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Introduction

The objective of this laboratory exercise is to demonstrate the basic principles of immunology by observing the action of rabbit antisera on red blood cells derived from different animals. Two antisera are evaluated in parallel, one from a rabbit given sheep red cells as a single injection with antisera collected 2 weeks later and one from the same rabbit given two additional “booster” inoculations of sheep red cells. Certain antigens found on sheep red cells are also found on goat red cells, thus a fraction of the antibodies found in the rabbit bind with goat cells, albeit with a lower titer (because the amount of these cross reactive antibodies is much lower than all the antibodies capable of binding sheep red cells or antigens). In the absence of blood complement, the action of these antisera is to bridge antigens between two adjacent red cells. This causes the cells to clump together or agglutinate. When an antibody attached to red cells activates complement, the resulting reaction leads to holes in the red cell membrane. Hemoglobin leaks out of the red cells, resulting in hemolysis. With a minimum of reagents, students can quickly grasp the principle of primary versus secondary antibody responses, conservation of proteins between closely related species, and the dramatic enhancement of immune function affected by complement.

This exercise has been tested for several years in both high school AP biology labs and in freshman college biology labs. It is best performed by students in small groups because some dilutions tend to be tedious and “taking turns” helps by reducing errors associated with repetition. The entire laboratory should take about 40 minutes to complete by students. The plates can be left out on a bench and read in 2 hours or refrigerated and read up to 1 week later. Laboratory preparation time will vary depending on how the reagents are supplied. If your institution is making the reagents, considerable preparation time is involved; however, commercially-available reagents which are pre-tested and supplied with precise dilution instructions reduces preparation time appreciably (see Notes for the Instructor).

Notes for the Instructor

The materials used in this exercise may be produced by the institution or purchased from a commercial vendor. Some of the reagents are commercially available now (e.g., rabbit anti-sheep red cell antibody, guinea pig complement and red cell solutions) from various animals (all are available from Organon Teknika Corp., Durham, NC). We have produced all the reagents at Cornell University and it is quite easy and practical if large numbers of students will conduct the laboratory. Each year we prepare reagents for freshman biology, the Cornell Institute of Biology

Teachers, and Ithaca High School AP Biology. We furnish reagents for 311 laboratory groups each year, for a total reagent cost of \$192.00 US; however, the red cell donors are available at no charge. Rabbit antisera and guinea pig serum are collected once, pretested to determine the optimal dilution and frozen until the week of the laboratory. Pretesting these reagents typically takes one technician or TA 1 day. Posters, pipets, microscope slides, plastic disposable tubes, and 96-well plates will vary in price depending on the manufacturers and volume purchased. Typically, the disposable supplies for one group cost less than \$2.00 US. Carolina Biological Supply Company has expressed an interest in marketing this entire laboratory complete with reagents and consumable supplies.

In the laboratory exercise performed at the ABLE conference, all reagents were pre-tested and dilution instructions were known. A pH 7.0 phosphate buffered saline solution containing 1% bovine serum albumin (BSA) was used as a diluent for all solutions (e.g., antisera, red cell suspensions, and complement). The 1% BSA is added to stabilize red cell membranes and prevent non-specific sticking of red cells to plastic. For each laboratory group of 2–3 students the following dilutions are made into plastic test tubes labeled using a felt pen: sheep, goat, dog, and rabbit red cells were all supplied as 50% cells and this stock solution was diluted 1:150 to produce 0.33% suspensions of each; guinea pig complement diluted 1:50 and kept on ice (or refrigerated) until used; rabbit antisera diluted 1:40. The final volumes of these reagents are quite small for single groups (e.g., 1.25 to 5 ml), and thus it is much less time consuming to calculate the total volume needed for all groups, make a single large dilution, and then dispense the solutions into individual plastic tubes for groups. Dispensing solutions and making dilutions is achieved using disposable plastic pipets (the 25 ml size is very handy for laboratories involving many groups). For a laboratory involving 16 groups, approximately 2 hours are needed for total preparatory time. All suspensions except complement can be aliquoted the day before and maintained in a refrigerator. Complement should remain frozen (if serum) or refrigerated (if lyophilized) and dilutions made the morning of the laboratory. We have been successful at thawing fresh guinea pig serum, making dilutions, and freezing them overnight, however, you should allow for a two-fold drop in complement activity using this method (which means the initial dilution should be 1:25 rather than 1:50). As part of the laboratory preparation we label all tubes and assemble them in beakers for each laboratory group. This way a tray containing the beakers can be removed from the refrigerator and brought out to the students 5 minutes before the laboratory begins. Laboratory set up is thus insignificant.

Preparation of reagents is straightforward. All red cells are collected in heparinized blood tubes and refrigerated (up to 2 weeks) until used. A rabbit must be injected intravenously with 2 cc of 50% sheep red cells (washed once in sterile phosphate buffered saline and resuspended in sterile PBS to 50% packed cells). Red cells are injected into the marginal ear vein of the rabbit and blood is collected from the central ear artery directly into a clot tube. This blood is allowed to clot (2 hours at room temperature) then centrifuged at 200 xg for 10 minutes. The straw-colored serum is carefully aspirated from the tube, placed in a 50 cc tube and incubated at 56°C for 30 minutes, and then frozen. The heating inactivates rabbit complement. Red cells are injected into the rabbit at intervals of 3 weeks with blood removed from antisera 2 weeks following the first and third injection. There is no need to euthanize the rabbit and more high-titer antisera can be developed by giving one additional “booster” injection and bleeding the rabbit in 2 weeks. Special precautions are necessary for handling guinea pig complement (see Introduction). Phosphate buffered saline is made by combining the following chemical grade reagents (all available from Sigma Corp.) in 1 liter of distilled water: 8.5 g of NaCl, 0.386 g of NaH₂PO₄·H₂O, and 1.02 g of Na₂HPO₄.

Several parts of this exercise have posed problems for students in the past. It is critical that all students understand the configuration of reagents to be added to the 96-well plate before the laboratory begins. We actually set up one plate the day before the class and bring it in for the students to see. A diagram of the plate with all added reagents is included in this chapter (Figure

9.1) and should be given to each laboratory group. Other problems occur during dilution of antisera across a row of wells. Typically 2 drops of antisera are placed in wells 1 and 2 of a row. Then the contents of well 2 (which has now diluted the antisera 1:1 in PBS) is sucked up into a pasteur pipet, 2 drops are added to well 3, and the remainder returned to well 2. This procedure continues by sucking up the contents of well 3, transferring 2 drops to well 4, and returning the remainder to well 3, etc. Two problems occur during this step: the students forget where they are in the dilution series and the contents remaining in the pipet are discharged too quickly into the wells, resulting in significant bubble formation. We have students working in pairs with one holding a pipet on the next well to receive antisera to avoid mistakes. Students should also take turns making these dilutions to break up the tedium of repetitive titrations. Students should be cautioned to discharge the contents of pipets gently to avoid bubbling in the wells. If you end up with bubbles in wells, read the HA titer later (24 hours) when the bubbles have broken down and hemagglutination has occurred.

Students may also experience problems interpreting the HA and hemolytic titers. The last well in each row is designed to be the negative control for that row. If it is not a perfect button of red cells at the bottom of the plate, then none of the other wells in that row will have a button either. Make all comparisons based on well 10. Occasionally red cells will form a doughnut or bulls-eye ring at the bottom of the negative control. Simply call this a 0 reaction and score the first reaction as having a much larger ring. If you want to be very conservative, call diffuse matting of red cells (carpet) across the bottom of the well as a positive and any pattern less than diffuse as negative. You are only likely to misjudge the actual titer by one or two dilutions. Be sure, however, to use this same scoring method throughout the plate. Uncommonly, wells containing rabbit red cells will develop a pattern of hemagglutination in which wells 1 through 6 are negative and wells 7 through 9 become increasingly positive, with well 9 showing a full mat (carpet-3). This is not an immunological phenomenon. Here ionic charges on the red cell are becoming expressed due to the lack of binding proteins in the rabbit antisera which normally neutralize them. Thus, as you dilute the antisera, you remove the binding proteins leaving red cells charged and these charged cells stick to the plastic surface. You can eliminate this effect by the addition of 0.5% normal rabbit serum to the PBS. This false positive never develops the 4+ HA pattern.

Occasionally, the complement has not been handled properly, and no hemolysis occurs in any well. This could be due to miscalculation of the dilution, inappropriate storage, or inactivation at room temperature. A slight drop in complement activity will cause only the wells with the most antisera to hemolyze. If the addition of complement caused hemolysis of goat cells but not sheep cells, a technical error was made setting up the plate. Occasionally guinea pig serum (the complement source) will lyse red cells directly (with no added rabbit sera). This results when the guinea pig has anti-red cell antibodies or when some factor on red cells besides antibody initiates the complement cascade. These non-specific (anticomplementary) reactions are usually avoided by diluting the guinea pig serum to the lowest concentration which gives a positive reaction with rabbit antisera on sheep cells. Companies which supply complement usually provide the purchaser with this dilution. When scoring hemolysis, tell students to forget the red cell button and just look for the last well where the solution is detectably pink. Students can gently aspirate the contents of a well into a pasteur pipet leaving residual red cells on the bottom of the well. If the contents of the pipet are ejected on a microscope slide, the pink colour may be determined more accurately.

Safety

Safety should always be a concern in any biology laboratory. The reagents in this laboratory, when properly handled, do not contain microorganisms capable of causing infection in humans. Leaving blood out at room temperature for days may lead to overgrowth by bacteria, but these organisms are rarely human pathogens. If the plates are going to be read after 24 hours and refrigeration is not available, the PBS should contain 0.01% sodium azide as a preservative. Under these circumstances, students should be asked to wash their hands before leaving the laboratory.

We provide two beakers to each group. One contains all the supplies, the other is used to collect waste as each reagent is used. Sharp objects (e.g., pasteur pipets and microscopic slides) should be discarded in a plastic-lined cardboard box, and never discarded in a wastebasket. Excess red cell suspension and antisera may be discarded down a drain and disposable plastics discarded with other waste (or preferably incinerated).

Students who accidentally cut themselves during the lab should wash the injured site immediately under running water and a disinfectant (e.g., betadine, iodine) and report the incident to the school nurse or laboratory supervisor. Deep cuts may require sutures and a tetanus vaccination. In our experience with over 1,000 students, this event has never occurred.

Materials**Immunology Lab A: Slide Agglutination Test**

Microscope slides (4 per person)
 Toothpicks (4 per person)
 Pasteur pipets with bulb (or eyedroppers) (4 per person)
 Pipet for antibodies (1 per person)
 Microscope for viewing slides
 Vial of anti-sheep red cell antibodies
 Washed red cells from: sheep, goat, dog, and rabbit (50% red cells)

Immunology Lab B: Hemagglutination and Hemolysis

Each group of two should obtain the following equipment and supplies:

Round-bottom, 96-well plastic agglutination plate (1)
 Small beaker with 7 pasteur pipets (be sure none of the tips are broken)
 Rubber bulbs (several)
 Phosphate buffered saline (labeled PBS) (5 ml)
 Antisera from once-injected rabbit diluted at 1:40 (labeled 1') (200 μ l)
 Antisera from thrice-injected rabbit diluted at 1:40 (labeled 3') (1,200 μ l)
 Guinea pig serum as a source of complement (labeled CP), fresh frozen (500 μ l)
 RBCs from sheep (3 ml), goat (2 ml), dog (1 ml), and rabbit (1 ml) at 0.3% concentration (labeled S, G, D, and R) in four separate containers
 Permanent marker (1)
 Small beaker marked "used pipets"

Student Outline

Introduction

Broadly speaking, host defenses may be divided into innate defenses and specific or adaptive immune mechanisms. All organisms possess some form of innate protection whether it be the integrity of the cell wall in bacteria or the skin in higher organisms. Innate defenses are non-specific because they do not protect against a specific microorganism. They include mucous membranes, mucus secretions, cilia on epithelial cells of mucous membranes such as those found in the respiratory system, and the non-specific inflammatory processes that include phagocytosis, release of histamine, and the activation of the blood complement protein that aids in phagocytosis.

Primitive protective phagocytic cells first appeared among sponges and coelenterates and have evolved into two separate pathways. One involved arthropods, annelids, and mollusks and the other involved the tunicates and echinoderms. It was the latter pathway that continued to evolve into the immune system of the vertebrates. In higher animals, namely mammals, reptiles, birds, and fish, these innate mechanisms interact with specific immune mechanisms to achieve a more selective and powerful attack by the host.

The adaptive immune mechanisms evolved later in the animal kingdom with clear evidence for their presence first appearing in echinoderms and mollusks and later becoming increasingly complex and precise in vertebrates. All vertebrates have evolved a system of interacting cells and molecular substances that operate in a coordinated fashion to deliver an attack against foreign invaders of exquisite specificity. Two hallmarks of adaptive immune mechanisms are *specificity* and *memory*. Unlike innate mechanisms which mount a similar attack against a parasite on each subsequent intrusion, immune mechanisms amplify and remember each subsequent encounter with a specific parasite. The concept of amplification and memory were taken under consideration in man's strategy for using vaccinations to protect against specific infectious diseases.

Vertebrates have evolved a highly efficient adaptive immune system that is characterized by receptor molecules with great specificity for foreign, non-self molecules called antigens. The immune system is often divided into two interacting, interdependent components: humoral and cellular. Cellular immunity depends on the activities of a class of specialized white blood cells called T-lymphocytes or T-cells. We will not be considering cellular immunity in this laboratory. Humoral immunity depends on proteins called antibodies or immunoglobulins (Igs). Antibodies are produced by another class of white blood cells called B-lymphocytes or B-cell. Through a complex diversity-producing genetic mechanism, each B-cell produces a unique antibody and it displays these antibodies on its surface. After the B-cell encounters an antigen that can bind to its surface antibody receptor, the B-cell is stimulated to give rise to clones of both memory cells or plasma (effector) cells. Plasma cells immediately start to produce and secrete their antibody; memory cells are long-lived and can respond to the same antigen in the future by producing clones of B-cells which may become new plasma cells. The presence of memory cells allows the second and subsequent response to the antigen to be more rapid and effective.

Antibodies are Y-shaped protein molecules that have two identical receptor sites on the tips of the molecule's forked end. Thus, each antibody can bind two antigens. Antibodies protect the body from foreign antigen through one of three mechanisms: neutralization, precipitation, and agglutination. An antigen is neutralized if in forming the antigen-antibody complex, the antibody masks the antigen's harmful chemical groups. Soluble antigens like bacterial toxins can be precipitated out of the plasma by the cross-linking of antibodies to form large networks of antigen-antibody complexes. Finally, if the antigen is part of a cell, antibodies may bind to the cell and activate complement proteins (leading to membrane damage) or direct the cell to phagocytes (which

may engulf and destroy the cell). When antibodies cross-link two or more red cells, agglutination occurs. This is manifested by red cells clumping together.

The complement system includes over a dozen different blood proteins that are activated when an antibody binds to an antigen that is part of a cell. Antigen binding exposes a site on the antibody that activates the first complement protein which then activates the next complement protein in the sequence, and so forth. Among other effects, this complement cascade leads to the formation of the membrane attack complex resulting in the formation of a transmembrane pore (hole) and lysis of the cell. If the target cell is a red blood cell, hemolysis results and hemoglobin and other cell components are released. When complement has been activated it is said to be fixed because it is now not able to react to other antigen-antibody complexes. Because the complement system can be activated by as few as two adjacent antigen-bound antibodies, complement fixation (CF) is a very sensitive assay for antigen-antibody complexes.

Since you will be working with blood in this laboratory there are several terms whose definitions are important. *Plasma* is the liquid part of the blood without any of the blood cells. Plasma contains all of the blood proteins including the blood-clotting protein fibrinogen. Plasma is produced by allowing the blood cells, in anticoagulant treated blood, to settle or by use of centrifugation to pelletize the cells. If blood is allowed or induced to clot, the clear liquid that is expressed from the clot is serum. *Serum* is plasma without fibrinogen. If an animal is injected with an antigen, its immune system will respond by producing antibodies specific to the antigen. Serum from that animal is called antiserum because it contains antibodies that bind specifically to the original antigen.

In this laboratory you will study the special form of agglutination called hemagglutination (HA) in which the agglutinated cells are red blood cells (RBCs) and the antigens are proteins associated with the RBC membrane. Hemagglutination is useful for studying native blood cell proteins (for example, the ABO blood proteins), but it can also be used to study a variety of free antigens since many antigens can be made to bind to red blood cells. Hemagglutination is a very sensitive assay technique for detecting antigen. It is estimated that as little as 0.005 μg of antibody-N per ml will cause observable agglutination of red blood cells. However, for an antibody to cross-link adjacent RBCs sufficient to cause hemagglutination, the antibody concentration must still be higher than that which is required to cause complement induced hemolysis. Thus, hemagglutination is less sensitive than complement fixation.

Using hemagglutination, this lab will demonstrate the principles of antibody-antigen binding, the secondary immune response, cross reactivity, and complement fixation. The materials to be used include antibodies from a rabbit that was injected once with red cells from a sheep and antibodies from a rabbit that was injected three times with the red cells from a sheep. Red blood cells from sheep, goat, dog, and rabbit will be used as antigens to detect the specificity of antibody. Certain antigens are conserved on the red cells of different species, thus cross reactive antibodies will be manifested by agglutination of non-sheep red blood cells. The slide agglutination test is used to detect antibodies in the human Rh negative mother which may react against Rh antigens found in her fetus.

Procedure for Slide Agglutination Test

1. Obtain four microscope slides. Label them with a marking pen: sheep, goat, dog, and rabbit.
2. Obtain a tube of anti-sheep red cell antibodies. With a clean pipet, place a drop of the antibodies in the center of the slide marked "sheep".

3. Obtain a tube of sheep red blood cells and a sterile pipet and clean toothpick. Place a drop of sheep blood on top of the drop of antibodies and stir gently with the sterile toothpick for three circular motions.
4. Allow the slide to stand for up to 5 minutes. If there is going to be agglutination, you should see clumping of the red cells. Check the slide under the microscope on low power and without the use of a coverslip to observe agglutination. Record in the chart below (+) for positive results and (-) for negative results.
5. Repeat this procedure for all the blood sources. Be sure to use a clean pipet for each red cell source. The pipet used for the antibody can be used for each test, as long as you do not accidentally contaminate the pipet with red cells. Clean toothpicks should be used for each procedure.

Procedure for Hemagglutination and Hemolysis

The extinction dilution method is commonly used to estimate the antibody concentration of an antiserum. In this method a serial dilution procedure is used to produce a range of dilutions of the original antiserum. The antigen source is added to each solution and one determines the greatest dilution that shows a positive response. This dilution is called the end point of the titration and the titer of the antiserum is the reciprocal of the end point. In this study, you will be performing serial dilutions of specific antisera in the rows of a special 96-well plastic agglutination plate. A suspension of red blood cells is then added to each well and after several hours you will determine if hemagglutination occurred. Hemagglutination forms a carpet of cells that covers the bottom of the well; non-agglutinated cells slide down to form a doughnut-shaped “button” of cells at the center of the curved tube bottom. Hemagglutination is scored as +4 if a mat is formed that turns over around the edges. A score of +3 is given for a mat only. A score of 0 is given if a pellet is observed indicating no hemagglutination. Scores of +2 and +3 are intermediate scores where some hemagglutination was observed. See the key for observations Figure 9.1. The last well of each titration serves as a control and contains the red blood cell suspension with PBS substituting for antiserum. This well should always show a negative response and receive a score of 0. The titer of an antiserum is the reciprocal of the last dilution before a well showing a negative response (a score of 0). So, if the end point of a particular antiserum titration is 1:1280, the titer of that antiserum is 1280. Sera with greater antibody concentration will show hemagglutination at a greater dilution and have a greater titer than sera with lower antibody concentration.

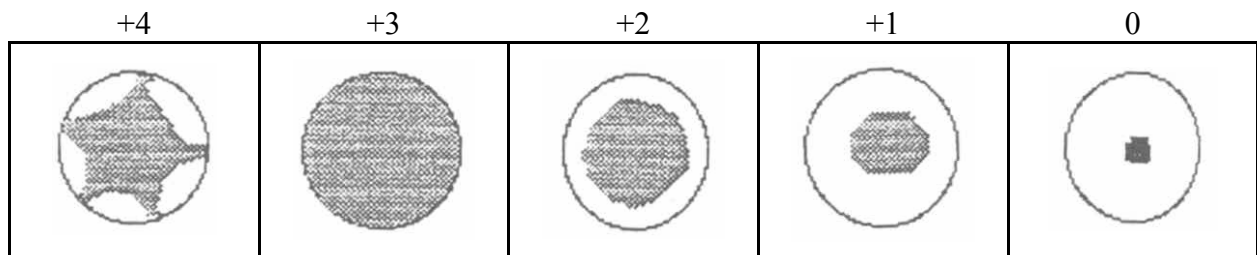
The titer of the antisera of once-injected and thrice-injected rabbits will be determined to compare the difference between the primary and secondary immune responses. You will also be exposing suspensions of red blood cells of goat, dog, and rabbit to thrice-injected rabbit antisera in order to determine if any RBC membrane antigens of red blood cells from these species will cross-react with anti-SRBC antibodies. Finally, a mixture of complement proteins from a guinea pig will be added to some reactions so that you can compare the sensitivity of hemagglutination and complement-induced hemolysis for determining the titer of an antiserum.

Refer to Figure 9.1 for setting up the hemagglutination plate and for scoring your results.

1. Obtain one 96-well plate and label it with a permanent marker according to that shown in Figure 9.1.
2. You will be using wells 1 through 10 of rows A through G in this study. To help you focus better on this part of the plate, remove the cover and use the marker to draw a border around this group of wells.

3. Add 100 μl (2 drops) of phosphate buffered saline (PBS) to wells 2 through 10 of rows A through G. Proceed one row at a time. When you are finished, hold the plate up to a light to ascertain that all wells except well 1 wells have liquid in them.
4. ROW A. Use a new pipet to add 200 μl (4 drops) of 1:40 once-injected rabbit antiserum (1') to well 1 of row A. Follow the procedure for performing serial dilutions shown below to dilute and distribute the antiserum in well 1 to wells 2 through 9.
5. The following serial dilution procedure is best done by two people working together. One person performs the pipetting operation, while the second person keeps place by pointing to the target well with a pencil. This approach will minimize the danger that wells are missed or treated twice.
 - (a) Using the original pipet you used to set up well 1, suck up the contents of well 1.
 - (b) Add 100 μl (2 drops) into well 2. Return the remainder to well 1. Very gently mix the contents of well 2 by stirring the contents with the end of the pipet – *avoid making bubbles!*
 - (c) Repeat this procedure for wells 3 through 9. Discard the 2 drops removed from well 9.
Note: Well 10 should not receive any antisera. This is your negative control for the row.
6. ROWS B through G. Use a new pipet to add 200 μl (4 drops) of 1:40 thrice-injected rabbit antiserum (3') to well 1 of row B. Use the same pipet to repeat this procedure for well 1 of rows C through G. Follow the procedure for performing serial dilutions shown below to dilute and distribute the antiserum in well 1 to wells 2 through 9 for each row B through G.
7. ROWS C and E. Use a new pipet to add 50 μl (1 drop) of complement proteins (Cp) to wells 1 through 10 of rows C and E. Mix the contents of each well by gently shaking the agglutination plate.
8. ROWS A, B, and C. As you perform the following operation, periodically gently tap the container with the 0.3% sheep RBCs (S) to mix the suspension of sheep red cells. Use a new pipet to add 100 μl (2 drops) of the RBC suspension to wells 1 through 10 of rows A, B, and C. Mix the contents of each well by gently shaking the agglutination plate.
9. ROWS D and E. As you perform the following operation, periodically gently tap the container with the 0.3% goat RBCs (G) to mix the suspension of cells. Use a new pipet to add 100 μl (2 drops) of the RBC suspension to wells 1 through 10 of rows D and E. Mix the contents of each well by gently shaking the agglutination plate.
10. ROW F. As you perform the following operation, periodically gently tap the container with the 0.3% dog RBCs (D) to mix the suspension of cells. Use a new pipet to add 100 μl (2 drops) of the RBC suspension to wells 1 through 10 of row F. Mix the contents of each well by gently shaking the agglutination plate.
11. ROW G. As you perform the following operation, periodically gently tap the container with the 0.3% rabbit RBCs (R) to mix the suspension of cells. Use a new pipet to add 100 μl (2 drops) of the RBC suspension to wells 1 through 10 of row G. Mix the contents of each well by gently shaking the agglutination plate.
12. When your plate has been properly set up, place the cover on it and store it in the designated area. Hemagglutination requires at least 2 hours; alternatively the plate may be refrigerated for the following week.
13. Discard the pasteur pipets in the “used pipet” container.

14. Discard all plastic containers into the trash.



	1	2	3	4	5	6	7	8	9	10	
A											
B											
C											
D											
E											
F											
G											

Figure 9.1. (Top) Key for scoring hemagglutination observations. (Bottom) Chart for scoring your results.

Interpretation of Results

1. The slide agglutination test is scored as a simple plus (+) or negative (-) based on the separation of red cells from the surrounding plasma.
2. The hemagglutination plate is scored using the following steps:
 - (a) Obtain your agglutination plate and remove the cover.
 - (b) Score the 10 wells of each row using the criteria shown in the key for observations in Figure 9.1. *Note:* Well 10 in each row should show no hemagglutination and serves as your standard for a score of 0. Enter the scores in the appropriate wells in the corresponding spaces in Figure 9.1.
 - (c) For each row, the end point of the titration is the highest dilution producing a positive score. So, if your first score of zero was observed in well 7, the dilution in well 6 is the end point. Enter the hemagglutination end points of the titrations for each row.
 - (d) The titer of an antiserum is the reciprocal of the end point. Record the hemagglutination titers.
 - (e) Complement-induced hemolysis may be observed in rows C and E. If the plasma has a pink tint, hemolysis occurred; if the plasma is clear, it did not occur. For each well in rows C and E, enter in Figure 9.1 an “H” if hemolysis was observed and an “N” if no hemolysis was observed. Was hemolysis observed in any other rows?
 - (f) The hemolytic end-point of the titration is the highest dilution producing observable hemolysis. Determine the hemolytic end-point for rows C and E. Record the hemolytic end points and hemolytic titers.

Questions for Further Discussion

1. Compare the hemagglutination titers of the antiserum from the once-injected rabbit (from row A) with the antiserum from the thrice-injected rabbit (from row B). Which antisera has the greatest anti-SRBC antibody concentration?
2. Now examine the results you observed for rows B and C to compare the hemolytic and hemagglutination titers of the antiserum from the thrice-injected rabbit. Does complement-induced hemolysis or hemagglutination seem to be more sensitive for detecting an antibody? Recall that the complement system can be activated by as few as a pair of antigen-bound antibodies whereas hemagglutination requires cross-linking by antibodies between the antigens on different RBCs.
3. Compare the titers of sera of the thrice-injected rabbit with anti-SRBC antibodies when mixed with sheep (row B), goat (row D), dog (row F), and rabbit (row G) RBCs. Do the results indicate any cross-reaction of anti-SRBC antibodies with RBC antigens in these mammals? Cross-reaction suggests a similarity in antigen structure. If you observed any cross-reaction to goat, dog, or rabbit RBCs compare the titer of the cross-reaction to the titer estimated in row B. Do your results make sense in terms of the known phylogenetic relationships between the sheep, goat, dog, and rabbit?
4. Consider the results you observed in row G. Would you expect the rabbit to have antibodies against any of its own RBC proteins? Review what you know about the mechanisms that the body uses to distinguish self from non-self.

5. Do your estimates of the hemolytic titer and hemagglutination titer of the thrice-injected rabbit with anti-SRBC antibodies when mixed with goat RBCs and complement (row E) agree with your earlier conclusions for sheep (row C)?
6. What purposes were served by the final wells (10) in each row containing everything but the antiserum?

Acknowledgements

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APPENDIX A

Background Information

Introduction

The study of immunology encompasses many other concepts in modern biology. All organisms have developed a mechanism to aid in their protection against foreign intruders. To this end, it is important to realize that all inhabitants of the earth live in the continued presence of microbes, many of which are parasitic. A major determinant of survival for any species relates to its ability to live in harmony with other microorganisms; infectious or parasitic microorganisms may be viewed as the ultimate predator and the immune system as one of the major naturally selected adaptations.

We think of the infectious (viral, bacterial, rickettsial, fungal, protozoal) agents in the context of disease in humans. When asked to name an infectious bacterial agent, we remember tuberculosis (*Mycobacterium tuberculosis*) or syphilis (*Treponema palladium*). Protozoal agents first remind us of malaria (*Plasmodium* spp.) or Toxoplasmosis (*Toxoplasma gondii*). However, it is important to understand that all living organisms are parasitized by infectious agents. Dogs suffer from Parvovirus and rabies, cows suffer from tuberculosis, frogs suffer from herpes viruses, Gypsy moths die from Nucleopolyhedrosis virus (NPV) infection and phage viruses attack and kill bacteria. It is easy, therefore, to comprehend the significance of host defenses on species survival.

Complexity of Host Defenses

Broadly speaking, host defenses may be divided into innate and adaptive immune defenses. All organisms possess some form of innate protection whether it be the integrity of the cell wall in bacteria or the skin in higher organisms. Innate defenses are non-specific because they do not protect against a specific microorganism. They include mucous membranes, mucus secretions, cilia or epithelial cells of the mucous membranes such as those found in the respiratory system, and the non-specific inflammatory processes that include phagocytosis, release of histamine, and the activation of the blood complement protein that aids in phagocytosis.

Primitive protective phagocytic cells first appeared among sponges and coelenterates and have evolved into two separate pathways. One involved arthropods, annelids, and mollusks and the other involved the tunicates and echinoderms. It was the latter pathway that continued to evolve into the immune system of the vertebrates. In higher animals, namely mammals, reptiles, birds, and fish, these innate mechanisms interact with adaptive immune mechanisms to achieve a more selective and powerful attack by the host.

The adaptive immune mechanisms evolved later in the animal kingdom with clear evidence for their presence first appearing in echinoderms and mollusks and later becoming increasingly complex and precise in vertebrates. All vertebrates have evolved a system of interacting cells and molecular substances that operate in a coordinated fashion to deliver an attack against foreign invaders of exquisite specificity. Two hallmarks of adaptive immune mechanisms are specificity and memory. Unlike innate mechanisms which mount a similar attack against a parasite on each subsequent intrusion, adaptive immune mechanisms amplify and remember each subsequent encounter with a specific parasite. The concept of amplification and memory were taken under consideration in man's strategy for using vaccinations to protect against specific infectious diseases.

Coordinated Action of Host Defenses

In vertebrates, where immune mechanisms are the most complex, innate and adaptive defenses against foreign antigens operate in a coordinated fashion to deliver the maximum effective result. Barriers to parasitic invasions such as the skin and mucous membranes are a primary defense that constantly protect us throughout our lives. Most microorganisms cannot penetrate these barriers when they are intact. When disrupted, a pathway is available for bacteria to follow into the tissues of the body. Inflammatory cells are a second arm of the innate defenses. These cells (macrophages, neutrophils) recognize bacteria as foreign agents and attempt to phagocytize and digest them. These non-specific defenses are usually successful in eliminating most foreign invaders.

If bacteria survive the non-specific attack and begin to replicate in the body, they are recognized by cells of the adaptive immune system. These cells may be found in the skin, circulating in the blood or lymph, or stationary in the lymph nodes and spleen.

Antigens, peptide and carbohydrate fragments of the parasite which stimulate immunity, elicit the production of antibodies and the proliferation of lymphocytes both of which may attack the specific bacterium. Antibodies, circulating glycoproteins synthesized by B-lymphocytes, can augment phagocytosis by macrophages and neutrophils, can activate blood complement for cell lysis, or can attach to other cells such as monocytes and mast cells to give specificity to their non-specific killing mechanisms.

Many cells and substances participate in immune responsiveness. Among the cellular elements, lymphocytes play a central role. Roughly speaking, two classes of lymphocytes are found and they are named after the organ in which they mature. Those that mature in the bone marrow of mammals or the bursa of Fabricius in birds are called B-cells and those which mature in the thymus are named T-cells. In addition to killing parasites by themselves, some T-cells also regulate the activity of the B-cells.

The function of lymphocytes is governed by other substances of the body. Hormones such as insulin, thyroxine, growth hormone, cortisol, prolactin, and endorphin all play a role in immunoregulation. Early evidence demonstrated that when humans and other mammals were under severe stress, their immunity to infectious agents was lowered. Cortisol, a stress activated hormone derived from the adrenal gland, was shown to be elevated in these animals and to have a suppressive activity on T-cells. In some species such as guinea pigs and humans, high levels of cortisol actually kill T-cells in the thymus gland. Later, it was found that some animals with disturbances in growth, such as dwarfs, had deficient levels of growth hormone and an immature immune system. Thyroxine and insulin regulate the metabolism of lymphocytes. The brain hormones prolactin and endorphin have a profound effect on enhancing or suppressing the activity of cells in the immune system. Interleukins are hormones produced by lymphocytes and monocytes which bind to specific cellular receptors on other inflammatory and immune cells (B-cells, T-cells, mast cells) to produce a transient response.

The Interleukins

We currently recognize 12 different lymphokines which are synthesized by and act upon cells of the immune system. They have been given consecutive numbers, such as IL-1, IL-2, IL-3, etc., and vary in activity and in the target cell, upon which they exert their effects (see Table 9.1). Another cytokine, tumor necrosis factor (TNF), shares many functional properties with the interleukins. Interleukin-1 and TNF are structurally different glycoproteins which are synthesized by the same cells and behave in a similar fashion. Cells that synthesize these molecules are macrophages and endothelial cells. Following bacterial invasion, macrophages recognize the intruders, become activated, and secrete IL-1 and TNF. These hormones alter the lining of blood vessels, activate the T-cells, and circulate to the brain. In the brain, IL-1 and TNF promote the synthesis of a lipid called prostaglandin E_2 which alters our internal regulation of body temperature. Because the brain now feels that 102°F is normal, it suppresses mechanisms which allow heat to escape from the body, such as sweating, and stimulates the generation of heat through shivering until the new temperature is reached. We refer to the 102°F body temperature as fever and most of us are uncomfortable in this state. Fever is, however, one of our most primitive defenses against foreign agents. Since many bacteria and viruses have a restrictive temperature range in which they multiply, raising the body temperature by 4–5°F retards bacterial growth and may even kill some of them. Syphilis is one of the agents which is sensitive to elevated body temperature. Since prolonged fever may not always be useful, we have learned to control it with the administration of aspirin and similar products that work through the inhibition of prostaglandin E_2 synthesis.

The effect of IL-1 on blood vessels is also extremely important since it causes the blood vessel lining cells (endothelium) to express new proteins on their surfaces which are recognized by circulating immune cells. Activated phagocytes and lymphocytes can then attach and penetrate the vessel and localize in the tissues. This phenomenon is called *homing* and allows cells responsible for immunity and inflammation to migrate precisely to sites where foreign agents have penetrated the body. The penetration of the blood vessel by lymphocytes is accomplished without disruption to the vessel wall because the cells actually squeeze between adjacent endothelial cells. Once in the tissues, inflammatory cells actively migrate to the bacteria by following a gradient of increasing concentrations of chemicals called chemotaxins. Chemotaxins are

substances produced by the macrophages, which are the first cells in contact with the bacteria, and the activated components of the complement system.

IL-1 and TNF also activate lymphocytes to express new cytokine receptors and to secrete the cytokines necessary for their future development. In addition, they are known to cause cells to express histocompatibility antigens necessary for proper cell to cell interaction and discrimination between self and non-self.

Table 9.1. List of known interleukins and their functions.

Interleukin (cytokine)	Source	Target cell	Effect
IL-1	Macrophage, lymphocytes, endothelium, fibroblasts, astrocytes	T-cells, B-cells, macrophage, endothelium, tissue cells	Lymphocyte activation, leukocyte-endothelial adhesion, fever, regulates sleep
IL-2	T-cells	T-cells	T-cell growth factor
IL-3	T-cells	Bone marrow cells	Stimulates bone marrow growth
IL-4	T-cells	B- and T-cells	B-cell growth factor
IL-5	T-cells	B-cells	B-cell growth factor
IL-6	T- and B-cells, macrophages, fibroblasts	B-cells and hepatocytes	B-cell differentiation and synthesis of acute phase reactants
IL-7	Lymphocytes	B- and T-cells	Stimulates proliferation of immature cells
IL-8	T-cells, macrophages	Granulocytes, endothelium	Stimulates the activity of neutrophils, acts as chemotaxin, inhibitor of endothelial cell-leukocyte adhesion
IL-9	T-cells	T-cell	T-cell and mast cell growth enhancement
IL-10	T-cells	Macrophage	Suppresses the development of T-cell subpopulations (TH ₁) by inhibition of macrophage IL-12 production
IL-11	Bone marrow stromal cells	Hepatocyte	Induces synthesis of acute phase proteins
IL-12	Macrophage	T-cells	Enhances the B-cells expression of IFN- γ during T-cell activation; also stimulates a lymphocyte subpopulation (NK cells)

Perpetuation of the Species

Perhaps the two greatest forces driving the perpetuation of any species are reproductive efficiency and host defense mechanisms. How many offspring/replicates an organism can successfully leave on the earth before it dies certainly has a profound impact on the ability of its lineage to exist over many generations. Perhaps equally important, however, is how well the organism combats the myriad of predators, particularly microorganisms, that it encounters throughout its life. There are two important considerations relevant to host defenses: (a) can the defenses of an organism keep it alive long enough to reproduce, and (b) can the defenses extend the survival of the individuals to allow multiple replications. For the immune system, addressing these considerations requires different strategies.

The survival of newborns makes the greatest impact on the average life expectancy of most species. How well the offspring of a species combats microorganisms shortly after birth will determine, to a great extent, whether it will become an adult. Hundreds of genes are necessary for proper immune function in mammals. Over 30 mutations in these genes are known to occur in mice and many more have been found in humans. These mutations can result in immunodeficiencies. In many instances, the immunodeficient offspring cannot survive more than a few weeks. The immune system of vertebrates is not completely developed at birth even in healthy infants with no genetic mutations. In order for the infant to survive long enough for its immunity to become established, the mother must transfer her immunity to the offspring. In egg laying vertebrates, the antibodies of the mother are transferred through the yolk. In placental mammals, maternal antibodies are either transferred across the placenta, as in rodents and primates, or through the mother's milk, as in cattle, swine, dogs, cats, and horses. Special antibodies have evolved which can cross the placenta or penetrate the newborn's intestine. The ability to passively transfer immunity from mother to offspring dramatically improves neonatal survival.

Once the infant establishes its own immunity, which takes several weeks for dogs, cats, and rodents and several months for primates, survival will depend on the ability of the immune system to respond with specificity to invaders. During the first exposure to an antigen the immune system mounts a weak response, but commits antigen-specific T- and B-cells such that upon second exposure to the same antigen an augmented immune response, known as the anamnestic response, occurs. Higher animals have developed an extremely complicated mechanism to achieve these functions and in doing so are better prepared to combat infection and to increase longevity and thereby the number of reproductive cycles.

As previously mentioned, there is an evolutionary hierarchy in host defense mechanisms. Primitive animals may depend entirely on innate mechanisms for protection and survival, while primates have developed many different specific immunologic mechanisms, some redundant, which help ensure better protection and survival. A variety of immunocyte types are found in invertebrates, including leukocytes and coelomocytes found in mollusks, annelids, and arthropods. There are early evolutionary trends of phagocytes to aggregate in tissues. Cell-mediated immunity, as exhibited by cytotoxic reactions responsible for the rejection of foreign organ transplants, has been demonstrated in sponges, coelenterates, annelids, and echinoderms. In the horseshoe crab, hemolymphatic factors have been found that are similar to the vertebrate complement system.

Among vertebrates, there seems to be basic cellular and molecular components of immunity throughout phylogeny, with increased specialization of tissues and organization as one progresses up the phylogenetic tree. The functional equivalents of B- and T-cells, memory mechanisms, and immunoglobulins are found in all vertebrates. Complement proteins have been demonstrated in all vertebrates. Lymph nodes with superficial resemblance to the nodes of warm-blooded animals are first found in amphibians. Gut-associated lymphoid tissue is found in the intestine of frogs.

One should keep in mind, however, that in all organisms the integrity of host defenses are contingent on proper nutrition. Much of the increased longevity of humans during the last 200 years, which increased the life expectancy from 40 to 77 in the U.S., has been due to correcting malnutrition thus allowing the immune system to operate optimally. Two other major contributions to human survival were the discovery of antibiotics and vaccinations.

Immunogenetics

In 1946, Sir Peter Medawar observed that freemartin cattle would not reject each others skin when reciprocal skin transplantation was performed. Freemartin calves are male and female twins which share the circulation of the placenta. As a result, both twins received each others blood during gestation. The antigens present in the blood were present in each calf while its immune system was developing and as a result both types of blood were considered “self” to the immune system. Although the blood of one twin contained cellular antigens which would normally stimulate immunity and graft rejection in the other calf, the immune system in this instance was incapable of discriminating between proteins responsible for graft rejection (histocompatibility antigens). Medawar coined the term *immunologic tolerance* to explain this phenomenon.

In an attempt to explain tolerance to antigens responsible for tissue graft rejection, a large complex series of genes were discovered. The segment of chromosome containing these genes was called the major histocompatibility complex (MHC) and the protein products of these genes fall into three classes. Class 1 proteins, frequently called class 1 antigens, are found on all nucleated cells and on macrophages these molecules are responsible for presenting foreign antigens to receptors found on lymphocytes. Viruses are recognized by lymphocytes in conjunction with class 1 proteins on the cell surface. Class 2 antigens on the macrophage and dendritic cell surface present other foreign substances, such as proteins from bacteria, to the cells of the immune system. The class 1 and 2 proteins present on grafted tissue that differ from those on the host tissues will elicit immunologic attack, destruction, and eventual rejection of the graft. We can identify various class 1 and 2 antigens on tissues by using a test called tissue typing. Grafts from donors which share identical class 1 and 2 molecules with the recipient will not be rejected, but such “matches” are uncommon because of the large number of different class 1 and 2 proteins. Grafts which only differ by a few proteins may survive rejection if immunosuppressive drugs are given initially during surgery. It may be possible to discontinue such therapy after years of treatment because the immune system tends to become tolerant to the foreign proteins over time. Cyclosporin-A has been a very effective drug to prevent graft rejection because it specifically inhibits T-cell function. For obvious reasons, persons taking immunosuppressant drugs are vulnerable to pathogens and often must take large doses of antibiotics.

A third class of proteins derived from the MHC are complement proteins. These proteins are also involved in the immune system and assist in the lysis of cells bound by antibodies. The complement system consists of nine serum proteins that react with antibody-antigen complexes and are involved in a series of proteolytic reactions that result in destruction of cell membranes of the cells bound by antibody. The genes encoding several of these proteins are found in the MHC.

T- and B-cells recognize foreign substances via receptors on their surface which specifically bind to non-self antigens. The receptors for T-cells are structurally different from those found on B-cells, yet they share certain characteristics. Because certain similarities exist between the structure of the immunoglobulin (antibody) molecule, the T-cell receptor, class 1 and 2 proteins, homing receptors, and differentiation proteins on lymphocytes, genes encoding these proteins are said to belong to the immunoglobulin-superfamily of genes.

The genes encoding immunoglobulin molecules are located on two separate chromosomes. Likewise, immunoglobulin genes and T-cell receptor genes are located on separate chromosomes. In the mouse, and certainly also in humans, every chromosome has regions dedicated to genes regulating the immune system. Some chromosomes contain fairly large complex segments of genes dedicated to immunity. When one adds all the genetic material responsible for generating the hundreds of proteins integral to the immune system, the amount of DNA dedicated to this one system is enormous. However, given the advantage in survival conferred by this host defense system, the allocation of DNA seems appropriate.

Antibody Production

B-cells contain receptors which are capable of recognizing at least a million different peptide configurations. If every receptor were encoded by a separate gene, at least two entire chromosomes would be needed for those receptors alone. Recently, the mechanism for generating all the receptor-antibody diversity has been discovered. The B-cell receptor is biochemically the same as the antibody that the B-cell will synthesize after activation. It differs only in a transmembrane peptide which holds it in place in the cell

membrane of the B-cell. Activation of the B-cell takes place when the receptor binds to the foreign antigen. The B-cell then proliferates and differentiates into antibody producing cells.

Antibodies (immunoglobulins) are four-chained molecules held together by disulfide bonds. Two chains are approximately twice the size of the other two chains and are referred to as the heavy chains, while the other chains are referred to as the light chains. Antibody molecules have portions of each chain which do not bind antigen and are relatively constant in amino acid sequence and are called the constant regions. The other end of the molecule is called the variable region and includes the regions which bind foreign antigen. Each antibody has at least two antigen binding sites per molecule, which allows the antibody to cross-link multiple antigenic sites. This is especially important to the process of erythrocyte agglutination by antibodies.

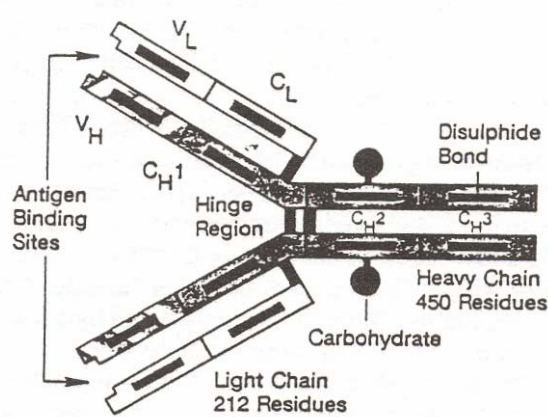


Figure 9.2. The basic structure of IgG.

Figure 9.2 illustrates the basic structure of IgG. The amino-terminal end is characterized by sequence variability (V) in both the heavy (H) and the light (L) chains, which are referred to as the V_H and the V_L regions, respectively. The rest of the molecule has a relatively constant (C) structure. The constant portion of the light chain is termed the C_L region. The constant portion of the heavy chain is further divided into three structurally discrete regions: C_{H1} , C_{H2} , and C_{H3} . These globular regions, which are stabilized by intrachain disulfide bonds, are referred to as domains. The sites at which the antibody binds antigen are located in the variable domains. The hinge region is a vaguely defined segment of heavy chain between the C_{H1} and C_{H2} domains. Flexibility in this area permits variation in the distance between the two antigen-binding sites, allowing them to operate independently. Carbohydrate moieties are attached to the C_{H2} domains (National Research Council, 1989).

The genes for heavy and light chains are quite complex and involve multiple gene segments which rearrange on the chromosomes somewhat randomly to produce many different combinations. Gene segments contain multiple variable genes (V), diversity genes (D), and joining genes (J) which recombine into a single VDJ region gene complex. This rearrangement of DNA constitutes the variable portion of the light or heavy chain, both of which require this gene rearrangement. The rearranged VDJ gene complex is transcribed together with a single constant region to produce messenger RNA for the specific B-cell receptor and antibody (Bankert and Mazzaferro, 1989).

The number of B-cells having a receptor for a specific foreign protein is very small before the initial binding with the foreign antigen. Following a successful binding interaction which is a matter of chance encounters, the B-cell proliferates and replicates itself. This "clone" of B-cells thus amplifies the immune response to the specific foreign antigen. Some B-cells differentiate into antibody secreting cells while others remain undifferentiated, but are capable of binding antigen and of replication. The undifferentiated B-cells are referred to as memory cells which will be responsible for the high titer (amplified) antibody produced upon second exposure to the antigen. T-cells are responsible for B-cell differentiation.

The T-Cell System

The stem cell progenitor of all lymphocytes is found in the bone marrow of mammals. However, cells destined to be T-cells must migrate to the thymus gland to develop in the presence of the class 1 and 2 MHC antigens. It is during this critical time that the T-cell acquires its antigen receptor which can differentiate self from non-self proteins. The process for production of the two chained T-cell receptor molecule (TCR) is similar to the mechanism used for antibody production by the B-cell. The product of the genes for variable, diversity, joining and constant regions show very little homology, however, with the antibody molecule. As the TCR molecules are formed, they interact with class 1 and 2 proteins found on the thymus cells. Those TCRs which bind tightly to the class 1 and 2 proteins expressed on the thymus cells are eliminated before entering the blood stream. This is a time for “educating” the T-cells and preventing those with receptors which bind self proteins from entering the circulation.

T-cells leaving the thymus have acquired a CD4 or CD8 surface glycoprotein. These glycoproteins are important recognition elements allowing the T-cells to adhere to other cells. They also functionally identify the T-cell. Those T-cells which carry the CD8 glycoprotein directly bind and kill viruses, some tumors, and reject incompatible grafts. Those T-cells carrying the CD4 glycoprotein synthesize lymphokines necessary for T-cell and B-cell differentiation. This CD4⁺ population thus controls the number of B-cells which will differentiate to secrete antibody. They are referred to as helper T-cells. The absence of CD4⁺ lymphocytes would greatly compromise the ability of the immune system to protect us against bacterial, protozoal, fungal, and to some extent viral diseases. It is no surprise then that if a virus could bind to the CD4 glycoprotein, invade and kill that cell, that immunodeficiencies may result. This is exactly how Human Immunodeficient Virus (HIV) causes Acquired Immunodeficiency Syndrome (AIDS).

The CD4⁺ T-cells elaborate many different interleukins necessary for proper immunologic functions. While some are primarily involved in B-cell differentiation (IL-4, IL-5, and IL-6), others effect the proliferation of CD8⁺ T-cells (IL-2). Since T-cells cannot react with self antigen due to thymic education, they will not recognize “self” as foreign. B-cells, due to their “random” acquisition of receptors may react against self antigens; however, without T-cell help they cannot differentiate to produce antibodies. This mechanism protects us against autoimmunity. Occasionally a foreign antigen can stimulate B-cells to differentiate without the necessity for T-cells. Although rare, this can lead to the production of autoantibodies. If autoantibodies bind normal tissues and activate complement, destruction of the tissue takes place. Diseases known to occur in this manner include lupus erythematosus, rheumatoid arthritis, and some forms of thyroid diseases. Certain hereditary abnormalities causing alterations in T- and B-cell function can also lead to these autoimmune disorders.

The antibodies secreted by B-cells have one of five possible constant regions. The constant region determines whether the antibody can cross into the intestinal tract, can cross the placenta, can bind to the surface of neutrophils or mast cells, or can activate complement proteins. It is therefore extremely important that the “class” of antibody be appropriate for the task it must fulfil. Interleukins are known to specifically control the class of antibody a B-cell will synthesize.

Immunoglobulins of the IgE class are controlled by Interleukin 4. They bind to the surface of mast cells in the mucous membranes. Mast cells contain the chemical histamine, which, when released, causes bronchial smooth muscle to constrict resulting in difficult breathing. When antigens specific to IgE on mast cells bind to the antibody, the mast cells secrete (degranulate) vacuoles filled with the histamine. The antigens of worms in our intestines may stimulate such a reaction which results in smooth muscle constriction in the intestine and expulsion of the worm from the gut. Unfortunately for many people, inhaled pollen may also activate mast cells that are found in the nose and lungs, which causes nasal discharge, shortness of breath, and tightness in the chest. These are the allergic symptoms of asthma. Antihistamines can counteract these unwanted side effects and are the drug of choice.

IgM and some types of IgG are classes of antibodies which activate complement. B-cells synthesize these antibodies under the influence of IL-2. The beneficial effects of complement include disruption of the bacterial cell wall leading to leaking and death, attraction of inflammatory cells to sites of infection, and enhanced phagocytosis of foreign substances by macrophages. Coupling of complement activation with antibody-antigen binding gives specificity to an otherwise non-specific host defense mechanism.

In 1894, Jules Bordet of the Pasteur Institute in France, demonstrated two components found in the serum of guinea pigs recovering from cholera infection. One component was inactivated by heating serum at

56°C for 30 minutes and the other was not. The “immune serum” which was heat inactivated could not protect guinea pigs alone. However, fresh non-immune serum replenished the component lost by the heat inactivation and allowed the immune serum to become bacteriolytic once again. We now know that the heat stable component in the serum was antibody and the heat sensitive component was protein complement. Dr. Bordet was awarded the Nobel Prize in Medicine and Physiology in 1919. For further reading see Abbas et al. (1991).

The Complement System

The complement system is comprised of numerous proteins which exist as proenzymes in the blood. During enzymatic cleavage, a complement proenzyme becomes converted to an active enzyme capable of modifying another complement protein. Activation of complement may take place when antibody binds to the antigen, thereby opening a complement binding site on the antibody molecule. Binding of the first component of the complement cascade leads to its activation to an enzyme capable of activating the next component. This is termed the classical pathway of complement activation. Another more primitive pathway involves the direct activation of the third component of complement by bacteria themselves without the necessity of any antibody (Quimby and Dillingham, 1989).

The classical pathway (Figure 9.3) is composed of nine components which are activated in the following sequence: C1, C4, C2, C3, C4, C5, C6, C7, C8, and C9. During activation, fragments of the proenzymes are cleaved off the molecule. Many of these fragments also have biological activity. When C3 is activated the proenzyme C3 is changed to the active C5 convertase, C3b. A small peptide fragment, C3a, is cleaved off from the C3 molecule and it acts as a mediator of inflammation by inducing smooth muscle contraction, changing the permeability of blood vessels and causing mast cells to degranulate. Additional fragments cleaved from C4 and C5 also have biological activity.

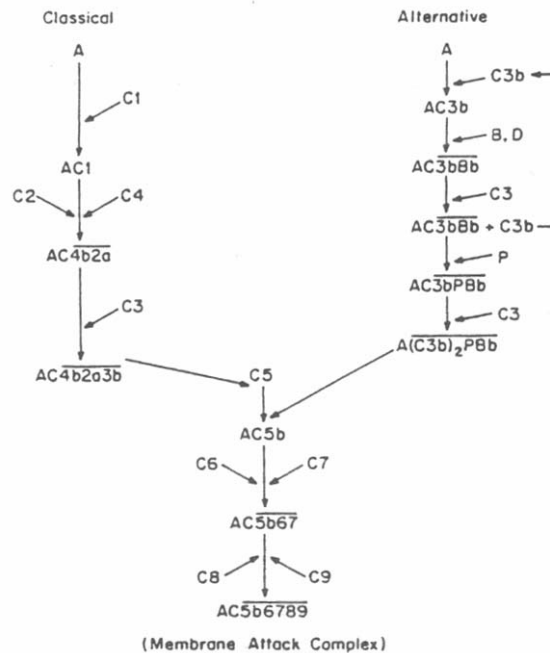


Figure 9.3. The complement pathway.

The hallmark of complement activation is the production of the membrane attack complex (MAC). This is a macromolecular complex composed of the active forms of each molecule from C5 to C9. Once this macromolecule forms on biological membranes, it produces a transmembrane channel which exposes the

cytoplasm to the outside environment. If this occurs on a red blood cell, the cell ruptures and releases its cytoplasmic hemoglobin. The process is called hemolysis and results in jaundice or yellowing of mucous membranes. This condition is most easily seen in the sclera of the eyes. Hemolysis is seen when antibodies attack red cells due to an incompatibility of blood groups during transfusions of blood. The recipient must be compatible with the donor's blood or the recipient's immune system will recognize the introduced cells as foreign and mount an immune attack against them. A repeated transfusion with the same incompatibility stimulates more antibody in the recipient which eventually will bind to the transfused red cells and activate complement. Hemolysis will result.

Antibodies in the ABO system are produced naturally without immunization and are normally found in persons with A, B, or O blood types. This is why it is particularly important to match blood types prior to any transfusions. Transfusions of incompatible blood into a recipient will induce an immediate transfusion reaction and the production of more IgM antibodies that can cause red cell agglutination. Once antibody has bound to the red cells, complement proteins can be activated and intravascular hemolysis will occur, resulting in anemia and jaundice.

A similar condition may occur in a newborn human if the mother's red cells are missing the Rhesus factor and the father's red cells contain the Rhesus factor which is termed Rh⁺. Genetically, the child could be Rh⁺ if it inherits that genotype from the father. Blood from the child mixes with the maternal circulation at or before birth and the mother will produce antibodies against the Rh⁺ red cells of the infant. These cells will circulate in the mother. During the second pregnancy, the mother's IgG antibodies cross the placenta and enter the fetal circulation, subsequently attacking the fetal Rh⁺ red cells. When this happens, the newborn displays jaundice at birth and will require a total body exchange blood transfusion to survive. Many Rh⁺ fetuses exposed to maternal anti-Rh antibodies die in utero. This condition, called hemolytic disease of the newborn or erythroblastosis fetalis, is now prevented by typing spouses during the mother's first gestation. When incompatibility is found, the mother is warned of the potential problem. During the first pregnancy, the mother is given antibodies against the Rh factor. This antibody quickly binds to any Rh⁺ red cells released by the infant during birth and destroys the red cells before the mother's own immune system is stimulated. As a result, hemolytic disease of the newborn is now a rare event in America (Luban, 1993). An interesting side note is that the antigen on human red cells responsible for this disorder is also found in the rhesus monkey, hence its name. When proteins found in lower animals are present in animals phylogenetically more advanced, we refer to the phenomenon as evolutionary conservation. Proteins that impart a strong survival advantage such as those found in the immune system are often highly conserved and are found to be structurally similar between species.

Hemagglutination

Hemagglutination is a phenomenon observed when antibodies bind to the surface of red blood cells which are also called erythrocytes. They bind to the red cell membrane proteins and carbohydrates or they may bind foreign antigens which attach to red cell membranes. Many viruses are known to attach to red cell membranes. If virus-coated cells are incubated with the serum from a patient sensitized to that virus, the patient's antibodies will cause clumping of the introduced red cells. Visualization of this clumping is called hemagglutination (HA) and is used in human and veterinary hospitals to diagnose viral disorders. However, for an antibody to cross-link adjacent red cells sufficiently to demonstrate hemagglutination, the concentration of antibody must be very high. Complement may be activated by two adjacent antibody molecules on a red cell even when the antibody concentration is too low to agglutinate the cells. This makes complement fixation (CF) more sensitive than the HA test. Tests that are more sensitive than either HA or CF include the enzyme linked immunosorbent assay (ELISA which is used also to detect HIV infection) and radioimmunoassay (RIA).

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APPENDIX B
Glossary

Autoimmunity: A condition presumed to be caused by sensitization or loss of tolerance to products produced by the body. A failure of the immune system to discriminate between self and non-self antigens.

Chromosome: Intranuclear elements composed of DNA and protein that carry the hereditary factors (genes) and are present at a constant number in each species.

Class I MHC antigens: Antigens encoded by the major histocompatibility complex (MHC) of genes that are found on all nucleated cells and are composed of two polypeptide chains of 45 and 12 kilodaltons.

Class II MHC antigens: Also known as Ia antigens, they are found on antigen-presenting cells and are composed of two polypeptide chains of 28 and 32 kilodaltons.

Complement: Any one of a group of at least nine factors, designated C1, C2, etc., that occur in the serum of normal animals and enter into various immunologic reactions. Complement is generally activated when antibody binds antigen; activation of complement can lyse erythrocytes, kill or lyse bacteria, enhance phagocytosis and immune adherence, and exert other effects. Complement activity is destroyed by heating the serum at 56°C for 30 minutes. This is done for experiments of hemagglutination when you don't want the cells to lyse.

Cyclosporine A: An immunosuppressive drug commonly used in organ transplantation. It appears to mediate its suppressive effect mainly through CD4+ (helper) T-cells.

Histamine: 4-(2-Aminoethyl)-imidazole (an amine occurring as a decomposition product of histidine that stimulates visceral muscles) dilates capillaries and stimulates salivary, pancreatic, and gastric secretions. It is found in the granules of the basophils and mast cells responsible for anaphylactic reactions (severe allergic reactions).

Immunologic memory: A phenomenon characterized by the presence in the body of an expanded set of clonally derived antigen-specific lymphocytes that can be rapidly recruited to produce an augmented immune response on subsequent exposure to the specific antigen.

Immunoglobulins: Proteins of animal origin with known antibody activity or a protein related by chemical structure and hence antigenic specificity. They can be found in plasma, urine, spinal fluid, and other body tissues and fluids.

Macrophages: A phagocytic cell (that is found in many tissues) that is derived from a blood monocyte, and that has an important role in host-defense mechanisms.

Mast cell: A small cell similar in appearance to a basophil and found associated with mucosal epithelial cells. These cells are dependent on T-cells for proliferation. They contain cytoplasmic granules which are released when antigen binds to membrane-bound IgE; these granules are laden with heparin, histamine, slow reactive substances of anaphylaxis, and eosinophil chemotactic factor of anaphylaxis.

Membrane attack complex. A multimolecular complex formed by the activation of the terminal components of the complement pathway and responsible for cytolysis.

MHC (major histocompatibility complex): A genetic region found in all mammals whose products are primarily responsible for the rapid rejection of grafts between individuals and that function in signaling between lymphocytes and cells expressing antigen.

Phagocytosis: Ingestion of foreign or other particles by certain cells.

PGE 2 (prostaglandin E2): An unsaturated fatty acid 20 carbons in length with an internal cyclopentane ring. It causes vasodilation, inhibits gastric secretion, induces labor and abortion, and is immunosuppressive. A derivative of arachidonic acid.

T-cell or thymic-derived lymphocytes: One of the two major classes of lymphocytes with important immune regulatory and effector functions.

TCR (T-cell antigen receptor): The antigen receptor of T-cells composed of two polypeptide chains and closely associated with the T3 surface membrane molecules.