Serologic reactions / Dr.Malik sallam / sheet 22

*موجود كل حكي الدكتور , ف يفضل تتبعو مع الفيديو

- **Basic immunologic and serologic** procedures are used to help us in the diagnosis of autoimmune disease of immune deficiencies, it can help in diagnosis of allergies.

Application of some techniques that will be presented, like production of monoclonal antibodies, that can be used as drugs for treatment of cancer of autoimmune diseases of allergies.

The main idea is about the wide range uses of immunologic and serologic procedures either **diagnosis** or **management** of patients who have immunologic diseases, or **uses of preventive techniques** to manage illnesses like cancer.

- **Serology** [definition]: serology comes from serum مصل

- لو اخذنا عينة دم وفصلناها عن طريق الطرد المركزي (Centrifugation) , راح يكون عنا جزء سفلي بتتجمع فيه الخلايا RBC mainly وبعدين WBC والـ platelet , والجزء اللي بضل fluid و فيه الـ proteins & other components بنسميه **الـSerum**

Serum with other components,
Mainly proteins

الـ antibodies موجودين هون

- إذن الـ **Serology** هو علم دراسة ال **antibodies** الموجودة في **الـSerum**

What's the difference between serum and plasma?

They're the same , except that the plasma contains fibrinogen and coagulation factors.

Fibrinogen and coagulation factors are consumed in the serum because of clotting, while in plasma there are anticoagulants like ethylene diamine tetra acetic acid (EDTA) and Citrate, those factors prevent coagulation.

What's the importance of studying antibodies ?

- **we can use it in serum , to quantify antibodies in total , to know if there's underlying B-Cell deficiency (As B-Cell produces antibodies)**
  So ☞ diagnosis of immune deficiencies
- **We can use serum to serologically diagnose certain infectious diseases في بعض الامراض المعدية اللي بتسببها الفيروسات والبكتيريا , عزل الـ organisms صعب وبوخذ**

فترة طويلة , او انه الـ infection صار اله فترة وبدي اتأكد اذا الشخص عنده مناعة , بستخدم الـ serology و بدوّر ع specific antibody against infectious agent

Example: pregnants Torch antibodies test using serology

Torch antibodies: antibodies against toxoplasma, CMV, herpes, rubella .

Hybridomas and monoclonal antibodies

Here I want to detect antigens or antibodies (which are proteins)

The main idea in serologic reactions is using highly specificity nature of antigen-antibody binding... antibody is going to bind a very specific epitope that is found in an antigen

- الـ Antigens مش بس عبارة عن منطقة واحدة , بكونو بروتينات فيها multiple epitope.. يعني البروتين ممكن يكون فيه hundreds of amino acids
  - Short stretches of amino acids give different epitopes

So when I pick an antigen and I give it to an animal or a human ⇢ immune system will react to this antigen by production of polyclonal antibodies,

because antigen have multiple epitopes, when entering the body more than one cell recognizes the specific site in this antigen, and will start producing antibodies.

⇢ Every single one of them is going to give us antibody with single specificity, but the **sum** of those antibodies when taking a blood sample and separating the serum is **polyclonal antibodies**

But here, I lost the specificity... because every one of them will bind a site in this antigen
So I can't look at a single hormone or protein... we should have single specificity so **NO cross reaction occurs**

← لنفترض مثلا في Antigen معين موجود ب فيروس معين.. و في 10 epitopes .. 9 منهم خاصين بالفيروس والعاشر موجود ب فيروس ثاني .. الـ9 راح يكون في الهم specific antibody , والعاشر اله specific antibody برضو , لكن راح يتداخل ب epitope فيروس ثاني

↳ SOOO if we use polyclonal antibodies against that antigen for detection of virus disease number **1**, and the patient has virus disease number **2**, antibodies will react to that antigen in virus number **2** and will give us **false positive** result.

➜ Now, how can we solve this problem?

Simply we increase the specificity by doing detection using monoclonal antibodies
I know that there is a specific epitope that is **ONLY** found on the target either infectious agent, protein (to diagnose autoimmune disease) or to check the presence of specific antigens of cancer cells either by flow cytometry or immunohistochemistry techniques.

➜ Remember, if we have monoclonal antibodies, detection is going to be specific

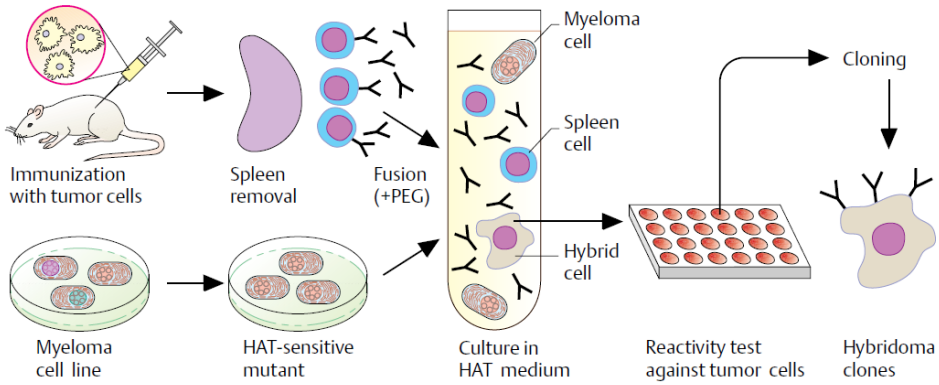
Now, when to use polyclonal or monoclonal antibodies?

➜ **Polyclonal AB**: when we need visible reaction like agglutination

➜ **Monoclonal AB**: most technique that need highly specific reactions depend on monoclonal antibodies (that are produced by hybridoma technology – invented in 1970s)

How to produce those monoclonal antibodies?

Please consider watching this short video **https://www.youtube.com/watch?v=XrUW54Ea598**



➜ Simply I take cells that are producing **antibodies** which are plasma cells, I collect those plasma cells from animals after injecting them with **antigen** of interest.
so we will have a huge number of plasma cells, every single plasma cell will give **antibodies** of single specificity

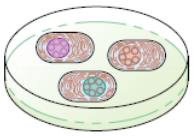
➜ We also have myeloma cell line (immortal cancer cells) their origin is plasma cells…They don't die, those myeloma cells give **antibodies** of single specificity

⇢ We use myeloma cell line that is deficient from certain enzyme which is **HGBT**.

HGBT Enzyme: this enzyme makes the cell able to synthesize nucleotides ON ITS OWN. (this is called DeNovo synthesis of nucleotide)

⇢ **Why we choose myeloma cell line that is deficient from this enzyme?**

Because if there's no nucleotides in the medium, those cells will die.


Myeloma cell line

While plasma cells that came from animals have this enzyme
So when we take those plasma cells after immunization, and we put them with myeloma cells, they'll fuse

➜ So we choose a medium that is deficient from nucleotides, so **myeloma cells** that lack this enzyme (that is responsible for producing nucleotides) will die,

➜ also **B-cells** (plasma cells) have certain lifespan so they'll die soon...

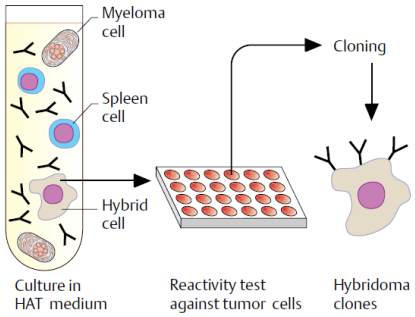
BUT, cells that are produced from **fusion between myeloma cells and plasma cells**, are the only to <u>survive and they won't die.</u>

*** Why those cells are immortal? ← أخذو هاي الميزة من الmyeloma cell line**

***و ميزة الـ production of antibodies of interest ← انأخذت من الـ Plasma cells اللي اخذناها من الحيوان**

Why we took spleen بالذات?

Because it's where B-cells are concentrated after immunization of animal.



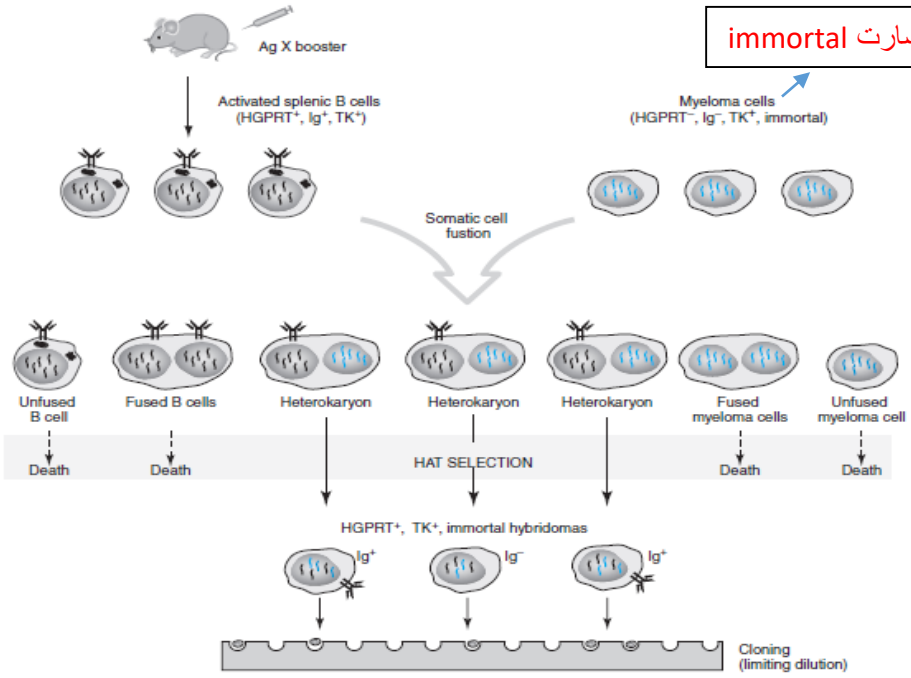
➜ So now the media contains antibodies produced by Hybrid cells as a result of B-cell fusion with Myeloma cell-line
Then we can collect those antibodies,

And because the different dilution of those antibodies, every Well will have antibodies of single specificity.
Because every well contains one single cell producing antibody of single specificity then we test this antibody, to know its nature (which antigen caused its production), then we can keep this Hybrid cell as it's immortal, so it keeps producnig monoclonal antibodies that'll be used to specifically detect certain antigen, either analyzed in lab,cancer antigen or autoantigen in certain autoimmune diesease

- You **should** memorise the major concept:

عنا **plasma cells** و **myeloma cells** .. بندمجو مع بعض وبصير في selection للخلايا اللي بتعمل على الانتاج المستمر لل **monoclonal antibodies** .. ف بنقدر نصنّع كميات كبيرة من هاي الـ antibodies اللي بتكون جدا جدا specific to the antigen of interest

أصلها plasma cells بس صارت immortal



Clinical applications of Monoclonal antibodies

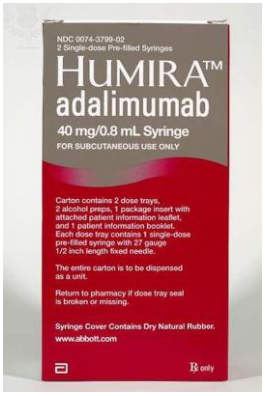
- تستخدم في فحوصات مختلفة , من ضمنها (Point-of-care-testing) أي نقطة الرعاية السريرية مثل:
  1. Pregnancy test
  2. Quick antigen test for certain infectious diseases

- Can be used as treatment option, for example: certain types of cancer like Hereceptin.

Hereceptin: Monoclonal antibody against HER2-Receptor in **breast cancer**.

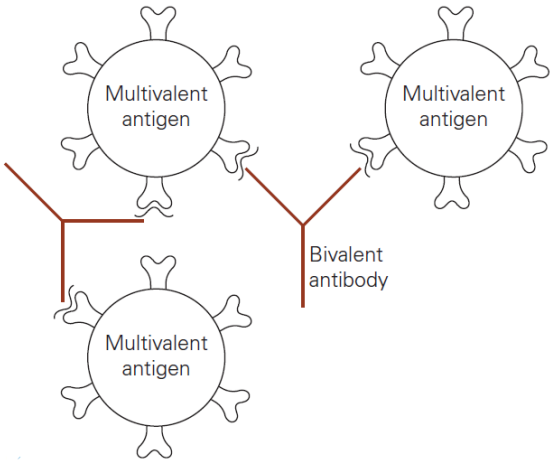
Humira: used in certain autoimmune diseases like rheumatoid arthritis

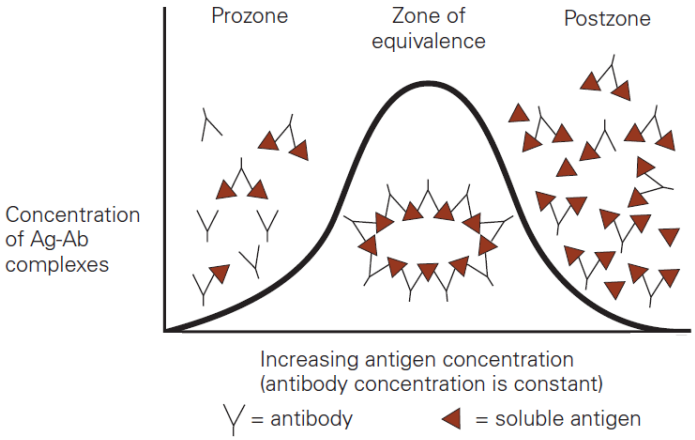
mab: monoclonal antibody



Antigen-Antibody binding features

➔ the main idea in serologic and immunologic reactions depends on specificity of binding between antibody with their cognate antigens (specific binding between antibodies and epitopes)





اذا بدي اكشف عن وجود إشي معيّن (peptide, hormone, certain antigen or whatever)

بستخدم ال **specificity of binding** بين الإشي اللي بدي أكشف عنه و its cognate **antibody** .. و بكون مربوط مع signal (عشان يعطيني إشارة)..

Signals can be: - Fluorescent molecule

- Enzyme (بعمل تفاعل وبعطي لون معيّن في حال كان الإشي اللي بنكشف عنه موجود)

- Radiolabel (that gives radioactivity, we measure it to decide whether we have the antigen or not)

Sometimes, antigen can be antibody… **WHEN**?

➔ for example when we're searching on evidence of previous infection by certain infectious disease… we look for antibodies IgG against antigens of infectious agents (virus,parasite,etc…)

the antibody here is called **anti-human antibody**

في قوى ارتباط متعددة .. ممكن تكون: **van der waals forces , Hydrogen bonding**

ما عدا ال **covalent bonding** غير موجودة ⟵ ف بكون easily **reversible**

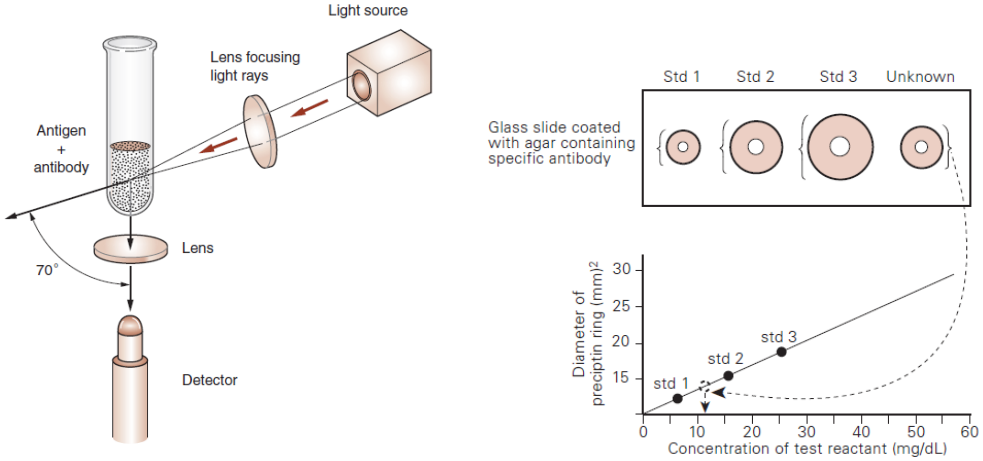
- To get visible reaction, concentration of Ag-Ab complexes should be in **zone of equivalence**

If Antigen concentration is **very high**… it gives false negative ⇾ because all binding sites in Antibodies get saturated… so it'll not form a large visible agglutinate or precipitate to be visible after the reaction

Precipitin, Turbidimetric and Nephelometric Immuaoessays

We detect antigen-antibody reaction using different methods, and the first method is

Detection of precipitation by measuring turbidity.



If the solution is clear, the presence of Ag-Ab complexes changes its light scattering characteristics (خصائص تششت الضوء) for this solution.
When antigen binds with antibody, the solution becomes turbid

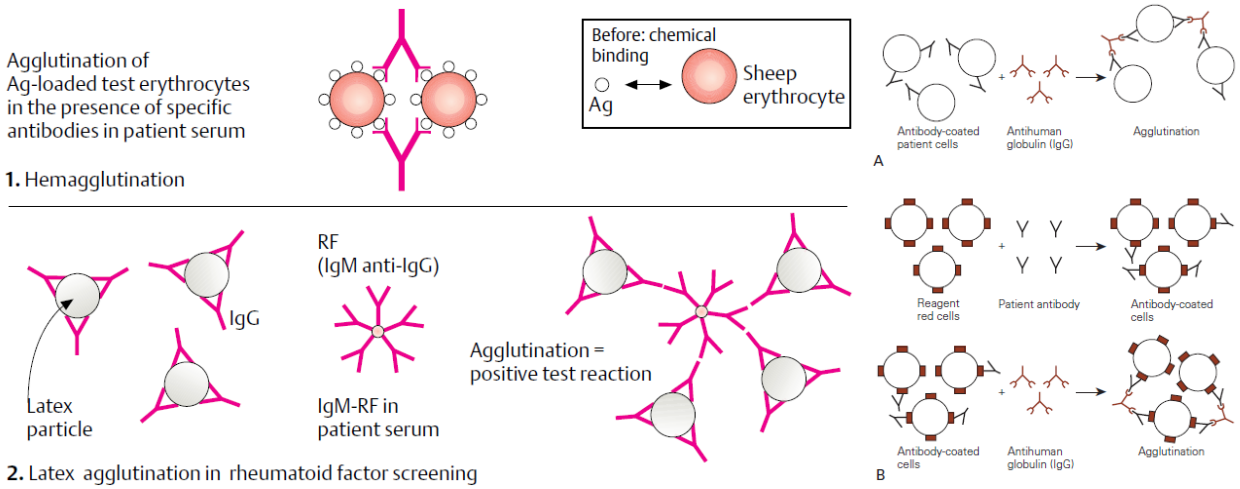
إذن لما أسلّط مصدر ضوء على العينة , وحطيت بالجهة المقابلة detector بزاوية 180 درجة عن مصدر الضوء , هذا يسمى قياس الTurbidimetry

أما إذا كان الـ Detector موجود بزاوية محدّدة , تسمى هاي الحالة Nephelometry

- النتائج بالأعلى Qualitative , في حال بدنا إياها تكون Quantitative لازم يكون عنا مجموعة من الـ Standards بقيس من خلالها التغيّر في امتصاص الضوء أو انعكاسه, وبقارنها بالعيّنة للوصول إلى الـ Exact concentration

Agglutination, Coomb's Tests

- إرتباط الـ Antigen مع الـ Antibody يتم الكشف عنه من خلال وجود Particle .. لذا لازم **reporter**



➔ In Precipitation there was no need for reporters, binding of Ag with Ab is enough to make complexes that change light scattering characteristics... so **we can detect them without REPORTER molecules**

➔ In agglutination, there is reporter molecule that detects when Ag-Ab binds together… and this reporter molecule is particle

Also, agglutination reactions are **cheap** and **very fast**!

This particle can be:

- RBC particle: which is called hemeagglutination
- Latex particle: latex agglutination
- It can be a type of cell, like Staphylococcus Aureus (that has protein A) which is the receptor of FC portion of the antibody: this is called CoAgglutination (when the reporter is staphylococcus Aureus bacterial cell)

➔ One of the most famous Hemagglutination reactions is **Coomb's reaction** (which is found in blood banks)

Coomb's test can be :

- **Direct**: Measures if antigens are **already** found on RBCs

➡ Used to detect hemolytic disease of the newborn (Because RBCs are already covered by antibodies)

- in this case, we take RBCs from suspected patients (who have hemolytic disease) of the newborn or drug induced hemolysis,
- then we only add antihuman globulin,
- in this case if antibodies are already found on RBCs, antihuman globulin will help them to **agglutinate** (to make visible agglutination) that can be detected by naked eye.

- **Indirect**: Measure if there are antibodies in serum
  in this case we will bind antibodies with RBCs, and detect them with Antihuman globulin
  *(Antihuman globulin is looking for antibodies in serum)

➡ Most famous use: **cross matching of blood**

- **Before blood transfusion**, we take recipient RBCs with donor serum, to make sure that this donor **doesn't** have antibodies that enter recipient's body, and destroy their RBCs...
- So in cross match before transfusion, we collect recipient RBCs (washed) to make sure to make sure that nothing is bound to them, maybe the recipient himself has autoimmune hemolytic anemia or drug induced hemolytic anemia, that's why RBCs should be washed.
- Then we get serum of blood donor, and we do incubation…
- after incubation, if there were alloantibodies that will bind from donor to recipient RBCs, they'll will have bounded after incubation.
- Then we want to detect this reaction, so we use Antihuman globulin which will detect alloantibodies in donor (so it doesn't destroy recipient's RBCs) **and in this case no blood will be transfused from donor**

In conclusion: Direct test detects RBCs that are already bound to antibodies, while Indirect test involves 2 steps to detect whether there's antibodies in the serum or not.

(قل الله ينجيكم منها ومن كل كرب)

Another examples on agglutination:

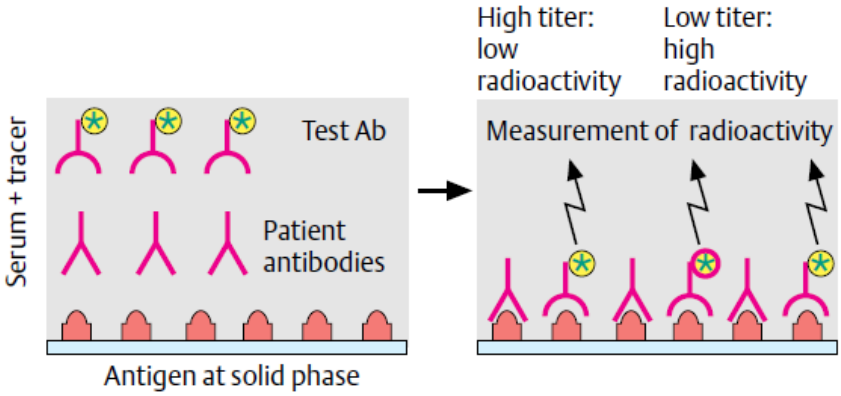
- in clinical lab, CSF diagnosis of meningitis, a fast diagnosis that detects bacterial antigens of most common agents of septic meningitis which are: streptococcus pneumonia, haemophilus influenzae, E. coli K1, strep B group (Streptococcus agalactiae) and neisseria meningitides.

➞ we take a sample of CSF and mix it with reagent (which is antibodies that detect antigen of every common causative agent), if we get visible agglutinate (in less than one hour) we know that this patient probably has meningitis due to this positive agent and it could guide empiric therapy quickly, results are **faster than real time PCR**, but **less sensitive and specific** compared to molecular detection.

- monospot test for epstein barr virus.
- widal test, for example in salmonella
- alagglutination is also used in detection of brucella (maltese fever)

Radioimmunoassay (RIA)

Little uses nowadays, but it was the point when sensitivity of detection of minute quantities of certain analytes has increased (in early 1970s)



Principle: antibody in patient serum competes with radioactively labeled test antibody

Why radioimmunoassay is rarely used?

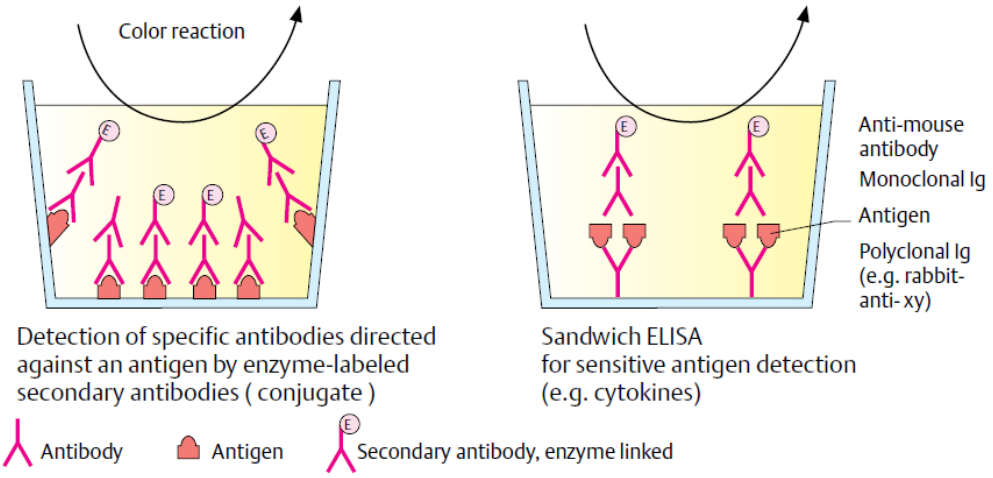
Because the reagent is **unstable**, and **shelf life is short** and it forms **radiologic hazard**

So it was replaced by enzyme immunoassay.

➞ RIA principle is the same as enzyme immunoassay but the label is radioactive material, while enzyme immunoassay is **more safe**, **longer shelf life**, reagent is antibodies **labeled with an enzyme**

Enzyme Immunoassay

➡ Safer than radioimmunoassay



In conclusion, the most enzyme immunoassay is **ELISA** (Enzyme linked immunosorbent assay),

➡ we have wells coated with antigens or antibodies

- then we add the sample that we're looking for antibody in
- for example, I want to do **ELISA** to detect Parvo virus **IgG**
- Then we get a well that is already coated with parvovirus antigens. (they're commercially available)

➡ If the sample has Antibodies due to **previous infection** with parvovirus it'll bind with antigen after incubation period (30 minutes,60 minutes or whatever the manufacturer say)

- Then we do washing to remove unbound antibodies... so we ONLY keep Antibodies that are specific to parvovirus antigen
- Then we add antihuman antibody which is labeled with an enzyme, and again we do incubation another time, so if the first antibody is found, the second antibody (which is labeled with an enzyme, it's called enzyme conjugate) will bind to the first antibody… so it'll stay even after washing.

  *If the first antibody wasn't found, enzyme conjugate will be removed.

- Finally, we add a color less substrate that enzyme will work on and convert it to colored compound, usually blueish, and we eliminate the reaction with a strong acid (usually) and we measure it with spectrophotometry.
  NOTE: Strength of the color is directly related to the amount of antibodies that were originally found in the sample.

JUST TO KNOW مش مطلوب تفاصيل:

There is competitive and non-competitive ELISA

In competitive ELISA there is competition between the sample and the reagent, and the strength of color is inversely related with amount of antibodies.

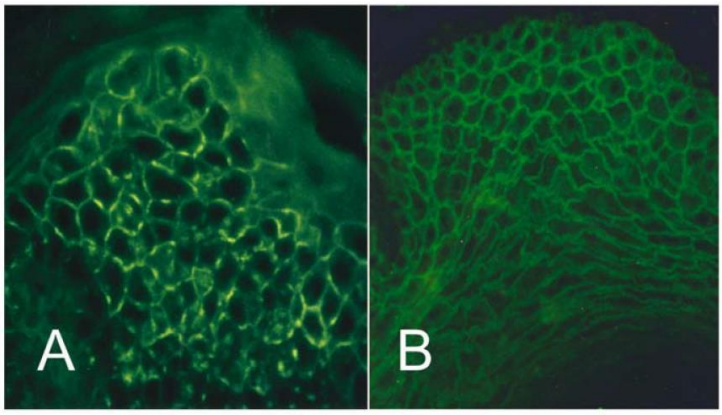
Fluorescent immunoassays

It can be:

- **Direct**:

Fluorescent immunoassays (Skin Ab)

في حال الكشف عن وجود Antibody بـ Tissue , مثلًا إذا أخذت biopsy من الـ Skin أو Kidney و انت بتدوّر عن وجود Antigen-Antibody complex أو Auto antibodies ← Direct immunofluorescence assay



Used to diagnose bullous diseases of the skin like **pemphigus vulgaris** and **bullous pemphigoid**

➜ Every single disease has different characteristic pattern in Direct Immunofluorescence,

- either as a network in epidermis if the antibodies are against intracellular substances in epidermis like **picture A,**
- or against the basement membrane like in **bullous pemphigoid** as shown **in picture B**
- Also it can be at tips of dermal papillae like **dermatitis herpetiformis**

- **Indirect**:

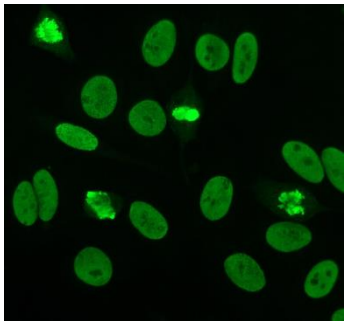
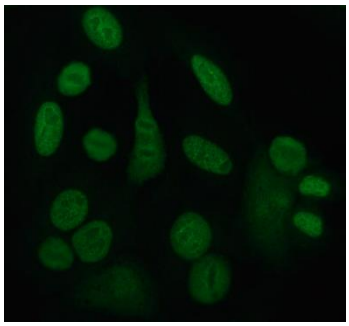
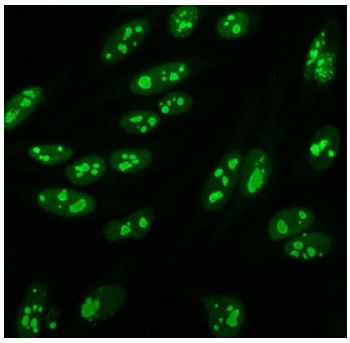
Fluorescent immunoassay (ANA Testing)

- في حال بدوّر عالـ antibody بالـ Serum بتكون indirect...
- بناخذ عينة من الـ Serum وبنكشف عن وجود Auto antibodies

أشهر الـ **fluorescent immunoassay** ← ANA (Antinuclear antibody testing) :

Antinuclear antibody exists in many systemic autoimmune diseases like: systemic lupus erythematosus (الحمة الذؤابية)

Pattern in Systemic lupus erythematosus can be:

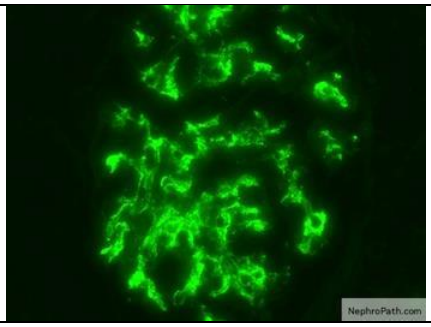
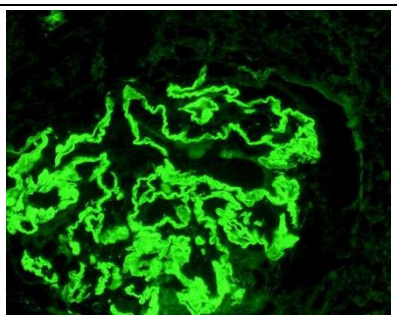
▪ **Homogenous**: Because we have Antidouble stranded DNA Antibodies	▪ **Speckled**:	▪ **nucleolar**:
		

*also can be centromere

Every single pattern gives indication of different variant from this systemic auto immune disease

Fluorescent immunoassay (Glomelular AB)

The same thing applies for renal disease

▪ Glomelurali Mesangial deposition of Ab-Ag complexes	▪ Antibasement membrane antibodies like in Goodpastur syndrome (GPS)
	

Flow cytometry (FCM)

Flow cytometry is analysis to cells that are found in a fluid, it's used for sake of enumeration of certain types of cells like CD34+ (stem cells) and diagnosis of immune deficiencies

flow cytometer is used to analyze cells based on their properties like size and their complexity (like if they contain granules or multilobed nuclei)

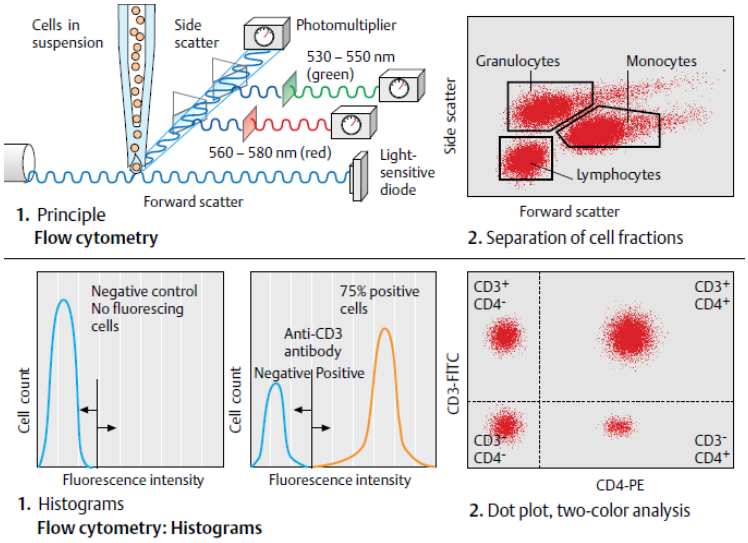
1. **First step** depends on **flow of cells**, cell by cell

   And by their **light scattering characteristics**, they are divided into populations.

   population that has small size on forward scatter,

   population that has minor complexity and granularity on side scatter

   ➜ those are lymphocytes because they have no granules and less complex than every other cell, also they're the smallest cells in peripheral blood



   Then comes monocytes and granulocytes, **SOOO** we can classify cells in the sample to populations

2. And by binding of **monoclonal antibody** (that are labeled with fluorescent molecule) we can know the **immunophenotype** for every single population (normal or no)

   in lymphocytes we can divide them by monoclonal antibodies, for example against CD3 to T-cells, against CD19 to B-cells, against CD16 and 65 to NKC, etc…

➜ Flow cytometry not only help in classifying populations, also through **immunophenotyping** we can know the percentage of $CD4^+$ T cells (T helper cells), $CD8^+$ T cells (Cytotoxic T lymphocytes), NKC and B-cells.

If there's **apparent expression**, like expression of certain CD molecules on cells that normally do not have them, **so we can diagnose certain types of leukemias** depending on immunophenotyping... but diagnosis doesn't depend only on flow cytometry

➜ hematopathologic examination are very important, they're irreplaceable.

Hematophathologist depend on morphologic features + flow cytometry might aid in diagnosis

Another applications of flow cytometry:

- enumeration of CD34+ of stem cells
- It can be used for DNA content analysis using **propidium iodide dye**