, which makes it inhospitable to many pathogens. Mucous membranes are another physical barrier. These membranes line certain body cavities like the respiratory tract, producing mucus, a thick substance which traps particles, including microbes. The body also produces chemicals that help keep organisms healthy. Lysozymes are antibacterial enzymes found in many fluids, including tears and saliva. Another chemical is stomach acid, which has a pH of about 2, making it impossible for many microorganisms to live. These defenses are non-specific in that they do not target a particular invader.

Once an invader has breached the barriers described above, leukocytes that patrol tissues like security guards are called into action. You are probably familiar with these leukocytes called macrophages ("big eaters"). These cells, like all blood cells, originate in the bone marrow. Once made the cells travel through the blood stream and then to tissues to detect invaders. The cells recognize something as foreign by detecting surface molecules on the invader that the host would not have. When an intruder is encountered, the macrophages surround trespassing bacteria much the way an amoeba engulfs a particle of food. This process is called phagocytosis ("cellular eating"). Unlike the amoeba, an immune cell is not trying to derive nutrition from its prey; its job is to destroy the cell. As

one writer put it, "During infection, our tissues become a battleground."

Macrophages give off chemical signals to recruit other immune cells and to increase permeability of blood vessels for their entrance. In response to these chemical signals, another type of leukocyte appears on the scene: the neutrophil.



Stained neutrophil

The cells are called polymorphonuclear (multi-lobed nuclei) granulocytes (having granules in the cytoplasm). The nucleus is initially uniform in shape and then constricts to form the lobes, which are connected by filamentous strands. The granules contain various substances for killing microbes, including lysozymes. Neutrophils also contain secretory vesciles. These leukocytes contain a large amount of glycogen in the cytoplasm, which provides neutrophils with a source of energy. Neutrophils are approximately 10-15 um in diameter.

Neutrophils are the most numerous cells of the innate system. They are found in the bloodstream and migrate to tissue only after getting a chemical signal from the macrophages that help is needed (this movement in response to a chemical is called chemotaxis). Like macrophages, neutrophils have the ability to recognize cells as "non-self" and phagocytose them. Neutrophils are short-lived and die after a few rounds of phagocytosis. In fact, dead neutrophils are a major component of pus. If bacteria have already been recognized as foreign and coated with molecules (ex. antibodies) in a process called opsonization, the neutrophils are more likely to phagocytose them. Neutrophils will even phagocytize artificial particles that have been opsonized.





After Phagocytosis

After engulfing the bacterium, neutrophil granules fuse with the membrane of the corralled pathogen whereby toxic chemicals reach it. Leukocytes take up lots of oxygen (called a respiratory burst) in order to produce certain of these toxic chemicals. Neutrophils also routinely release their granules in order to kill microbes extracellularly. The chemicals, however, also kill some surrounding body cells and tissues.

In addition to neutrophils, other cells of the innate system are activated when a foreign cell is detected. One of these cells is the dendrite. It engulfs microbes and they displays the antigens on its surface. Macrophages also display antigens. Antigen display is the processes that first engages the adaptive immune system.

In this activity, we will consider only the activity of neutrophils.

In Part 1, fresh, whole non-human blood will be treated with heparin to keep it from clotting. The blood will then be treated chemically and centrifuged to separate the neutrophils from the other blood components. After centrifugation, the neutrophil layer will be removed and then mixed with microspheres (latex beads) which have been opsonized in serum. The cells will be examined under the microscope for phagocytosis.

In Part 2, fresh, whole non-human blood will be treated in the manner above to collect the neutrophils. The neutrophils will be divided so that one group is not stimulated (control) and one group is stimulated (experimental). Nitro blue tetrazolium (NBT) will be added to each group of neutrophils. This chemical causes the neutrophil granules to turn blue/black if an oxidative burst is occurring. The color change occurs when NBT (colorless) is reduced to formazan (blue/black). The cells will be examined microscopically for evidence of dyed granules.

Relevance to Students' Lives:

Everyone gets sick at some point in his/her life. In addition to acute illnesses, many students today struggle with chronic asthma or allergies. A knowledge of the immune system may help a person better understand the treatment a doctor is giving.

In the first part of this lab, the students will be able to see the immune system in action. Many students may have seen amoebas engulf particles, but they have probably not seen phagocytes engage in this activity.

In the second part, the students will conduct a test that is given to detect an immune system disorder: chronic granulomatous disease (CGD).

CGD is an inherited abnormality of phagocytic cells of the immune system. It causes repeated infections by certain types of bacteria. Symptoms include frequent and difficultto-clear skin infections and swollen lymph nodes in the neck with abscess that may require surgical drainage. People with this disease have neutrophils which are able to phagocytize bacteria, but not kill them since their granules lack toxic chemicals.

The NBT test is used as an indicator of CGD. When the dye is added to a patient's neutrophils and they don't turn blue/ black, the person is given further tests to confirm CGD.

III. Student Outcomes / Learning Objectives

In this lab the students will:

observe neutrophils phagocytose beads find evidence of the respiratory burst in neutrophils using the NBT test

IV. Time Requirements

Teacher lab prep:	About an hour (not counting time spent to get blood from
	source)
Student work:	Phagocytosis: 50 minutes of lab time
	Respiratory Burst: 50 minutes of lab time

V. Advance Preparation / Materials and Equipment

The materials will easily be enough for a class of 24 with 4 people/ group.

Equipment

aprons goggles latex gloves incubator micropipettor + tips or disposable plastic pipets (1 ml size) centrifuge centrifuge tubes microscope slides glass coverslips microscope with 400 X magnification small plastic vials with caps plastic petri dishes coffee stirrers

Chemicals

bleach

Prepare a 10 % solution by diluting household bleach. Keep a container of this dilute solution at the front for used slides.

Phagocytosis:

5 ml of non-human blood Heparin (Sigma D4776) \$30 Polymorphprep (Axis-Shield) RPMI 1640 Media (Sigma RO883) \$15 Phosphate Buffered Saline (Fisher SH3025601) \$8 Bovine serum (Sigma B8655) \$12 Fluoresbrite Carboxylate Microspheres 3 μ m in diameter (Polysciences, Inc.) \$175 or Baker's Yeast (I did not have as much luck with this). \$1

Note about blood:

The blood must be fresh since neutrophils live only about a day. This blood may be obtained from a USDA inspected slaughterhouse or perhaps a veterinarian. If you live near a medical research facility, you might be able to get some non-human blood. If it has not been done for you, you will need to add the heparin as soon as the blood leaves the animal. In the alternate activity, you will not add heparin and fresh blood drops will be put on a slide.

To heparinize the blood:

- 1. Add 10 units of heparin / 1 ml of blood.
- 2. Mix slowly.

To isolate neutrophils:

- 1. Slowly dispense 5 ml of heparinized blood into a test tube so as not to damage the cells. Neutrophils are fragile.
- 2. Place 5 ml of Polymorphprep in a 12 ml centrifuge tube.
- 3. Carefully layer the heparinized blood over the Polymorph in the centrifuge tube so as not to mix the layers.
- 4. Centrifuge at room temperature at 1500 rpm for 30 minutes.
- 5. Remove the top yellow layer (supernatant) and discard with the micropipetor or plastic pipet.

- 6. Remove the PBMC layer and discard with the same pipet.
- 7. Using a new pipet, remove the neutrophils and place in a test tube.



- 8. Centrifuge at room temperature at 1300 rpm for 5 minutes.
- 9. Add media in a 1:1 ratio.
- 10. Gently mix.

To activate the yeast:

- 1. Mix 1.0 g sucrose into 100 ml of warm water.
- 2. Add 0.1 g of dry yeast.
- 3. Let the mixture stand for 1 hr prior to opsonization.

To opsonized the beads:

- 1. Dilute the beads from the company stock bottle as follows :
 - Add 1 drop of beads from bottle to 2.5 ml distilled water.
- 2. Dilute serum by half with phosphate buffered saline (PBS).
- Add the diluted bead solution to the serum in a 1:1 ratio and incubate at 37^oC for 30 minutes before using.

Respiratory Burst:

Non-human fresh blood Heparin (Sigma D4776) \$30 Nitro blue tetrazolium (Fisher ICN10041680) \$25 Distilled water Stimulant: Phorbol 12-Myristate 13-Acetate (PMA) (Fisher BP685-1) \$65 Note: you might also try the microspheres or yeast as a stimulant. Opsonize them first. To prepare nitro blue tetrazolium solution:

This chemical comes as a powder and should be prepared the day of the lab since it has a short shelf life as a solution. Distilled H₂O is added to the powder to make a solution.

VI. Student Prior Knowledge and Skills

The students should have learned some basic chemistry, particularly the role of enzymes in living things.

They should know basic cell structure, including lysosomes, golgi and endoplasmic reticulum.

Optional:

This unit could be used to teach microscope skills or to reinforce them. You may consider teaching them how to use the oil immersion lens.

The students may or may not be familiar with the concepts of osmosis and diffusion and phagocytosis. One could use this unit to teach those concepts.

VII. What is Expected from Students

The students will be required to turn in a lab report and participate in discussion. An extension activity would be a report on immune disorders (see Section X.).

VIII. Anticipated Results

Phagocytosis

It is expected that students will be able to observe phagocytosis. If they are not particularly patient, they may only be able to see the beads after they have been engulfed. In the picture below, one bead has been engulfed and the other 2 are being being "investigated" by the neutrophils.



Respiratory Burst

It is expected that students will be able to see the darkened granules in the neutrophils, as in the photomicrograph below. The neutrophils in the above picture are not stained.



400 X

IX. Classroom Discussion

Teachers should talk to students about precautions that health care providers take when working with bodily fluids (universal precautions).

X. Assessment

The lab report should be a graded assignment.

Teachers may want to assign reports on immune disorders. Below are a few examples:

Chronic Granulomatous Disease (CGD) Severe Combined ImmunoDeficiency Syndrome (SCIDS) IgA deficiency complement deficiency asthma allergies Acquired ImmunoDeficiency Syndrome (AIDS)

Students may want to investigate autoimmune disorders. Below are a few examples:

pernicious anemia Crohn's disease Graves' disease rheumatic fever rheumatoid arthritis multiple sclerosis psoriasis vitiligo

Students may want to investigate organ transplants and problems with maintaining a body part that is "not self".

XI. Resources

American Association of Immunologists <u>http://www.aai.org</u>

Centers for Disease Control <u>http://www.cdc.gov</u>

Janeway, Charles et al. <u>Immunobiology: The Immune System in Health and Disease</u>, 6th Ed. New York: Garland Sci. Publishing, 2005.

National Institutes of Health <u>http://www.nih.gov</u>

Schmit, Thomas E. <u>Laboratory Activities to Enhance the Study of Whole Blood</u> <u>Components and their Role in the Immune Response.</u> Hamilton, MT, 2004.

Sullivan, James A. Quill Graphics (personal communication) http://www.cellsalive.com

Student Section

I. Rationale

The body has many protective mechanisms to keep things from harming it. The immune system is responsible for defending the body against intruders that are constantly attacking it.

The immune system is usually divided into 2 parts:

1. innate: system of defense that is non-specific and available before exposure to antigens (molecules recognized as foreign)

2. adaptive: system of specific defense that develops when one is exposed to invaders. For example, chemicals called antibodies are made by certain leukocytes (white blood cells) to specifically match and bind to antigens that are presented to them.

In this lesson, we are going to learn about 2 aspects of the innate system:

- 1. phagocytosis (immune cells engulfing foreign material)
- 2. respiratory burst (evidence of immune cells being stimulated)

II. Materials (per group)

apron microscope slides glass coverslips detergent distilled H₂O alcohol 2 coffee stirrers 1 petri dish 20 ul neutrophils 20 ul opsonized beads

III. Procedure

Observing Phagocytosis

Preparing the coverslips for neutrophils:

- 1. Gently wash a glass coverslip with dilute granular dishwasher detergent (e.g. Cascade) in very hot water.
- 2. Rinse the coverslip several times in distilled water.

- 3. Cover with absolute alcohol.
- 4. Dry the coverslip by hand just before use using lens tissue backed by a paper towel.
- 5. Put moist paper towels in the bottom of a petri dish.
- 6. Place 2 plastic coffee stirrers parallel to each other in the dish.
- 7. Lay the coverslip onto the coffee stirrers.



Challenging the neutrophils:

- 1. Obtain 20 μ I of isolated neutrophils from your teacher and drop onto the coverslip center.
- 2. Cover the petri dish with the lid and incubate at 37^o C for about 20 minutes.
- 3. Invert the coverslip bottom onto a slide on which you have placed a drop of media with opsonized beads.
- 4. Observe microscopically under 400 X magnification.
- 5. Put your used slide in the bleach solution at the front.

Alternate procedure using fresh non-heparinized blood (demonstration):

- 1. Add a generous drop of non-heparinized blood to the coverslip instead of a neutrophil solution.
- 2. Cover and incubate for about 20 minutes or until a good clot is formed.
- 3. Pick up the coverslip with forceps, tip and touch edge to paper to remove the clot, then rinse in warm RPMI 1640 medium.
- 4. Carefully dry the coverslip bottom and invert it onto a slide on which you have placed a drop of medium with opsonized beads.
- 5. Observe microscopically under 400 X magnification.
- 6. Put your used slide in the bleach solution at the front.

Observing Evidence of Respiratory Burst

Cells not stimulated:

- 1. Obtain 200 μ l of isolated neutrophils from your teacher.
- 2. Transfer 120 μ I NBT solution to a small vial.
- 3. Add 200 μ l of well-mixed neutrophils. Cap tightly. Mix gently by tilting slightly and "rolling" the vial. Do not invert the vial.
- 4. Incubate at 37^oC for 10 minutes. Remove and let stand at room temperature for an additional 10 minutes.
- 5. Mix the neutrophil-NBT mixture again as in Step 3.
- 6. Transfer approximately 20 μ l of the mixture onto a glass slide. Using a cover slip, gently spread out the mixture. Do not spread thin or you will damage the cells.
- 7. Allow the slide to air dry.
- 8. Add 1 drop of distilled water and a coverslip.
- 9. Observe microscopically under 400 X magnification.
- 10. Put your used slide in the bleach solution at the front.

Cells stimulated:

- 1. Transfer 100 μ l NBT solution to a small vial.
- 2. Add 50 μ l of well-mixed neutrophils and 5 μ l of opsonized beads to the vial. Cap tightly. Mix gently by tilting slightly and "rolling" the vial. Do not invert the vial.
- 3. Incubate at 37^oC for 10 minutes. Remove and let stand at room temperature for an additional 10 minutes.
- 4. Mix the neutrophil-NBT mixture again as in Step 2 above.
- 5. Transfer approximately 20 μ l of the mixture onto a glass slide. Using a cover slip, gently spread out the mixture. Do not spread thin or you will damage the cells.
- 6. Allow the slide to air dry.
- 7. Add 1 drop of distilled water and a coverslip.
- 8. Observe under 400 X magnification.
- 9. Put your used slide in the bleach solution at the front.

Alternate procedure using non-human heparinized whole blood (demonstration).

The procedure is the same except non-human heparinized whole blood is used instead of isolated neutrophils.