Chapter 6

Identification of T Lymphocytes

James M. Bader

Department of Biology
Case Western Reserve University
Cleveland, Ohio 44106-7080
jxb14@po.cwru.edu

Jim received his B.S. from the University of Notre Dame and M.S. from Case Western Reserve University where he is the Manager of the Graduate and Undergraduate Biology Teaching Laboratories and the Assistant Director of the Center for Biology Education. He teaches courses in Introductory Biology, Microbiology, and Aquatic Biology.

Reprinted From: Bader, J. M. 1996. Identification of T lymphocytes. Pages 113-123, *in* Tested studies for laboratory teaching, Volume 18 (J. C. Glase, Editor). Proceedings of the 18th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 322 pages.

Contents

Introduction

This laboratory exercise is offered as a component of a 3 week immunology sequence in our upper level Microbiology Laboratory course. It was developed in an attempt to focus on two major concepts in immunology, namely cell mediated vs. humoral immunity and the populations of cells involved in the immune response. In order to successfully comprehend the experimental design of this laboratory, it is necessary that the student have a clear understanding of the sequence of events and the cells involved in both humoral and cell mediated responses. They should understand that this is a cell mediated response and that T lymphocytes are proliferating in response to an antigen challenge. This could lead to a discussion of the role of cytokines in the immune response and their applications in treating immune disorders.

This exercise provides students with an opportunity to actually identify and enumerate T lymphocytes. Although this particular technique is no longer used routinely in a clinical setting, as recently as 8–10 years ago it was the only way to identify populations of T cells. With the development of monoclonal antibodies, subpopulations of T cells can now be identified and enumerated. However, this process uses fluorescently labeled antibodies which must be detected with a fluorescence microscope or a fluorescence activated cell sorter, both items that may not be available to most teaching laboratories. This exercise offers the advantage of being a relatively simple procedure that can be successfully completed by individuals who do not consider themselves immunologists.

This exercise also offers the possibility of modification and variation. The T lymphocyte populations of the spleen may be compared to those found in the thymus, lymph node, blood, or thoracic duct lymph and correlated with the immunological function of those organs. It would also be interesting to compare T lymphocyte counts in immunocompromised vs. healthy animals.

Materials

For each group of students

Rat (2) either sex, matched for weight
Syringe 1 cc and 10 cc
Needle 21 gauge
Microcentrifuge tubes, 1.5 ml graduated (10)
Micropipettors 1–20 µl, 20–200 µl, 200–1000 µl
Sterile blue and yellow tips
Hemacytometer
Compound microscope
Scissors
Sheep red blood cells in Alsever's solution (5 ml)
Sterile phosphate buffered saline (PBS) (10 ml)

Basal medium Eagle supplemented with 5% fetal bovine serum (20 ml)
Alcohol washed iron powder (preweighed 4 mg aliquots)
Horseshoe magnet

For the class to share

Microcentrifuge
Cheesecloth
37°C water bath with racks for microcentrifuge tubes
Some means for sacrificing rats
0.1% toluidine blue (50 ml)
0.2% nigrosin (50 ml)
Rocker platform

Notes for the Instructor

This exercise takes part of one 3 hour laboratory period and all of another. The preparation of the vaccine requires about 1 hour during the first laboratory period. The vaccine may be prepared ahead of time and refrigerated to save time. I like to have the students prepare the vaccine because they become familiar with dilutions and use of the hemacytometer which they will use extensively the following period.

A number of potential obstacles should be considered before attempting this exercise. Since animals are used, it is likely that an Institutional Animal Care and Use Committee form will have to be filed with the appropriate university representatives. Please do not let the red tape discourage you from attempting this exercise or any other using animals. If you would like to cut down on the number of animals required, we have successfully shared one experimental rat per four students (rather than two) and on occasion we have also reduced the number of control rats by sharing a few for the whole class.

This procedure was originally designed for use in mice, so mice can be substituted for rats without any change in the protocol. The T-cell activating cell-surface antigen equivalent to CD2 and Ox49 is designated Ly-37 in mice (Hudson and Hay, 1989). We use rats because we have a rat colony that is maintained within our department rather than across campus at the animal resource center.

The animals may be sacrificed by overdose of ether as we do or by overdose of CO_2 as was done at the ABLE conference. Other methods may be suitable without significant side effects. It is best that the animals be sacrificed immediately before use. If the students are to sacrifice the animals, this usually creates a traffic jam unless some large vessel is available for sacrificing many animals at once. Try to get all the animals sacrificed and the single cell suspensions generated within 1 hour to complete the laboratory within a 3 hour period.

The initial single cell suspension that results from passing the spleen through cheesecloth need not be a homogeneous solution. We have seen excellent results from samples that were not well broken up. Although we use cheesecloth, nylon wool is preferred for generating single cell suspensions because it binds some of the B lymphocytes in the process. We have had difficulty obtaining nylon wool and so have substituted cheesecloth with no apparent loss of sensitivity.

An alternative to using iron powder to remove macrophages is to run them through a column. Sephadex G-10 may be used to selectively remove phagocytic cells, but the iron-magnet combination is faster, less expensive, and technically less demanding than running a column.

Student Outline

Introduction

Vertebrates are constantly exposed to and in continual contact with millions of microorganisms. Many of these may be part of the normal flora and cause no harm; in fact they may be beneficial and some are actually essential to the overall health of the individual. However, as vertebrates move through their environments, they encounter many bacteria, viruses, fungi, and protozoa that may be pathogenic, that is, these microorganisms may cause damage to the host.

Vertebrates have evolved a wide range of defense mechanisms against these invaders. In humans, much of the anatomy and physiology of the skin, mouth, and gastrointestinal tract serves to favor beneficial organisms and select against pathogens. In addition, vertebrates possess a number of innate mechanisms for combating pathogenic organisms. Nonspecific host-resistance mechanisms, or natural resistance as it is sometimes known, include inflammation and phagocytosis among others and target any invading microorganism, even if the host has never been previously exposed to that pathogen. Specific host-resistance mechanisms produce a directed immune response as a result of invasion by a pathogen. The pathogen acts as an antigen which stimulates a specific response by the host cells. This response may involve only the production of antibodies (humoral response) or it may be a cell-mediated event.

In humans, the immune response includes a number of organ systems such as the spleen, thymus, bone marrow, lymph, and blood. White blood cells or leucocytes are the cells of the immune system, constantly monitoring for antigens. Both specific and nonspecific host-resistance are mediated by these cells. The two major groups of leucocytes are (1) phagocytic cells consisting of polymorphonuclear leucocytes (PMN) and macrophages which participate in both specific and nonspecific responses, and (2) lymphocytes consisting of B cells and T cells.

Lymphocytes are widespread throughout the body, circulating in the blood and lymph and residing permanently in the spleen and lymph nodes. In humoral immunity, B cells with antigen specific antibodies on their cell surface, recognize circulating soluble antigens and under the chemical influence of T cells secrete antibodies against the antigen. B cell binding of the antigen stimulates the production of short lived plasma cells which secrete the antibodies and long lived memory cells which recognize and respond to the antigen if it is encountered again.

T lymphocytes also have antigen-specific molecules on their surface which are evolutionarily similar to antibodies. The T cell receptor (TcR) has a constant region that is anchored in the T cell membrane and a variable region that extends from the cell. Two major subpopulations of T cells have been identified based on their functions and surface proteins.

CD4 lymphocytes
- T helper cells (T_H) stimulate B cells to produce antibodies
- Delayed type hypersensitivity T cells (T_D) activate macrophages

CD8 lymphocytes
- Cytotoxic T cells (T_C) destroy antigen presenting cells
- T suppressor cells (T_S) may suppress the immune response

In cell mediated immunity, T cells recognize antigens only when they are presented by another cell in combination with proteins of the major histocompatibility complex (MHC). The MHC proteins serve as molecular markers, allowing the T cells to discriminate between cells that are self and nonself. MHC proteins on the surface of antigen presenting cells, together with a bound antigen, signal the T cell that this is a nonself cell and is targeted by the immune response.

Monitoring the T cell population or subpopulations is important in the diagnosis of a large number of cell mediated hypersensitivities and auto-immune diseases. Type IV hypersensitivity usually involves T_D cells primarily and to a lesser extent T_C cells and macrophages. T_D cells are stimulated to produce lymphokines which can contribute to an inflammatory response by attracting macrophages and activating them. This reaction is the basis for the skin test commonly administered for tuberculosis.

Auto-immune diseases involving cell mediated immunity include lymphocytic choriomeningitis in which the body responds to the lymphocytic choriomeningitis virus by producing T cells that fail to protect the membranes surrounding the brain resulting in fatal neurological damage. Hashimoto's thyroiditis occurs when T cells attack and destroy the thyroid gland; destruction of the adrenal gland by T cells causes Addison's disease. Not to be overlooked are immune deficiency diseases such as AIDS and DiGeorge syndrome. In AIDS, the HIV targets the CD4 T helper cell population which plays an important role in both humoral and cell mediated immunity. In DiGeorge syndrome, the thymus is defective causing a deficiency in the number of T cells. The lack of sufficient T cells is usually fatal, often in infancy.

Clinically, monoclonal antibodies have been raised to most of the major proteins on the surface of T cells. These can be used to track specific subpopulations of T cells and determine their numbers. An alternative takes advantage of the fact that populations of activated lymphocytes differentially bind to foreign red blood cells to form rosettes. Human T lymphocytes possess a 50 kDa surface protein designated CD2 which is the sheep red blood cell receptor. Binding at the CD2 receptor stimulates T cell activation against the antigen. However, this method is technically sensitive because sheep red blood cells bind weakly with human T cells. Experimental success depends largely on proper laboratory technique, especially if results are to be compared between labs. To offset the problem of weak binding and to avoid the dangers associated with working with human blood, we will demonstrate T cell rosette formation with rats. Rat T cells possess a protein functionally equivalent to the CD2 receptor. In rats it is designated Ox49 and it binds T lymphocytes in an antigen-specific manner and stimulates T cell activation. When activated T cells are incubated with sheep red blood cells, rosettes will form. Rosettes are defined as a single lymphocyte binding five or more erythrocytes (Myers, 1989). These may be stained, and viewed and quantified with the light microscope. This method provides a convenient way to separate T cells from B cells for identification and enumeration.

This exercise directs the student through (1) a procedure for the separation of T cells from rat spleen and (2) the rosette assay for the determination of the percentage of T cells in blood.

Procedure

Part A : Preparation and administration of sheep red blood cell vaccine

1. Immunize a rat 7 days before the experiment with a sheep red blood cell vaccine given intraperitoneally as described below. Prepare a sheep red blood cell (SRBC) vaccine by first washing the cells.
   a. Collect approximately 1 ml of sheep blood in Alsever's solution from the vacutainer. Place the blood into a sterile graduated 1.5 ml-microcentrifuge tube and centrifuge at 1500 ×*g* for 5 minutes. Remove the fluid portion (plasma) and discard.
   b. Resuspend the pellet up to 1 ml with sterile PBS. Mix and centrifuge at 1500 × *g* for 5 minutes. Discard the supernatant after the spin. Resuspend the pellet to 1 ml in sterile PBS.

2. Next, prepare a saline suspension of the washed SRBC to approximately 10^8 cells/ml as follows.
   a. Perform four tenfold serial dilutions (10^{-1} through 10^{-4}) of the washed SRBCs in sterile phosphate buffered saline (PBS) by serially diluting 100 µl of SRBCs into 900 µl of sterile PBS.
   b. Fill one chamber of a hemacytometer with approximately 20 µl of one dilution (perhaps 10^{-1}) and fill the other chamber with 20 µl of another dilution (perhaps 10^{-3}). Let the cells settle for a few minutes before counting.
   c. Using the high dry objective of the microscope, count the cells in the large triple ruled squares designated 1 through 5 in the Figure 6.1 below. Cells falling on the left border lines and across the top are considered to be in that square while cells on the bottom and right borders are excluded.
   d. To determine the number of cells/ml, use the formula given below.

$$\text{Cells/ml} = \frac{\text{No. cells counted}}{\text{No. triple ruled squares}} \times 25 \times 10^4 \times \text{dilution factor}$$

3. Select the dilution which is closest to 10^8 cells/ml and inject 1 ml intraperitoneally with a 1 cc syringe and 21 gauge needle. This is done most easily by first anesthetizing the rats in an ether jar for approximately 2–3 minutes. You want the rats unconscious, but not dead. Once the rats have been properly anesthetized, turn them on their backs and pull at the skin at the lower abdomen. The skin will pull away from the body and vital organs. Insert the needle until you feel it penetrate the skin and inject the vaccine. Monitor the rat after injection to be sure that it recovers.

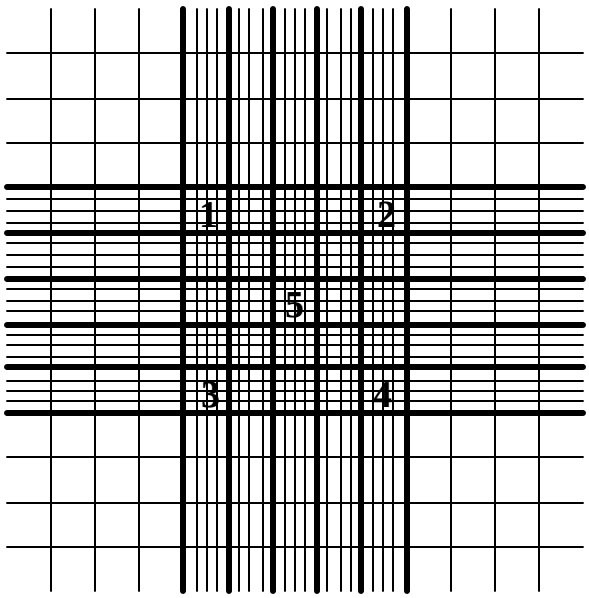

Figure 6.1. The figure shows the appearance of a hemacytometer with the improved Neubauer ruling as it would appear under low power of a compound microscope. The depth of the chamber is 0.1 mm and each of the 25 triple ruled central squares measures 0.04 mm^2 in area. Each of the 25 squares is subdivided into 16 smaller squares which measure 0.025 mm^2 in area.

Part B : Isolation and enumeration of T lymphocytes

1. On the day of lab, you will sacrifice the rats that have been immunized and one that has not and prepare a single cell suspension from their spleens. Prepare a 10 cc syringe by placing 2–4 layers of cheesecloth in the barrel, just covering the opening. Too much cheesecloth will prevent the cells from passing through and too little will not have the proper shearing effect. Wet the cheesecloth with Basal medium Eagle supplemented with 5% fetal bovine serum (BME with 5% FBS). Place the rat into a jar with ether. After the rat has died, make a midline incision up the abdomen. The spleen will be a tongue of dark red tissue on the left side of the rat next to the stomach. Cut the spleen out with scissors and mince it into small pieces. Place the pieces into the barrel of the 10 cc syringe with the cheesecloth soaked in BME with 5% FBS. Force the tissue through the cheesecloth into a microcentrifuge tube. The homogenate may be lumpy after passing through the cheesecloth. This is not a problem as long as some shearing did take place. Resuspend the cells to 1.5 ml with BME with 5% FBS. Lymphocyte viability tends to vary with the amount of fibrous tissue in the organ and the skill level of the operator. Approximate cell yields from the spleen are 35% T lymphocytes, 38% B lymphocytes and 25% "null" cells.
2. Set aside an aliquot of each lymphocyte suspension for the viable lymphocyte count (Part C) by removing 0.5 ml of the lymphocyte suspension to a clean microfuge tube and bringing the volume to 1 ml with BME with 5% FBS. Keep on ice.
3. It is necessary to remove other cells of the immune system from our suspensions. We will take advantage of the phagocytic properties of macrophages by feeding them iron and then using a magnet to draw them out of suspension.
   a. Wash 1 g of iron powder in ethanol and then in distilled water. Allow to air dry overnight or place in a 60°C oven for a few hours.
   b. Adjust the single cell suspension to $2–3 \times 10^7$ cells/ml using the hemacytometer as described in Step 2 of Part A. First count an undiluted specimen and then, if necessary, adjust to the proper concentration by serially diluting 100 µl of the lymphocyte suspension into 900 µl of BME with 5% FBS. Notice that the vast majority of cells you are counting are red blood cells.
   c. Once the proper dilution as been prepared, add 4 mg of iron powder to the tube and mix thoroughly.
   d. Incubate at 37°C for 30 minute in a water bath, mixing occasionally. During this time, proceed to Part C, the viable lymphocyte count.
   e. After the 30 minute incubation, place a horseshoe magnet in a bucket of ice so that the poles are just below the surface and facing upward. If positioned properly, each magnet can work for four tubes of cell suspension. Place four tubes (two from your group and two from another group) in a 2×2 configuration in a rack such that the bottom of each tube is exposed. Place the tubes so that they are resting directly on top of or next to either pole of the magnet. Stand the tubes on ice for 10 min. During this time, the macrophages which took up the iron during the 30 minute incubation will be drawn toward the magnet.
   f. After 10 minutes, with the tubes still standing on the magnet, carefully remove the cells that are still in suspension and transfer them to a clean microfuge tube. These cells should be erythrocytes and lymphocytes which did not take up the iron. Be careful to stay away from the side of the tube in contact with the magnet to avoid macrophages. After removing the cells in suspension, discard the tubes with the iron.
   g. Adjust the volume in the tube with the lymphocytes to 1 ml with BME with 5% FBS.
4. Spin the lymphocytes at $150 \times g$ for 10 minutes at 4°C. Draw off and discard the supernatant and resuspend the pellet to 0.5 ml with BME with 5% FBS. This concentrates the lymphocytes.
5. Label two new tubes and add a 0.1 ml aliquot of the lymphocytes from the immunized rat to tube 1, and add a 0.1 ml aliquot of the lymphocytes from the non-immunized rat to tube 2

6. Add 0.1 ml of SRBC which has been adjusted to a concentration of 2.4×10^8 SRBC/ml to tube 1 and tube 2. Mix well by inversion.
7. Centrifuge tubes 1 and 2 at 150 × g for 10 minutes at 4°C. Discard the supernatant. This concentrates the cells and brings the SRBCs into close contact with the lymphocytes. Rosettes begin to form at this point.
8. Add 0.3 ml of BME with 5% FBS to both tubes and resuspend the cells by placing them in a rack on an orbital shaker at 75–100 rpm for 5 min. The use of a pasteur pipette to resuspend the cells is discouraged because the shear forces generated by this action reduces the number of rosettes. During this time, the rosettes are forming as SRBCs bind to activated T lymphocytes. Since this binding is not terribly strong, the resuspension must be done very gently on an orbital shaker or even by hand.
9. Add 0.1 ml of 0.1% toluidine blue to better visualize the cells and *gently* mix the cells by hand.
10. Count the number of rosettes in each suspension using a hemacytometer. Count at least four samples per tube.

Results

1. Calculate the number of rosettes/ml of suspension and from this the number of rosettes per 10^6 lymphocytes
2. Compare the number of rosettes per 10^6 lymphocytes from the normal and immune animals and calculate the factor of immunization.

Part C : Viable lymphocyte count

Chances are good that you are not familiar with the morphology of viable lymphocytes, so this is an opportunity for you to try to differentiate between lymphocytes and other leukocytes. Also, analysis of this sample yields an approximation of the number of viable lymphocytes and may reflect your success at generating a decent single cell suspension. Nigrosin is a non-electrolyte dye and is not able to pass through the plasma membrane of viable cells. Cells whose nucleus has been stained by this procedure would be dead.

1. Centrifuge the lymphocyte suspension set aside in Part B, Step 2 at 150 × *g* for 10 minutes at 4°C.
2. Carefully remove and discard the supernatant without creating turbulence in the tube.
3. Resuspend the pellet in 1 ml PBS.
4. Mix 0.1 ml of each cell suspension with 0.1 ml of 0.2% nigrosin solution and incubate at room temperature for 5 minutes.
5. Count the number of viable lymphocytes using a hemacytometer. Small lymphocytes have a diameter of about 10 µm and a large nucleus:cytoplasm ratio with the nucleus being very round and regular in shape. Large lymphocytes may have granules in the cytoplasm. They are characterized by a lower nucleus:cytoplasm ratio. Plasma cells may be identified by their characteristic large cytoplasm with the nucleus pushed to the edge of the cell.
6. Compare the number of viable lymphocytes with the number of cells counted in Part B, Step 3b to determine the percentage of cells which are lymphocytes.

Review Questions

1. Why did we immunize the rats with SRBCs one week before the experiment? Are T cell sensitized to the antigen the same way B-cells are?
2. What chance would we have of seeing rosettes if we had not immunized the rats? In other words, is this specific or non-specific binding of SRBCs to lymphocytes.
3. Getting rid of the phagocytic cells by feeding them iron was a neat trick. But why bother?
4. Do B cells that have been stimulated by the presence of SRBC antigen bind to the SRBCs the same way we are trying to demonstrate for T cells? Are some of the rosettes we saw actually B cells and not T cells? How can we distinguish between the two populations of lymphocytes?
5. What if you added the following variation to your protocol?
   a. Prepare a single cell suspension and remove the phagocytic cells from an immunized rat as described in the protocol.
   b. Add 0.1 ml of the lymphocyte suspension to two tubes; into one tube add 0.1 ml of rabbit anti-rat immunoglobulin serum, into the other add 0.1 ml of normal rabbit serum.
   c. Add 0.1 ml of the SRBC suspension to each tube, centrifuge, mix on rotary shaker. Would you expect rosette formation in these two tubes.

Literature Cited

Hudson, L. and F. C. Hay. 1989. Practical immunology. Third edition. Blackwell Scientific Publications, Oxford, England, 507 pages.

Myers, R. L. 1989. Immunology. Wm. C. Brown Publishers, Dubuque, Iowa, 99 pages.

APPENDIX A
Suppliers and Recipes

Suppliers

Sheep blood is available from a number of sources. Two popular sources are:

Carolina Biological Supply
2700 York Road
Burlington, NC 27215
1-800-334-5551

Lampire Biological Laboratories
PO Box 170
Pipersville, PA 18947
(215) 795-2838

Fetal bovine serum	Sigma	Cat # F2442
Basal medium Eagle	Sigma	Cat # B9638
Magnets	Fisher	Cat # 12-012

Recipes

Phosphate buffered saline (PBS)

NaCl	8g/l
KCl	0.2 g/l
Na_2HPO_4	1.15 g/l
KH_2PO_4	0.2 g/l

0.1% Toluidine blue

Toluidine blue	0.1 g
dH_2O	100 ml

0.2% Nigrosin

Stock solution

Nigrosin	2 g
PBS	100 ml

Dilute stock solution 1:10 before use.

APPENDIX B
Suggested Answers to Review Questions

1. While T cells involved in a cell mediated response are not sensitized to antigen presentation in the same way B cells are during a humoral response, we wanted to allow sufficient time for the proliferation of T cells.
2. The Ox49 cell surface antigen serves as a T cell activator and as the erythrocyte rosette receptor, so the binding is antigen-specific.
3. We wanted to remove as many other types of leucocytes as possible so that we would have primarily lymphocytes in our cell suspension. By taking advantage of the phagocytic properties of macrophages, we were able to selectively remove them from the suspension.
4. Experienced cytologists may be able to distinguish between the two populations of lymphocytes morphologically. Although there is homology between the B cell antigen receptor (surface antibodies) and the T cell receptor (TcR), the T lymphocyte receptor for antigen includes the CD3 surface protein as well as the CD4 or CD8 proteins.
5. Rosette formation should be blocked in the tube with anti-immunoglobulin serum, but should be unaffected in the tube with normal serum.