Chapter VII. The main tests in immunology

The study of the immune system requires the use of a large number of techniques and methods based in particular on antibodies, whose applications are very important in biology.

Immunological techniques that have been identified or are being researched:

- **Antigens** present in cells - in tissues - in biological fluids (serum, urine, etc.) using monoclonal – polyclonal – antibodies.
- **Antibodies** mainly in the serum - more rarely in other biological fluids (saliva...) using corresponding antigens.

The choice of technique is based on:

- The concentration of the antigen or antibody.
- The form of the antigen (soluble or particulate)
- The location of the antigen or antibody

VIII. 1 Basic principle

Immunological techniques rely on **an antigen-antibody reaction**

The Ag-Ac reaction is an exothermic, reversible and specific reaction.

- **Exothermic:** the reaction is characterized by the formation of an energy-releasing bond, which results in an influence of temperature on the smooth running of the reaction.
- **Reversible:** the bond between Ac and Ag are weak bonds (electrostatic, hydrogen, hydrophobic ...), so it can be broken quite easily by varying physico-chemical parameters (pH, temperature, ionic strength).
- **Specific:** the antibody site of an immunoglobulin (paratope) can combine with one and only one epitope; there is stereochemical specificity between the paratope and the epitope.

The Ag-Ac reaction results in the formation of a complex called the **immune complex**. The - immune complex is the product that will be highlighted in all immunological techniques.

VIII.2 Methods for detecting antigens and antibodies

The aim of these immunological techniques is to be able to detect the presence (or absence) of an Ac or Ag in a medium (qualitative method). Some methods can, in addition to the qualitative aspect, inform us about the amount of Ac or Ag present in the medium, thus allowing assays (quantitative method). There are 2 main reaction groups:

- **Reactions producing a directly visible " signal ":** precipitation and agglutination method
- **Reactions that do not produce a " signal ":** marking method.

VIII.2 1. Precipitation methods

Immunoprecipitation reactions use soluble Ag which will react *in vitro* with an Ac having several antigenic sites (at least bivalent), to form a large macromolecular network. A macromolecule (or polymer molecule1) is a very large molecule, which has a relatively high molecular weight. The larger the macromolecular network, the more likely it will precipitate. For this, there is an optimal Ag/Ac ratio for which the size of the network will be maximum (equivalence zone). When one of the reagents is in large excess compared to the other, there is a risk of re-dissolution of the immune complex (zone phenomenon).

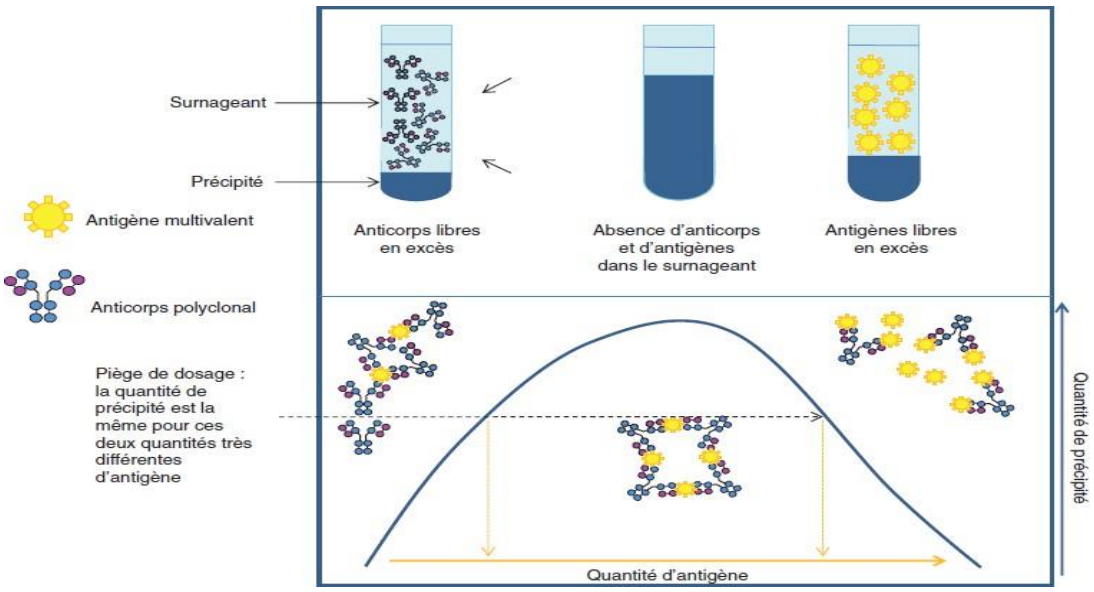

Figure 29: Precipitation curve

Two optical principles of automatable equipment, based on the deflection of a monochromatic laser beam in contact with precipitate particles, have been developed:

1. **Turbidimetry:** Transmitted light measurement produces a decreasing signal with antigen concentration.

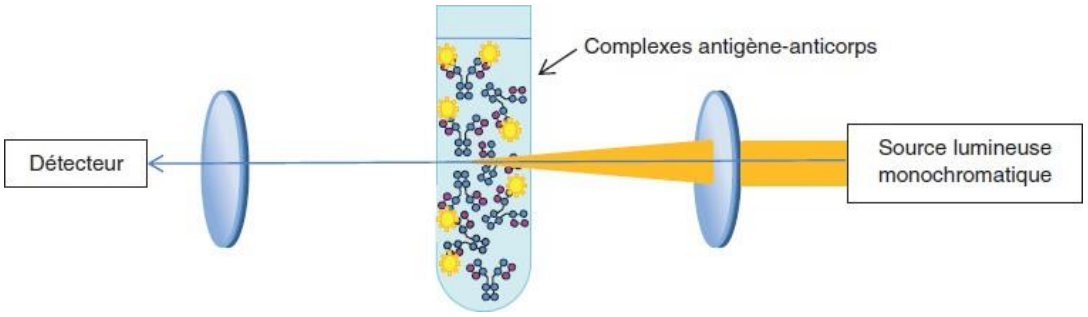

Figure 30: Principle of Turbidimetry.

2. **Nephelometry:** Measuring light scattering produces a signal that increases with antigen concentration.

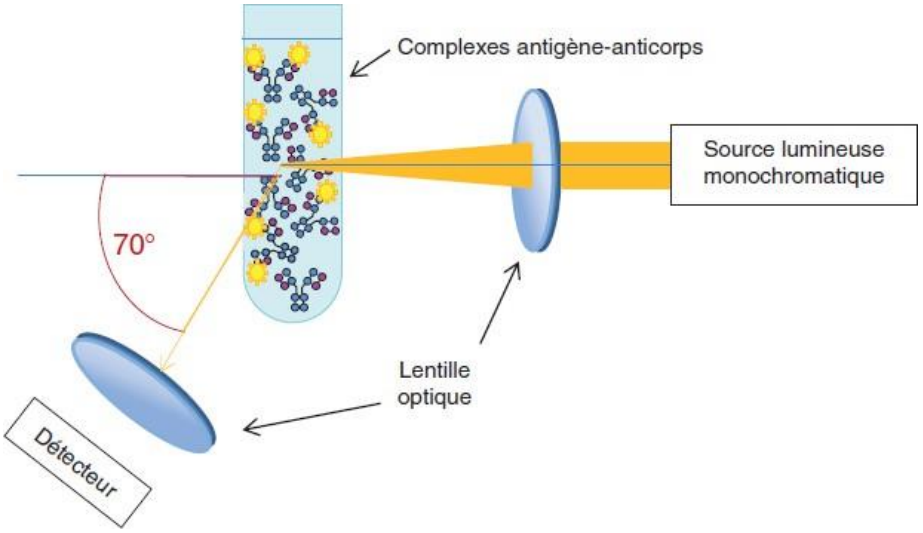

Figure 31: Principle of Nephelemetry.

Application Examples

- **In research:** Immunoprecipitation in a liquid medium is commonly used for the study of proteins. The protein extracts to be analyzed (e.g. culture medium, cell lysate) are incubated with the antibodies. This step allows the formation of antigen-antibody complexes. The complexes are separated

by simple centrifugation, or by means of an anti-immunoglobulin antibody or a protein binding the Fc of immunoglobulins, coupled to beads.

- **In the clinic:** nephelometry is used in common practice for the assay of serum proteins, including major immunoglobulin isotypes (IgG, IgA, IgM and incidentally IgD) and certain components of complement (C3, C4, C1-inhibitor and factor B). Latex particle techniques allow the assay of rheumatoid factors, certain IgG subclasses and free light chains.

VIII.2 2. Agglutination methods

Immunoagglutination reactions use insoluble Ag in suspension (not in solution). These Ag will react specifically with multivalent Ac to form networks of insoluble particles which then become visible to the naked eye. This method is widely applied to the determination of blood groups and the detection of Ac. Hemagglutination

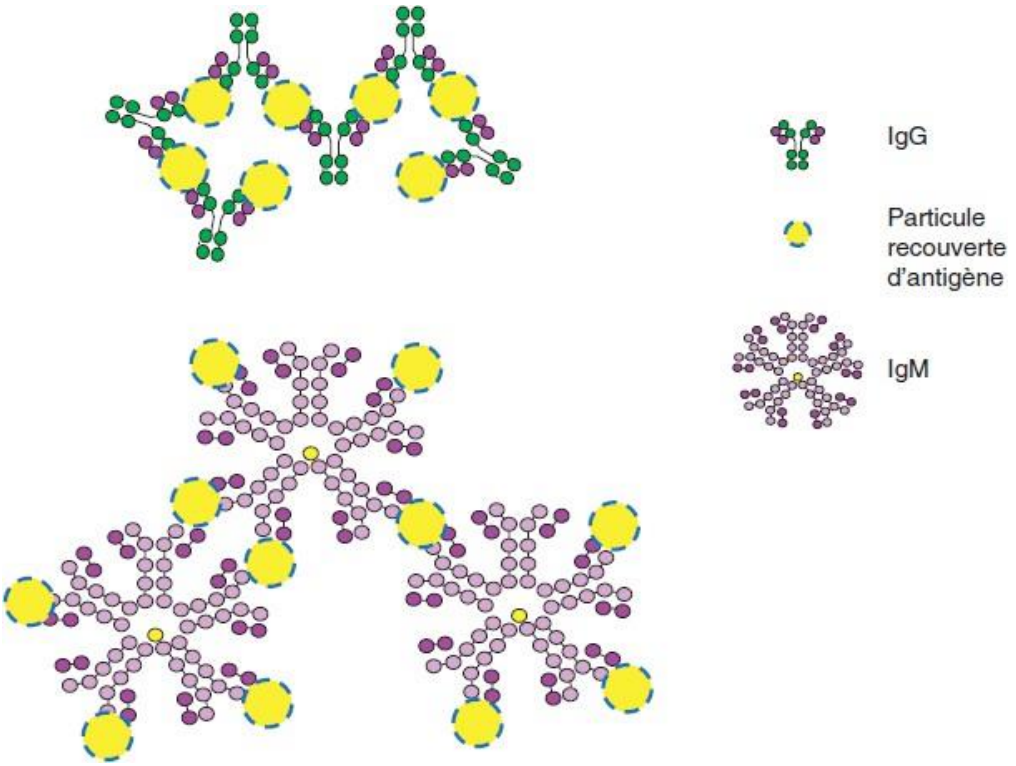

Figure 32: Example of direct agglutination with IgG and IgM agglutinating antibodies.

Application Examples

- **In research:** this technique can be applied to many antibody antigen combinations.
- **Clinical studies**
  - blood groups are determined by active or direct agglutination. This type of reaction is also applied to bacterial serogroups (e.g. Salmonella, Shigella, enteropathogenic E. coli);
  - the search for anti-hematocyte antibodies is carried out in the context of haemolytic anaemias by direct or indirect Coombs tests;
  - passive agglutination is used, for example, for the detection of antitoxoplasma antibodies in a serum after binding of a soluble toxoplasma antigen to latex beads;
  - microparticulate nephelometry is used for the determination of rheumatoid factors.

VIII.2 3. IEP

This method is a combination of protein electrophoresis and immunoprecipitation. The sample and a reference standard are first separated by electrophoresis. Then an antiserum is diffused perpendicularly to the electrophoretic separation. In the equivalence region, the formation of immune complexes leads to the appearance of precise precipitation lines. The intensity, shape and location of precipitation arcs are used to identify proteins. Immunoelectrophoresis is applied in case of suspicion of mono- or polyclonal gamma pathies. The polyclonal immunoglobulins are homogeneously distributed in the gamma globulin fraction after electrophetic separation. On the other hand, monoclonal immunoglobulins form a local peak in the gamma globulin fraction (M gradient); the latter manifests itself as a curvature in the rainbow

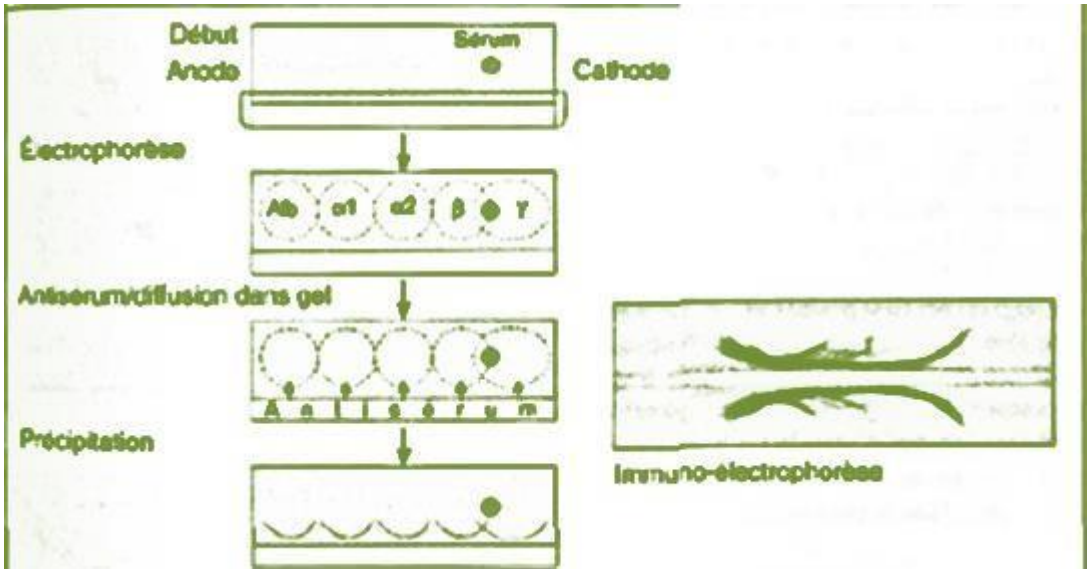

Figure 33: Schematic representation of immunoelectrophoresis steps.

VIII.2 4. Methods using a marker

Immunolabelling techniques are used to analyse Ag or haptens in very small quantities (sensitivity of the order of nmol/L). This principle is also used to study non-precipitating and non-agglutinating immune complexes. The main principle of labelling is to attach to one of the reagents a substance that will identify the immune complexes sought. The most used markers are:

- Fluorochromes (fluorescein or rhodamine)
- Isotopes, Radioactive
- Enzymes (alkaline phosphatase or peroxidase)

The emitted signals are detected (qualitative method) and can be measured (quantitative method).

A. Immunoenzymology: better known as ELISA (Enzyme Linked Immuno Sorbent Assay). In this reaction, the antigen or antibody is labelled with an enzyme, which makes it possible to transform a colourless substrate into a coloured product. The completion time varies from less than an hour to several hours. The reading is carried out using a spectrophotometer.

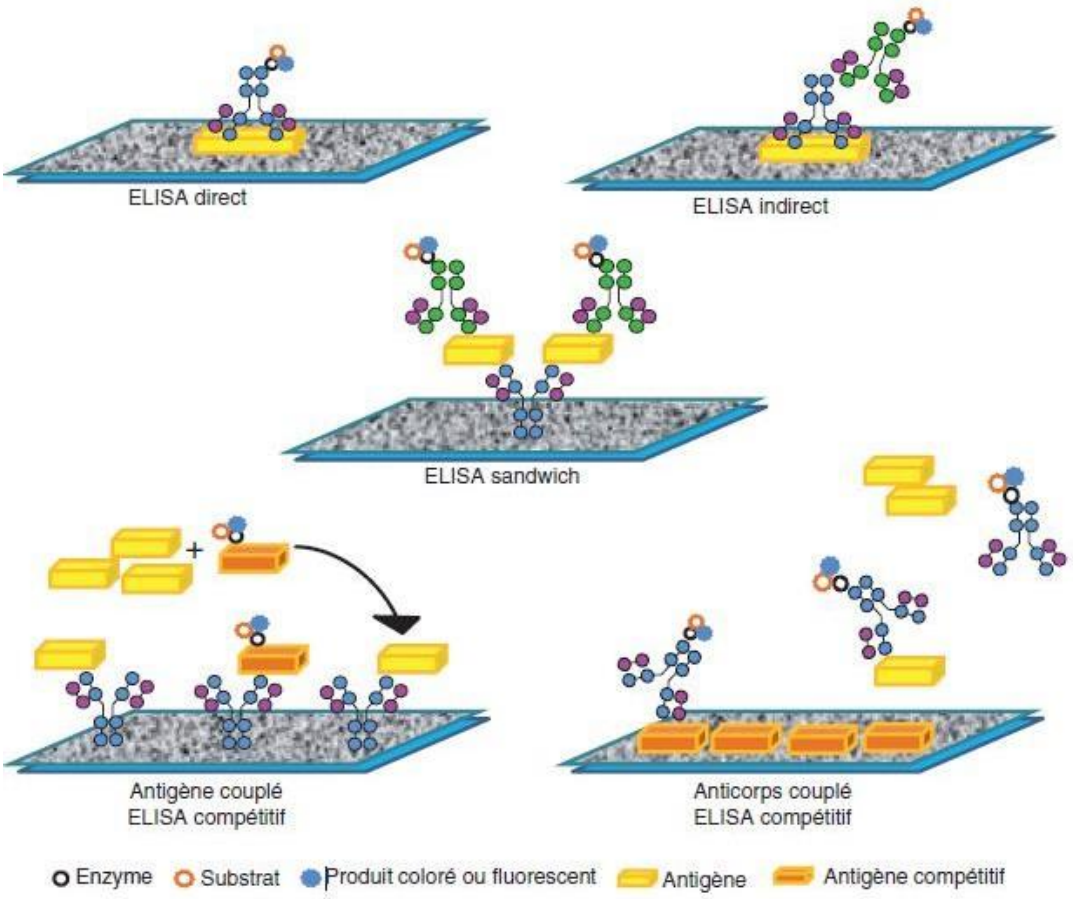

Figure 34: Schematic representation of the different types of ELISAs.

Application Examples

ELISA is a technique very commonly developed in the field of biological diagnosis or in basic research.

- **In research:** the determination of proteins of interest in cell supernatants or lysates is frequently carried out by ELISA.

- **Industrial and veterinary applications:** these techniques are also applied in the quality control of finished or in-process products, as well as in epidemiology and veterinary control.
- **Clinically:** ELISA is widely used for the serodiagnosis of infectious diseases and for the diagnosis and monitoring of autoimmune diseases.

B. Radioimmunology: also known as Ria (Radio-Immuno-Assay) based on the use of a radioactive marker. Radioimmunological techniques have been developed following the discovery of radioisotopes, which are very sensitive tracers. These techniques were initially developed for the determination of insulin and then other hormones. For a long time, iodine-125 has been the only radioactive marker used in the development and conduct of immunoassays for substances present at very low concentrations in biological fluids and tissues. It has been gradually supplanted by the use of non-isotopic markers (enzymatic and luminescent) offering greater ease of use without the regulatory restrictions related to the handling of radioactive products. The sensitivity of radioimmunological techniques is of the order of pmol/L or ng/mL. This sensitivity allows the assay of certain steroid hormones, vitamins, drugs, tumor markers or autoantibodies.

C. Immunofluorescence: Direct immunofluorescence uses antibodies labeled with the fluorescent reagent, while indirect immunofluorescence relies on a secondary antibody labeled with a fluorochrome to reveal the binding of the antigen-specific primary antibody. Direct immunofluorescence can detect two or more antigens simultaneously. On the other hand, indirect immunofluorescence has a higher sensitivity for the detection of weakly expressed antigens, since several molecules of the secondary antibody can bind to a primary antibody molecule. The cell membrane can be permeabilized by fixation to allow detection of cytosolic antigens. Immunofluorescence is also used for the analysis of suspended cells, tissue sections or preparations obtained using cytocentrifuges.

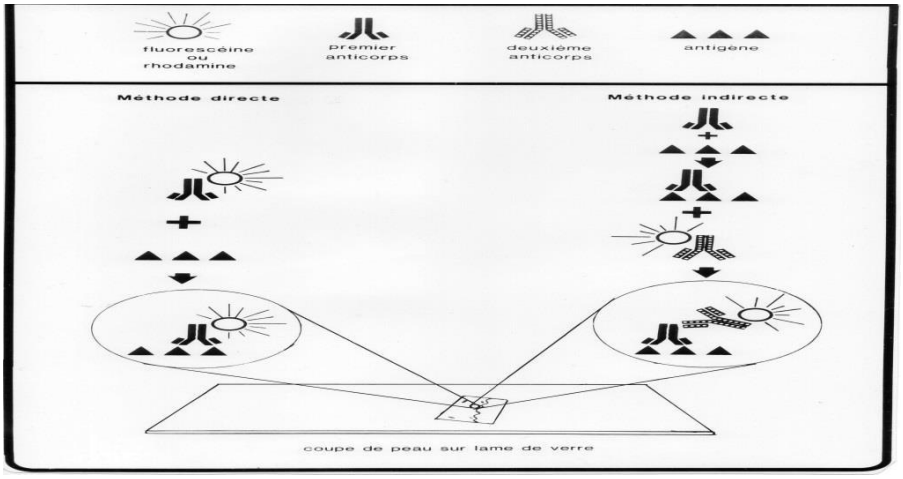

Figure 35: Schematic representation of the different types of Immunofluorescence.

Table 4. Characteristics of the different fluorochromes and tandems most used in immunofluorescence

Fluorochrome	Tandem	Couleur	Laser d'excitation	Longueur d'onde d'excitation (nm)	Longueur d'onde d'émission (nm)	Sensibilité
FITC : isothiocyanate de fluorescéine	Non	Vert	Bleu	495	519	Moyenne
PE : phycoérythrine	Non	Jaune	Bleu	480/565	578	Forte
PE-Cy5 : phycoérythriine-cyanine 5	Oui	Rouge	Bleu	480/565/650	670	Bonne
PE-Cy7 : phycoérythriine-cyanine 7	Oui	Rouge	Bleu	480/565/743	767	Bonne
PerCP-Cy5.5 : peridinin-chlorophylle/cyanine 5.5	Oui	Rouge	Bleu	490	675	Moyenne
ECD : phycoerythrine-Texas red	Oui	Orange	Bleu	486	620	Forte
Cy5 : Cyanine 5	Non	Rouge	Rouge	650	670	Moyenne
Cy5.5 : Cyanine 5.5	Non	Rouge	Rouge	675	694	Moyenne
APC : allophycocyanine	Non	Rouge	Rouge	650	660	Forrte
APC-Cy7 : allophycocyanine-cyanine 7	Oui	Rouge	Rouge	650/755	767	Faible
Pacific blue	Non	Bleu	UV/violet	403	455	Faible
Krome Orange®	Non	Orange	UV/violet	398	528	Forte
Horizon V500	Non	Bleu-vert	UV/violet	415	500	Forte