# IMMUNOLOGY

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# 8

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# COOMB'S TEST AND LABELED IMMUNOASSAYS

Salam every one (I rearranged things differently than the record).

In the previous lecture we started talking about Coomb's test, we said that when we are dealing with an **incomplete** antibody (IgG) that is unable to cross link RBCs together (due to gaps i.e because of the negative charge between the cells, so it can adhere/sensitize to one but can't put the other cells together), we need an antiglobin or anti- IgG in order to bring these cells together and produce agglutination. Now if we take RBCs that already have the antigen and the antibody (attached to the epitope) on their surface and we cross link them with the **anti human immunoglobulin** this is known as a **Direct Coomb's** test.

Application for Coomb's Test: we use this test when we want to detect the presence of a hemolytic disease in the newborn (rhesus incompatibility disease). If the born baby is $RH^+$ and the mother is $RH^-$ so we suspect that the mother has produced immunoglobulins IgG against it (IgG can cross the placenta except igG class 2) they will cross the placenta and sensitize the RBCs. Thus we take a blood sample from the newborn baby and add Anti-IgG to the sample, if the cells agglutinate, this means that the antibody is there (i.e. the RBCs are **sensitized** so when we add the Anti-immunoglobulin, **agglutination** happens). If they don't agglutinate, this means that RBCs aren't sensitized and they don't have **anti-D** immunoglobulin on their surface. This is known as **Direct Coomb's test**.

So if the baby is born with the suspected disease, the RBCs in that baby will have **IgGs** against the **D** antigens on the RBCs surface (those IgGs come from the mother as IgG can cross the placenta except IgG class 2), so the cells are **sensitized** {cells that have antibodies on their surfaces are known as **sensitized cells**}.



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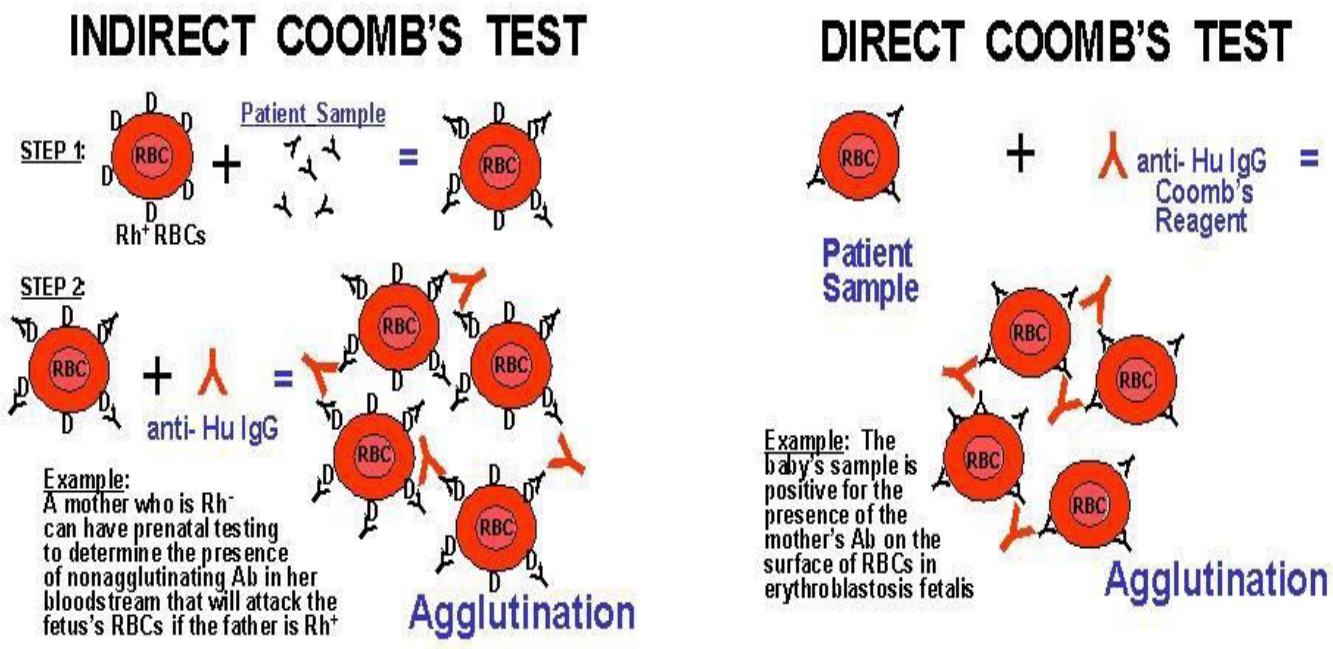
As we have a **Direct Coomb's** test, we have an **Indirect Coomb's** test as well, what is the indirect coomb's test?

This is really a little bit longer since we have to **sensitize** the cells themselves, for example: we have a lady, and we want to know whether she has been immunized and sensitized against D antigen (if she has antibodies against D antigen; i.e. does she have **anti-D IgG** in the blood) so we take a sample from the **serum** and we mix it with RBCs that are **$Rh^+$,** what happens now? These cells will become **sensitized**, then we add the **anti IgG** and we will get agglutination. If agglutination doesn't happen then she doesn't have Anti-D IgG in her serum.

In **Direct Coomb's** test we agglutinate RBCs that have been naturally sensitized, But for **Indirect Coomb's** test we want to detect the presence of the antibody in the **serum**, we mix the serum with the RBCs that are Rh+, if the antibody is present in **serum,** it will sensitize the RBCs and when we add the anti immunoglobulin (anti IgG), these cells will agglutinate.

**Direct and Indirect Coomb's** tests are used very extensively in blood banking especially for the detection of hemolytic diseases in newborns and unexpected antibodies (antibodies that are attached to RBCs and you don't know about them because there is no agglutination) so we use direct Coomb's test to detect the presence of these unexpected antibodies. We will talk about rhesus incompatibility and these unexpected antibodies later on.




## Labeled Immunoassays:

We mentioned two phenomena that can occur when we mix antigen and antibody: (1-**agglutination, 2-precipitation**) that we have seen already, but we have other phenomena that can occur when the antigen and antibody are mixed together (they don't occur in the body but used in the lab) and they are known as **labeled immunoassays:**

## A- Radio immunoassays:

We can label antibodies (or antigens) with radioactive substance "usually they use iodine (1,25-I)", and then we mix them together (the known-in quantity- with the unknown), if they (the antigen and antibody) are really compatible with one another and there is antigen-antibody reaction; we can measure the amount of radioactivity in immuno-complexes (by a **gamma counter**) and that will give us an indication of how much there is of the antibody **or** the antigen (depending on which of them we are looking for).

Radio immunoassays are very sensitive, they are used for measurement of **very very small amounts** of reagent, because sometimes when the

concentration is so small you can't really detect it by means of precipitation, so when we have very small amounts of antigens or antibodies we use this assay .

Radio immunoassays are not used very extensively because there are hazards related to them; after all we are dealing with radioactive materials, it is difficult to get rid of waste products; when we finish, we should dispose them in a safe way in order not to contaminate water supply and so on. They also tend to have very short shelf life; most probably a month or two months so after that you can't use them because the radioactivity decays very quickly, also the gamma counter is an expensive equipment so it is not used that much.

The doctor said he's not going through details about this subject, but if you are interested, read the following☺:

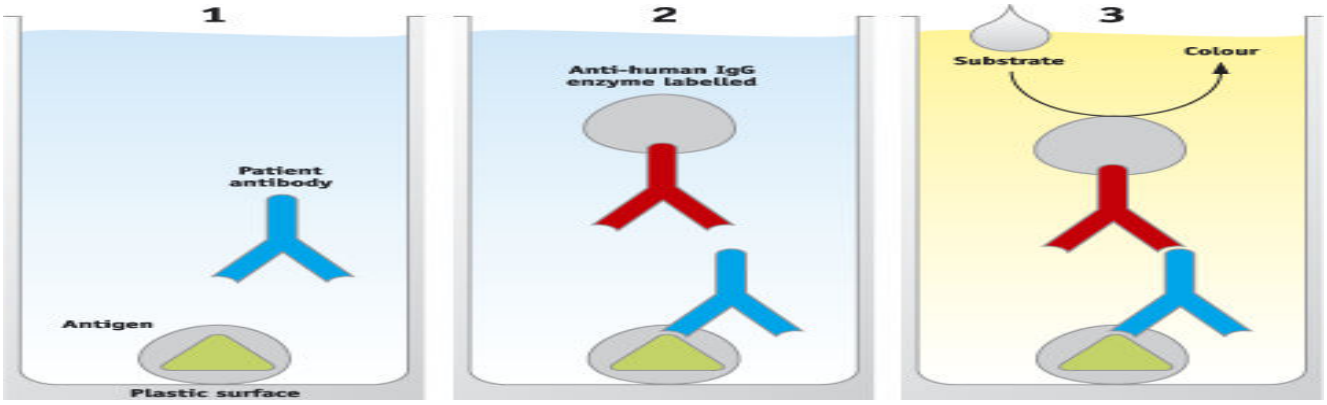
To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by iodine,. This radiolabeled antigen is then mixed with a **known** amount of antibody for that antigen, and as a result, the two specifically bind to one another. Then, a sample of **serum** from a patient containing an unknown quantity of that **same** antigen is added. As the concentration of patient antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to **free** radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is **measured** using a **gamma counter**

## B- ELISA (enzyme-linked immunosorbent assay):

Here the label is actually a kind of **enzyme**, we add an enzyme to the antibody, it will react with antigen and the resultant immuno-complex can be mixed with the **substrate** of the enzyme, so what happens----> if the enzyme is there "in the immuo-complex", the enzyme will work on the substrate, the enzyme action on the substrate usually produces a **color** which can be measured. You should stop the reaction, because after mixing them together the enzyme will carry on breaking down the substrate producing more deeper colors, so after a fixed time (about a minute), you add an **acid** that will stop

the enzyme from working any more, and then you look for the presence of the color which is measured by means of a device (colorimeter or something like this) and the amount of the color that has been produced will be either proportional or disproportional depending on the method you are using to give you an indication of how much substance you are looking for.

( Elisa is as sensitive as the Radio Immunoassays, cheaper, safer, easy to use, less hazardous and has longer shelf life, so basically it is the best method used in labs).




## C- Immunofluorescence:

If you shine a laser (a light beam) to fluorescent substances like **fluorescein** and **rhodamine**, what will happen? They will absorb some of the energy that is present in the beam and transmit the rest in a longer wave length {so: a light with certain wave length --- > some of the energy will be absorbed by the substance itself --- > and the rest will be transmitted **as a longer wave transmission**}. When you shine this special light on them you get a **color** (green in case of fluorescein, red in case of rhodamine and there are also others). So we have the fluorescent material attached to the **antibodies**, and they are used for the detection of antigens in tissue.

For example:- in case of glomerulonephritis disease, you have immune-complexes deposited in the glomeruli themselves, and that activates the complement system, we get complement break-down products there, so if we want to know whether there is complement activation or not; we look for complement components like **C3** or **C3b** in the tissue, we can have it in a

tissue biopsy and fix it on a slide (don't stain it, you have to take it as fresh frozen section ) then we add the immunoglobulin (antibody against the antigen that we suspect to be present in the tissue) (like **anti-C3 antibody**) that has been labeled by fluorescent substance, then we wash the excess of antibody (to ensure that we don't have any excess of the antibody that haven't been attached and may give us false positive results), and then we examine it under the fluorescent microscope, if the complement is there in the section; the antibody (with the fluorescent) will be actually attached to it and we can see it (whenever there's a green light; it means that there's antibody labeled by fluorescein and attached to the antigen (complement C3 for e.g.). If the antigen of the complement C3 is not there, the labeled antibody will not attach {so we can use this method for the detection of antigens in tissues}.

There is a procedure we use in the lab called:

## D- Flow Cytometry:

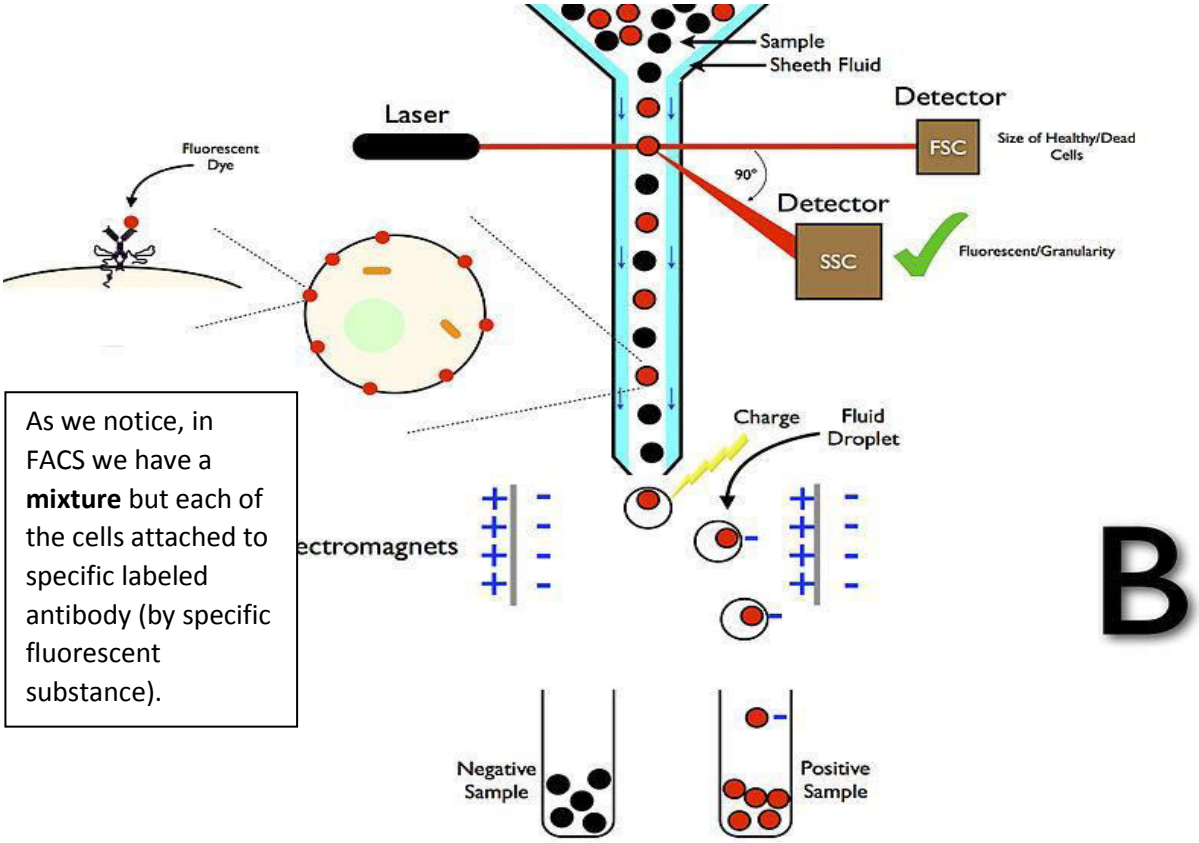
As the name indicates we have cells that are flowing. You mix the cells that you are interested in in a liquid and you put them through this system, then they come out one after the other (they come out in line one cell at a time), and as they come out you can hit them with a laser, this light laser will be reflected according to the cells, so there is scattering of light at (an angle) and forward scattering of light. The **forward scattering of light** is really an indication of **the size** of the cell {the bigger the cell, the less it will scatter light forward} and the **scattered light to the side** (in an angle) is an indication of the **granularity** of the cell. So once the cell comes in, you can judge (from the amount of scattered light forward and to the side) how big and how granular the cell is, so by the flow cytometry we can count and enumerate the cells, for example: lymphocytes (small, about 10 micron in diameter, and not granular since they have very little cytoplasm and big

nucleus), neutrophil or granulocyte (big and granular), macrophages (big but not that much granular).

## E- FACS (fluorescence-activated cell sorting):

From the name: it relies on fluorescence (fluorescence- activated) and it is a **cell sorter** (it sorts the cells) how? According to the type of antigens (epitopes) on their surface; B-cell receptor (BCR) on B-cell surfaces, a cell that has CD4 is a helper T-cell, a B-cell usually has CD19 on the surface, CD8 is present on cytotoxic T cell, CD2 is present on T lymphocytes and NK cells. So when we have a mixture of B and T lymphocytes, we can separate T from B lymphocytes and helper-T cells from the cytotoxic T-cells by this method.

What do we do? We bring antibodies against the relevant antigen, for e.g. we have antibody against major BCR, antibody against CD4, antibody against CD19, antibody against CD8, before we mix these antibodies we label them accordingly (the first one with fluorescein, the second one with rhodamine and so on) so we put different fluorescent agents on each of the antibodies (anti CD4, anti CD8, anti CD19……). Then we mix these labeled different antibodies with the cells in the mixture. After mixing them, the CD19 on the B cell will attach to the antibody (anti CD19) that has green color which is fluorescein, and CD4 will attach to antibody that has rhodamine and so on. Then you wash the excess and you actually pass these cells accordingly in line just the same in the flow cytometer, and as they go downwards they will be subjected to a laser so if the antibody which has been labeled is attached to the cell, it will fluoresce. This device will be adjusted so that every time the cell shines (fluoresces) green, it will direct the cell to the right (for example). If it shines red, it will direct it to the left (because of the electric charge that is given to it). You will end up with: the test tube on the right has only the CD19 cells (B cells), the test tube on the left will have cells with CD4 (helper T cell). So if we want to study the cells separately we sort them by this mean and examine them under Immunofluorescence microscope.




As we notice, in FACS we have a **mixture** but each of the cells attached to specific labeled antibody (by specific fluorescent substance).

Illustration of FACS (a specialized type of flow cytometry )

And that's it :) This sheet is dedicated to : (my sister Sara Alzalabyeh :P ) and Noor Abu Gnim.

Written By: Anan Abu Qaddoum .

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