

DIAGNOSING THE IMMUNE SYSTEM

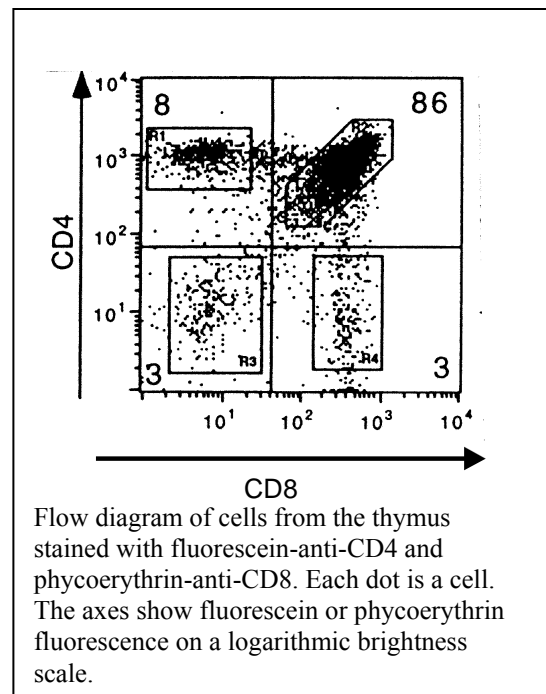
IMMUNODIAGNOSIS. This refers to two things: the diagnosis of immunological diseases, and the use of immunologic techniques in the laboratory for the diagnosis of other conditions. Most of the methods used in a big reference or hospital laboratory (ours for example) are described here. Some more specialized and primarily research methods are considered in the Techniques 2 unit.

Things we'll look at: B cell numbers and function
 T cell numbers and function.
 Detecting and measuring antibodies
 Detecting and measuring antigens

First, let's consider how the lab evaluates lymphocyte numbers. Of course, we start with a CBC—complete blood count: white cell total and differential (percentage of each type), platelets, and red cells. These are nowadays done by smart robots that scan a stained blood smear, identify cells, and then run algorithms that tell them what kind of cell it is and whether it's within normal parameters. If it isn't, the robot flags that cell for a human to review.

B & T CELL NUMBERS. B cells in blood can be measured by counting cells with surface immunoglobulin. Most labs use instead the markers CD19 or CD20 because they are more specific (a macrophage or PMN could have immune complexes on its surface, bound to its Fc receptors, and score as a false positive). A fluorescent molecule is coupled to a monoclonal antibody (mAb) to CD19 or 20, which is then mixed with a blood sample. For T cells we use mAbs to CD3 (total T cells), or to CD4 or CD8. Fluorescent cells can be counted under a microscope that has a UV light source and appropriate filters, or by flow cytometry.

Flow Cytometry. The machines are called flow cytometers because they take cells in suspension and pump them through an orifice so small that the cells emerge in single file in a very fine stream. Lasers illuminate the cells and light emitted or scattered by each cell is collected by photomultipliers connected to a fast computer system. Light scatter gives information about cell size and cytoplasmic granularity, and if the cell has bound a fluorescently-tagged antibody, the fluorescent light emitted is quantified. Monoclonal antibodies to many cell surface molecules are available, and can be bought tagged with different dyes—or even quantum dots—so that they can be used simultaneously (see the diagram). Other dyes can be used, like propidium iodide, which reacts quantitatively with DNA, becoming fluorescent, so that you can tell where a cell is in the cell cycle by its DNA content. By using multiparameter cytometry, you can ask questions like, What percentage of cells in the blood bears the CD34 marker that is seen on hematopoietic stem cells? Are they cycling or resting? and the machine can determine this for you, examining 10,000 individual cells in a second.



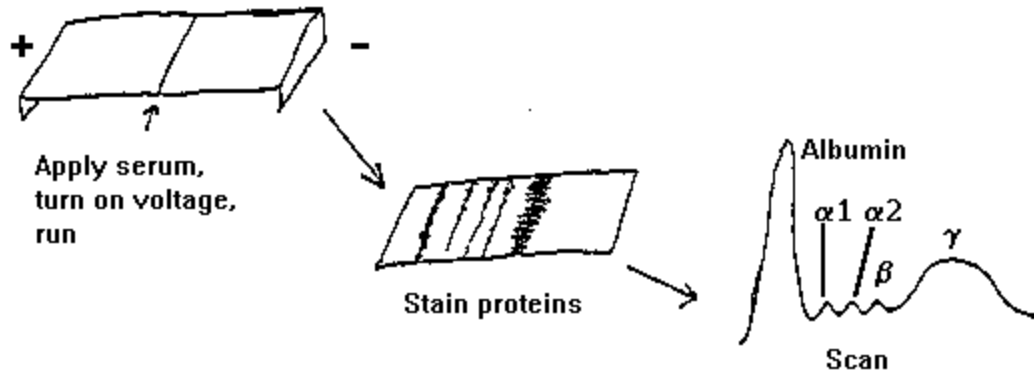
Problem 1: According to the flow diagram, what fraction of the total cells is "double-positive"? What fraction is "double-negative"? What fraction are helper cells? What fraction is CTL?

If cells are fixed and permeabilized, flow cytometry can be used to detect internal antigens, such as cytokines (not yet secreted) or transcription factors; this is how we identify Treg, for example.

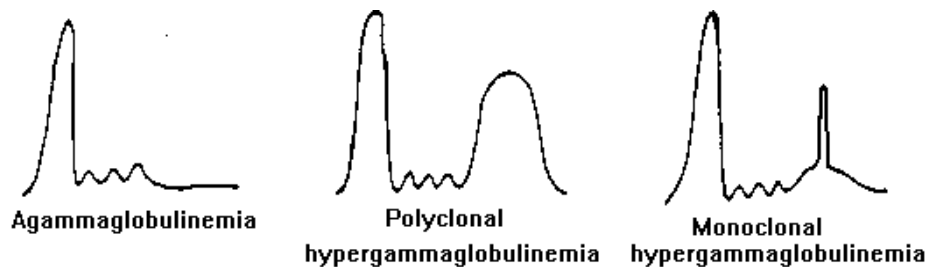
You might want to review the difference between pro-B, pre-B, immature and mature B cells as explained earlier. These are distinguished by fluorescence microscopy using Abs to IgD, IgM, and H or L chains, on both fixed (permeabilized) and intact cells, so you can distinguish whether a molecule is within a cell, or on its surface.

B CELL FUNCTION.

To test the humoral arm of the immune system, we begin with serum protein **electrophoresis**:

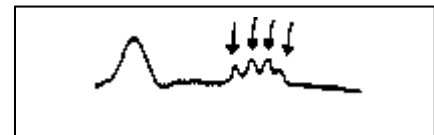


This is cheap (around \$40) and easily quantified, but not very sensitive to small abnormalities. For example, it could not pick up selective IgA deficiency, because IgA runs pretty much together with the much larger IgG (γ globulin) band.



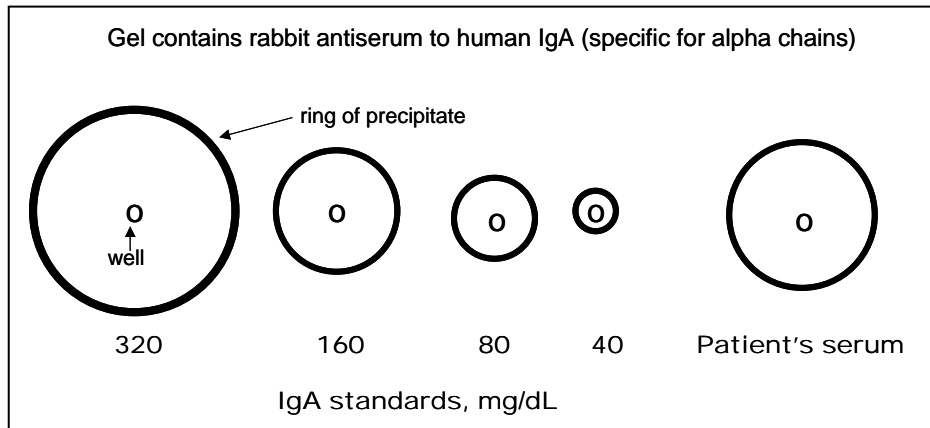
Monoclonal peaks like the one above on the right are seen when there is a vast overproliferation of one clone of activated B cells (plasma cells), as in the malignancy called **multiple myeloma**.¹

Electrophoresis can be done on urine (to look for “Bence Jones protein,” free immunoglobulin light chains seen in patients with multiple myeloma) and on cerebrospinal fluid, where oligoclonal (a few clones) peaks in the IgG region are sometimes seen in multiple sclerosis →



¹ When that diagnosis is suspected, the lab will follow up with a technique to determine the H and L chain types of the abnormal protein using a technique (immunofixation electrophoresis) that, if you’re interested, you can read about in the Supplemental Material file on line.

To measure levels of individual immunoglobulin classes or subclasses we can use **single radial immunodiffusion** (you can review immunodiffusion in gels in the Antibody Function notes):



Problem 2: What's the approximate concentration of IgA in the patient's serum?

Immunodiffusion can be used to measure any other multivalent antigen (one that can form a precipitate with an appropriate antibody), for example the individual complement or clotting components, if you have a specific antiserum. Gels of this type can be purchased ready-to-go. They're fairly cheap, but slow for a big hospital lab, which uses quicker tests that can be automated. For example, the capture ELISA.

T CELL FUNCTION.

1. The **best overall test** (for Th1 activity): skin test with common antigens to which most people will have DTH. A good set is: Candida, streptokinase/streptodornase (SK/SD,) trichophytin, mumps, tetanus, tuberculin. Read at 24-48 hours.

Problem 3: List all the steps that must take place to get a positive test.

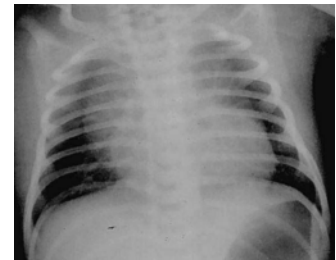
2. Challenge DTH test: over 98% of normals will become "sensitized" (immunized) to DNFB (dinitrofluorobenzene) in about 10 days if it's painted on their skin. This is like intentionally inducing poison ivy.

3. Stimulate T cells in mononuclear leukocyte preparations (lymphocytes + monocytes) with the T cell **mitogens** PHA or Con A, and observe either proliferation or IL-2, IL-4, or IFN γ production. Mitogens are plant (usually) proteins that bind certain sugar sequences; they are probably part of the plant's defense system against things like fungi. Some of these sugar sequences are also found on human cells; ConA and PHA both bind sugars associated with the T cell receptor complex, fooling T cells (all of them!) into thinking they are recognizing antigen. These tests are sometime useful to do because total numbers may be normal while function is impaired.

4. In infants, a chest x-ray may be very useful; is the thymus there?

5. Lymphoid biopsy may be necessary in suspected primary immunodeficiency. A biopsy of rectal mucosa, though it sounds nasty, is often less traumatic to the patient.

6. Killer cell assays are done in research labs. See Supplementary Materials if you are interested in learning how.



AUTOIMMUNE DISEASES.

Antinuclear antibodies. Antibodies against autoantigens in the nucleus are best observed using human cells grown on a slide. To do the test, the slide is fixed with an agent that makes the cells' plasma membranes permeable (alcohol or acetone work fine) so that antibodies can penetrate to the interior, and patient's serum is dropped on the slide. After washing, fluorescein-labeled goat anti-human IgG (occasionally, anti-IgA or –IgM) is added. A further wash, and the slide is examined under the UV microscope. Experienced rheumatologists can tell much not only from the presence of fluorescence (indicating antinuclear antibodies,) but also from the pattern—speckled, diffuse, nucleolar, etc.

Rheumatoid factor (IgM anti-IgG) is detected by its ability to agglutinate latex particles if they have been coated with IgG (passive agglutination). This is a simple and cheap test which anyone can do.

Immune complexes in serum often are insoluble in the cold. If you suspect a Type III disease, put a sample of serum in the fridge and examine it after 1 – 7 days for a precipitate; this precipitate is called a mixed cryoglobulin, to distinguish it from the pure (monoclonal) cryoglobulin that is occasionally seen in multiple myeloma.

Problem 4: A patient's serum contains cryoglobulin. You spin it down in your centrifuge, and now want to know if it's mixed or monoclonal. What will you do?

Immunofluorescence has already been described in the notes for Type II and Type III immunopathology. It can be used to identify antibody in a patient's tissues (direct immunofluorescence) or in their blood (indirect).

Immunohistochemistry is very like immunofluorescence, but uses a final antibody labeled instead with an enzyme, typically peroxidase, which produces a brown or black product. These slides can be observed with an ordinary microscope and archived for a long time (fluorescence fades with time.)

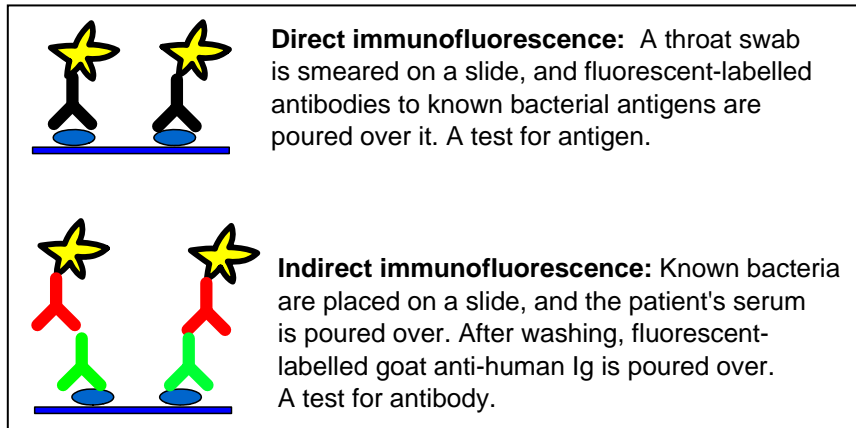
TESTS FOR SPECIFIC ANTIBODY.

We often want to measure antibody to a particular antigen, as opposed to total levels of immunoglobulin, as evidence of current or prior infection or immunization with a particular pathogen. And people with some conditions like Common Variable Hypogammaglobulinemia may have Ig in their plasma but respond poorly to a specific new antigen, so you'd like to be able to test for that.

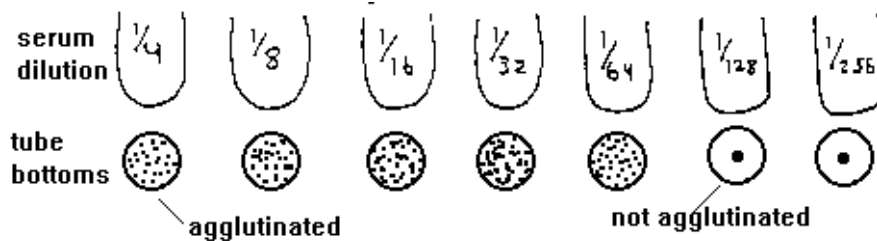
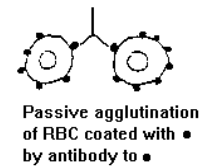
You could mix serum plus antigen, and look for **precipitation** (as in the quantitative precipitin test): it's possible, but hopelessly insensitive, and never done for diagnosis.

Simple ELISA. As is done in the HIV antibody screen, antigen is coupled to a plate, then the test serum is added; if there is antibody to the antigen it will bind. It is then identified using an enzyme-coupled antibody to the specific class of the expected serum antibody (see next section for more details about ELISA).

Antibodies to bacteria can often be detected quickly, sensitively, and specifically by **immunofluorescence**: A smear of the suspect bacterium, grown in culture, is prepared, the patient's serum is layered over it, and the slide is then washed. Next, fluorescein-labeled goat anti-human Ig is added; the slide washed again, and looked at under a microscope by UV light. If the bugs fluoresce, there was antibody in the serum. As just described this is an indirect fluorescence test.



Passive agglutination: The bigger the effective size of the antigen, the more sensitive the test, so we couple small Ags to red blood cells or latex beads, and add dilutions of the patient's serum, looking for an agglutination titer (titer is the reciprocal of the highest dilution that will still do something).



Problem 5: Titer here is _____?

IMMUNOLOGICAL TECHNIQUES FOR MEASURING ANTIGENS.

We often want to detect or measure a substance in blood or other fluid, or cells; this could be a normal component (say, VEGF), or a pathogen (*Chlamydia*), or a drug (methamphetamine). Clever immunologists can almost always find a way to produce antibodies against the substance of interest (well, not against the *real* little ones like Na^+ or glucose).

If an antigen is at least *divalent*, the favorite technique is a sandwich or **capture ELISA** for it. We've discussed it already. We use the enzyme-labeling technique because it's easy to detect and doesn't involve radioactivity. Many antibodies to viruses are measured by ELISA. A more sensitive version of this test uses a substrate that becomes a fluorescent product; these are called FIA, fluorescent immunoassays.

Problem 6: Give three reasons why a lab would want to avoid radioisotopes.

Small molecules, with only one epitope, can't be measured in a capture assay. For them we use a variety of **competition assays**, which are covered in Immunological Techniques 2.

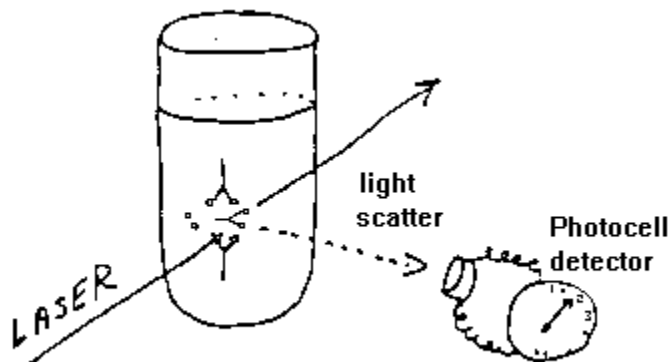
Rapid screens. In the old days a kid with a sore throat got it cultured and had to wait 3 days before he found out if he was growing *Streptococcus*. Now, using a kit, the throat swab is extracted in a tube of buffer, and the extract passed through a membrane to which Strep antigens stick because there's a dot of anti-Strep antibody coupled to it. To detect this binding, the kit provides another antibody against Strep to which liposomes (little fat droplets with a water interior) have been bound; the water contains a dye. This preparation is also passed through the membrane, and it sticks if antigen has been trapped by the dot. Detergent is added to pop the liposomes, and so the spot turns color if there was Strep antigen in the extract.

Problem 7: This is very like a test already described in these notes. Which?

Something similar to this goes on in a dipstick pregnancy test (in which antibodies are used to detect chorionic gonadotrophin in the urine).

Reverse passive agglutination. Here's a final example of a clever technique to detect antigen: for screening suspected bacterial meningitis, you can obtain sets of tiny latex beads coated separately with antibodies to 4 common bacterial culprits. Add beads to a drop of the patient's cerebrospinal fluid; if any agglutinate, it's because they have been cross-linked by bacterial antigen that was in the CSF.

Nephelometry is used for measuring many proteins: individual immunoglobulin classes, complement components, lipoproteins, and other blood and fluid proteins:



Mix patient's serum with antibody to the component of interest, and immediately shine the laser through the tube (all done automatically).

Small complexes form in milliseconds; they're not visible to the eye yet, but they scatter light (Greek: *νεφέλη*, cloud) which is measurable and proportional to antigen concentration. The antigen must be able to form immune complexes, i.e., it must be at least divalent.

More exotic assays are used in large clinical labs: they are discussed under Immunological Techniques 2.

Answers to problems:

Problem 1: 86% are double-positive CD4+/CD8+, upper right
 3% are double-negative CD4-/CD8-, lower left
 8% are CD4+/CD8- helper cells, upper left
 3% are CD4-/CD8+ CTL, lower right

Problem 2: First, the subject must have more Th1 cells specific for the antigen than a non-immune person. The injected antigen binds to dendritic cells; gets taken up; is processed into peptides; reappears on the cell surface sitting in Class II MHC; Th1 come by; they bind, get activated; they release IFN γ ; macrophages are attracted; they arrive in numbers, and release their own inflammatory mediators. All that happened when a test is positive!

Problem 3: The ring is bigger than the 160 ring but smaller than the 320, so it's somewhere in between. Note that the dilutions are not linear. I'd say it's about 200 mg/dL. Is this within the normal range for serum IgA?

Problem 4: Ask the lab to do protein electrophoresis and look at the gamma area. If it looks narrower than normal, ask for immunofixation electrophoresis. This could reveal a monoclonal immunoglobulin. On the other hand, if it's a mixed cryoglobulin, there should be many clones represented in the complexes, and you wouldn't see a narrow, monoclonal pattern.

Problem 5: Titer is 64, the reciprocal of the serum dilution (1/64) in the last tube where the cells are still agglutinated.

Problem 6: They can be dangerous to the technician's health. And to her fetus, if she has one. They are expensive. They often have short half-lives and have to be discarded before full use; this wastes money. The state and federal regulations are horrible to deal with.

Problem 7: You don't have to look very far: it is identical in principle to the Capture ELISA. The differences are: it's done on a membrane, not a well; and the dye is in liposomes coupled to the second antibody, not generated by an enzyme coupled to the second antibody.

Learning Objectives for 25. Diagnosing the Immune System

1. Describe the procedure used in serum protein electrophoresis, and the underlying principles.
2. Discuss the serum protein electrophoretic pattern which would be expected if a patient:
 - a. was normal
 - b. had selective IgA deficiency
 - c. had multiple myeloma
 - d. had severe pyogenic (pus-producing) infections
 - e. was hypogammaglobulinemic
3. Discuss single radial immunodiffusion, with regard to the types of antigens that can be quantified with it, and the way that quantization is done.
4. Outline the principles involved in nephelometry. Compare to single radial immunodiffusion for cost and speed.
5. Describe tests that are used for determining if a patient has antibody to a soluble or particulate antigen.
6. Distinguish between direct and indirect immunofluorescence techniques.
7. Discuss the advantages of passive agglutination (e.g., with antigen-coated latex particles) over precipitation, and outline the technique.
8. Describe in principle the ELISA test. Diagram the reactants involved when the ELISA is used to measure antibody, and to measure antigen.
9. Describe a test which can be used to evaluate T cell immunocompetence in a clinic or on the ward.
10. Describe tests to evaluate T cell numbers and function in the lab. Describe flow cytometry.