IMMUNOLOGY BASIC LABORATORY TESTS

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PREFACE

The textbook „Immunology – basic laboratory tests“ is targeted for medical students studying in English as well as for doctors, scientific research and health care workers. Immunology – basic laboratory tests is the outcome of the project KEGA 038UK-4/2019 Ministry of Education, Science, Research and Sport of the Slovak Republik.

Immunology – basic laboratory tests provide theoretical knowledge on the one hand. On the other hand, the most common practical methods used in immunological laboratories. It allows readers to choose their own pace of study and to get feedback through control questions included at the end of each chapter.

Laboratory techniques used in immunology make it possible to identify components of innate and acquired immunity, humoral and cellular immunity. The reasonable indication of laboratory tests and the ability to make a correct diagnosis on time is significant in terms of patient prognosis and prevention of irreversible tissue and organ damage with the need for increased long-term treatment costs. That is possible only thanks to quality preparation, acquisition of the necessary knowledge in the field of immunology and their interconnection with knowledge from other medical areas.

The acquisition of in-depth knowledge of laboratory diagnosis possibilities will be particularly beneficial in practice so that diseases can not only be identified and effectively treated, but also that individual components of the immune system can be appropriately stimulated if the patient's condition requires that.

Based on erudite knowledge, it is possible to rationally indicate laboratory examinations of direct and indirect diagnostics, reduce traumatization of the patient by unnecessary examinations and sample collections, and thus improve the quality of patient’s life.

MUDr. Martina Neuschlová, PhD.

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CELLS OF THE IMMUNE SYSTEM

1

1.1 HEMATOPOIESIS

Cells that provide an immune response are called **immunocompetent cells**. The cells of the immune system have different lifespans. Their loss is complemented by hematopoiesis. **Hematopoiesis is the formation and development of red and white blood cells from a pluripotent hematopoietic stem cell**. It is a process of creating and renewing blood cells, which go through the stages of proliferation, differentiation and maturation. **The myeloid and lymphoid lineages** of the multipotent cells are differentiated **from the pluripotent stem cell** (Figure 1).

Lymphocytes develop from the **lymphoid lineage**, other blood cells (granulocytes, monocytes, erythrocytes and thrombocytes) **from the myeloid lineage.** Adult hematopoiesis is performed in the bone marrow and is regulated by numerous cytokines (blood growth factors GM-CSF, M-CSF, G-CSF, erythropoietin, thrombopoietin, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9), also by some hormones and nerve effects. Vitamins and minerals are required for proper hematopoiesis.

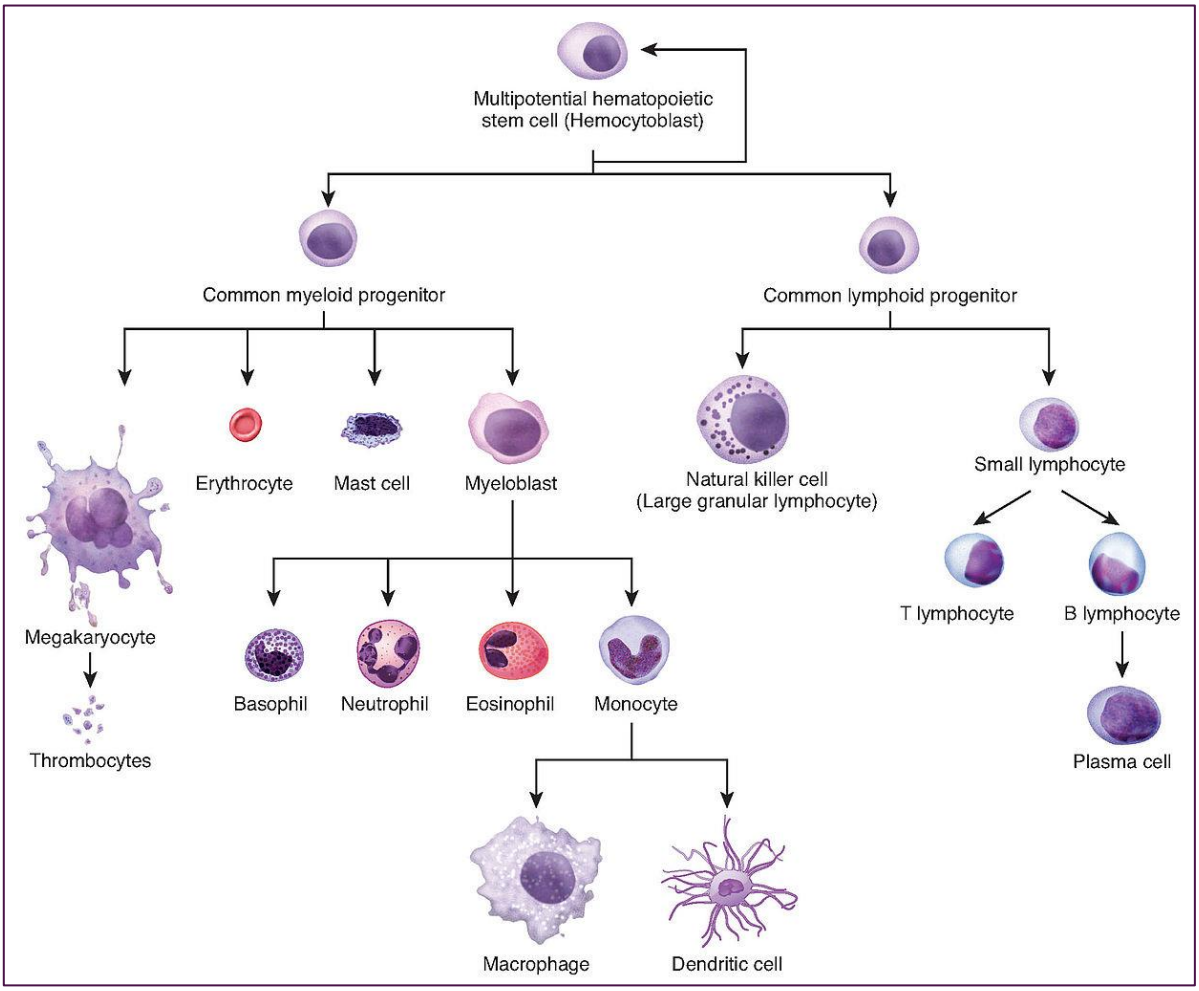


Fig. 1 Hematopoiesis

(Source:*https://upload.wikimedia.org/wikipedia/commons/thumb/9/9f/0337_Hematopoiesis_new.jpg/1246px-0337_Hematopoiesis_new.jpg)*

The percentage of individual blood leukocytes is shown in Table 1.

Tab 1. The percentage of blood leukocytes in a human (Source: Authors)

Myeloid linie	Granulocytes (PMNs)	Neutrophils	60 %
		Eosinophils	4 %
		Basophils	< 0,2 %
	Agranulocytes	Monocytes	8 %
Lymphoid linie	Lymphocytes do not contain granules	T-lymphocytes B-lymphocytes	25-40 %
	NK cells contain granules	5-10 % of lymphocytes	

1.2 CD MARKERS OF CELLS

Cells of the immune system are characterised by the presence of membrane differentiation antigens (glycoprotein antigens). These appear in the cell membrane during their development and are referred to as CD (**C**luster of **D**esignation).

A CD marker is a unique molecule or complex of molecules that a cell expresses. These molecules help to differentiate the cell type or subtype, its maturation stage, degree of activation, etc.

The name consists of the letters CD and numbers (e.g. CD1, CD2, ...). Some CD markers are specific to certain cells only and can, therefore, be used to isolate different cell populations more accurately. An overview of some important CD markers and cell types is given in Table 2. By November 2021, 417 CD markers have been officially known, and more are being added. An up-to-date overview of officially recognized CD markers is available at http://www.hcdm.org/index.php/molecule-information.

Tab 2. Overview of CD markers and cell types (Source: Authors)

CD2	immature T-lymphocytes
CD3	all T-lymphocytes (part of TCR)
CD4	T helper lymphocytes
CD8	T cytotoxic lymphocytes
CD14	Monocytes and macrophages
CD15	Neutrophils and eosinophils
CD16	NK cells, neutrophils
CD19	B-lymphocytes
CD38	Plasma cells
CD56	NK cells
CD58	Endotelial cells, antigen-presenting cells
CD68	Dendritic cells, macrophages
CD80 a 86	Antigen-presenting cells
CD203	Basophils, mast cells

1.3 POLYMORPHONUKLEAR LEUKOCYTES

Those are cells of the immune system originating from the myeloid lineage. They are characterized by a multilobed nucleus and named according to the presence of numerous granules in the cytoplasm, while divided based on different staining granules in microscopic evidence into three types:

- Neutrophils: their granules do not stain with either alkaline or acidic dyes
- Eosinophils: their granules are coloured red with acidic dyes
- Basophils: their granules are dyed with basic dyes to dark blue.

1.3.1 Neutrophils

Neutrophils (neutrophil granulocytes, microphages) account for more than half of all leukocytes (60% of leukocytes), 15 μm in size. Healthy people have about 5000 neutrophils in every drop of blood. Immature neutrophils have a nucleus in the shape of a rod; mature neutrophils have a segmented multilobed nucleus. There are two types of granules in the cytoplasm containing antimicrobial substances:

- **Primary azurophilic granules** contain bactericidal enzymes such as hydrolases, myeloperoxidases. neutral proteases
- **Secondary granules** contain lactoferrin, lysozyme, alkaline phosphatase, collagenase and others.

They belong to the most active leukocytes with a highly developed capacity of phagocytosis, are classified as professional phagocytes and are the primary cells of the 1st defence line against pathogens, foreign cells. They have an important role in engulfing and killing extracellular pathogens.

Neutrophils are potent cells of the immune system ("foot soldiers"). They are important in the defence against infection because they engulf and kill the bacteria. Neutrophils play an important role in the inflammatory process, as they rapidly mobilize on the site of inflammation; they are **the first cells arriving at the site of injury**. At the site of inflammation, molecules appear on the vascular endothelium to which neutrophils adhere firmly and cross through the blood vessel wall (diapedesis). Subsequently, they migrate to the tissue where the chemotactic signals come from. Substances released from the neutrophil granules have a strong bactericidal effect and serve to destroy the absorbed particles. **They can phagocytose but are not antigen-presenting cells** (Figure 2)**.**

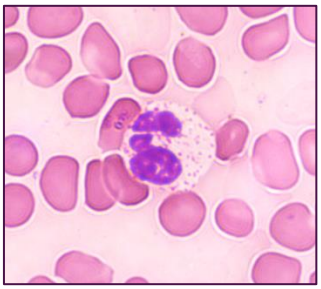


Fig. 2 Neutrophil (Source: *Martina Neuschlova*)

Neutrophils live only for a short time, less than a day, and do not have mitochondria. They use cytoplasmic glycogen as an energy source, which is a source of glucose in anaerobic glycolysis. Neutrophils can also act at the site of bacterial infection - in damaged tissue with a low oxygen content.

1.3.2 Eosinophils

Eosinophils (18 μm in size) represent 4% of the leukocytes. Healthy people have several hundred eosinophils in a drop of blood. In the cytoplasm, they have about 200 granules containing specialized proteins: the main basic protein, the eosinophilic cationic protein, the eosinophil peroxidase ..., which have a toxic effect on parasites (mainly worms) and tumour cells.

Their ability to **phagocytose is weak**. However, **upon activation, the active metabolites are released from the granules, prostaglandins, cytokines and leukotrienes, which damage the surface of the parasite**. The number of eosinophils is increased in parasitic infection, which is one of the laboratory diagnostic features. On the cell membrane of eosinophils, there are **receptors for immunoglobulins IgE and complement components.** They play an **important role in allergic reactions** as well as in chronic inflammation (Figure 3).

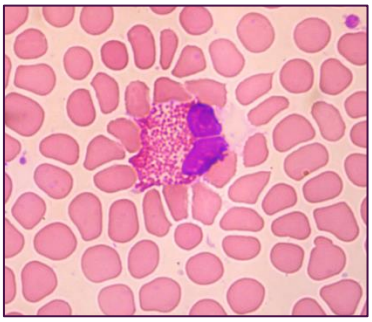


Fig. 3 Eosinophil (Source: *Martina Neuschlova*)

1.3.3 Basophils

Basophils represent only less than 0,2% of leukocytes. They circulate in the blood and are morphologically similar to mast cells. Basophils have granules in the cytoplasm that contain a large number of inflammatory mediators: histamine, heparin, etc. They do not contain peroxidase.

They are mainly activated in allergic reactions **(inflammation is a common part of allergic reactions). Histamine dilates blood vessels and is involved in the development of urticaria, which is often present in allergic reactions. There are receptors** for immunoglobulin IgE **in the cell membrane. Basophils** do not phagocytose (Figure 4).

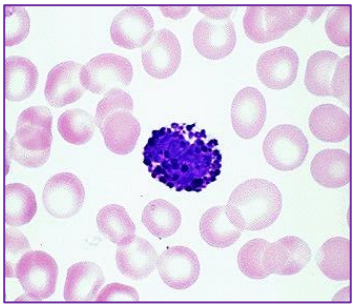


Fig. 4 Basophil (Source: *http://www.angelfire.com/ok3/apologia/drafts/basophil.jpg*)

1.4 MONONUCLEAR CELLS

1.4.1 Mast cells

Mast cells are tissue cells with a diameter of about 30 μm. They are mononuclear cells with metachromatically staining granules and have a relatively small core. They are found in connective tissue, especially in blood, lymphatic vessels and peripheral nerves. Mast cells are localized primarily near the epithelial surfaces of the respiratory, digestive system and skin exposed to allergens from the external environment. They are morphologically similar to basophils that circulate in the blood. Their granules contain many inflammatory mediators (heparin, histamine, etc.). These cells do not contain peroxidase.

Mast cells are **the most critical cells in allergic reactions**. In their cell membrane, there are **receptors for immunoglobulins IgE** (FcεRI). The first contact with the allergen sensitizes the individual, generating specific IgE antibodies that bind their Fc fragments to mast cells (or basophils) that have specific receptors for IgE antibody (FcεRI). Following subsequent exposure of an individual to the same antigen, the antigen binds to the bound IgE antibodies, causing degranulation of mast cells (or basophils) and **release of mediators of the allergic reaction into the extracellular space.** These mediators are of two kinds, primary and secondary:

- ✓ **Primary mediators** are those that are pre-formed and stored in granules: histamine, proteoglycans (heparin, chondroitin sulphate), proteases and some cytokines.
- ✓ **Secondary mediators** are those synthesized de novo. These include leukotrienes, prostaglandins, platelet-activating factor - PAF.

They result in contraction of smooth muscle, bronchi, significant vasodilation and fluid penetration into the extravascular space (Figure 5).

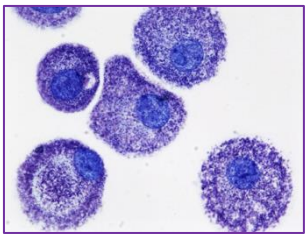


Fig. 5 Mast cells (Source: *http://www.mastcellaware.com/images/mastcells-lg-1.jpg*)

1.4.2 Monocytes

Monocytes (12-20 μm) represent ca. 6-8% of leukocytes (Figure 6) and do not contain granules – agranulocytes. They are mononuclear cells with vacuolized cytoplasm, which stains weakly basophilic grey-blue. They have a round or irregular shape with a large eccentrically located core, which is usually irregular (kidney-shaped). Healthy people have about 500 monocytes in a drop of blood.

They are equipped with several receptors for phagocytosis, through which they recognize particles to be absorbed and destroyed. The mature monocytes travel from the bone marrow into the blood, leave the bloodstream by diapedesis after three days, and move into tissues where they are converted to tissue macrophages that survive for several months (unless activated by the inflammatory process) and to dendritic cells.

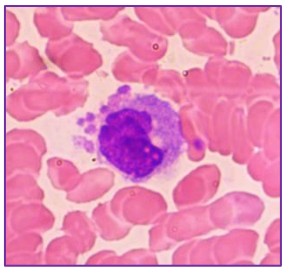


Fig. 6 Monocyte (Source: *Martina Neuschlova*)

1.4.3 Antigen-presenting cells – APC

Antigen-presenting cells (**A**ntigen **P**resenting **C**ells - APCs) include **dendritic cells, macrophages, and B-lymphocytes.** In particular, myeloid dendritic cells and macrophages can present antigen. B-lymphocytes also can present antigen but to a lesser extent.

1.4.4 Macrophages

Macrophages are motile mononuclear phagocytic cells. They are both professional phagocytes and **antigen-presenting cells**. The macrophage precursors are monocytes. Tissue macrophages are **important in the first line of defence against pathogens**. Fixed macrophages are immovable and are deposited in the binders, if necessary they can be converted to free macrophages that are capable of amoebic movement and phagocytosis.

They are found in different tissues and are named after their location. Most macrophages are located at strategic sites where microorganism invasion is expected, or foreign particle accumulation is likely. In 1892 Metchnikoff was the first one to describe macrophages based on their ability phagocytose microorganisms („large eaters“).

Macrophages:

- in connective tissue, they are called **histiocytes,**
- **Kupffer cells**in the liver**,**
- **osteoclasts** in the bones,
- **microglia** in the brain,
- **alveolar macrophages** in the lungs,
- **peritoneal and pleural macrophages** in serous cavities,
- in the mucosal tissue, they are part of **MALT** (mucosa-associated lymphoid tissue) and others.

Macrophages live long, several months unless activated by the inflammatory process or tissue damage. Their role is the phagocytosis, the presentation of antigens by T lymphocytes, regulation of inflammation, destruction of microorganisms, removal of dead cells, destruction of tumour cells and cells infected with fungi and parasites, production of various signal and antimicrobial proteins, support of wound healing and tissue restoration and maintains iron homeostasis. In the wound, they first engulf and kill bacteria, then produce the enzymes needed to break down damaged tissue and ultimately synthesize growth factors necessary for tissue remodelling.

1.4.5 Dendritic cells

Dendritic cells (DC) are **the most important antigen-presenting cells** (Figure 7), playing a critical role in initiating the acquired immune response. They act as the messenger cells of the immune system between the innate and the acquired immune response. Activated dendritic

cells represent an activation signal for naive T-lymphocytes. They were named for their characteristic appearance with numerous branchlike cytoplasmic projections (branches; dendron means a tree in Greek). Their precursors are monocytes (some dendritic cells may arise from lymphoid – also called plasmacytoid lineage cells).

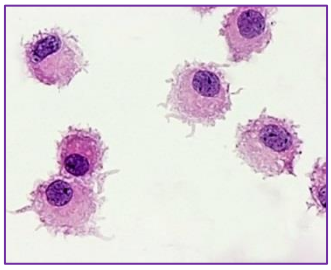


Fig. 7 Dendritic cells (Source: *http://www.amsvans.com/blog/wp-content/uploads/2013/06/tip-dendritic-cells-stained-purple-multiple-sclerosis-ms-study.jpg*)

They are localized primarily in non-lymphoid peripheral tissues at the site of contact with the external environment. **Dendritic cells** are found mainly in skin tissues (called Langerhans cells), respiratory and gastrointestinal mucosa (interstitial DC), afferent lymphatic vessels, in peripheral blood circulation, in secondary lymphatic tissues, in the medulla of the thymus (thymus DC). They can be divided into mature and immature.

Immature dendrite cells are not able to activate T-lymphocytes effectively. However, they capture large amounts of soluble and corpuscular particles by phagocytosis and macropinocytosis. In phagolysosomes, the absorbed material is processed into peptides. Dendritic cells serve as search engines. Their protuberances extend between epithelial cells. They are located at potential microbial portals of entry and **serve as sentinel**. When immature dendritic cells sense an invasive threat, they rapidly begin to mature. They migrate to regional lymph nodes if they capture the antigen. They also process the captured antigen and display it as a peptide bound to MHC class II. molecules (pMHC class II.) to Th-lymphocytes. This binding represents the first signal required for the activation of naive Th-lymphocytes. During this process, the dendritic cells mature. As a result, co-stimulatory molecules are overexpressed in dendritic cells that react with partner Th-lymphocyte molecules. These co-stimulatory interactions represent the second signal required for optimal activation of naive Th-lymphocytes into Th-lymphocyte subpopulations that are capable of releasing various cytokines and thereby regulating the immune response. However, not all antigens enter cells via phagocytosis. Some are bound to the surface of the target cell and subsequently degraded by proteasomes, then presented in complex with MHC class I. to cytotoxic Tc-lymphocytes.

Mature dendritic cells present an antigen according to whether it is:

- an extracellular antigen (exogenous antigen presentation pathway – DCs present antigen on their surface in complex with MHC class II. molecules) or
- an intracellular antigen (endogenous antigen presentation pathway – autologous, viral, tumour antigens are presented by dendritic cells in complex with MHC class I. molecules).

Dendritic cells are divided into myeloid and plasmacytoid cells. Myeloid dendritic cells (mDC) are found in lymphoid organs, sites where antigens are most likely to enter, in the skin,

respiratory and gastrointestinal tract. Their most essential biological role is antigen presentation and subsequent polarization of the immune response. Plasmacytoid dendritic cells (pDC) circulate in the blood and have also been found in peripheral lymphatic organs. They produce large amounts of interferons (mainly alpha), which leads to the activation of cells of the immune system, namely activation of NK cells and macrophages, and leads to the intensification of phagocytosis. Interferons are important for generating an antiviral immune response. Another group is follicular dendritic cells, which are so named according to their location in lymphatic follicles, where they form a microarchitecture (network), and although they also have long "dendritic" outgrowths, they are not really dendrite cells. They do not have a hematopoietic origin, do not express MHC class II. molecules, do not phagocytose or even present an antigen. They capture antigens opsonized by complement or antibodies. They are involved in the formation of future B memory cells.

1.4.6 Lymphocytes

Lymphocytes (Figure 8) represent 25-30% of all leukocytes. They are agranulocytes derived from the lymphoid lineage.

Lymphocytes mediate **specific immune responses** and occur mainly in lymph fluid and all organs of the lymphatic system. They are round cells with a large round to oval nucleus and a narrow border of the cytoplasm that stains light blue. The nucleus occupies almost the entire cell, consisting of a dense, compact network of chromatin. These cells can be small, medium or large. A small mature lymphocyte is the smallest leukocyte (8-12 μm). Depending on the function, membrane markers (CD markers) and surface antigen-specific receptors (TCR, BCR), different types of lymphocytes are distinguished: T lymphocytes, which make up 65-75% and B lymphocytes, which make up 20-30%. In a light microscope, they are indistinguishable, but can be distinguished by a flow cytometry-based on surface membrane markers. **The basis for classification** is the presence of membrane markers (CD markers) and expression of antigen-specific receptors (TCR, BCR).

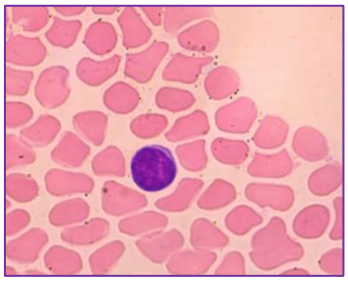


Fig. 8 Lymphocyte (Source: *Martina Neuschlova*)

T-lymphocytes

T-lymphocytes develop from a stem pluripotent hematopoietic cell in the bone marrow and gain their final morphological and functional form upon migration to the thymus.

They are responsible for cell-mediated specific immunity (cellular). T-lymphocyte antigen receptors recognize peptide fragments of protein antigens that are bound to HLA molecules on the surface of antigen-presenting cells. By expressing a different antigen receptor (TCR), T-lymphocytes are divided into two basic populations: the TCRαβ receptor and the TCRγδ receptor. TCRαβ receptor T-lymhocytes are predominant (95%) compared to TCRγδ receptor T-lymphocytes (5%). T-lymphocytes having a TCRαβ receptor are further divided into helper (Th, helper), cytotoxic (Tc) and natural regulatory (nTreg) lymphocytes. They all have CD2 and CD3

membrane markers. For helper Th-lymphocyte subpopulation the CD4 is a typical marker, for cytotoxic Tc-lymphocyte subpopulation it is CD8 marker and regulatory nTreg-lymphocytes have CD25 marker.

- Helper Th-lymphocytes assist B-lymphocytes in the production of antibodies and assist phagocytes in destroying the ingested microorganisms.
- Cytotoxic Tc-lymphocytes kill surviving microorganisms intracellularly.
- Another group of T-lymphocytes are natural regulatory Treg-lymphocytes, which suppress the immune response and contribute to maintaining tolerance to self-antigens and preventing the development of autoimmune processes.

T-lymphocytes differentiate into **effector cells (Te) and memory cells (Tm)** upon antigen activation. Naive T cells must be activated by an antigen presented to them by activated APC, which also expresses co-stimulatory molecules. **The antigen is recognized by the TCR receptor in the form of a peptide fragment bound to the MHC molecule (pMHC) on the surface of the APC**. TCRs cannot recognise soluble molecules. The TCR can only recognize an antigen in association with MHC class I. (endogenous antigen presentation pathway) or MHC class II. molecules (exogenous antigen presentation pathway). T-lymphocyte activation can then be performed if the APCs simultaneously express the co-stimulatory molecules needed as a second signal for naive T-lymphocytes activation. The interface between APC and the naive T cell is called the **immunologic synapse.**

Effector T-lymphocytes directly attack and eliminate virus-infected or tumour-altered cells. They direct the cooperation of different immune cells by synthesizing molecules that help other cells to destroy the pathogen.

Memory T-lymphocytes survive in the body for many years. Upon repeated encounters with the antigen, they provide a rapid and more intensive immune response. A characteristic feature of T-lymphocytes is their constant movement. They leave the thymus and enter the secondary lymphoid organs (spleen, lymph nodes) with blood, from where they travel to the tissues, return to the lymph nodes and finally into the bloodstream. This cycle is performed at an average of 24-48 hours.

NK cells

NK (natural killer) cells - are a special lymphocyte population, derived **from the lymphoid lineage**. They make up 5-15% of the total lymphocyte population in peripheral blood. They differentiate in the bone marrow and some may also form in the thymus. Morphologically, it is a heterogeneous population with the highest proportion of large granular cells with a diameter of 10-12 μm. They have the membrane marker CD16, CD56, CD57, they do not have the CD3 marker (which is otherwise typical for all T-lymphocytes). Characterized as non B and non T cells, they do not express TCR, BCR. They have the ability to non-specifically recognize and kill certain foreign cells.

NK cells are **components of the innate immunity (cellular component**). They are able to rapidly attack infected cells without previous sensitization. NK cells play **an important role in anti-tumour and anti-viral immunity** and are involved in the **regulation of immune**

processes through their production of numerous cytokines. They are a major effector of antibody-dependent cytotoxicity (ADCC – **a**ntibody-**d**ependent **c**ellular **c**ytotoxicity) and contain an antibody receptor (Fc). The released cytokines IL-2, IFN-γ increase the proliferation and cytotoxic activity of NK cells. Under the influence of interleukin IL-2 production, they differentiate into a more aggressive group of cells, the so-called LAK cells (lymphokine-activated killer cells). IFN-γ activates macrophages to more efficient phagocytosis of absorbed microorganisms. NK cells and macrophages are one example where two cell types cooperate effectively to eliminate intracellular pathogens. Macrophages engulf microorganisms and produce IL-12. IL-12 activates NK cells to secrete IFN-γ, which in turn activates macrophages to destroy engulfed microorganisms.

NK cells play a key role in tumour rejection, immune surveillance, resistance to infections, and regulation of immune responses. Destruction of tumour and virus-infected cells involves their recognition (NK cells empty the contents of their cytoplasmic granules into the extracellular space at the site of contact with the infected cell). Released proteins such as perforin, granzyme, serine esterase penetrate into cancer or infected cells and ultimately destroy them. NK cell is then re-released and can re-bind to another cancer cell, so the whole process can be repeated (Figure 9).

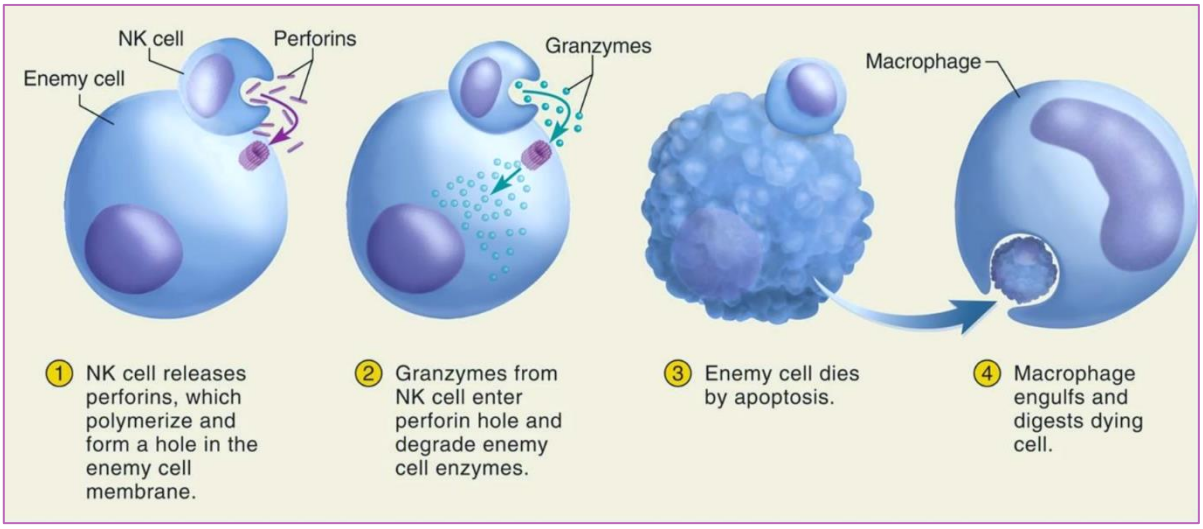


Fig. 9 Destruction of tumour and virus-infected cells by NK cells (Source: according to: *https://classconnection.s3.amazonaws.com/933/flashcards/2828933/jpg/immune_surveillance--nk_cells-144BCA0EDA02A2CDE51.jpg*)

NKT cells

NKT cells represent a smaller separate T cell subpopulation (about 0.01-1% in the blood). They mature in the thymus, recognize different types of antigens and are involved in the modulation of the immune response. After stimulation, they can produce many cytokines very rapidly. They have the ability to lyse cells, similarly to NK cells, which are particularly useful in anti-tumour and antimicrobial immunity.

K cells

K cells (killer cells) are rather a functional type of leukocytes that are capable of destroying a cell in conjunction with an antibody bound to it. K cells participate in ADCC response - antibody-dependent cellular cytotoxicity. K cells are components of innate immunity. They have FcR (Fc receptor) on their surface and are therefore able to bind Fc part of the antibody. If this antibody has bound to the antigen on a cell surface, effector ADCC cell can bind to the Fc part of this antibody and kill the antigen-bearing cell.

B-lymphocytes

B-lymphocytes are found in peripheral blood at the amount of 20-30% of lymphocytes. They develop from a stem pluripotent hematopoietic cell in the bone marrow, where they also mature. They contain the membrane feature CD19.

The mature B cells carry **immunoglobulin molecules on their surface that serve as** a specific receptor for the **BCR antigen**.

They are **responsible for the humoral part of specific immunity**. In the presence of thymus-independent antigen (which activates B cells without help from T cells) or in the presence of thymus-dependent antigen (which activates B cells with help from T cells), B-lymphocytes are differentiated to **plasma cells** that produce a large number of specific antibodies (B-lymphocytes alone do not have the ability to produce antibodies). Part of the activated B-lymphocytes does not differentiate into plasma cells, but they remain in the body for a relatively long time as **memory B-lymphocytes**.

Plasma cells

Plasma cells have an eccentrically located nucleus, a large amount of cytoplasm with numerous mitochondria, a lot of massive endoplasmic reticulum, and a Golgi apparatus.

Plasma cells are differentiated effector cells of the B-lymphocyte line adapted for efficient antibody production. Their role is to **produce antibodies of the same specificity as the original B-lymphocyte from which they developed**. Plasma cells have their own antigens that are not found on its B cell precursors, do not have surface Ig receptors and have membrane CD38 marker on their surface. They only live for a few days. The antibody that is produced by a particular plasma cell (and by its specific clone) may be of any class (IgM, IgG, IgA, IgE, IgD), but will only bind the antigen that induced its production (Figure 10).

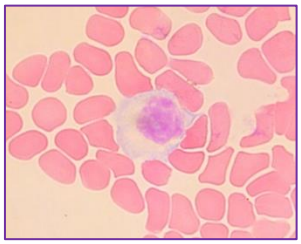


Fig. 10 Plasma cell (Source: *Martina Neuschlova*)

1.5 ISOLATION OF MONONUCLEAR CELLS FROM PERIPHERAL BLOOD

Isolation of mononuclear cells from peripheral blood is accomplished **by a separation procedure by gradient centrifugation** with the aim to separate mononuclear cells (lymphocytes and monocytes) from erythrocytes and granulocytes.

Procedure: Uncoagulated venous blood is used, which is diluted 1:1 in physiological saline in a test tube and then gently layered over a separation dilution (e.g. ficoll-verographin, telebrix) and centrifuged (2000 rpm). During centrifugation, cells with a lower weight than the separation solution remain above its level and cells with a higher weight drop to the bottom of the tube. Plasma is located above the separation solution and the mononuclear cell layer (Figure 11).

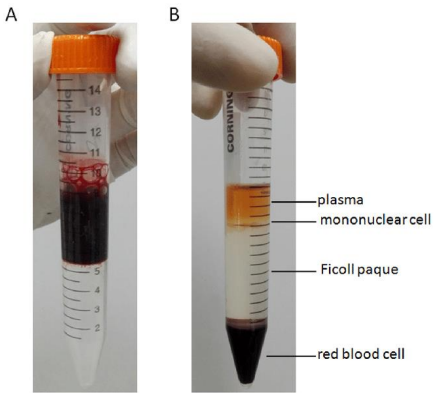


Fig. 11 Isolation of mononuclear cells by separation solution (Source: according to: *https://www.researchgate.net/figure/Images-of-cells-collected-from-the-interface-between-the-Ficoll-Paque-PLUS-and-plasma-A_fig1_308754834)*

Result: The mononuclear cells are carefully harvested from the interface between the separation solution and plasma using a sterile Pasteur pipette, and transferred to sterile centrifuge tubes. The suspension thus obtained is centrifuged, the sediment is washed twice with saline, and the cells are counted in a Bürker chamber. Under physiological circumstances, 1-2 x 10^6 mononuclear cells are obtained from 1 ml of blood. Currently, there are several modifications of this classical method that allow more accurate isolation of various cell populations.

1.5.1 Separation of T-and B-lymphocytes by rosette formation assay

T- and B-lymphocytes have different membrane receptors for binding to erythrocytes of other animal species. **T-lymphocytes have a receptor** (CD2) on their surface **for sheep erythrocyte** (LFA3) and form **E-rosettes** (Figure 12). **B-lymphocytes have a receptor for mouse erythrocytes** and form **M-rosettes.**

Procedure: First, erythrocytes are treated chemically (e.g. neuraminidase) to achieve a stronger binding to human lymphocytes. The animal erythrocytes, when mixed with human lymphocytes, bind to the lymphocyte surface and form a star-like formation called rosettes. The lymphocyte is considered as a rosette with at least three erythrocytes bound. Erythrocytes are arranged around a central lymphocyte to form rosette (that looks like a flower).

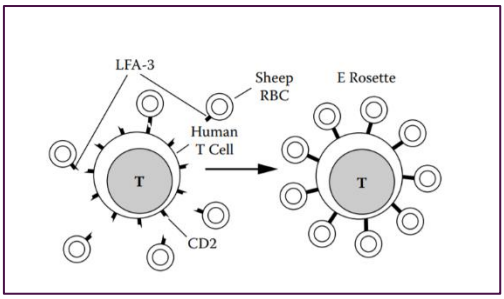


Fig. 12 E-rosette-forming T-lymphocyte, scheme

(Source: according to: *Cruse JM, Lewis RE. Illustrated Dictionary of Immunology, Third Edition. CRC Press Taylor & Francis Group 2009; 816 pp. ISBN 978-0-8493-7987-1*)

The formed rosettes are then separated from lymphocytes that have not bound erythrocytes (by gradient centrifugation). In this case, non-rosetting lymphocytes remain above the separation solution and the rosetting lymphocytes sediment below the separation solution.

Result: This procedure yields a lymphocyte suspension with a purity of about 95%. A number of lymphocytes and rosettes is counted in the Bürker chamber (Figure 13).

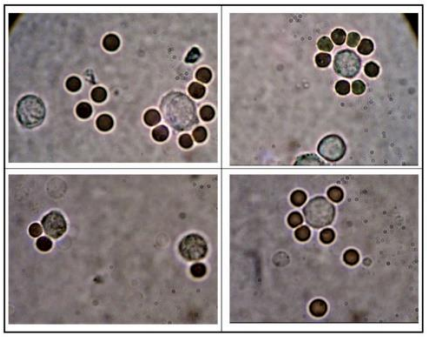


Fig. 13 E-rosette-forming T lymphocyte. Lymphocyte binned with more than 3 sRBC considered positive cell (1000x)

(Source: according to: *https://www.researchgate.net/figure/E-rosette-forming-T-lymphocyte-Lymphocyte-binned-with-more-than-3-sRBC-considered_fig1_304558232*)

The rosette was used as an early method to enumerate T cells (i.e., in the formation of E rosettes, in which CD2 markers on human T lymphocytes adhere to and surround LFA-3 molecules on sheep red blood cells to give a rosette arrangement).

Rosette tests allow distinguishing two types of rosettes, active and total, depending on the ratio of erythrocytes to lymphocytes and incubation time.

- **Active rosettes** have higher avidity of receptors for sheep erythrocytes (even with a small number of erythrocytes) and are formed immediately after the addition of erythrocytes.
- **Total rosettes** are detected after 18-24 hours of incubation time with an excess of erythrocytes (reflecting approximately the number of T-lymphocytes).

Active rosettes are an early marker for lymphocyte activation. They are **better-reflected patient's immune status.**

1.5.2 Separation by flow cytometry

Flow cytometry (**Flow** – cells in motion, **Cyto** – cell, **Metry** – measure) is a standard method for analyzing a single cell population. The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected, counted and sorted. The cell suspension is most often labelled with monoclonal antibodies bind to a fluorescent molecule. Fluorescently labelled monoclonal antibody molecules bind to the antigens of the examined cells. So labelled suspension is placed in a flow cytometer. A suspension of the labelled cells is excited by the laser to emit light at varying wavelengths. Up to thousands of particles per second can be analysed as they pass through the liquid stream. Several detectors are carefully placed around the stream, at the point where the fluid passes through the light beam. Obtained data is then analyzed by a computer that is attached to the flow cytometer using special software (Figure 14).

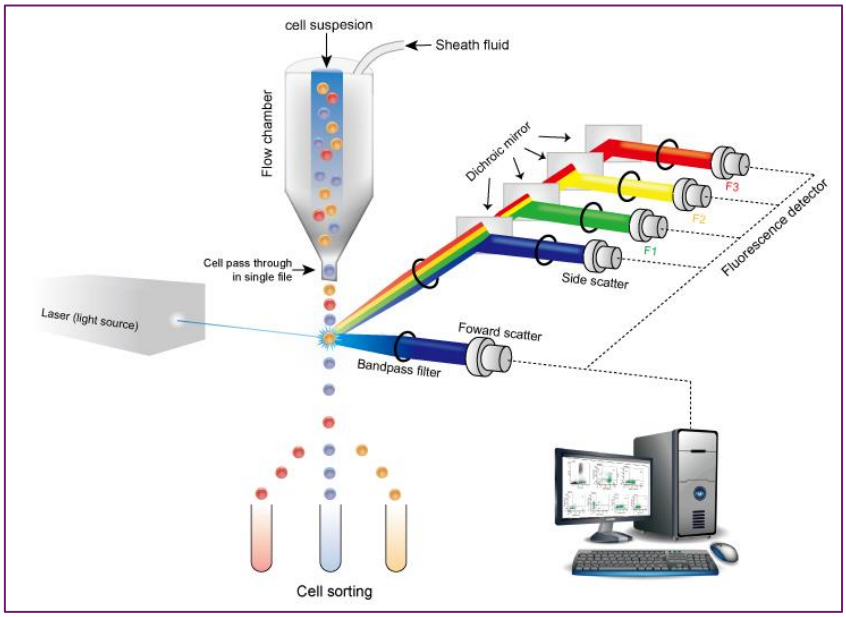


Fig. 14 Flow cytometry – schema

(Source: according to: *https://www.creative-diagnostics.com/images/Flow-cytometry-guide-1.jpg*)

Flow cytometry is a sophisticated device measuring multiple physical characteristics of a single cell such as size and granularity simultaneously as the cell flows in suspension through a measuring apparatus. Its working depends on the light scattering features of the cells under investigation, which may be derived from several dyes or monoclonal antibodies targeting either extracellular molecules located on the surface or intracellular molecules inside the cell. This approach makes flow cytometry an important tool for detailed analysis of complex cell populations in a short period of time.

Flow cytometry represents the gold standard for the determination of cell populations. It is a fast, sensitive, automated method for statistical processing.

Questions for self-assessment

1. What is hematopoiesis? Which cells of the immune system originate from the myeloid lineage and which from the lymphoid lineage?
2. What is characteristic of polymorphonuclear leukocytes? Which of them comes first to the place of damage? Which ones have receptors for IgE immunoglobulins on the membrane?
3. What is typical of mast cells? What responses do they play an important role in and why? Which mediators do they release into the extracellular space?
4. Which cells differentiate from monocytes? Which cells are antigen-presenting (APCs)? Where are dendritic cells located mainly? What is their role?
5. Which cells originating from the lymphoid line are without granules? Which immune responses do they mediate? What is the basis for lymphocyte classification?
6. What are the T-cell subpopulations? To what cells do T-lymphocytes differentiate after antigen activation? For which part of the immune response are T-lymphocytes responsible?
7. Which lymphoid lineage cells are important in anti-tumour and anti-viral immunity?
8. Which lymphoid lineage cells are responsible for the humoral part of specific immunity? What receptors do they carry on their surface? To what cells do they change in the presence of antigen?
9. Describe the principle of isolation of mononuclear cells from peripheral blood. Describe the principle of separation of T- and B-lymphocytes based on rosette formation and with the help of the flow cytometry.

Cells of the immune system https://portal.jfmed.uniba.sk/articles.php?aid=395

The interactive presentation contains study material about cells that provide immune responses and a set of test questions. Through these questions, the students can verify the acquired knowledge with feedback.

LABORATORY METHODS IN IMMUNOLOGY

2

Laboratory techniques used in immunology are either aimed at detecting and finding the number of antigens or antibodies in body fluids and tissues, or for determining immune system cells and their functions. Immunological examination laboratory techniques are also divided into humoral and cellular.

- In humoral immunoassays, serum or plasma may be most frequently assessed. Measurement of total immunoglobulins is a first-line screening investigation in suspected humoral immunodeficiency.
- Cellular immunoassays are more problematic than humoral assessment; most often, whole blood is collected in a tube with an anticoagulant. Both can undertake measurement of cell-mediated immunity *in vitro* (flow cytometry, interferon-gamma release assays, ELISPOT) and *in vivo* (delayed-type hypersensitivity skin testing) methods.

However, in addition to these materials, various body fluids (cerebrospinal fluid, effusions, bronchoalveolar lavages and others) or tissues (biopsy specimens) may be examined for needs of immunological diagnosis.

2.1 COMPLEMENT

Complement is a complex of approximately 30 serum glycoproteins that form **one of the essential effector systems of innate non-specific immunity**. They are mainly synthesized in the liver, less in mononuclear phagocytes, spleen and bone marrow. The **main components** are serum proteins **C1 to C9**. These serum proteins **are activated step by step by converting from the inactive form to the active enzymes** that act on other components of this cascade. The proper functioning of the complement system depends on the perfect coordination of all components (Figure 15).

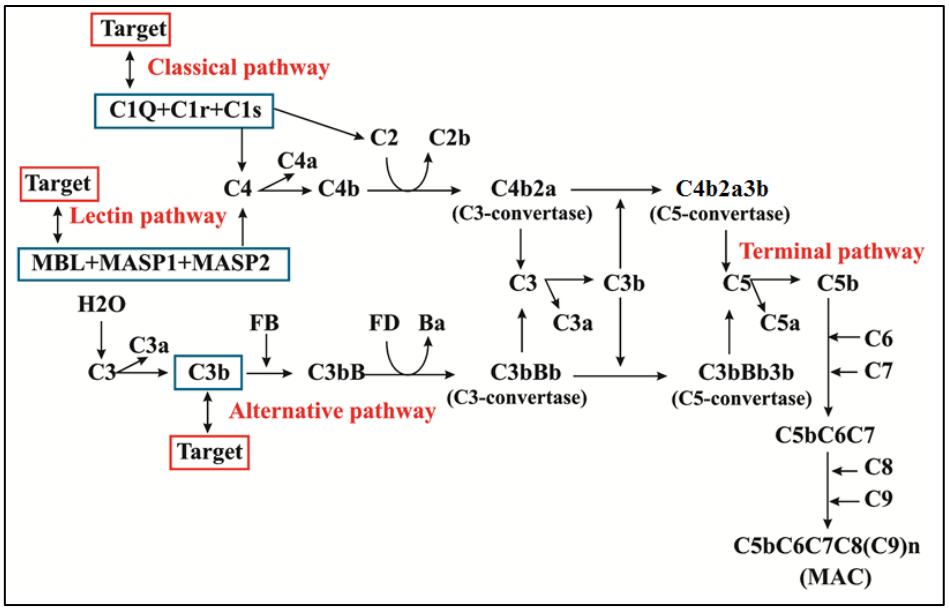


Fig. 15 Complement activation pathways
(Source: *http://www.intechopen.com/source/html/43780/media/image1.jpeg*)

The essence of the activation of the individual components of complement is the conversion of the first inactive component C1 into an active proteolytic enzyme, which cleaves the molecule of the following component into two fragments (larger fragment b, smaller fragment a - except C2 component). One of them has proteolytic activity and cleaves the following complement component. This fragment with proteolytic activity is designated fragment b, is larger and remains bound to the antigen surface. The smaller fragment referred to as the fragment a, has a different biological activity. It is smaller than fragment b and acts in the liquid phase. The horizontal line above the complement component or complex indicates enzymatic activity. Some components are not numbered but labelled as factors (factor B, D, P = properdin, H, I). A fragment with lost biological activity (inactive) is designated at the beginning as "i" (e.g. iC3b). Some components are labelled by function (MBP, MASP).

There are three complement pathways: **classical, alternative, or mannose-binding lectin pathways**.

The central component of complement is glycoprotein C3. All three pathways merge at C3, which is then converted into C3a and C3b. C3b fragment binds to the surface of microorganisms. **The end result of this cascade is a massive amplification of the response and activation of the cell-killing membrane attack complex (MAC) and lysis of the target cell.**

2.1.1 The classical complement pathway

The classical complement activation pathway begins with C1 activation. It is **triggered by the binding of IgM or IgG antibodies to the antigen surface**. Upon binding of the antibody to the antigen, a conformational change in the immunoglobulin molecule occurs and the binding site for the C1q complement component (this binding site is found in the Fc portion of the IgM or IgG molecules) is uncovered. Activation occurs when C1q binds to these molecules, then the conformation of C1r and C1s, which become serine proteases, is altered to form an activated complex containing all of these C1qrs components (see Figure 15). This C1-complex cleaves C2 and C4, which then form C3 convertase (C4b2a). C3 is then cleaved by the C3 convertase and forms C5 convertase in association with C4b and C2a (C4b2a3b). C5 convertase cleaves C5 into C5a and C5b. C5b binds to C6, C7, C8, C9 and membrane attack complex is generated. **In the classical activation pathway, there is a "start-up" time shift of 4-5 days.**

2.1.2 The alternative complement pathway

The alternative activation pathway is evolutionarily the oldest, **triggered by external factors, especially of bacterial origin** (see Figure 15). It can be activated by components of bacterial cell walls of gram-positive and gram-negative bacteria (lipopolysaccharides, peptidoglycan), bacterial exoproducts, fungal surfaces (zymosan), viruses and virus-infected cells, tumour cells, some parasites etc. The alternative complement pathway begins with the activation of C3 and requires factor B and factor D.

2.1.3 The mannose-binding lectin pathway

The mannose-binding lectin pathway was the last one to be discovered. This pathway is similar to the classical pathway of activation, but **the activator is** not the antibody but **the serum mannose-binding lectin** (MBL - mannose-binding lectin). **MBL** is an acute phase inflammatory protein that increases during inflammation. It **binds mannose, which is a typical surface component of many microorganisms**. MBL binds to the surface of the microorganism, the MASP-1 and MASP-2 proteinases bind to it, thereby forming a complex similar to C1qrs, and a cascade of activation may continue as in the classical pathway (see Figure 15). However, in this case, the presence of antibodies is not required as in the classical pathway of activation.

2.1.4 Biological effects of complement

In the activation of complement, many mechanisms are activated that are of great importance in defence of the body, especially against infection. Individual components of the complement have the properties of chemotaxins, opsonins, they regulate the process of phagocytosis and inflammatory reaction, induce histamine release, participate in lysis of microorganisms, in the release of biologically active substances, in the formation of second signals for initiation of the specific immune responses (see Table 3).

Tab. 3 Biological effects of complement (Source: Authors)

Biological effects of complement	Complement components
Lysis of microorganisms	C5b-C9, MAC
Inflammation:	
- degranulation of mast-cells and basophils	C3a, C4a, C5a
- degranulation of neutrophils	C5a
- degranulation of eosinophils	C3a, C5a
Extravasation and chemotaxis of leukocytes	C3a, C5a, C5b
- platelet aggregation	C3a, C5a
- release of hydrolytic enzymes from neutrophils	C5a
- increased expression of CR1 and CR3 on neutrophils	C5a
Opsonization, stimulation of phagocytosis	C3b, C4b, iC3b
Neutralization of viruses	C3b, MAC

Some major functions of complement are:

1. Opsonization and phagocytosis

C3b, bound to immune complex or coated on the surface of the pathogen, activate phagocytic cells. These proteins bind to specific receptors on the phagocytic cells to get engulfed.

2. Cell lysis

Membrane attack complex formed by C5b6789 components ruptures the microbial cell surface, which kills the cell.

3. Chemotaxis

Complement fragments attract neutrophils and macrophages to the area where the antigen is present. These cell surfaces have receptors for complements, like C5a, C3a, thus, run towards the site of inflammation, i.e. chemotaxis.

4. Activation of mast cells and basophils and enhancement of inflammation

The proteolytic complement fragments, C5a, C4a, and C3a induce acute inflammation by activating mast cells and neutrophils. All three peptides bind to mast cells and induce degranulation, with the release of vasoactive mediators such as histamine. These peptides are also called anaphylatoxins because the mast cell reactions they trigger are characteristic of anaphylaxis. Binding to specific complement receptors on cells of the immune system, they trigger specific cell functions, inflammation, and secretion of immunoregulatory molecules.

5. Production of antibodies

B cells have a receptor for C3b. When C3b binds to B-cell, it secretes more antibodies. Thus C3b is also an antibody-producing amplifiers which converts it into an effective defence mechanism to destroy invading microorganism.

6. Immune clearance

The complement system removes immune complexes from the circulation and deposits them in the spleen and liver. Thus it acts as an anti-inflammatory function. Complement proteins promote the solubilization of these complexes and their clearance by phagocytes.

2.2 DEFECTS OF COMPLEMENT SYSTEM

Increased activity of the complement system due to unregulated activation, as well as reduced activity due to genetically determined or acquired deficiency of any component of the complement may lead to pathological manifestations.

Elevated levels of individual components are found very rarely. In most cases, **only C3 and C4 components** are detected. Their elevated levels may be an expression of inflammatory activity because complement components react similarly to acute-phase inflammatory proteins.

Decreased complement activity may be caused, on the one hand, **by inherited complement deficiencies** that are classified into two general categories:

1) integral component defects (the lack of some components of the complement) and
2) regulatory component defects.

On the other hand, decreased complement activity may be caused **by acquired disorders** of the complement components, inhibitors or complement receptors.

Complement related Diseases

Diseases associated with complements can be due to the deficiencies in any of the protein components or regulatory components.

Some examples of complement protein deficiencies are:

Deficiency of C2 and C4 can cause SLE (systemic lupus erythematosus). Deficiency of C3 and factor D can cause pyogenic bacterial infection. Deficiency of C5-C9 (or MAC deficiency) may lead to neisserial infections, like gonorrhoea or meningitis.

Deficiencies of regulatory proteins lead to too much activation of complements in the wrong time and place, which leads to unwanted inflammation and cell lysis. Pyogenic bacterial infection and glomerulonephritis are the results of such deficiencies.

Mutations in the complement regulators factors may lead to the atypical hemolytic uremic syndrome, age-related macular degeneration or hereditary angioedema, etc.

The complement system can also be stimulated by abnormal stimuli, like persistent microbes, an antibody against self-antigens or immune complexes deposited in tissues. Even when the system is regulated correctly and activated, it can cause significant tissue damage.

Total complement activity (CH50) may be ordered to look at the integrity of the entire classical complement pathway. If an alternative component defect is suspected, an AH50 test may be performed. In the case of suspected hereditary angioedema (recurrent oedema of unclear aetiology), we examine the concentration of C1-INH in serum (because C1-INH inhibitor deficiency is present).

2.3 DETERMINATION OF COMPLEMENT COMPONENTS

After complement activation by the classical, alternative or lectin pathway, the cascade reaction proceeds very rapidly and the individual components of the complement have only a short half-life and mostly low serum concentrations. Therefore, the determination of the individual components is a problem. Collected blood of 5 ml is sent for examination on the day of collection, rapid transport of the patient's blood to the laboratory has to be ensured. In practice, only **two key complement components C3 and C4 are usually determined.** The methods used during the examination are the reaction of the protein with a specific antiserum. Single radial immunodiffusion or ELISA methods can be used. Nowadays, nephelometry is often used. Components C3 and C4 have normal values, but these are modified by laboratories depending on the methods used and antisera. Values for serum C3 component are given in the range 0.8-1.2 g/l and for the C4 component 0.15-0.4 g/l.

2.3.1 Determination of complement components by single radial immunodiffusion assay

Complement tests, most commonly C3 and C4, are used to determine whether deficiencies or abnormalities in the complement system are causing, or contributing to, a person's disease or condition.

Procedure: Single radial immunodiffusion (RID) is performed on a glass slide or Petri dish. In this assay, the antiserum with specific antibodies against the examined component (C3 or C4) is added to temperated (about 50 ° C) solution of agarose or agar, which is poured into a glass slide or Petri dish and allowed to cool. Upon cooling, round wells are cut in the cooled agar. The antigens solution (e.g. C3) with known standards in increasing concentrations are added to the wells with a pipette. The tested serum with an unknown concentration of complement component C3 is added to the next wells with a pipette and allowed to diffuse. The glass slide is placed horizontally in a wet chamber to avoid drying and subsequent cracking of the gel during the reaction (Figure 16). In this system, only one component diffuses, which in this case is an antigen (single immunodiffusion). An antibody that has been uniformly mixed in the gel cannot diffuse. The antigen diffuses from the well in all directions into the gel (radial immunodiffusion). As the antigen C3 and specific antibody interact, they form in a zone of equivalence a precipitation ring. In the method of Mancini et al. (1965), the precipitation ring diameters are measured after immunodiffusion (Figure 17, 18).

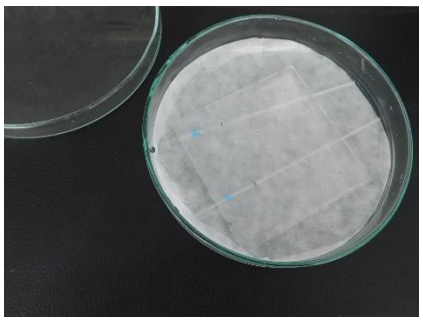


Fig. 16 Wet chamber and RID
(Source: *Martina Neuschlova*)

Fig. 17 Precipitation rings
(Source: *Martina Neuschlova*)

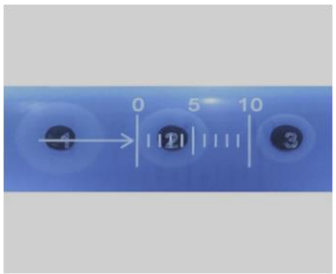


Fig. 18 Precipitation rings - measurement
(Source: *http://www.idbiotech.com/rid-srid-kit-test-radial-immunodiffusion/57-horse-igg-idr-rid.html*)

The diameter of the precipitation ring is directly proportional to the concentration of the antigen. By measuring the zones of precipitation produced by samples of known concentration, we can prepare a standard curve to determine the antigen concentration of the unknown sample. First, the diameters of the precipitation rings around the wells with known antigen concentrations, are

measured. Then the diameters of precipitation rings around the wells with unknown antigen concentration, are measured. The diameter of the ring (or its square) is directly proportional to the antigen concentration (Figure 19).

Result: The unknown antigen concentration in the sample is determined with the use of the diagram. **Single radial immunodiffusion – Mancini method is the basic method for the quantitative determination of antigens.**

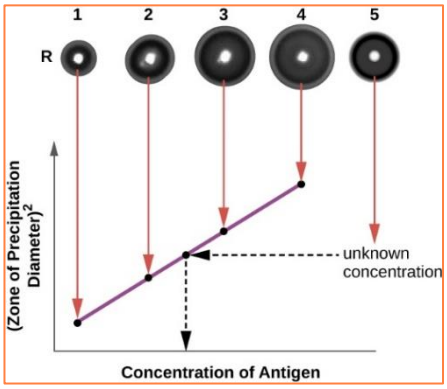


Fig. 19 Analytical line in single radial immunodiffusion (Source: *https://bio.libretexts.org*)

(A graph for the diameter of the precipitation ring on y-axis vs. concentration of antigen on x-axis and determination of the unknown concentration of C3 or C4)

2.3.2 Determination of total complement activity

The total complement activity, or CH50 blood test, assesses the overall activity of the complement system, and mainly evaluates the classic complement activation pathway. If an alternative component defect is suspected, an AH50 test may be performed. The CH50 blood test is often ordered to evaluate complement activity in cases of many autoimmune or severe infectious diseases. A total complement measurement, which is also known as a total hemolytic complement or a CH50 measurement, checks how well the complement system is functioning.

This test is usually ordered for people with a family history of complement deficiency and those who have symptoms of rheumatoid arthritis, kidney disease (glomerulonephritis), systemic lupus erythematosus, immune complement disease, myasthenia gravis, a serious infectious disease, cryoglobulinemia (the presence of abnormal proteins in the blood).

Procedure: 5 ml of clotted blood is sent to examination. Rapid transport of the patient's blood to the laboratory must be ensured as the blood must be processed within one hour of collection. The CH50 test is the functional capability of serum complement components of the classical pathway to lyse sheep erythrocytes pre-coated with rabbit anti-sheep erythrocytes antibody. When antibody-coated (sensitised) sheep erytrocytes are incubated with test patient's serum, the classical pathway of complement is activated and haemolysis results. A fixed volume of optimally sensitised sheep erythrocytes is added to each serum dilution. After incubation, the mixture is centrifuged. The degree of haemolysis is quantified by measuring the absorbance of the haemoglobin released into the supernatant at 540 nm. The amount of complement activity is determined by examining the capacity of various dilutions of test serum to lyse sensitised sheep erythrocytes. If a complement component is absent, the CH50 level will be zero; if one or more components of the classical pathway are decreased, the CH50 will be decreased. CH 50 and AH 50 are **screening tests sensitive to inactivity, absence or reduction of any component of the complement system.**

Result: A CH50 test is used to help determine any protein abnormalities and deficiencies in the complement system. Values are reported in hemolytic units: **one hemolytic unit determines the amount of complement that is able to lyse 50% of the standard concentration of sensitized sheep erythrocytes.** They may range from 41-90 hemolytic units.

If test results show increased levels of complement, this could indicate serious health problems such as cancer, ulcerative colitis and infections. Decreased levels of complement may indicate acquired deficiency, rheumatoid arthritis, vasculitis, systemic lupus erythematosus, glomerulonephritis, liver disease or cirrhosis, cryoglobulinemia, recurrent infections. Low CH 50 levels were found in malnutrition, renal transplant rejection, hereditary angioedema, hepatitis. It may also be hereditary. Low levels of total complement hemolytic activity are physiologically present in newborns and the first months of life.

2.4 LYSOZYME (muramidase)

Lysozyme is a protein with low molecular weight (14,6 KDa) that consists of a single polypeptide chain of 129 units. It is an important **hydrolytic enzyme with an antibacterial effect that destroys the cell wall of the gram-positive bacteria.** Lysozyme **breaks the beta-1,4-glycosidic linkages** between N-acetylmuramic acid and N-acetylglucosamine **in the murein** (peptidoglycan), which is the major component of gram-positive bacterial cell wall.

Murein (peptidoglycan) is an essential and specific structural component of the bacterial cell wall found on the outside of the cytoplasmic membrane of almost all bacteria. The main structural features of peptidoglycan are linear glycan strands cross-linked by short peptides. The glycan strands are made up of alternating N-acetylglucosamine and N-acetylmuramic acid residues linked by beta-1,4-glycosidic bonds. The cell wall of gram-positive bacteria may contain up to 40 layers of peptidoglycan. As a result of lysozyme, the bonds are cleaved, and lysis of the cell wall from the outside occurs. Since the cell wall is essential for bacteria, its damage leads to impairment of its function.

Lysozyme was discovered in 1922 by Sir Alexander Fleming. **It is a part of specialized cellular organelles - lysosomes, also found in granules of leukocytes.** It is involved in degradation and microbicidal processes and kills many gram-positive bacteria. Lysozyme is more effective against gram-positive bacteria than gram-negative bacteria because gram-positive bacteria contain far more peptidoglycans in their cell wall and peptidoglycan layers of gram-negative bacteria are under an outer lipid bilayer that acts as protection against lysozyme. Therefore lysozyme requires cooperation with complement for its action. Complement disrupts the outer membrane. It creates holes in the outer membrane, thanks to the membrane attack complex.

Lysozyme is found in relatively high concentrations in granules of neutrophils, blood serum and most body secretions, such as nasal mucus, saliva, tears, and breast milk, where it reaches high concentrations. In general, this non-specific humoral factor is found in both invertebrates and vertebrates but is also released from the flagellin of the bacteriophage, thereby causing host cell perforation. It then applies even when new bacteriophages are released from the cell. Egg white also contains a lot of lysozymes. In the case of infectious processes taking place in the oral cavity or pharynx, it can be administered as a local antiseptic therapy, e.g. also in the form of pills.

Lysozyme protects us from the ever-present danger of bacterial infection. It functions as an antimicrobial agent of non-specific immunity. Lysozyme together with complement is important for the bactericidal activity of serum. Fresh serum kills bacteria, but practically only non-pathogenic.

2.4.1 Determination of lysozyme – lysozyme detection simple assay

This simple assay to detect lysozyme activity uses **gram-positive bacteria *Micrococcus lysodeikticus*** as the substrate. Lysozyme activity results in the lysis of the *Micrococcus lysodeikticus* cells.

Procedure: Initial suspensions of the *Micrococcus lysodeikticus* is prepared. Agar or agarose mixed with the suspension of *Micrococcus lysodeikticus* is poured onto a glass plate and allowed to cool. Upon cooling, round wells are cut in the cooled agar. The known concentration of standard lysozyme solution is pipetted into these wells at increasing concentrations. Into the other wells are pipetted samples, in which the concentration of lysozyme is detected. The incubation period is 24 hours in a wet chamber at room temperature.

Lysozyme diffuses into the agar and **causes lysis of the *Micrococcus lysodeikticus***. The diameters of lysis are then measured (Figure 20). First, the diameters of the lysis around the wells with known lysozyme concentrations, are measured. Then the diameters of lysis around the wells with patient's samples are measured. A standard curve is prepared to determine the lysozyme concentration of the sample. The diameter of the lysis is directly proportional to the lysozyme concentration.

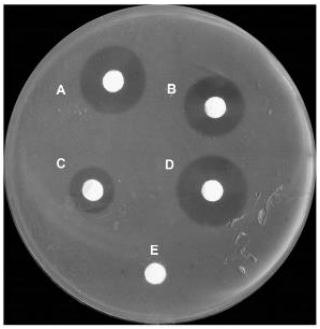


Fig. 20 Lytic activity of lysozyme against *Micrococcus lysodeikticus*

(Source:*https://journals.plos.org/plosone/article/figure/image?size=medium&id=info:doi/10.1371/journal.pone.0017593.g003*)

Results: Using the standard curve, the concentration of lysozyme in the patient's samples is determined. The reference values are in the range of 20,8 ± 3,2 mg/l. However, each laboratory creates its reference values, which are dependent on the lysozyme standards used.

2.5 DETERMINATION OF BACTERICIDAL ACTIVITY OF SERUM

Antibacterial agents such as **lysozyme together with complement are important for the natural bactericidal activity of serum.** Fresh serum kills bacteria, but practically only non-pathogenic. This natural bactericidal activity is different from the bactericidal activity in the patient's serum during treatment with antimicrobials and can be measured.

Procedure: When determining the bactericidal activity of serum, the substrate must be a living microorganism. *Candida albicans, Staphylococcus aureus, Escherichia coli* or *Pseudomonas* are often used. The blood is incubated with a suspension of the microorganism, and then the procedure shall follow according to selected microorganism. When large microorganisms such as e.g. *C.albicans* are used, their survival is assessed microscopically after vital staining by trypan blue. Phagocytic cells should be lysed (e.g. by osmotic lysis) before self-evaluation. The killed candida are stained blue, and the living ones are uncoloured because they eliminated the blue dye. If smaller microorganisms (e.g. staphylococci, escherichia) are used, inoculation is performed on solid medium in a Petri dish. Those bacteria that have survived, form colonies.

Result: Subsequently, the grown colonies are counted from the sample and control, then the bactericidal activity is calculated according to the formula:

$$\frac{\text{Number of grown colonies from the examined sample} \times 100}{\text{Number of grown colonies from control}}$$

This method is relatively labour-intensive and difficult to standardize. Fresh blood is required and must be processed within 2 hours of collection. The cultivation of the surviving microorganisms follows, so the test usually takes 2-3 days. However, the application of bactericidal tests for routine clinical diagnosis is limited. We use them more often in research. It is an important parameter in immunological studies given that it allows the assessment of innate immune defence systems.

Bactericidal activity of serum may be affected by treatment with antimicrobial agents. In some infections, it is not enough to determine whether the microorganism is ATB-sensitive, but also the bactericidal effect of serum is necessary to achieve the therapeutic effect.

Principle: serum collected during the therapy with ATB is diluted in the geometric series of 1: 2, 1: 4, 1: 8, 1:16, etc. Each dilution is inoculated with patient's own strain of microorganism obtained from the patient's blood culture. Each of these dilutions is inoculated on a sterile solid medium without antibiotics.

Result: Bactericidal titer is considered to be the highest serum dilution that reduces the bacterial population by at least 99.9% compared to the inactivated serum control. The control is a serum that is inactivated by heating to 56°C for 30 minutes. Appropriate antimicrobial treatment should also achieve the bactericidal effect of serum even when diluted 8 to 16-fold (up to 32-fold in staphylococcal endocarditis) (Figure 21).

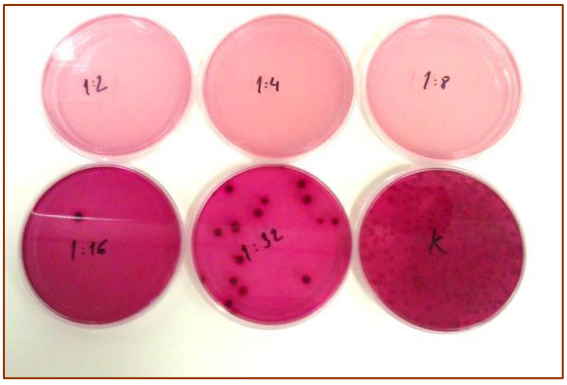


Fig. 21 Determination of bactericidal activity of serum (Source: *Martina Neuschlova*)

2.6 DETERMINATION OF ACUTE-PHASE PROTEINS

Acute-phase proteins are a class of proteins whose plasma concentrations increase in the acute phase of inflammation in the body (positive acute-phase proteins). For this reason, their measurement can be used for diagnostic purposes. They are primarily synthesized in the liver in response to upstream inflammatory signals. Their synthesis is enhanced by inflammatory cytokines (IL-1, IL-6, TNF α). Frequently investigated proteins of the acute phase of inflammation include α-1-antitrypsin (A1AT), α-2-macroglobulin (A2M), orosomucoid (ORM), ceruloplasmin (Cpl), fibrinogen, C-reactive protein, procalcitonin.

All proteins of the acute-phase proteins have a specific function in the body. They are directly involved in defensive reactions, opsonize bacteria (e.g. CRP), regulate the inflammatory response by blocking proteases released from granulocytes and thus to some extent protect tissues from massive damage (α-1-antitrypsin). In addition, they may have the function of ion transporters (transferrin, ceruloplasmin); they participate in haemocoagulation (fibrinogen).

The most frequently examined acute-phase proteins include **CRP, procalcitonin** and **orosomucoid**.

- **CRP** is a sensitive and very dynamic indicator of the acute phase of inflammation. CRP levels rise dramatically during inflammatory processes occurring in the body.
- **Orosomucoid** (also called α-1 acid glycoprotein) is synthesized mainly in the liver (in hepatocytes), (also called α-1 acid glycoprotein) is synthesized mainly in the liver (in hepatocytes), and lymphocytes, monocytes, neutrophils also produce it. It increases during inflammation with similar dynamics to CRP.
- **Procalcitonin** is a precursor of calcitonin (a hormone produced by the thyroid gland that is involved in the regulation of calcium levels). This substance is produced by many types of cells in the body. The level of procalcitonin can increase only after inflammation (bacterial, fungal). Procalcitonin levels are determined in patients suspected of severe infections, which increase significantly in bacterial but not viral diseases. For this reason, its elevated levels are used to differentiate the bacterial origin of inflammation from inflammation of other aetiology.

2.6.1 Determination of CRP – C-reactive protein

CRP is one of the most commonly assessed acute-phase proteins in the context of inflammation monitoring. It is sensitive indicators of inflammation. Its serum levels in the blood usually are low and increase when there is a condition causing inflammation somewhere in the body. Its increase can be either mild or dramatic.

It is a very dynamic parameter. **Its dynamics are faster than erythrocyte sedimentation rate (ERS),** that means, it reacts much more rapidly in inflammation by increasing the concentration. Therefore, it reflects on the severity of inflammation, but since it is a non-specific parameter, it does not contribute to the determination of the nature of the disease. The result must be interpreted with other data obtained on the disease.

Significantly high CRP levels are nearly always a sign of a severe underlying medical condition. The most common cause is a severe infection, **infection of bacterial aetiology,** CRP concentration increases significantly (10-fold or more). However, increased CRP may also be detected in non-infectious inflammations, such as **heart attack,** a poorly controlled **autoimmune disorder, arthritis, inflammatory bowel disease,** severe tissue damages or in **postoperative periods.** In rheumatic diseases, on the one hand, CRP levels may be significantly increased (in the acute phase of rheumatoid arthritis) or, on the other hand, only slightly increased in the active phase of SLE, which contrasts with marked inflammatory activity and high erythrocyte sedimentation rate. If high CRP is found in SLE patients, this is a complicated, mostly infectious process. CRP is an important marker of inflammation in patients with hypogammaglobulinemia since they do not usually have an erythrocyte sedimentation rate increase.

CRP test principles

The C-Reactive Protein test is based on the principle of the latex agglutination. When latex particles complexed human anti-CRP are mixed with a patient's serum containing C-reactive proteins, a visible agglutination reaction will take place within 2 minutes.

Qualitative Test procedure:

1. Bring all reagents and serum sample to room temperature and mix latex reagent gently prior to use. Do not dilute the controls and serum.
2. Place 1 drop of serum, positive control and negative control on separate reaction circle on a glass slide.
3. Then add 1 drop of CRP latex reagent to each of the circles.
4. Mix with separate mixing sticks and spread the fluid over the entire area of the cell.
5. Tilt the slide back and forth slowly for 2 minutes observing preferably under artificial light.
6. Observe for visible agglutination.

Result interpretation of CRP Test (Figure 22):

- *Positive result*: agglutination of latex particles, indicating the presence of CRP at a significant and detectable level.
- *Negative result*: no agglutination.

Semi-Quantitative Test procedure:

1. Prepare dilution of the specimen with physiological saline 0.9%, (1:2, 1:4, 1:8, 1:16, 1:32, 1:64)
2. Then proceed for each dilution as in qualitative test.

Result interpretation of CRP Test for the semi-quantitative method:

- *Positive result*: the last dilution of serum with visible agglutination is the CRP titre of the serum.

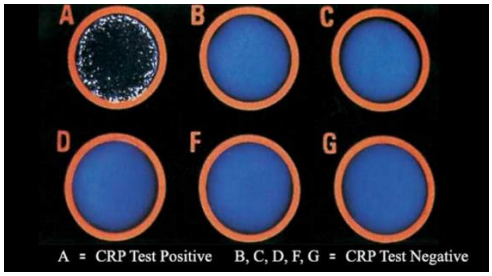


Fig. 22 Result interpretation of CRP Test

(Source: *https://microbiologyinfo.com/c-reactive-protein-crp-test-principle-uses-procedure-and-result-interpretation/*)

Quantitative Test procedure:

Single radial immunodiffusion (Mancini method) may be used to **the quantitative determination of CRP.** The principle is similar to the determination of complement components by precipitation in a gel environment.

In this assay, the anti-CRP antibody against the examined C-reactive protein is added to temperated (about 50° C) solution of agarose or agar, which is poured into a glass slide or petri dish and allowed to cool. Upon cooling, round wells are cut in the cooled agar. The CRP with known standards in increasing concentrations are added to the wells with a pipette. The tested patient's serum with an unknown concentration of CRP is added to the next wells with a pipette and allowed to diffuse. The glass slide is placed horizontally in a wet chamber to avoid drying and subsequent cracking of the gel during the reaction. In this system, only one component diffuses, which in this case is CRP (single immunodiffusion). An anti-CRP antibody that has been uniformly mixed in the gel cannot diffuse. The CRP diffuses from the well in all directions into the gel (radial immunodiffusion). As the CRP and specific anti-CRP antibody interact, they form in a zone of equivalence a precipitation ring. In the method of Mancini et al. (1965) the precipitation ring diameters are measured after immunodiffusion. The diameter of the precipitation ring is directly proportional to the concentration of the CRP. By measuring the zones of precipitation produced by samples of known concentration, we can prepare a standard curve to determine the CRP concentration of the unknown sample. First, the diameters of the precipitation rings around the wells with known CRP concentrations, are measured. Then the diameters of precipitation rings around the wells with unknown CRP concentration, are measured. The diameter of the ring (or its square) is directly proportional to the CRP concentration.

Result: The unknown CRP concentration in the serum is determined with the use of the diagram.

Determination of CRP by nephelometry or turbidimetry

In clinical practice, **nephelometry or turbidimetry** is the most commonly used method for CRP examination. Convenient small devices are used for this purpose; they are simple and are adapted for CRP examination from capillary blood. They operate in POCT (point of care testing) mode, which means that the determination of this parameter is made directly at the patient's treatment site. As a result, this method is used in many healthcare facilities. It is available by default in paediatrician, adolescent and adult clinics and many other outpatient clinics. Since the results are within a few minutes, it is a beneficial method for practice.

The **principle of nephelometry and turbidimetry** is based on the scattering or absorption of light by solid or colloidal particles suspended in solution. When light is passed through the suspension, part of incident radiant energy is dissipated by absorption, reflection, and reaction while the remainder is transmitted.

When light strikes on particles in solution, some of the light will be absorbed by the particles, some will be transmitted through the solution and some of the light will be scattered or reflected. The amount of light scattered is proportional to the concentration of insoluble particles (antigen-antibody complexes). Nephelometry deals with measurement of the intensity of scattered or reflected light. Turbidimetry deals with measurement of the intensity of transmitted light. Nephelometry, a method to detect the concentration of serum proteins including immunoglobulin,

is based on the concept that particles in solution will scatter light passing through the solution rather than absorbing the light. Turbidimetry is based on measuring an absorbance change.

A recent development is the so-called microparticle-enhanced nephelometric or turbidimetric immunoassay where antigen or antibody is conjugated to spherical, hydrophilic microparticles (latex particles). This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry or turbidimetry. For the quantification of CRP, latex particles coated with anti-CRP antibodies are used. The test sample is mixed with latex particles coated with anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles. Light scattering (measured by a nephelometric procedure), or an absorbance change (measured by a turbidimetric procedure), is proportional to the concentration of the analyte present in the sample. CRP concentrations are calculated by using a calibration curve.

Nephelometry and turbidimetry are applied to the quantitative determination of various proteins and other antigens. They both are methods of choice in the clinical laboratory to measure the concentration of immunoglobulin isotypes as well as other proteins including C-reactive protein.

2.7 PHAGOCYTOSIS

Phagocytosis is the ability of professional phagocytes to recognize, absorb, kill and degrade foreign material. In non-specific defence, the most important barrier to the spread of microorganisms is their destruction by phagocytes.
Two types of phagocytes are classified as **professional phagocytes:**

- **polymorphonuclear leukocytes** (PMNL) - also called **microphages**
- and **macrophages**. All develop from bone marrow hematopoietic stem cells.

The function of phagocytes is on the one hand **effector** – phagocytosis and elimination of microorganisms in the presence of bactericidal substances and hydrolytic enzymes and the **regulatory function** – mediated by the production of cytokines, prostaglandins, leukotrienes, etc.

Microphages are mainly neutrophils and eosinophils. They circulate in the blood and are ready to move promptly to the site of inflammation. The advantage of these granulocytes is that they are ready to perform their effector functions immediately.

2.7.1 Neutrophils

Neutrophils are a type of white blood cells (WBC or granulocyte) that protect us from infections, among other functions. They live for a very short time, about 24 hours**.** They are the largest group of leukocytes in humans. They represent up to 60-70% of leukocytes (4.3-11 x 10^9/litre of blood), are very active and play an important role in acute inflammation. Their advantage is their promptly mobilization and release of inflammatory mediators. Their **main role is phagocytosis and destruction of pathogens.**

They are the **basic cells of the 1st line of defence against pathogens**, foreign cells. Because of their abilities, they are referred to as "footsoldiers". Neutrophils, however, are **not antigen-presenting cells** (do not express MHC class II).

Inactive neutrophils are circulating in the blood for approximately 6-7 hours. After activation and migration into the tissues, they survive 1-2 days (pus contains living or dead neutrophils and killed bacteria). Neutrophils are about 15 μm in diameter. Immature young forms of neutrophils have a rod-shaped nucleus and have a lower phagocytosis capability than mature forms. The mature forms of neutrophils have a segmented nucleus that contains 4-5 lobes (Figure 23).

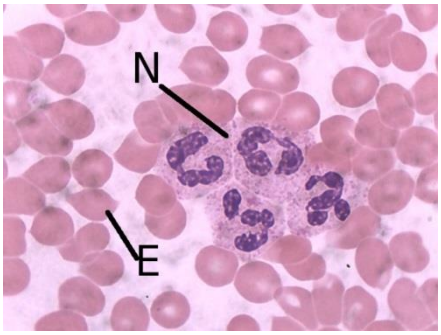


Fig. 23 Neutrophils (N), Erythrocytes (E)
(Source: *http://galleryhip.com*)

Neutrophils play an important role in the inflammatory process, as they rapidly mobilize on the site of inflammation, they are **the first cells arriving at the site of injury or inflammation.** Neutrophils come into contact with microorganisms faster than macrophages. However, they are rapidly followed by blood monocytes, which then differentiate at the invasion site into macrophages, which phagocytose and kill bacteria.

Leukocytes flow in the centre of small blood vessels, where blood flow is fastest. In the inflammatory sites, vessels are dilated and the slower blood flow allows the leukocytes to move out of the centre of the blood vessel and interact with the vascular endothelium. Under normal conditions, monocytes migrate continuously into the tissues, where they differentiate into macrophages. During an inflammatory response, the induction of adhesion molecules on the endothelial cells by the infection focus, as well as induced changes in the adhesion molecules expressed on leukocytes, recruit large numbers of circulating leukocytes, initially neutrophils and later monocytes, into the site of an infection. The migration of leukocytes out of blood vessels is a process known as extravasation.

These interactions enable leukocytes to squeeze between the endothelial cells. It then penetrates the basement membrane. The movement through the basement membrane is known as diapedesis, and it enables leukocytes to enter the subendothelial tissues. Next step is a migration of leukocytes through the tissues under the influence of chemokines that are produced at the site of infection (Figure 24).

There are two types of granules in the cytoplasm containing antimicrobial substances:

- **Primary azurophilic granules** contain bactericidal enzymes such as hydrolases, myeloperoxidases, neutral proteases
- **Secondary granules** contain lactoferrin, lysozyme, alkaline phosphatase, collagenase and others.

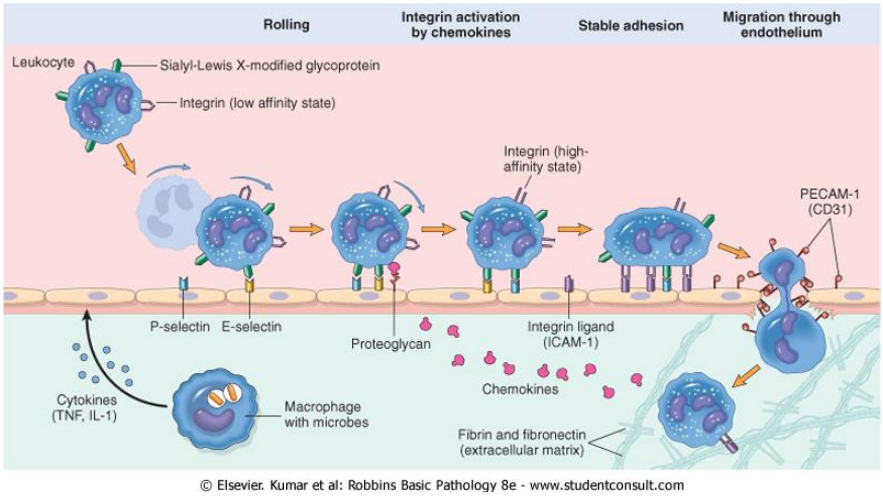


Fig. 24 Neutrophils leave the blood vessels and migrate to the inflammatory sites
(Source: *Kumar et al. Robbins Basic Pathology 8e, http://www.studentconsult.com*)

Neutrophils release enzymes from the granules into the extracellular space, allowing them to actively move to the site of inflammation. If they fail to meet the bacteria within a reasonable time, they will gradually die.

If more enzymes are released into the extracellular space, the tissue will liquify and some neutrophils will die, resulting in pus formation. Neutrophils that have fulfilled their role also die and are removed by other phagocytes (especially by macrophages). Larger amounts of dead neutrophils are part of the pus.

2.7.2 Eosinophils

Eosinophils represent 1-4% of leukocytes. They have a diameter of about 18 μm. In the cytoplasm, they have about 200 **acidophilic granules** containing specialized proteins: eosinophil cationic protein, eosinophil peroxidase, major basic protein, ribonuclease, lipase. They are coloured red with acidic dyes (Figure 25).

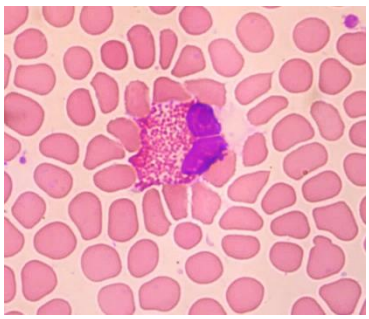


Fig. 25 Eosinophils (Source: *Martina Neuschlova*)

Eosinophils have **receptors for complement components** (for C3b) and **receptors for IgE antibodies** on their surface. They persist in the circulation for 8-12 hours, in tissues for 8-12 days. Eosinophils can phagocytose only weakly.

However, they form a large number of metabolites upon activation. They produce leukotrienes, prostaglandins and a number of cytokines. They play an important role in **allergic**

reactions, in the defence against **parasitic infections caused by multicellular worms** (mainly helminths in the gastrointestinal tract), in the **defence against tumour cells**. Eosinophilia is present in parasitic diseases, allergies and some types of bone marrow and lymph node tumours.

2.7.3 Monocytes

Monocytes represent 6-8% of leukocytes. They are large mononuclear cells, usually 12-20μm in diameter, which has a large, eccentrically positioned kidney-shaped nucleus. The cytoplasm is vacuolized, stains slightly basophilic (blue-grey), and also contains very fine azurophilic granules (Figure 26).

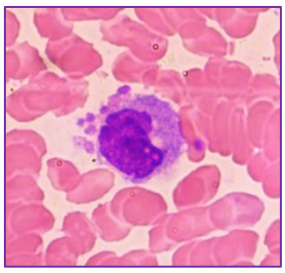


Fig. 26 Monocyte (Source: *Martina Neuschlova*)

They circulate in the bloodstream for approximately 1-3 days. They then adhere to the vascular endothelium and leave the bloodstream through a process called diapedesis. They migrate continuously into the tissue where they **differentiate into macrophages**. However, during inflammation, a large number of neutrophils and later monocytes migrate into the **site of an infection or injury**. Monocytes differentiate into several types of macrophages according to different anatomical localization. After activation macrophages phagocytose and kill the bacteria.

2.7.4 Macrophages

Macrophages are large mononuclear phagocytes that arise from blood monocytes when they leave the circulation and migrate into the tissues.

The tissue macrophages have a different name according to the different anatomical localization. For example, alveolar macrophages occur in the lungs, Kupffer cells in the liver, histiocytes in connective tissue, microglia in the central nervous system. osteoclasts in bones, Langerhans cells in the skin.

Macrophages are strategic cells in the first immunologic line of defence against pathogens. They are **professional phagocytes** and at the same time, they are classified as **antigen-presenting cells**. They express MHC class II. molecules (Figure 27).

Fig. 27 Macrophage

(Source: *http://www.medical-labs.net/neutrophil-macrophage-and-myeloid-derived-dendritic-cell-function-840/*)

They are found in especially large numbers in connective tissue, in the submucosal layer of the gastrointestinal tract, in the lung, along certain blood vessels in the liver and throughout the spleen.

Macrophages have **receptors for complement components** (for C3b) **and Fc receptors** on their surface. **They can repeat phagocytosis**. In their lifetime they can phagocytose more than 100 bacteria. Macrophages are also referred to as "large eaters". They live a long time, an average of 75 days, sometimes months to years. They capture, engulf and process foreign particles by phagocytosis and present antigens to specific T cells for processing (Figure 28).

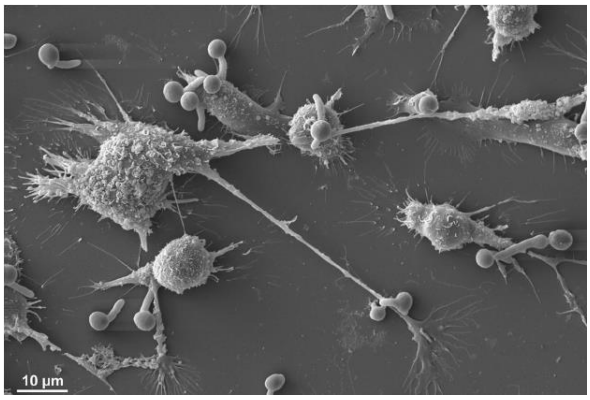


Fig. 28 Macrophages interacting with *Candida albicans*
(*scanning electron microscopy,* Source: *Bain et al., 2014*)

Macrophages are capable of recognizing, absorbing, processing and presenting antigen to T cells. They can be activated, for example, by the C3b complement fragment, interferon-gamma, endotoxin of gram-negative bacteria, which increases their ability to phagocytose.

Upon phagocytosis, macrophages also produce a variety of other toxic products that help kill the engulfed microorganism, such as a nitric oxide (NO), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2). In addition, they produce various immunoregulatory molecules, signalling and antimicrobial proteins. Some of these molecules can participate in triggering specific immune responses and result in a specific cellular immune response. Thus, macrophages represent the connection between non-specific and specific immunity.

2.7.5 Phagocytosis process

Phagocytosis is an active process, in which specialized cells of the immune system engulf, kill and break down microorganisms (or foreign material). It has a **key role in innate non-specific immunity** because phagocytes can recognize, ingest and destroy many pathogens without the aid of adaptive specific immunity.

Chemotaxis

In the earliest phases of infection are released by tissues **chemoattractant cytokines** called **chemokines**. **Chemokines induce chemotaxis**. They function as chemoattractants for phagocytes and recruit neutrophils, monocytes and other immune cells from the blood to the site of infection or injury.

The most potent chemokines include some complement components, leukotrienes, interleukin IL-8, but mainly bacterial components. Phagocytes are attracted to the site of inflammation according to a chemotactic gradient. The major chemokine for neutrophils is cytokine interleukin IL-8. For monocytes and eosinophils, these are MIP-1α, MIP-1β, MCP-1 and RANTES. Chemokines common to all phagocytes are C3a, C5a complement components,

leukotriene LTB4, PAF (thrombocyte activating factor), fMLP (formyl-methionyl-leucyl-phenylalanine – a bacterial protein). The receptors for all these chemokines are present on the surface of the phagocytes. Their stimulation leads to chemotaxis and subsequent activation of phagocytosis.

Adherence

The foreign material is attached to the surface of the phagocyte, this phase is called **adherence**. Neutrophils and macrophages recognize pathogens through their **cell-surface receptors PRR** (pattern recognition receptors) which can recognize molecules found **on the surface of microorganisms**, referred to as **PAMP** (pathogen associate molecular patterns). In this case, it is opsonin-independent phagocytosis.

Opsonization

Another important mechanism for recognizing foreign particles and ensuring their phagocytosis is opsonization. **The coating of a particle with proteins that facilitate its phagocytosis is known as opsonization. Opsonins are molecules that bind to the surface of microbes, making them more attractive to phagocytic cells to facilitate their phagocytosis.** Important opsonins include IgG antibodies (especially IgG1 and IgG3), complement components (especially C3 component of complement), mannose-binding lectin (MBL), fibronectin, acute-phase inflammatory proteins. Receptors for opsonins are present on phagocytic cells (especially for C3b, immunoglobulins IgG1 and IgG3, MBL, acute-phase proteins). Phagocytes can remove not only pathogens but also own apoptotic cells or damaged cells. They can recognize them by unusual structures on their surface (for example, phospholipids on the surface of apoptotic cells that normally occur on the inside of the membrane) or after their opsonization with antibodies or complement components. The subsequently enhanced phagocytosis is termed **opsonin-dependent phagocytosis.**

Ingestion

Ingestion is initiated after the recognition and attachment of microbes or other particles by phagocytes. **The phagocyte can engulf the particle by extending membrane protrusions around the foreign particle.** This process is known as **endocytosis**. Another mechanism is **macropinocytosis**, which is the nonselective uptake of extracellular material such as soluble molecules, antigens, nutrients. In this mechanism, **invaginated membrane ruffles of the plasma membrane are formed to engulf extracellular fluids** into a large intracellular vesicle.

Phagosome

A phagosome (or endocytic vacuole) is a **vesicle formed around a particle engulfed by a phagocyte**.

Phagolysosome

In the next step, **the phagosome fuses with one or more lysosomes to form a phagolysosome**. Lysosomes contain enzymes that can mediate an intracellular antimicrobial response. The lysosomal contents are released to destroy the microbes. In the next step, the phagosome fuses with one or more lysosomes to form a phagolysosome. Phagolysosomes possess

many sophisticated mechanisms directed to eliminate and degrade microorganisms. They have an acidic environment that enables the optimal function of many enzymes (pH 4–5). Lysosomes contain many degradative enzymes, including various cathepsins, proteases, lysozymes, and lipases that can mediate an intracellular antimicrobial response.

Respiratory burst

Upon phagocytosis, phagocytes also produce a variety of toxic products that help kill the engulfed microorganism. The most important of these is the rapid release of superoxide anion and hydrogen peroxide, which are directly toxic to bacteria. This mechanism is called the **respiratory burst** (also known as the **oxidative burst**). Respiratory burst plays an important role in the immune system because **is the principal effector mechanism for the production of reactive oxygen species used to kill internalized pathogens** following phagocytosis.

Nitric oxide

Another important mechanism that helps kill engulfed microorganisms is **nitric oxide**. It is produced by inducible nitric oxide synthase (iNOS) in the activated phagocytic cells. NO is an **important component of host defence against intracellular pathogens**.

Degradation and discharge of ingested matter

After degradation of ingested matter, **indigestible material remains in residual bodies**. Waste products are eliminated by **exocytosis** (Figure 29).

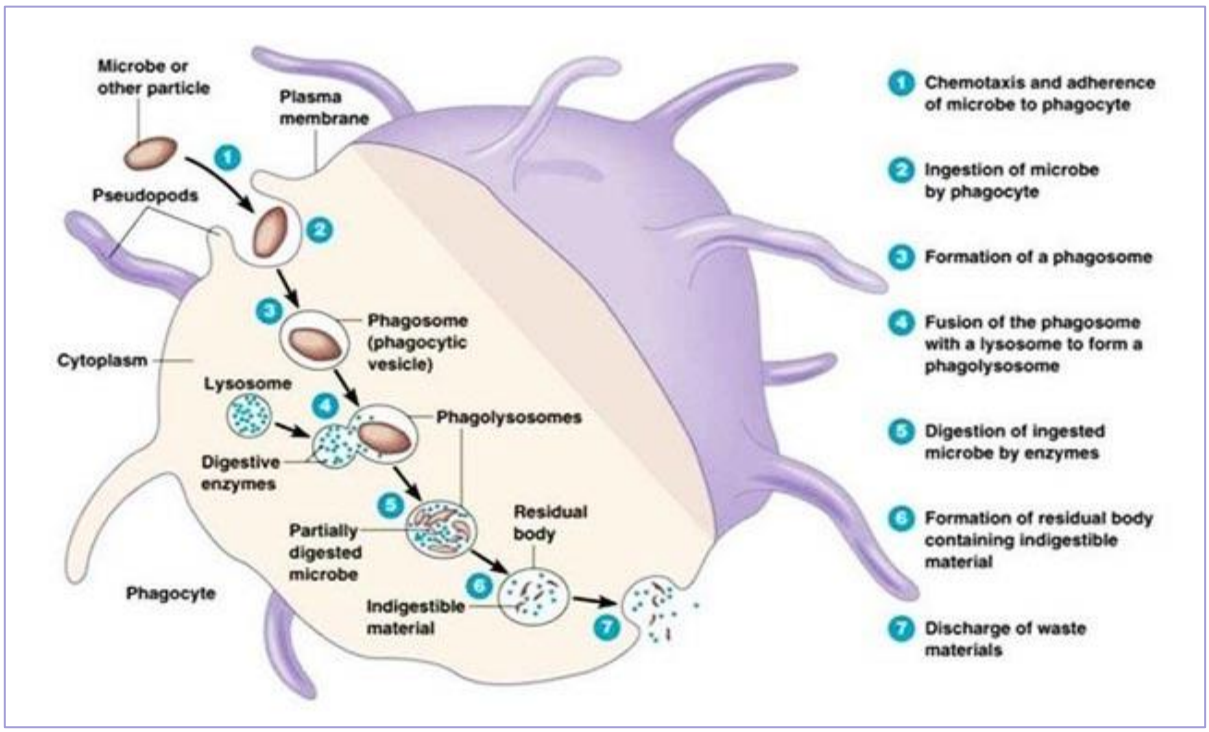


Fig. 29 Mechanism of phagocytosis (Source: *https://microbenotes.com/phagocytosis-introduction-mechanism-steps-and-example/*)

2.7.6 Phagocytosis assays – lab methods

To test the ability of phagocytes to engulf foreign material are used: bacteria, yeasts or microspherical hydrophilic particles. Uncoagulated whole blood is collected and the examination must be carried out within 2 hours after collection. The smear in a light microscope is evaluated. 100 cells are evaluated usually (Figure 30).

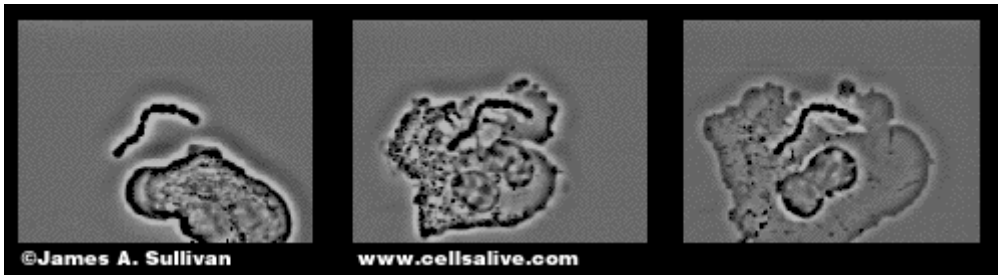


Fig. 30 Phagocytosis of *Streptococcus pyogenes* by a macrophage
(Source: *http://textbookofbacteriology.net/strep.fig4.jpeg*)

Phagocytic activity and phagocytosis index - using light microscopy

Bacteria, yeasts, or microspherical hydrophilic particles (MSHP) can be used to test the ability of phagocytes to absorb foreign material. Traditional assays require tedious cell counting under a microscope.

Uncoagulated whole blood is collected and the examination must be carried out within 2 hours after collection.

This standard phagocytosis assay enables to determine **phagocytic activity (FA) and phagocytosis index (FI)**

- **Phagocytic activity** determines the percentage of phagocytes that have engulfed particles. 100 phagocytes are counted.
- **Phagocytosis index** is the average number of engulfed particles per phagocyte.

Either latex beads or zymosan particles are an easy target as these can be seen by light microscopy as well. Commonly used pathogens are yeast *Candida albicans* and zymosan, which is prepared from the cell wall of yeast *Saccharomyces cerevisiae* and consists of protein-carbohydrate complexes. Engulfed particles are manually counted.

In these standard assays, 100 cells are usually counted and evaluated, which is time-consuming, and difficult when testing a large number of samples. Whole blood is used for the assay, the test lasts about an hour at 37 °C. Throughout the process, it is necessary to ensure gentle shaking so that the particles do not sink to the bottom, but at the same time, the phagocytic cells must have a relative calm for the ingestion of the particles. A blood smear is then made from the suspension and stained. The intensity of phagocytosis is then assessed under a light microscope (Figure 31).

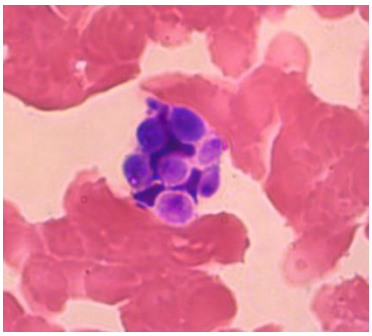


Fig. 31 Phagocytosis of *Candida albicans* by human neutrophils, 7 ingested candida in a neutrophil (Source: *Martina Neuschlova*)

Phagocytic activity expresses the percentage of phagocytes that can absorb microorganisms or microparticles. It is calculated according to the formula:

$$FA\ \% = \frac{\text{the number of phagocytic PMN´s}}{\text{the number of all PMN´s capable of phagocytosis}} \times 100$$

Phagocytosis index is the average number of microorganisms or microparticles absorbed by a single phagocyte. It is calculated according to the formula:

$$FI = \frac{\text{the number of phagocytosed (absorbed) particles}}{\text{the number of phagocytic PMN´s}}$$

Physiological values for the adult range: FA = 77,1 ± 8,8

FI = 3,6 ± 0,8

However, reference values may vary from laboratory to laboratory.

Phagocytosis assay – using oxidative burst tests, chemiluminescence, flow cytometry

In phagocytosis, there is a significant metabolic activity of phagocytic cells, which can be practically used in several tests. Oxidative burst tests are used – nitroblue tetrazolium test (NBT) and iod nitrotetrazolium test (INT). An alternative method to test the oxidative burst is chemiluminescence. Phagocytic cells emit light while ingesting bacteria or other particles. Luminol-dependent chemiluminescence produced by peripheral blood phagocytic cells is measured using a luminometer. In flow cytometry, bacteria are used to measure the engulfment and degradation of living bacteria by monocytes and granulocytes in human whole blood from fresh heparinized venous blood samples.

Phagocytosis can be assayed *in vitro* by measuring the engulfment of various substrates (e.g. E.coli, Zymosan, red blood cells) by phagocytic cells. Phagocytosis assays quantify the engulfment of one of these substrates by macrophages or other phagocytes. Quantitation is done in a 96-well plate on a standard colorimetric ELISA reader. It may be adapted for use with 24-well or 48-well plates. These assays fully quantify phagocytosis with no manual cell counting and provide a more accurate and user-friendly alternative to the standard phagocytosis assay.

2.7.7 Why is phagocytic activity being examined?

To confirm or rule out phagocytosis disorders in primary or secondary immunodeficiencies. Phagocytosis is an important mechanism in the microbial killing pathway of phagocytes. Deficiencies in phagocyte function likely predispose individuals to acquire or succumb to infectious diseases.

If a primary disorder of phagocytic activity is suspected, examination of phagocytosis is the method of the first choice. These are congenital immunodeficiencies that manifest clinically in early childhood. Rarely, lighter forms may appear in adulthood. However, these are very serious disorders that directly endanger the patient's life, without timely and appropriate treatment.

Of the primary immunodeficiencies, the phagocytosis disorder is in:

- chronic granulomatous disease (microbicidal defect due to lack of NADPH-oxidase, when insufficient hydrogen peroxide is produced). It is clinically manifested by serious infections and the formation of granulomas in the organs.
- Myeloperoxidase defect, which is accompanied by frequent candidiasis, is relatively widespread.
- Glutathione-synthetase defect with NADPH regeneration disorder.
- Chediak-Higashi syndrome with granule anomalies and degranulation disorder.
- Others are glucose-6-phosphate dehydrogenase defect and
- ingestion disorder.

Secondary immunodeficiencies are induced by environmental effects, some infections, general malnutrition, malignancy or therapeutic interventions. Investigation of phagocytic activity is recommended for monitoring the clinical condition during treatment with immunosuppressants (corticosteroids, etc.), cytostatics (cyclophosphamide and other myelotoxic drugs), cytokines, growth factors (G-CSF, GM-CSF, INF-γ, TNF). The examination is also recommended after stem cell haematopoietic transplantation.

Decreased phagocytic activity poses a higher risk of infection and sepsis.

2.7.8 When is phagocytic activity examined?

The examination is suitable for people who have experienced the following complications:

- Recurrent and chronic infections of the skin and mucous membranes (pyoderma, abscesses, skin impetigo, disseminated candidiasis, chronic furunculosis, localized purulent inflammation)
- Lymph node infections
- Gingivitis, aphthous stomatitis
- Respiratory tract infections (otitis, sinusitis, bronchitis)
- Gynecological inflammations
- Osteomyelitis, meningitis
- Sepsis
- BCG-itis after vaccination against tuberculosis

- If these infections are caused by pyogenic bacteria, fungi, yeasts and parasites. In particular, staphylococci (*Staphylococcus aureus, Staphylococcus epidermidis*), enterobacteria (*Escherichia coli, Klebsiella pneumoniae*), *Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pneumoniae*, intracellular bacteria.

As for the informative value of these tests, it is rather limited to the measurement of reduced values. The increase in values is difficult to identify because any phagocyte is capable of phagocytosis; if the substrate is supplied in excess. It may be present in ongoing acute infections.

The reduction in phagocytosis occurs in very severe conditions. For example, in the final stages of cancer, long-term bacterial infections, severe immunodeficiencies. Young immature forms of neutrophils also have a low ability to phagocytose. These tests are more useful in research, for example in toxicology, pharmacology.

Questions for self-assessment

1. Complement is one of the effector systems of which components of immunity? In what ways can complement be activated? What is the end result of cascade complement activation?
2. Which components of complement are usually determined in practice? Which laboratory method is used to determine them? Why is the total complement activity determined? What is a hemolytic unit?
3. What is lysozyme? What is the principle of the method for determining lysozyme? Which microorganisms can be used in the determination of lysozyme?
4. What is the natural bactericidal activity of serum? How is serum inactivated? Does fresh serum have stronger or weaker bactericidal activity than an inactivated serum? Can serum bactericidal activity be affected by antimicrobial treatment?
5. Which proteins of the acute phase of inflammation are most often determined in practice? What methods can be used to determine CRP levels?
6. What is phagocytosis? Which cells belong to professional phagocytes? What is the actual process of phagocytosis - its individual steps?
7. Describe the principle of determining phagocytic activity and phagocytic index using light microscopy. What is the phagocytic activity? What is the phagocytosis index?
8. What other methods are used to examine phagocytosis? Why and when is phagocytosis examined?

Complement https://portal.jfmed.uniba.sk/articles.php?aid=408

The interactive presentation contains study material about ways of complement activation, determination of its activity and a set of the test questions. Through these questions the students can verify the acquired knowledge with feedback.

Phagocytosis https://portal.jfmed.uniba.sk/articles.php?aid=403

The interactive presentation contains study material about the ability of professional phagocytes to recognize, absorb, kill and degrade foreign material and a set of test questions. Through these questions the students can verify the acquired knowledge with feedback.

METHODS FOR ANALYSIS OF HUMORAL IMMUNITY

3

3.1 DIRECT AND INDIRECT METHODS

Diagnosis of infectious diseases is performed by direct and indirect methods. Direct diagnosis is the visualization of the causative agent of infectious disease using microscopy, cultivation, antigen detection, detection of genetic material. Indirect diagnosis is the detection of the response of a macroorganism to the presence of a microorganism. This is done either in the field of cellular immunity by skin tests or in the field of humoral immunity by the detection of specific antibodies by serological reactions.

Serological reactions are reactions between an antigen (Ag) and an antibody (Ab) that take place in a specific environment (*in vivo* - in the body or *in vitro* - in the laboratory). Serological responses are classified as methods for the examination of humoral immunity. The property of antigens and antibodies is that **they bind specifically**. That means, one reaction component can be identified based on the other reaction component. For example, if we want to prove the antigen, we use sera with known antibodies (polyclonal, monoclonal antibodies). On the contrary, if we want to detect antibodies, we use a known antigen.

3.2 ANTIGEN

An antigen (Ag) is any substance that is recognized by the immune system capable of stimulating an immune response and can bind specifically to an antibody. Their name arises from their ability to **gen**erate **anti**bodies. Antigens can be, for example, bacteria, viruses, fungi, parasites or their parts, various organic substances with high molecular weight (proteins, glycolipids, polysaccharides), chemicals, pollen etc.

The smallest portion of an antigenic molecule against which an immune response is directed is called an **epitope** – the basic recognition unit. It is also known as an **antigenic determinant**. It is the binding site to which a given antibody binds specifically. The antigenic determinant can bind to a specific antigen receptor on the surface of B cell or T cell. Binding between the receptor and antigenic determinant occurs only if their structures are complementary (like a puzzle). Many antigens have a variety of distinct antigenic determinants on their surface, which are capable of reacting with different cell receptors. Antigens generally comprise multiple epitopes, each eliciting separate but specific immune responses that, in acquired immunity, may be mediated by antibodies (humoral immunity) or by T cells, which are responsible for cell-mediated immunity.

The specificity of an antigen is determined by the production of antibodies that react only with the antigen (which induced their production). Non-specific (heterophilic) antigens are those antigens that have induced the production of antibodies that react with various similar antigens (cross-reaction).

Immunogens and haptens

The antigens that elicit an immune response are called immunogens. They are complete antigens. Those antigens that can induce antibody production or produce cell-mediated immune responses are immunogenic. Not all antigens are immunogenic. Some small antigens with

low molecular weight do not elicit an immune response. These antigens are called haptens. Haptens can react with preformed antibodies but become immunogenic only when attached to large molecules, called carriers. Then they acquire the ability to induce an immune response. In this case, the carrier is responsible for the immunogenicity and the hapten for the specificity of the immune response. Although the haptens themselves are non-immunogenic, they are able to react with antibodies.

- immunogens - substances that are able to generate an immune response by themselves
- haptens - substances that are able to react with antibodies but are unable to stimulate their production directly

Proteins are generally good immunogens. Complex proteins with numerous epitopes are the greater immunogens than simple peptides that contain only a few epitopes. Carbohydrates, steroids and lipids are poor immunogens. Aminoacids are not immunogenic.

Divisions of antigens

Antigens are divided according to the relationship to the organism into:

- exogenous antigens, (or foreign antigens to the host immune system) originate from outside the body, from the external environment
- endogenous antigens (autoantigens, self-antigens, produces by the host) originate within the body, they are part of his own cells and tissues (e.g. nuclear, cytoplasmic antigens).

Antigens are divided according to origin into:

- natural (found in nature)
- synthetic (prepared artificially in the laboratory)

Superantigen is able to induce a non-specific activation of a large number of T cells and massive cytokine release. Superantigens are produced by some pathogenic viruses and bacteria most likely as a defence mechanism against the host immune system (e.g. staphylococcal enterotoxin, toxic shock syndrome toxin).
Allergen is capable of eliciting a strong pathological immune response in a susceptible person, known as allergic reaction. In some people, the immune system recognizes allergens as foreign and dangerous. Immune system reacts by making IgE antibodies to defend against the allergen. This reaction leads to allergy symptoms.

According to solubility, we distinguish between corpuscular (insoluble) and colloidal (soluble) antigens.

- **Corpuscular antigens** include, for example, bacteria, viruses, yeast, latex particles, erythrocytes, etc.
- **Colloidal antigens** include bacterial toxins, enzymes, extracts of microorganisms, etc.

3.3 ANTIBODIES

Antibodies (Ab), also called immunoglobulins, are complex protein molecules that are produced by plasma cell clones in response to the presence of antigens. Antibodies are able to identify and neutralize foreign substances that enter the body (bacteria, viruses, but also transplanted organs, etc.). The immune system can recognize them as non-self because on the surface of these antigens are molecules different from those found in the body (self). Antibodies specifically bind to the antigen and neutralize its function by inducing other reactions that remove the antigen from the body (activation of complement, macrophages, etc.). In the 19th century, Behring and Kitaso found that the blood serum of immunized animals contained specific neutralizing substances. This was the first demonstration of the antibodies. The determination of specific antibodies by serological methods is used in the diagnosis of many infectious diseases.

Antibodies are:

- glycoproteins
- present in serum and body fluids
- induced when immunogenic antigens are introduced into the lymphoid system of the host
- specifically attack and neutralize antigens that are identical to the one that triggered the immune response and induced their formation

The basic terms used in serological reactions include the affinity and the avidity of the antibody.

- **Affinity** is the strength of binding of one molecule to another at a single site, e.g. the binding of a monovalent Fab fragment of antibody to a monovalent antigen. The affinity of an antibody expresses the strength of the binding between one epitope of a particular antigen and the binding site of the antibody. It is related to the specificity of the antibodies. Affinity increases during the immune response to a specific antigen. The binding of antibodies to a given antigen becomes better over multiple exposures. This process is called affinity maturation.

- **Avidity** is the total strength of binding of two molecules or cells to one another at multiple sites. It is distinct from affinity, which is the strength of binding of one site on a molecule to its ligand. The term avidity is often used to describe the collective affinity of multiple binding sites of an immunoglobulin. For example, the IgM antibody is a pentamer and has 10 binding sites. It can, therefore, bind antigens with very high avidity, although the affinity of the individual binding sites is low. The avidity of an antibody expresses the strength of the interaction of a polyvalent antibody with a polyvalent antigen. It increases with affinity as well as with the number of binding sites.

Biological properties of Immunoglobulin Classes

Tab. 4 Biological properties of Immunoglobulin Classes (Source: *Rich et al., 2019*)

IgM

- Principal immunoglobulin of the primary immune response
- Generally restricted to the vascular compartment
- Antigen receptor (in form of monomer) for most naive B cells
- Fixes complement potently

IgG

- Principal immunoglobulin of secondary immune responses
- Binds to Fc receptors on neutrophils, monocytes/macrophages, NK cells
- Ig1 – Ig4 subclasses with different functions
- Fixes complement, except subclass IgG4

IgA

- Principal immunoglobulin of mucosal immunity
- IgA1 and IgA2 subclasses
- They can opsonize but they cannot activate complement

IgD

- Antigen receptor for mature B cells
- Typically coexpressed with membrane IgM

IgE

- Binds to Fc receptors on mast cells, basophils and eosinophils
- Antibody of immediate hypersensizivity, important in allergic reactions
- Important in defence against helmints

What is serum and plasma?

Either plasma or serum may be separated from the blood cells by centrifugation. The essential difference between plasma and serum is that plasma retains fibrinogen (the clotting component), which is removed from serum.

- ✓ **The serum is the yellow fluid obtained after centrifugation of clotted blood**. It contains many different molecules but no cells, fibrinogen or clotting factors. It is more common to use serum than plasma to the laboratory diagnosis in immunology such as detection of antibodies, because clotting factors may interfere with certain assays.
- ✓ **Plasma is a transparent yellow fluid component of blood** containing water, plasma proteins and electrolytes. In plasma are also present fibrinogen and clotting factors because it is **obtained after centrifugation of uncoagulated blood.**

Plasma from which fibrinogen and clotting factors have been removed is called serum. For serum preparation, venous blood is collected under aseptic conditions, in a volume of 5-10 ml into a test tube without anticoagulants (without heparin or EDTA).

Examined sera must not be hemolytic, bacterially contaminated, or repeatedly thawed and frozen. The serum needs to be processed immediately, as otherwise some parameters would be altered by their degradation. If serum processing cannot be performed immediately, it may be frozen for extended periods at –20°C. A frozen specimen should be held in a freezer at 0°C to –20°C but some specific tests require the specimen to be frozen at –70°C (dry ice).

3.4 SEROLOGICAL REACTIONS

Serological reactions are in vitro reactions of antigen with antibody in a particular environment. They are used in serological assays usually to detect the presence of antibodies in human serum against foreign antigens (e.g. microorganisms) or self-antigens (e.g. rheumatoid factor).

3.4.1 Phases of serological reactions

Each **serological reaction has two phases**: specific and non-specific.

- In a **specific phase**, the antibody reacts with a specific antigen.
- In the **non-specific phase**, the formed immunocomplex is visualized.

The ratio of antigen and antibody in the reaction, the environment in which the reaction takes place, and the method of visualization play an important role. Depending on these conditions, serological reactions are referred to by different names.

Depending on the used antigen, which can be **corpuscular** (insoluble) or **colloidal (soluble),** we distinguish several basic types of serological reactions, namely agglutination, precipitation and complement fixation. The components of serological reactions are labelled with different markers. **According to the technique** used to visualize the result, we distinguish, for example, fluorescence, enzyme immunoassay, radioimmunoassay, etc. **The environment** in which the serological reaction can take place is liquid or gel (prepared from agar or agarose).

3.4.2 Applications of serological reactions

Serological reactions can be used both in direct diagnostics and indirect diagnostics.

- **The direct diagnosis** is the detection of specific microorganisms or their parts (antigens) in a patient's biological sample or the determination of its antigenic properties, also known as their serotyping. Direct diagnosis means making visible or proving the presence of an aetiological agent or some of its components.
- **The indirect diagnosis** is identifying the specific antibodies in biological material from the patient, or individual classes of specific antibodies, using a known antigen. Indirect diagnosis involves the detection of specific antibodies of various isotypes and functions which are produced by a host in different quantities at different times in the immune response to infection.

3.4.3 Evaluation of serological reactions

Evaluation of serological reactions is qualitative and quantitative:

- **qualitative assessment** – positivity or negativity is determined by comparison with threshold values
- **quantitative evaluation** – the antibody titre is determined. A positive result indicates the highest serum dilution in which the antigen-antibody reaction is detectable (e.g. 1:160). The result can also be expressed as a titre, which is the inverted serum dilution value (titre 160).

Each detection of specific antibodies by a certain method has its own evidential value. The classical methods (agglutination, complement fixation test, precipitation etc.) detect a mixture of antibody classes and diagnosis requires observation of **the dynamics of their production in at least two samples taken over 14–21 days**. Different stages of the disease are characterised by the presence of specific antibodies of different isotypes:

- IgM antibodies are detectable from the 7th–10th day after infection, depending on the sensitivity of the assay. IgM antibodies are a sign of acute infection and remain for 3–6 months after primary infection. The detected IgM antibodies must be evaluated for each infectious agent individually. The presence of IgM in serum is temporary and indicates a recent acute infection. It can usually be confirmed with one sample.
- IgG antibodies appear later, around the 14th day of infection) and culminate in the 4th–6th week after infection. They persist for a long time. They are a sign of protective immunity. Tests for IgG antibodies often require paired samples. The first sample should be taken during the acute phase of infection and the second sample during the convalescence period.
- IgA antibodies are produced in the acute phase of infection, but they are also produced when a chronic disease is reactivated or on re-exposure to an infectious agent.
- IgE antibodies are associated with parasitic diseases.

The interpretation of laboratory findings requires experience and good theoretical knowledge because the persistence of antibodies for many years after acute infection and the existence of chronic infections that can become reactivated broadens the range of possible laboratory findings. The results of serological laboratory methods should be considered comprehensively with regard to the patient's clinical condition and the results of other examinations.

Confirmation of acute infection:

- a **fourfold increase of total antibody titer**
- **seroconversion** – from negativity to positivity, or
- **presence of IgM**

At the onset of the disease, indirect evidence of the causative agent by serological reactions may show a negative result even though the infection is ongoing. This is a **false negativity**. Antibodies can only be detected after a certain time. The time interval from the onset of infection to the period when the causative agent can be detected by serological examination of antibodies is called the **serological window period**. It depends on the length of the seroconversion. Knowing

the length of the window period is important from an epidemiological point of view in the spread of serious infections, also in the examination of blood donors, organ transplants, etc. **During the interval of the serological window period, it is not possible to reliably diagnose the infection in the patient by means of indirect serological examinations.** However, it is possible to use direct diagnostic methods (detection of antigen, nucleic acid, etc.).

3.5 AGGLUTINATION

Agglutination is a serological reaction in which a corpuscular (insoluble) **antigen** (called agglutinogen) **reacts with a specific antibody** (called agglutinin) **in a specific environment** (in liquid medium, e.g. saline) **to form an agglutinate** (Figure 32). An antigen reacts with specific antibodies to form non-dispersible clumps while simultaneously clarifying the liquid medium or suspension. Agglutinin is an antibody capable of inducing agglutination (aggregation) of corpuscular antigens (or antigen-bound corpuscular particles). Agglutination occurs optimally when antigens and antibodies react in equivalent proportions. This method depends on monitoring an antigen-antibody complex, which forms visible clumping.

Agglutination tests are easy to perform. These tests have a wide range of applications in the clinical diagnosis of non-infectious immune disorders and infectious diseases. Agglutination reactions have a wide variety of applications in the detection of both antigens and antibodies in serum and other body fluids. They are very sensitive and the result of the test can be read ease visually.

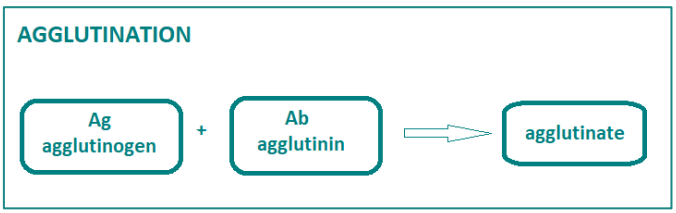


Fig. 32 Agglutination (Source: *Martina Neuschlova*)

3.5.1 Types of agglutination reactions

Various methods of agglutination are used in diagnostic immunology and generally are divided into direct and indirect.

In **direct agglutination**, the antigen suspension is formed directly by particles, which occur naturally on the surface of microrganisms (e.g. bacterial surface antigens).

In **indirect agglutination**, the antigen has been conjugated to the surface of suitable macromolecular particles, known as carriers. Suitable carriers could be biological (such as erythrocytes) or artificial (such as latex or charcoal particles).

3.5.2 Direct agglutination

In direct agglutination, the serum is diluted geometrically. **Then a standard amount of known antigen is added to the diluted serum.** It is used to prove the presence of antibodies. This method is used for the detection of antibodies in typhoid fever, paratyphoid fever, salmonellosis (Widal reaction), in spotted fever (Weil-Felix reaction), it also serves for the detection of antibodies in brucellosis, tularemia, listeriosis, yersiniosis, dysentery, cholera, etc.

Widal test

Widal test is an agglutination test, which detects the presence of antibodies in patient's serum produced against the causative agents of typhoid and paratyphoid fever especially in the endemic regions. The test was based on demonstrating the presence of agglutinin (antibody) in the serum of an infected patient, against the H (flagellar) and O (somatic) antigens of Salmonella typhi. Widal test is helpful and significant in typhoid fever cases in endemic regions only if patients have four-fold or greater increases in O or H agglutinin titres in serum specimens obtained 2–3 weeks apart.

Procedure: Widal test can be done in two ways: one is rapid test on slide and another is tube test in which result may be obtained after one night of incubation. Classical tube agglutination method is a quantitative method based on the detection of antibodies against individual salmonella antigens (flagellar H antigen, somatic O antigen). The serum is diluted geometrically. Dilutions 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 and control tube only with isotonic saline. Then a drop af appropriate widal test antigen is added to all the test tubes. The incubation occurs in termostat at 37°C for 16-20 hours. Antibody titre is the highest dilution of serum showing clear agglutination.

Result: The reaction in the tube is positive if the supernatant is completely clear and there is sediment at the bottom of the tube. It is a macroscopically visible agglutinate in the form of small fine grains or flaky clumps. In the control tube (serum-free), turbidity must be homogeneous in the absence of agglutinate (Figures 33, 34).

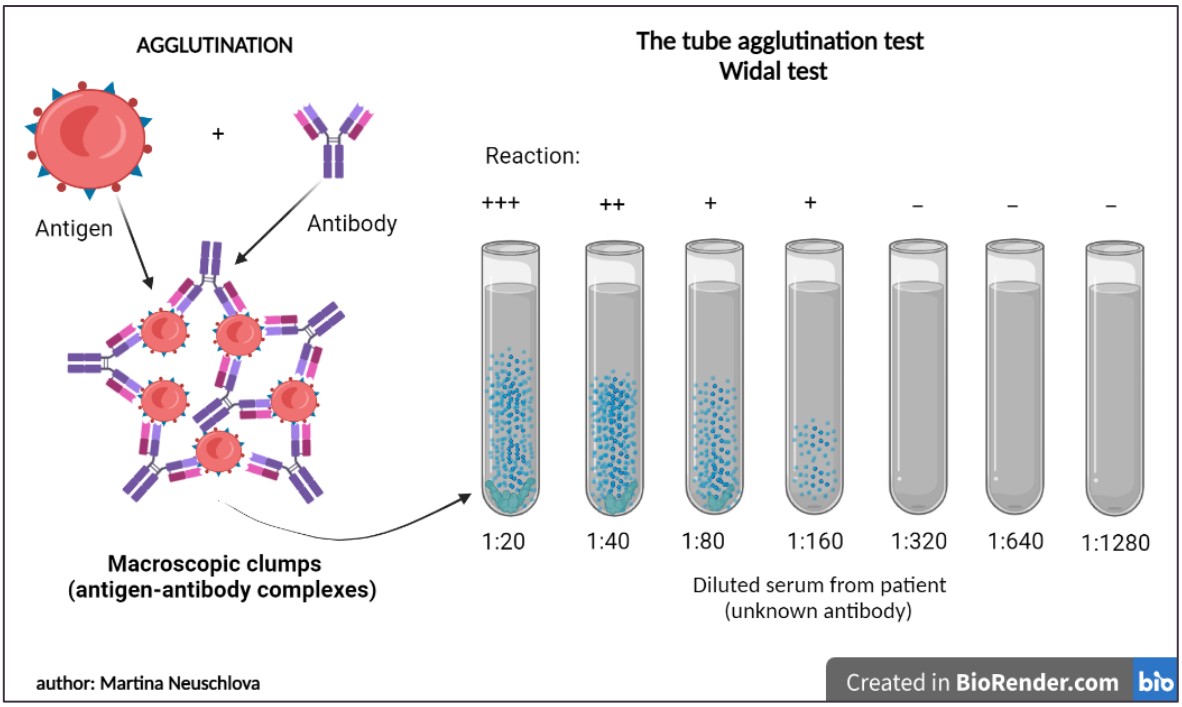


Fig. 33 Tube agglutination test, Widal test - schema
The titre = 160 (Source: *Neuschlova, 2021; created in BioRender*)

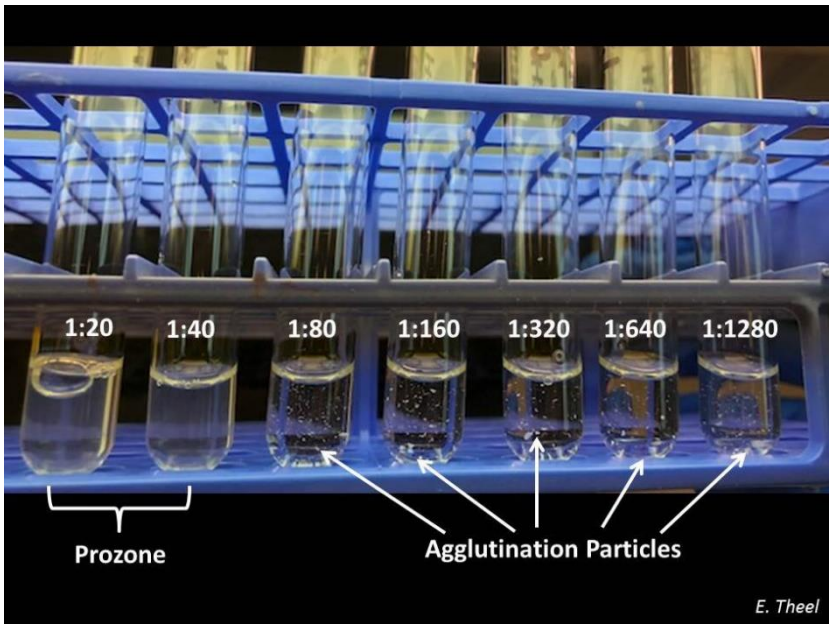


Fig. 34 Serum tube agglutination test (Source: *E. Theel*)

Newer methods that have been developed as a fast and reliable alternative to the Widal reaction include IDL Tubex® TF, which can detect IgM O9 antibodies in a matter of minutes. The principle is based on inhibition magnetic binding immunoassay – a semiquantitative colometric assay. Furthermore, Typhidot®, which detects specific IgM and IgG antibodies against Salmonella typhi antigen in human serum or plasma. A newer version of this Typhidot - M® test was developed only to detect specific IgM, the test is based on the binding of S. typhi to specific IgM antibodies.

Serotyping – slide agglutination test

It is a **qualitative agglutination reaction performed on a slide**. Microorganisms from the same species can differ in the antigenic determinants expressed on the cell surface. **Serotyping** is one of the classic tools for epidemiological study and is applied to numerous species that express different serotypes. Slide tests are commonly **used for rapid identification of bacteria** (e.g. strains of the genus *Salmonella, Shigella, Yersinia enterocolitica, Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa, Francisella tularensis, Haemophilus influenzae* etc.). By using antisera that are specific for individual antigens, it is possible to determine the antigenic structure of a bacterium. The method consists of mixing a drop of antibody and a pure colony of tested bacteria on a glass slide. A positive reaction is indicated by the formation of visible clumps within a minute. **Polyvalent and monovalent diagnostic agglutination sera** are available. Polyvalent antisera contain several antibodies in one bottle. Monovalent antisera contain only one type of antibody in one bottle. Agglutination is first performed with polyvalent antisera and, if positive, agglutination is continued with the individual monovalent antisera that were contained in the respective polyvalent antiserum.

Procedure: Serotyping is performed after identification of the species on a fresh, pure culture of tested bacteria isolated on a solid medium. Where polyvalent and monovalent antisera are available, serotyping is performed first with polyvalent sera, then with the specific monovalent sera corresponding to the mixture giving marked agglutination.

- Place one drop of antisera onto a slide for agglutination and add a pure colony of tested bacteria.
- Mix reagents thoroughly.
- Rock the slide in the circular motion for 30 seconds and observe for agglutination (Figure 35).

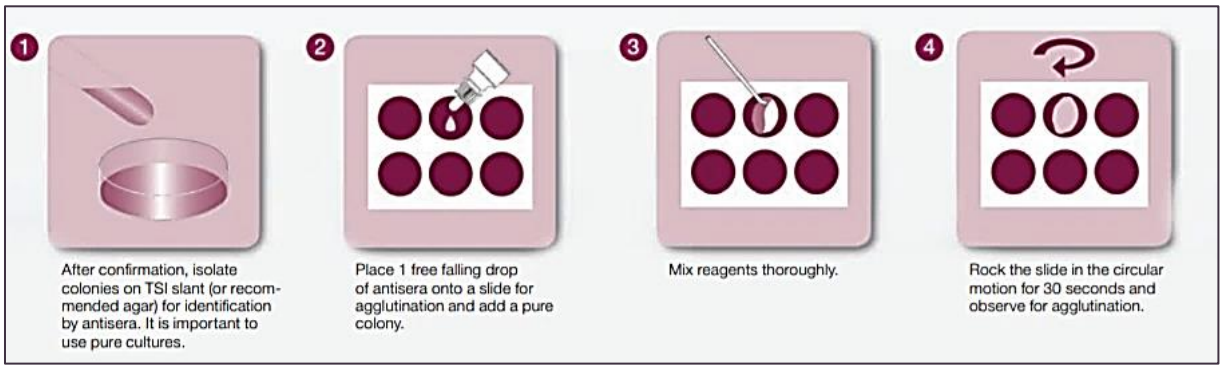


Fig. 35 Serotyping – a simple protocol

(Source: *https://www.bio-rad.com/webroot/web/pdf/fsd/literature/FSD_14-0699.pdf*)

Result: Positive agglutination is manifested by the formation of clumps and the fluid is completely or partially clarified. The evaluation is best against light or on a black background (Figure 36). Based on agglutination with monovalent serum, the serotype of the examined bacterium is determined.

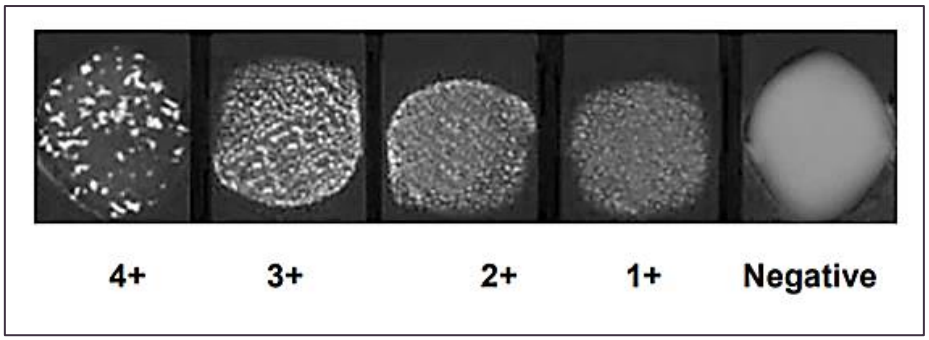


Fig. 36 Serotyping with the use of polyvalent antisera

A homogenous suspension is a negative reaction. If agglutination is observed with one of the polyvalent antisera, the serotype of the isolate is determined by testing the isolate with monovalent antisera specific to the serotypes / sub-factors found in the polyvalent sera.

(Source: *https://antimicrobialresistance.dk/CustomerData/Files/Folders/6-pdf-protocols/58_23-10-gfn-shigellaserotypification-final-29-06-10.pdf*)

Cold agglutination

Cold agglutinins are normally made by the immune system in response to some infections. Healthy people generally have low levels of cold agglutinins in their blood. But lymphoma or some infections, such as mycoplasma pneumonia, infectious mononucleosis, can cause the level of cold agglutinins to rise. **They cause red blood cells to clump together (agglutinate) at low temperatures (0–5°C). At higher temperatures, they lose this ability.** Blood is drawn from the patient for examination, allowed to clot at room temperature, and serum is collected. After diluting the serum in a geometric series, a suspension of group 0 human erythrocytes is added to each tube. It is placed in a refrigerator and the reaction is read after 24 hours. The presence of cold agglutinins results in the formation of agglutinate. However, this agglutinate disappears after incubation in a 37°C water bath. Sometimes high levels of cold agglutinins can destroy red blood cells throughout the body. This condition is called autoimmune hemolytic anemia.

Prozone phenomenon

A prozone phenomenon can sometimes be observed when evaluating agglutination reactions in test tubes. At a high concentration of antibodies, the number of epitopes is outnumbered by antigen-binding sites. This results in the univalent binding of antigen by antibody rather than multivalently. Only small immunocomplexes are formed, and therefore appear macroscopically negative. The condition of excess antibody is called a prozone phenomenon (Figure 34). This may be due to the presence of incomplete antibodies or blocking antibodies. Although incomplete antibodies can bind to the antigen, there is no second phase of the reaction and thus no visualization of the serological reaction. Occasionally, antibodies are formed that react with the epitopes of a cell but does not cause any agglutination. They inhibit the agglutination by the complete antibodies added subsequently. Such antibodies are called blocking antibodies.

3.5.3 Indirect agglutination

Indirect agglutinations are reactions employing **carrier particles that are coated with soluble antigens**. **Suitable carriers could be artificial (such as latex or charcoal particles) or biological (such as erythrocytes)**. The best known examples of indirect agglutination are **hemagglutination and latex agglutination**.

Hemagglutination tests

Passive hemagglutination assay and hemagglutination inhibition test are modifications of the agglutination tests that are used to detect antibodies against both bacterial and viral antigens (e.g. *Treponema pallidum*, respiratory syncytial virus, influenza viruses, rubella virus). Hemagglutination usually uses a suspension of formalized sheep erythrocytes, which can be sensitized with various soluble antigens.

In **passive hemagglutination assay**, red blood cells generally collected from sheep, chicks, or humans are coated with antigen and incubated with biological samples to detect the presence of corresponding antibodies and the degree of hemagglutination is assessed to detect the antibodies (Figure 37, 38, 39).

Procedure: The serum is diluted geometrically. Each well contains particulate antigens coated on red blood cells. In a positive (agglutinated) reaction sufficient antibodies are present in the serum to link the antigens together forming a mat of antigen-antibody complexes on the bottom of the well. In a negative (nonagglutinated) reaction, not enough antibodies are present to cause a linking of antigens. The particulate antigens roll down the slopping sites of the well, forming a pellet at the bottom. In the example (Figure 37), the antibody titer is 160 because the well with a concentration of 1:160 is the most dilute concentration that produces a positive reaction.

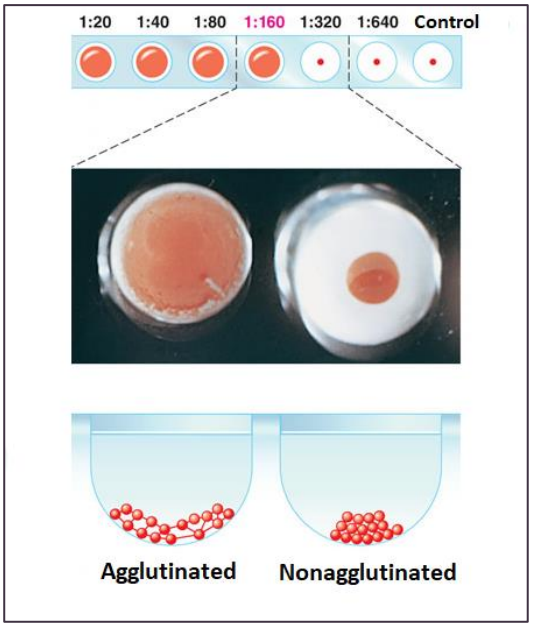


Fig. 37 Passive hemagglutination (Source: © 2013 Pearson Education, Inc)
(*http://classes.midlandstech.edu/carterp/Courses/bio225/chap18/lecture3.htm*)

The applications of passive hemagglutination are very wide. For example, it can be used in the diagnosis of sexually transmitted diseases, e.g. syphilis (lues). It is a TPHA test (*Treponema pallidum* hemagglutination test). It is a specific assay that captures total treponemal antibodies. It is used when syphilis is suspected. During pregnancy it is used to rule out infection in a pregnant woman because in a positive case there is a risk of intrauterine infection with teratogenic consequences.

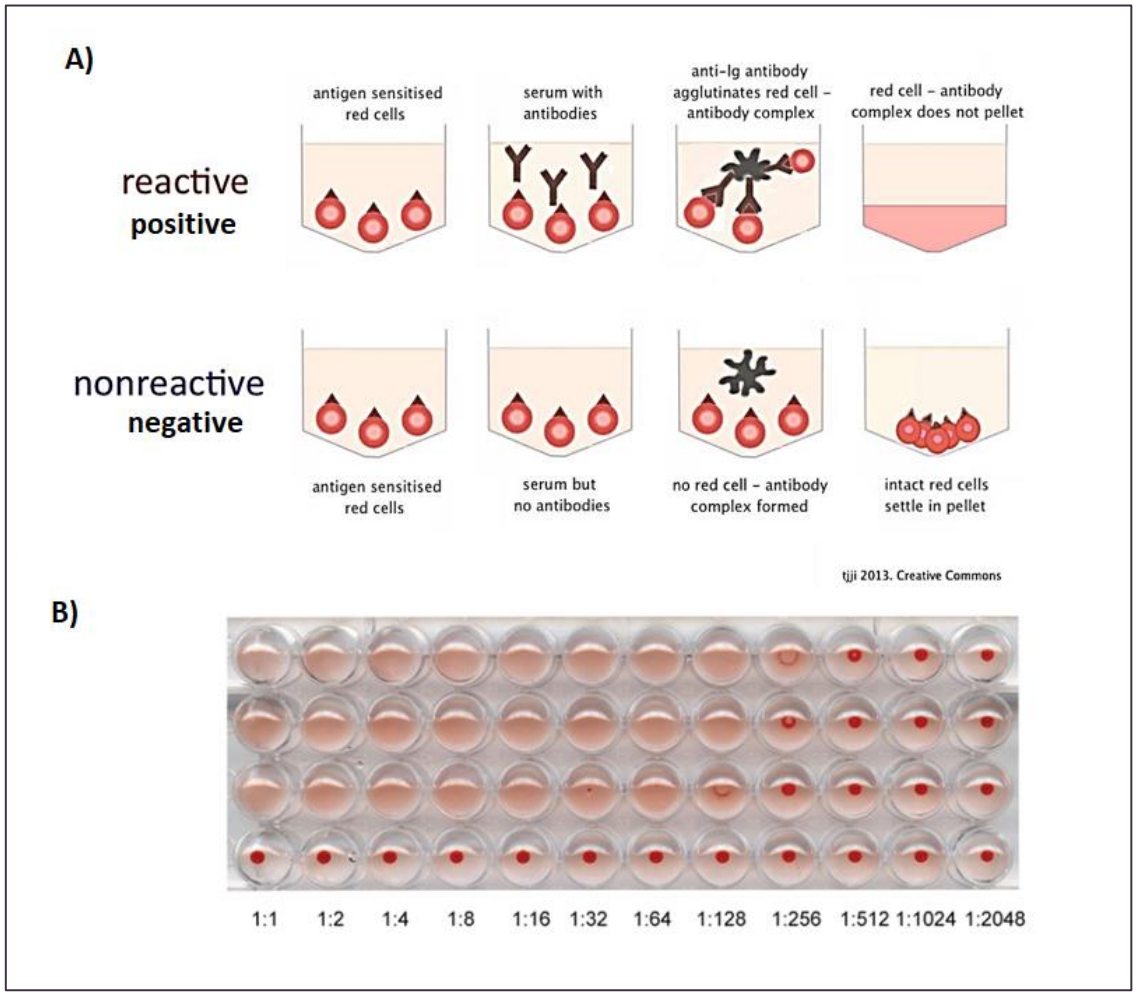


Fig. 38 Hemagglutination assay

A) reactive = positive, nonreactive = negative (Source: *http://www.medical-labs.net/wp-content/uploads/2014/11/Indirect-Haemagglutination-Test.jpg*)

B) the highest dillution of the sera showing complete agglutinating activity of erythrocytes was considered as hemagglutination titers (Source: *https://www.researchgate.net/figure/Hemagglutination-assay-Triplicates-of-purified-plant-produced-hemagglutinin-rHA0-were_fig11_221971435*)

Hemagglutination inhibition test is used to detect and titrate antibodies developed against a virus. This test detects the presence of antibodies based on the inhibition of hemagglutination, which some microorganisms cause spontaneously. A virus normally causes hemagglutination of red blood cells. If antibodies against the virus are present, they neutralise its function and inhibit hemagglutination – hemagglutination inhibition test, virus neutralisation test (Figure 39). The highest dilution in which hemagglutination is not visible determines the antibody titre in the tested serum.

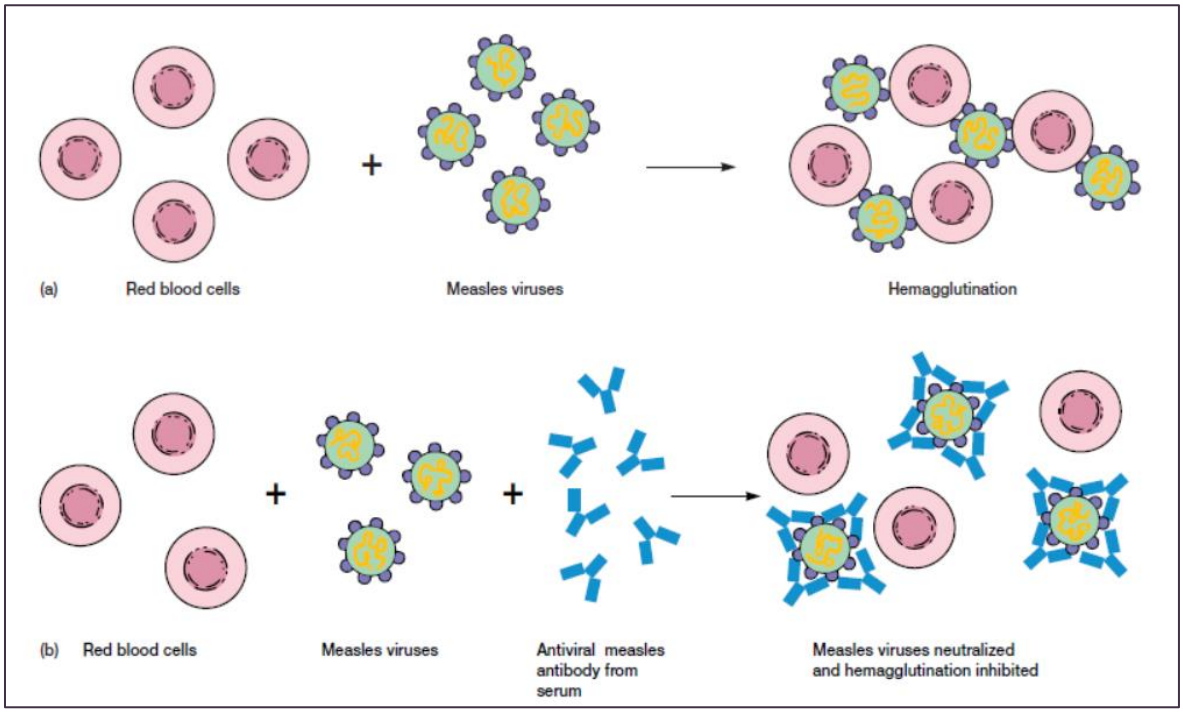


Fig. 39 Hemagglutination inhibition test (Source: *https://i2.wp.com/microbeonline.com/wp-content/uploads/2014/12/Hemagglutination-and-Hemagglutination-Inhibition-test.png?ssl=1*)

Latex agglutination

The latex agglutination test is a **laboratory method to check for certain antibodies or antigens in a variety of body fluids including saliva, urine, cerebrospinal fluid, or blood.** Latex agglutination is an **indirect agglutination** that is used as a rapid test to obtain the result within minutes. The test depends on what type of sample is needed. The sample is mixed with latex beads coated with a specific antibody or antigen. If the suspected substance is present, the latex beads will clump together (agglutinate). Latex agglutination results take about 15 minutes to an hour. Latex tests are very popular in clinical laboratories to detect etiology of bacterial meningitis from cerebrospinal fluid (*Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, Escherichia coli* a *Streptococcus agalactiae*). Agglutination occurs when the pathogen of interest is present in the cerebrospinal fluid. Latex tests are also available to detect *Clostridium difficile* toxins A and B and rotavirus from the stool, and serotyping of *Neisseria gonorrhoeae* strains and to confirm *Staphylococcus aureus* or betahemolytic streptococcus, etc.

The large particle size of latex facilitates the visualization of the antigen-antibody reaction. Latex agglutination tests are easy to do and rapid to perform. Latex agglutination can detect the presence of both antibodies and antigens.

- If we want **to detect specific antibodies**, known microbial antigens must be bound to the latex particles.
- If we want **to detect antigens** of microorganisms, known specific antibodies must be bound to the latex particles (Figure 40 and 41).

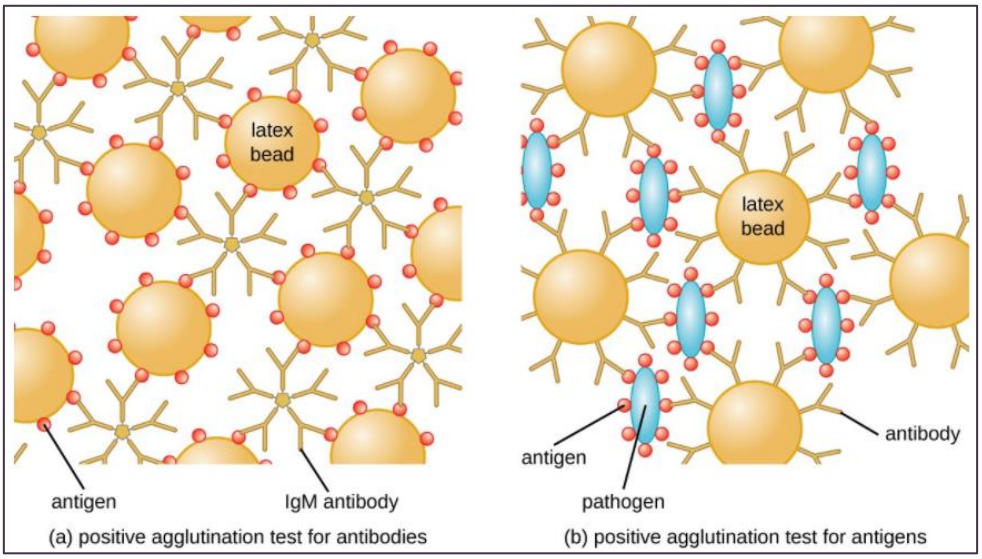


Fig. 40 Latex agglutination to demonstrate the presence of antibodies (a) or antigens (b)
(a) Latex beads coated with an antigen will agglutinate when mixed with patient serum if the serum contains antibodies against the antigen.
(b) Latex beads coated with antibodies will agglutinate when mixed with patient material if the material contains antigens specific to the antibodies.
(Source: *https://opentextbc.ca/microbiologyopenstax/chapter/agglutination-assays/*)

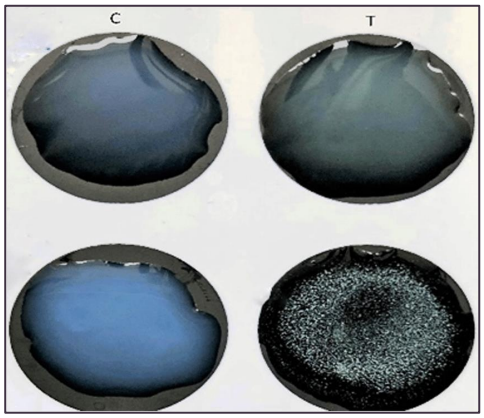


Fig. 41 Latex agglutination test
Agglutination of latex particles were occurred, when antigens present in sample and this agglutination interpreted with the naked eye in few min (bottom right).
(Source: *https://www.researchgate.net/figure/Latex-agglutination-test-of-fecal-supernatant-in-patients-with-diarrhea-Agglutination-of_fig4_333906288*)

3.6 PRECIPITATION

Precipitation is a serological reaction **in which a soluble (colloidal) antigen (called precipitinogen)** reacts **with a specific antibody (called precipitin) in a specific environment (in saline or gel) to form a precipitate** (Figure 42). Antibodies are the major component of the precipitate, so antigen is usually diluted in precipitation. Precipitation reaction can be broadly of three types: precipitation in solution, in agar or in agar with an electric field.

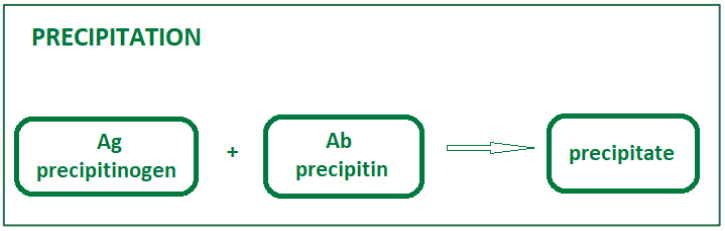


Fig. 42 Precipitation (Source: *Martina Neuschlova*)

3.6.1 Zone of equivalence

The precipitation reaction can take place in a liquid or gel medium. The size of the precipitate depends on the antigen and antibody concentration. **The amount of precipitate** decreases with both an excess of antigen and an excess of antibody in the reaction.

Most of the precipitate forms in the equivalence zone, where complete antigen-antibody binding occurs. A graph of precipitate formation as a function of antigen concentration using a constant amount of antibody is shown in the precipitation curve (Figure 43).

In the zone of equivalence, the concentrations of the antigen and antibody are equal and create the maximum number of immune complexes (precipitates).

In the zone of antibody excess (known as the **prozone phenomenon**), there is the insufficient antigen to create a visible number of immune complexes. Excess antibodies (in a highly positive sample) prevent particle crosslinking and the result appears to be negative (a false negative). In the prozone phenomenon, there is too much antibody for efficient lattice formation. This is because antigen combines with only a few antibodies and no cross-linkage is formed.

In the zone of antigen excess (known as the **postzone phenomenon**), there are insufficient binding sites and a sufficient number of immune complexes cannot form. In postzone phenomenon, small aggregates are surrounded by excess antigen and again no lattice network is formed.

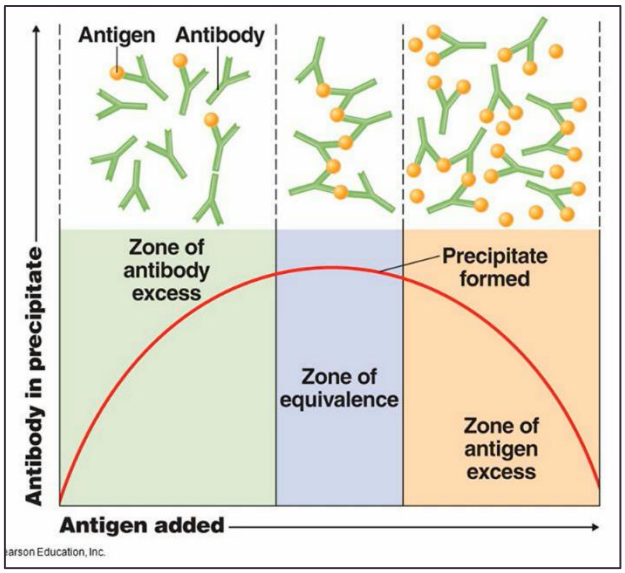


Fig. 43 Precipitation curve with prozone, zone of equivalence and postzone
The zone of antibody excess is known as the prozone phenomenon and the zone of antigen excess is known as postzone phenomenon. Precipitation reaction occurs optimally only when the proportion of the antigen and antibody in the reaction mixture is equivalent (in zone of equivalence)
(Source: *https://www.biosciencenotes.com/precipitation-test/*)

3.6.2 Precipitation in liquid medium

Precipitation reaction can be carried out in a liquid medium (in solution). An example of precipitation in a liquid medium is a **ring test, in which a precipitation ring is formed** at the interface. It is used, for example, in Ascoli's thermo precipitation test for the detection of *Bacillus anthracis* antigens. An extract is prepared from the examined material (a piece of tissue, mainly the spleen) by boiling in a physiological solution. *B. anthracis* polysaccharide antigen is detected in this extract. Ring test consists of layering the antigen solution over a column of antiserum in a narrow tube. A precipitation ring is formed on the interface (Figure 44).

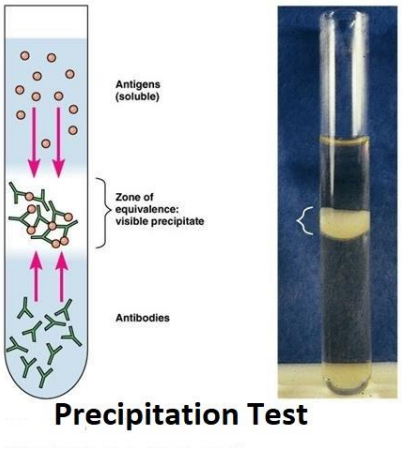


Fig. 44 Precipitation in solution (Source: *https://www.biosciencenotes.com/precipitation-test/*)

3.6.3 Precipitation in gel medium – immunodiffusion

Precipitation carried out **in a gel medium** is known as **immunodiffusion.** Immunodiffusions are highly developed and the most common variants currently in use include single radial immunodiffusion, double immunodiffusion and immunoelectrophoresis. These methods are often known by the names of their inventors (Mancini method, Ouchterlony method). Agar or agarose gel is most often used for immunodiffusion. A precipitation line or ring of precipitation are formed at the site of the most optimal reaction of the antigen with the antibody. Immunodiffusion methods are generally divided into single and double immunodiffusion:

- **single immunodiffusion** – only one component diffuses into the gel medium, the other component is present in the gel
- **double immunodiffusion** – both the antigen and the antibody diffuse into the gel medium at the same time

Single radial immunodiffusion

An example of single immunodiffusion is **the single radial immunodiffusion (RID)** or **Mancini method.** It is the basic method for the quantitative determination of antigens in a sample. A solution containing the antigen is placed into wells in a gel or agar surface evenly impregnated with an antibody. The diameter of the ring that precipitates around the well as a result of antigen-antibody reaction corresponds to the amount of antigen in the solution.

Principle: Radial immunodiffusion is a type of precipitation reaction that is based on the antigen-antibody interaction and formation of a visible cross-linked precipitate when antigen and antibody are present in equivalent concentrations. In the test, an antibody is incorporated into a gel medium and poured into a glass plate to form a uniform layer. Circular wells are cut into the gel and antigen is introduced into these wells. Specific antigens to the incorporated antibodies diffuse through the gel in all directions from the well and react with specific antibodies, forming a visible ring of precipitation in the zone of equivalence. The diameter of the precipitate ring corresponds to the amount of antigen in the solution (Figure 45, 46, 47).

Procedure:

- An agar containing an appropriate antibody is poured into a glass plate. Carefully circular wells are cut and removed from the plates.
- A single or series of standards containing a known concentration of antigen are placed in separate wells, while control and "unknown" samples are placed in other remaining wells.
- As the antigen diffuses radially, a ring of precipitate will form in the area of optimal antigen-antibody concentration. The ring diameters are measured and noted (Figure 46, 47).

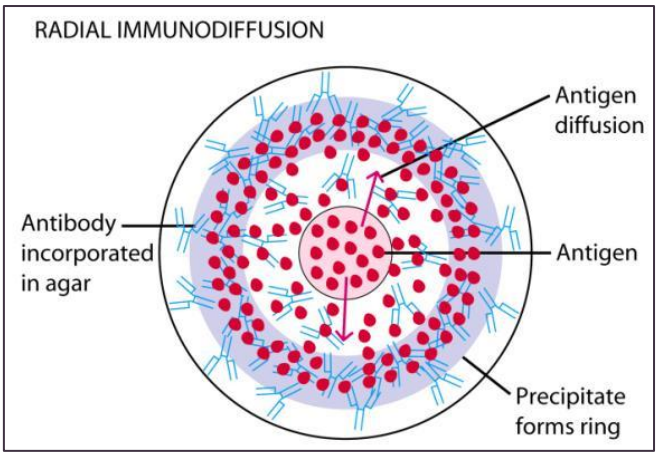


Fig. 45 Single radial immunodiffusion – principle
(Source: *http://www.sbs.utexas.edu/sanders/Bio347/Images/Lectur41.jpg*)

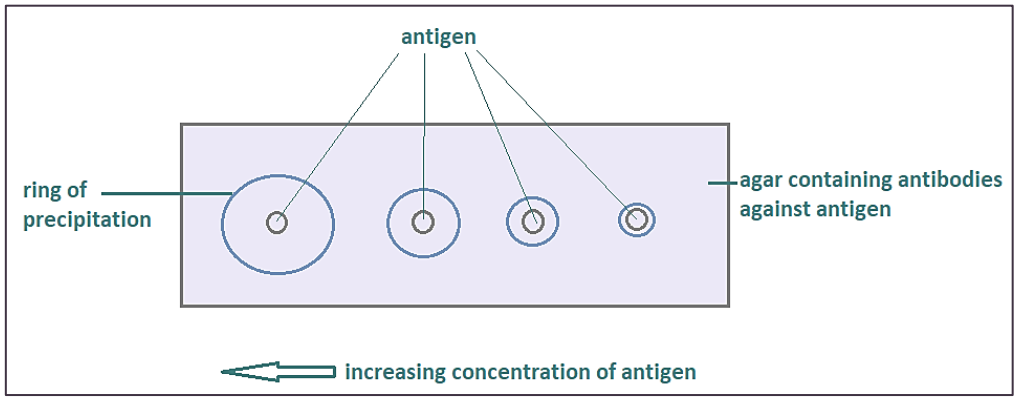


Fig. 46 Single radial immunodiffusion – scheme (Source: *Martina Neuschlova*)

Fig. 47 Single radial immunodiffusion – result
(Source: *https://assets.fishersci.com/TFS-Assets/CCG/product-images/F37532~p.eps-650.jpg*)

Result: The presence of a precipitin ring around the antigen wells indicate specific antigen-antibody interaction. The absence of the precipitin ring suggests the absence of reaction. The greater the amount of antigen in the well, the farther the ring will form from the well. A standard curve is prepared using the ring diameters of the standards versus their concentrations. This curve is then used to determine the concentration of the control and unknown samples (Figure 48).

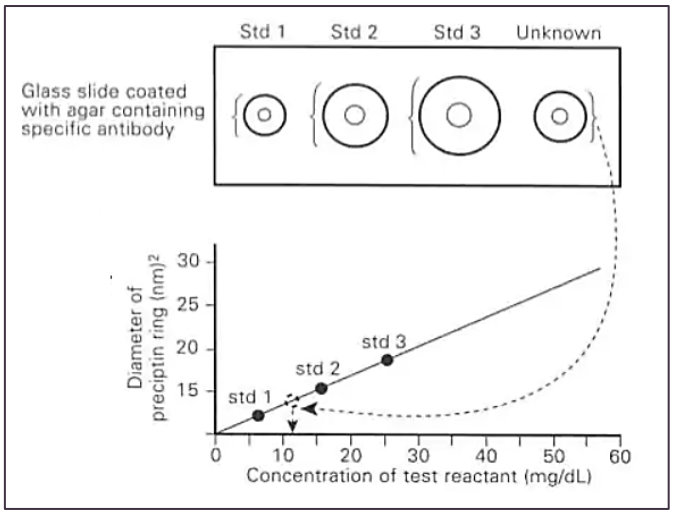


Fig. 48 Single radial immunodiffusion – graph

(Source: *http://image.slidesharecdn.com/immunodiffusion-130121011208-phpapp01/95/immunodiffusion-principles-and-application-9-638.jpg?cb=1359056050*)

Applications:

- mostly to determine the quantity or concentration of an antigen in a sample
- levels of immunoglobulin classes in sera
- levels of components of complement (C3 andC4)
- levels of proteins in sera, etc.

Double immunodiffusion

An example is a **double immunodiffusion** or the **Ouchterlony technique.** It is a **method for the qualitative determination of antigens. I**t is not a suitable method for determining the concentration of antigens. In Ouchterlony double immunodiffusion, both the antigen and antibody diffuse independently through agar gel in two dimensions, horizontally and vertically.

Principle: An antigen solution or a sample extract of interest is placed in wells bore on gel plates. Sera or purified antibodies are placed in other remaining wells. Mostly, an antibody well is placed centrally. On incubation, both the antigens in the solution and the antibodies each diffuse out of their respective wells. In the case of the antibodies recognizing the antigens, they interact together to form visible immune complexes which precipitate in the gel in the zone of equivalence to give a thin white line (called precipitin line) indicating a reaction (Figure 49).

Procedure: An agarose gel is poured into a glass plate and allowed to set. Wells are punched into the gel using a gel borer. Wells are filled with solutions of antigen and antiserum. Antiserum is usually placed in the central well and different antigens (or sample extracts of interest) are added to the wells surrounding the central well. The glass plate is incubated in a moist chamber overnight at 37°C.

Result: The presence of an opaque precipitation line between the antiserum and antigen wells indicates antigen-antibody interaction (Figure 50, 51). The absence of precipitation line suggests the absence of reaction.

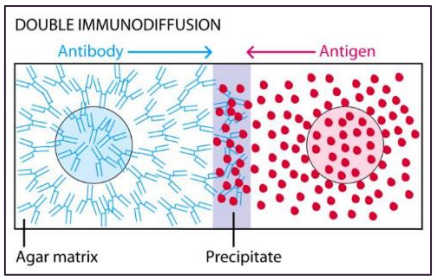


Fig. 49 Double radial immunodiffusion - principle (Source: *http://www.sbs.utexas.edu/sanders/Bio347/Images/Lectur42.jpg*)

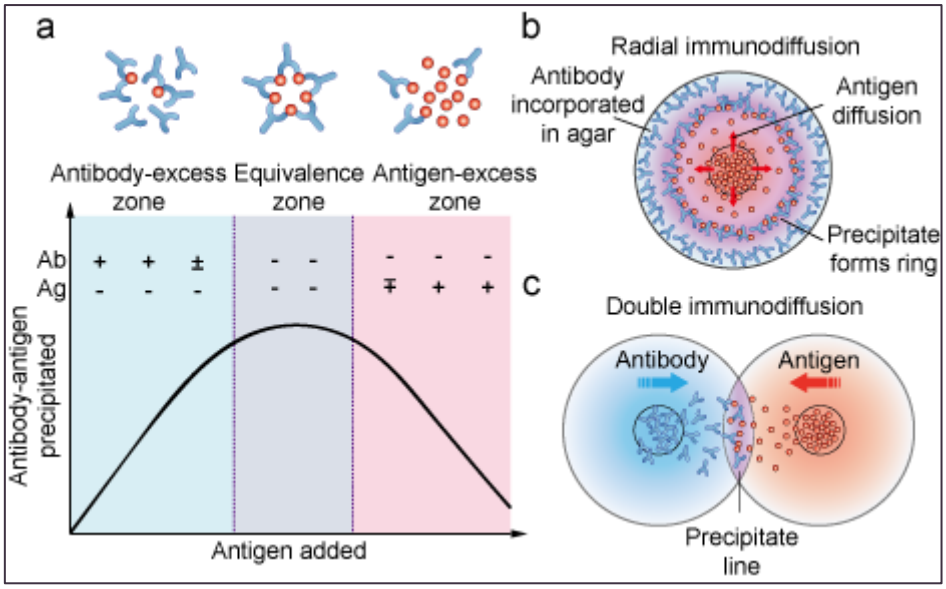


Fig. 50 Types of precipitates in zone of equivalence (Source: *https://www.creative-diagnostics.com/images/immunodiffusion.png*)

Applications: this technique is useful for the analysis of antigens and antibodies. The principle of double immunodiffusion is also used in Elek's test. Elek's precipitation test in the gel is a special test used for the demonstration of toxigenicity of *Corynebacterium diphtheriae*. The antitoxin (equivalent to antibody) is applied to the central well, the suspension of tested strain, where we want to detect the presence of toxin (antigen equivalent) to the peripheral wells. A positive result (toxin production) is manifested by the formation of a precipitation line (Figure 51).

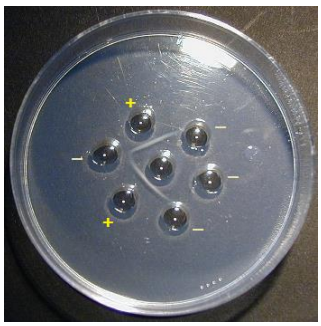


Fig. 51 The Ouchterlony test – double radial immunodiffusion technique: Positive (+) and negative (–) result
(Source: according *http://www.snv.jussieu.fr/bmedia/ATP/images/ouchb1.jpg*)

When more than one well is used there are many possible outcomes based on the reactivity of the antigen and antibody selected. The pattern of the lines can determine whether the antigens are the same. The results may be either of the following:

- Identical (a continuous line) – line of precipitation at their junction forming an arc represents serologic identity or the presence of a common epitope in antigens.
- Non-identical (the two lines cross completely) – a pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens are unrelated and share no common epitopes.
- Partially identical (a continuous line with a spur at one end) – the two antigens share a common epitope, but some antibody molecules are not captured by the antigen and traverse through the initial precipitation line to combine with additional epitopes found in the more complex antigen (Figure 52).

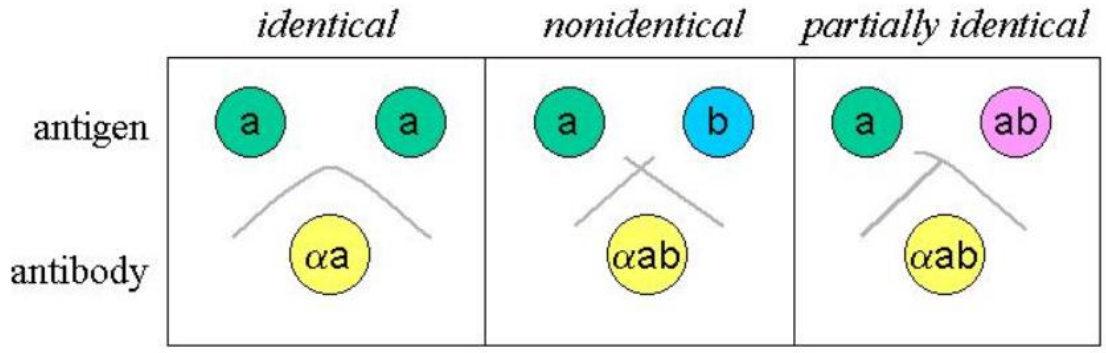


Fig. 52 Double radial immunodiffusion – antigenic relationship between two antigens
(Source: *https://science.umd.edu/classroom/bsci423/song/Lab5.html*)

3.7 ELEKCTROPHORESIS AND IMMUNOELECTROPHORESIS

3.7.1 Electrophoresis

The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with an opposite sign. **Electrophoresis through agarose or polyacrylamide gels is a standard method used to separate**, identify and purify **biopolymers** since both these gels are porous. The mobility of a substance in the gel **depends on both charge and size**.

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative and towards the positive electrode (the anode).

Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. By conventional electrophoresis, a **serum is separated into albumin, alpha1, alpha2, beta1, beta2 and gamma globulins.** Gamma-fraction is particularly important for immunology because it contains immunoglobulins (Figure 53).

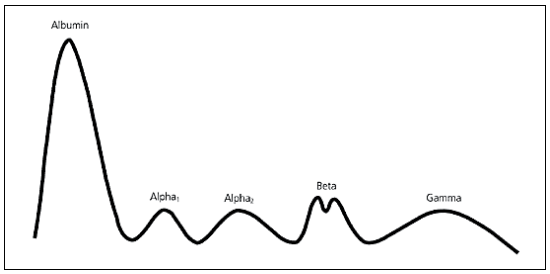


Fig. 53 Typical normal pattern for serum protein electrophoresis in healthy people
(Source: *http://www.aafp.org/afp/2005/0101/afp20050101p105-f1.gif*)

Electrophoresis can capture changes such as:

- **hypergammaglobulinaemia** (when the concentration of all immunoglobulins is increased)
- **hypogammaglobulinaemia** (decreased concentration or lack of immunoglobulins)
- and **monoclonal gammopathy** (deformations of the gamma fraction in the presence of monoclonal immunoglobulin, so-called peak, shown in Figure 54).

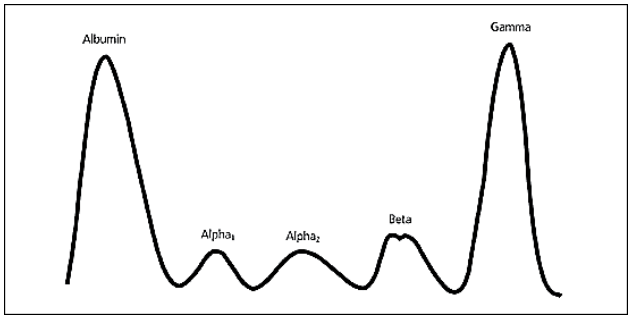


Fig. 54 Abnormal serum protein electrophoresis pattern in a patient with multiple myeloma. Note the large spike in the gamma region.
(Source: *http://www.aafp.org/afp/2005/0101/p105.html*)

3.7.2 Immunoelectrophoresis

Immunoelectrophoresis is a **combination of electrophoretic and immunodiffusion methods**. An antigen mixture is first separated into its component parts by electrophoresis and then tested by immunodiffusion. This technique is similar to the Ouchterlony technique, the only difference being that the antigen movement is facilitated by electrophoresis. An antigen is placed in a well in an agar gel. A current is applied to the gel causing the migration of antigens influenced by charge and size. An opening is then cut in the gel and filled with antiserum containing antibodies. Both antigens and antibodies diffuse into the gel (modification of double radial immunodiffusion) and form precipitates in the area where they meet in concentrations of optimal proportions (in the zone of equivalence).

Result: Precipitin line between the antigen and antisera wells indicate positive reaction or specific antigen-antibody reaction due to the presence of antibody specific to the antigen. The absence of the precipitin line indicates no reaction. The presence of more than one precipitin line indicates the heterogeneity of the antibody for the antigen.

Immunoelectrophoresis has several modifications:

- **classical immunoelectrophoresis** (combination of classical electrophoresis and double radial immunodiffusion) (Figure 55 and 56),
- **rocket immunoelectrophoresis** (the movement of antigen molecules is accelerated by a direct current) (Figure 57),
- **counter-current immunoelectrophoresis** (depends on the movement of antigen towards the anode and of antibody towards the cathode through the agar gel under the electric field),
- **immunofixation** (consists of a protein electrophoresis phase and a fixation phase; after electrophoretic separation of serum proteins, a specific antiserum against some light or heavy immunoglobulin chain is added). This method is used for the detection and typing of monoclonal antibodies or immunoglobulins in serum or urine. It is of great importance for the diagnosis and monitoring of certain blood-related diseases such as myeloma.

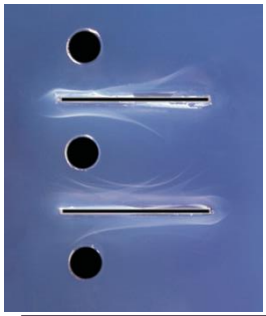


Fig. 55 Classical immunoelectrophoresis – result
(Source: *https://www.edvotek.com/Images/product-items/272_media-1.jpg?resizeid=3&resizeh=200&resizew=200*)

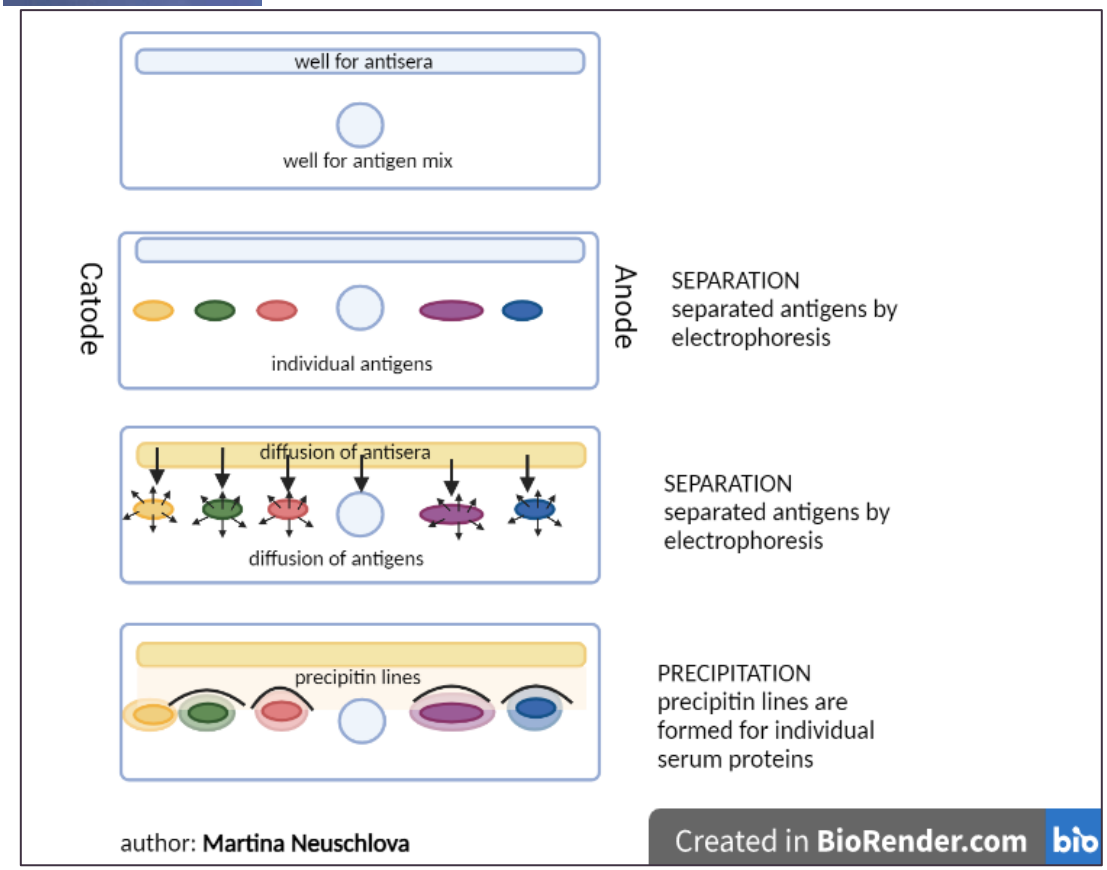


Fig. 56 Classical immunoelectrophoresis – schema
(Source: *Neuschlova, 2021; created in BioRender*)

Rocket immunoelectrophoresis is an adaptation of radial immunodiffusion using electrophoresis, developed by Larell. It is called so due to the appearance of the precipitin bands in the shape of cone-like structures (rocket appearance) at the end of the reaction. An antibody is incorporated in the gel and antigen is placed in wells cut in the gel. Antigen migrates in an electric field in a layer of agarose containing an appropriate antibody. The migration of the antigen toward the anode gives rise to rocket-shaped patterns of precipitation. The height of the rocket, measured from the well to the apex, is directly proportional to the amount of antigen in the sample. Rocket electrophoresis is mainly used for the quantitative estimation of the antigen in the serum (Figure 57).

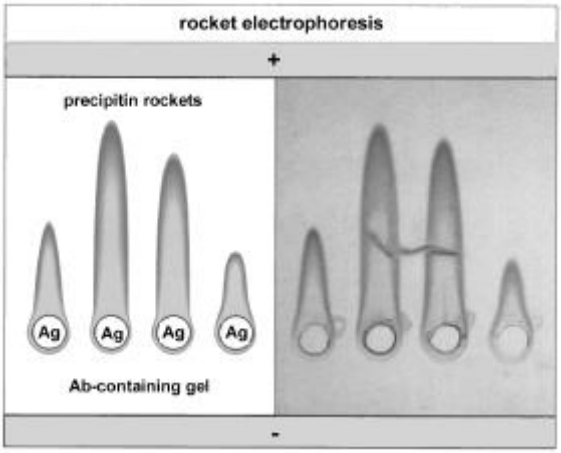


Fig. 57 Rocket immunoelectrophoresis
(Source: *http://www.microbiologybook.org/mayer/Image51.gif*)

3.8 COMPLEMENT FIXATION TEST (CFT)

The Complement Fixation Test (CFT) is the serological method most commonly used to detect the presence of specific antibodies. The principle is the binding of complement to the antigen-antibody complex. Before the reaction, the test serum should be inactivated by heating at 56°C for 30 minutes to remove patient complement activity in the serum sample.

3.8.1 Procedure of Complement Fixation Test

Complement Fixation Test consists of two stages: the **first step is specific,** and **the second step is nonspecific.**

The first step (complement fixation stage): a known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. Patient's serum is heated at 56°C for 30 minutes to inactivate endogenous complement which may disturb the test calibration.

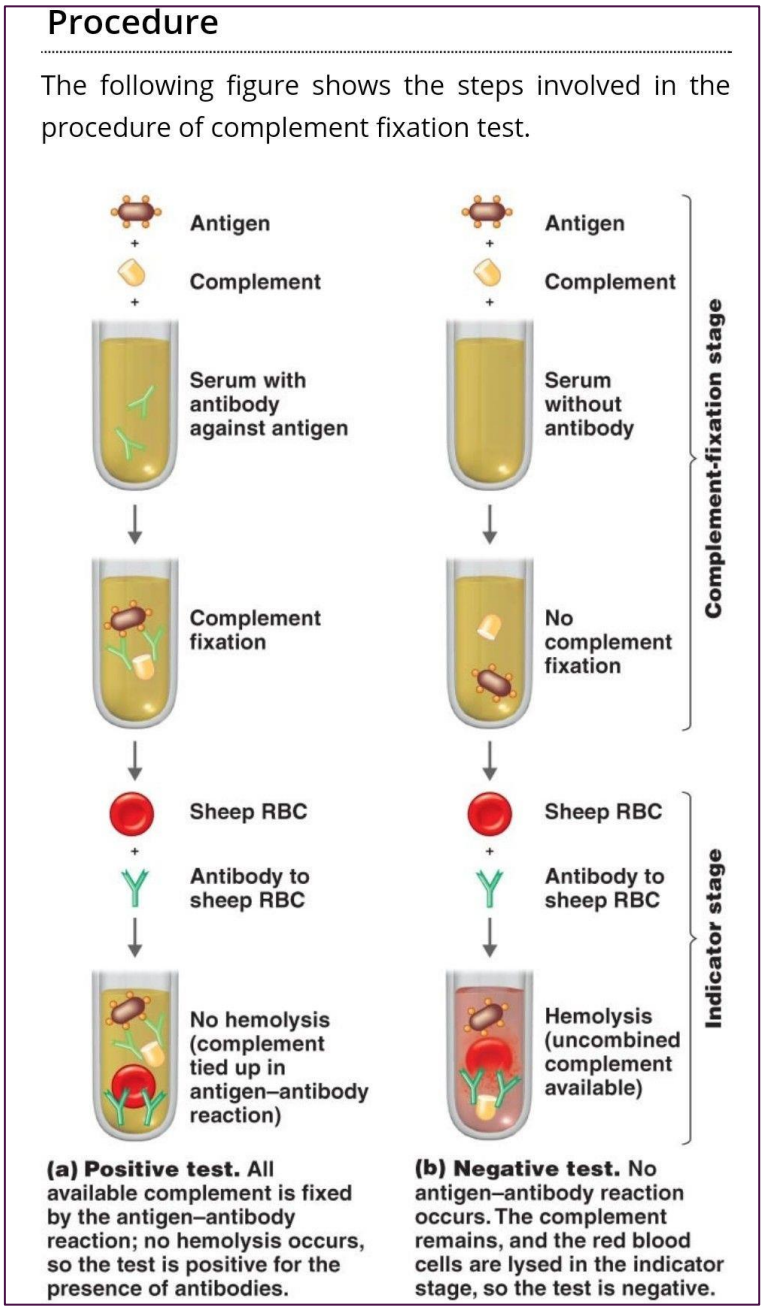
a) If the serum contains a specific antibody to the known antigen, the antigen-antibody complex will form, and the complement will be fixed by the antigen-antibody complex.

b) However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, thus complement will not be fixed but will remain free (In the indicator stage this complement will react with RBC coated with antibody to sheep RBC).

Second step (indicator stage): the second step detects whether a complement has been utilized in the first step or not. This is done by adding the indicator system.

a) if the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. There won't be any lysis of red blood cells.

b) however, if there is no specific antibody in the patient's serum, there will be no antigen-antibody complex, therefore, the complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody-coated sheep red blood cells to bring about their lysis (Figure 58).



Obr. 58 Complement Fixation Test

(Source: *https://laboratoryinfo.com/wp-content/uploads/2015/03/complement-fixation-test.jpg*)

3.8.2 Results and interpretation

The result of the reaction is evaluated according to the hemolysis of red blood cells in the hemolytic system.

- **positive CFT** (antibodies are present in serum), **no lysis** of sheep red blood cells indicates the presence of antibody in the test serum (sheep red blood cells sediment only).
- **negative CFT** (antibodies are not present in serum), **lysis** of red blood cells indicates the absence of antibody in the serum. The complement will remain free and will cause lysis of red blood cells.

Application: CFT is suitable for the detection of antibodies in various infectious diseases of bacterial, parasitic, fungal and viral etiology.

Questions for self-assessment

1. What are serological reactions? What is antigen, immunogen, hapten, superantigen, allergen? Which antigens are corpuscular and which are soluble?
2. What are antibodies? What is the difference between serum and plasma?
3. Which phases does the serological reaction have? How are serological reactions divided according to the antigen used, according to the technique and in what environment they can take place? What is the evidence of acute illness?
4. What is agglutination? What is the principle of direct agglutination? What is Widal's reaction? What is determined by serotyping?
5. What is the principle of indirect agglutination? What carriers are used in hemagglutination and latex agglutination?
6. What is precipitation? What is the characteristics of an equivalence zone? In what environment can precipitation take place and give examples.
7. What is the name of precipitation in a gel medium? Why is single radial immunodiffusion used and what is its principle? What is the purpose of double radial immunodiffusion and explain its principle?
8. What is electrophoresis and immunoelectrophoresis?
9. What is the essence of the complement fixation reaction? What are the phases of the complement fixation reaction? How is the CFR result evaluated? What does it mean when hemolysis occurs in CFR? And how is the result of CFR evaluated when hemolysis does not occur?

Specific immunity – Immunoglobulins https://portal.jfmed.uniba.sk/articles.php?aid=431

The interactive presentation contains study material about cells that provide immune responses and a set of test questions. Through these questions, the students can verify the acquired knowledge with feedback.

NEWER METHODS IN IMMUNOLOGY

4

Laboratory tests are procedures that involve testing of biological samples like blood, urine, or tissues of organisms in an environment where the appropriate types of technical equipment and professional human resources are available. Laboratory tests are responsible for the collection of the sample, their analysis, and evaluation. Tests are of different types. While some are providing reliable and precise information about the health condition and others provide more general information so that the physician can rule out possible health problems. The type of performed test depends on the symptoms observed in the patient. Rapid tests give results in minutes or seconds, while other tests take hours to days for the production of a result. Results from these tests are crucial as they determine the decisions to be made by the physician, including additional tests and treatment management. Important is that these tests must be performed with precision and with minimal chances of false results. Laboratory tests enable the identification of infectious agents or the detection of biomolecules involved in immune answers against the infectious agent. Each antibody can bind its specific antigen, forming antigen-antibody complexes (Figure 59). Different laboratory testing techniques are based on this principle. The knowledge of laboratory testing is imperative in clinical medicine, the development of new and advanced testing is also equally important.

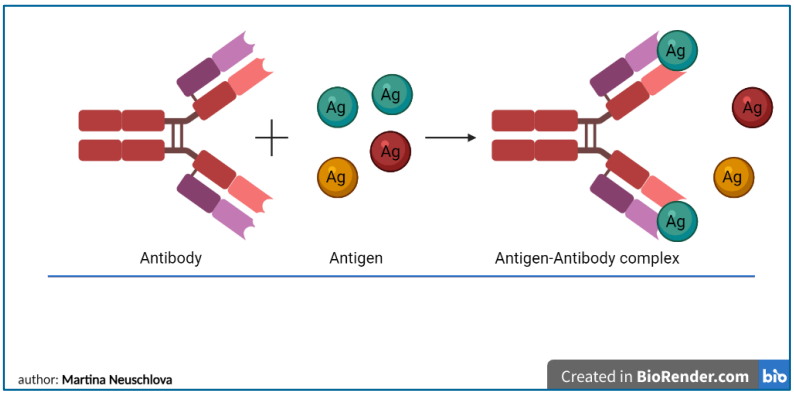


Fig. 59 Antigen-antibody reaction (Source: *Neuschlova, 2021; created in BioRender*)

4.1 IMMUNOFLUORESCENCY

Immunofluorescence is a technique that utilizes **fluorophores (fluorochromes)** to visualize various cellular antigens such as proteins. It can be utilized to visualize the localization of various cellular components within cells or tissues. Fluorophores are essential for immunofluorescence. They emit light when exposed to a certain wavelength of light. One of the best-known fluorochromes is fluorescein isothiocyanate (FITC) or cyanine dye. The biological sample of interest is incubated with an antibody specific to the antigen of interest. Immunofluorescence is known in two modifications: **direct and indirect immunofluorescence**. The antibody may be directly conjugated to a fluorochrome (direct immunofluorescence) or may be detected by a secondary antibody conjugated to a fluorochrome (indirect immunofluorescence). Direct immunofluorescence uses a single antibody directed against the target antigen. In contrast, indirect immunofluorescence uses two antibodies. Antigens can then be visualized by examination under a fluorescent microscope.

4.1.1 Direct immunofluorescence

Direct immunofluorescence involves the exclusive use of antibodies that have been coupled with fluorochromes. The detected antigen may be found in tissue, body fluid, cell culture, etc. The specimen is incubated with the fluorochrome-labelled antibody, the unbound antibody is removed by washing, and the specimen is examined.

Procedure: The test sample must be fixed in a thin layer on a slide. A specific fluorochrome-labelled antibody is then applied to the surface of the preparation thus prepared. If the antigen is present in the sample, it will bind to the antibodies during incubation. Subsequent washing removes excess unbound antibodies. After incubation and washing, the preparation is then evaluated in a fluorescence microscope (Figure 60, 62a).

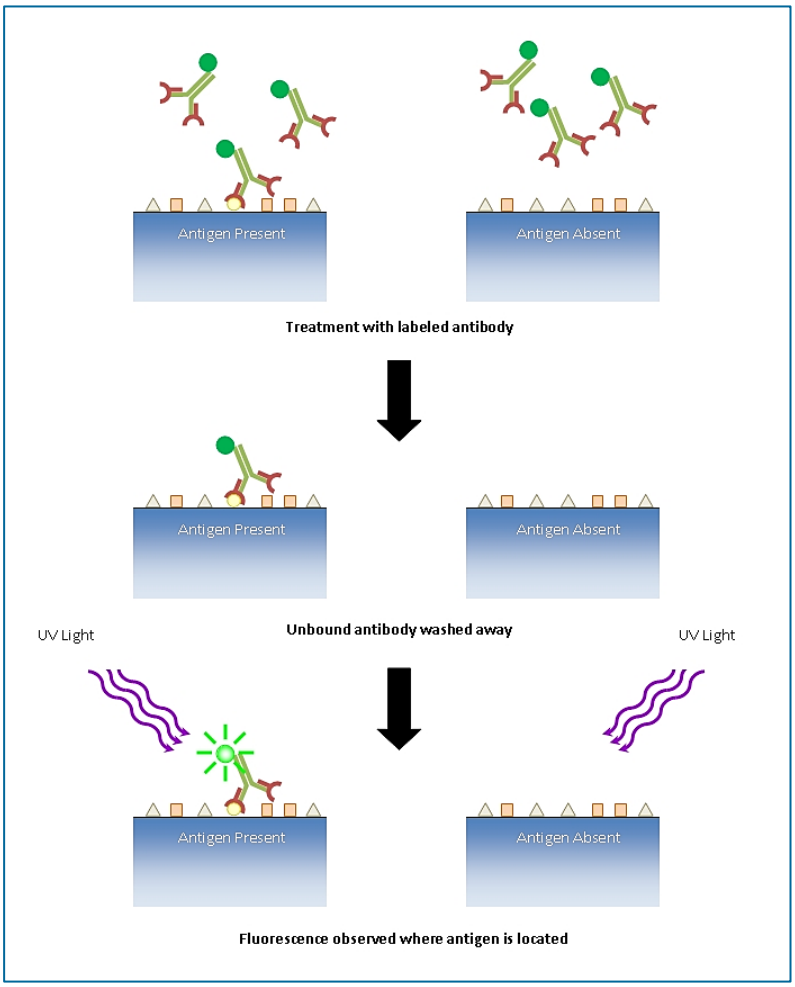


Fig. 60 Direct immunofluorescence (Source: *https://di.uq.edu.au/community-and-alumni/sparq-ed/cell-and-molecular-biology-experiences/immunofluorescence/background-immunofluorescence*)

Result: A positive result is manifested by fluorescence at sites where the antigen is present. If fluorochrome FITC is used, the fluorescence is yellow-green. Direct immunofluorescence is mainly used for the detection of antigens in tissue sections, for the rapid detection of pathogens in sputum, swabs, etc. Direct immunofluorescence is not often used now, as it would involve making a range of differently labelled antibodies for every protein studied in laboratories. In addition, the sensitivity of the technique is low, as there is only one label for each binding site.

4.1.2 Indirect immunofluorescence

Indirect immunofluorescence may be preferable in many cases. The specimen is incubated with an unconjugated antibody (primary antibody), then washed and incubated with a fluorochrome-conjugated anti-immunoglobulin antibody. This second antibody reveals the presence of the first. A positive result is shown by fluorescence when examined under a fluorescence microscope (Figure 61).

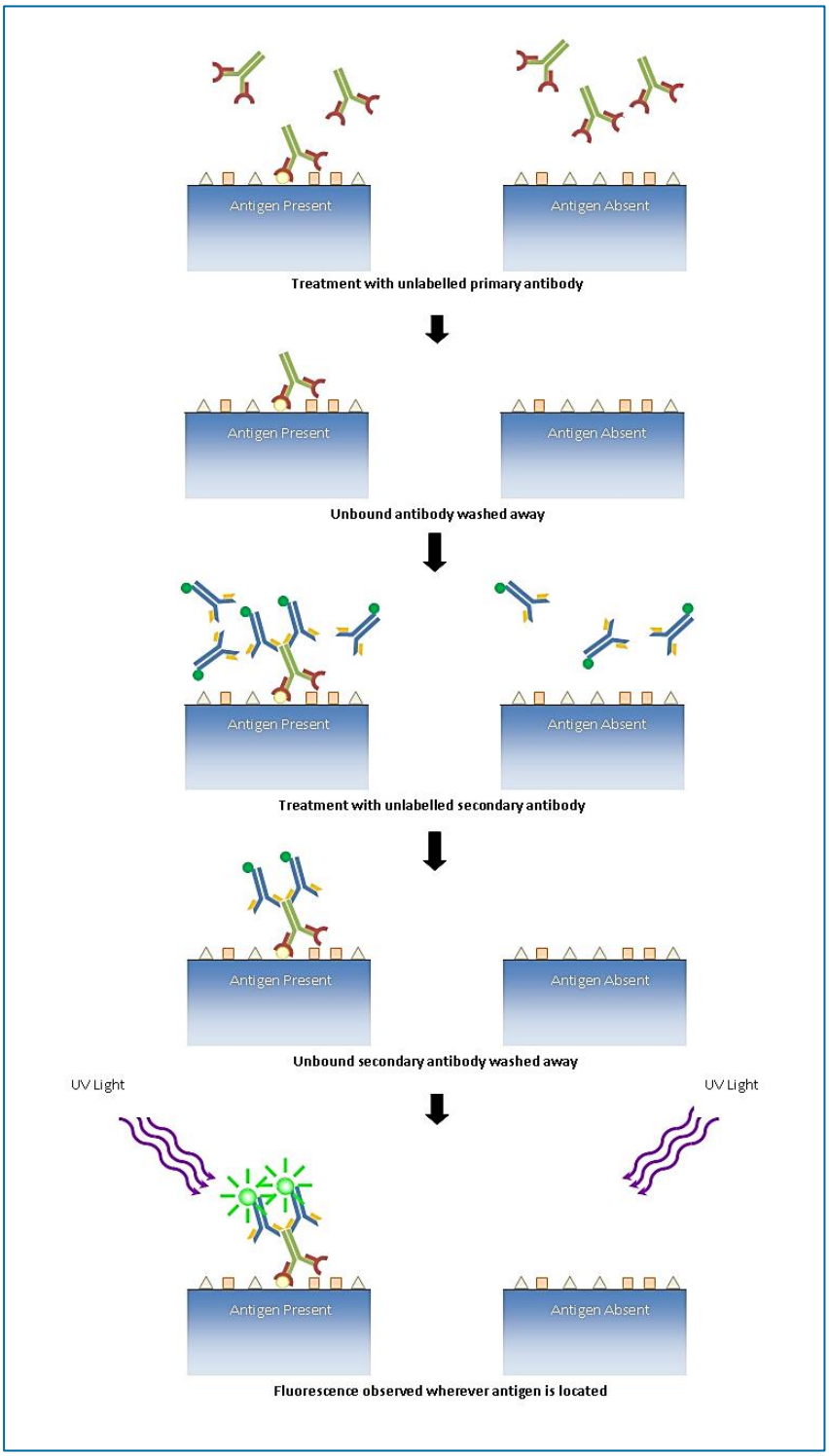


Fig. 61 Indirect immunofluorescence (Source: *https://di.uq.edu.au/community-and-alumni/sparq-ed/cell-and-molecular-biology-experiences/immunofluorescence/background-immunofluorescence*)

Procedure and result: The general protocol for antibody treatments is to expose the cells to the primary antibody for 1-2 hours, wash to remove all unbound primary antibodies, then expose them to the secondary fluorochrome-labelled antibody for 1 hour. The cells are washed to remove any unbound secondary antibodies. Fluorescence is observed under the fluorescent microscope wherever antigen is located. The signal strength is higher, resulting in greater sensitivity because multiple labelled secondary antibodies can bind to a single primary antibody.

Indirect immunofluorescence is used more frequently to detect antibodies in serum.

Procedure: in this case, the preparation is prepared from healthy tissue, cell or bacterial culture. A patient sample tested for the presence of antibodies is applied to this preparation. After the first incubation and washing, a fluorochrome-labelled secondary antibody is added. After the second incubation and washing, the preparation is viewed under a fluorescence microscope.

Result: a positive reaction is manifested by the fluorescence of antigen-containing structures against which antibodies have been present in the patient's serum. This method is most commonly used to determine anti-infective antibodies (Figure 62b) and autoantibodies in both organ-specific and systemic autoimmune diseases. For example, the presence of antinuclear antibodies in the examined serum is reflected by the fluorescence of nuclear structures in cells.

Advantage: The major advantage of indirect immunofluorescence is that one fluorochrome-conjugated anti-immunoglobulin antibody will suffice for many primary antibodies and it is not necessary to conjugate each new antibody individually. The indirect immunofluorescence usually gives brighter fluorescence than the direct because many secondary antibodies may bind to the first. Amplification is then possible. This may be an important advantage where monoclonal antibodies are used because their monospecificity results in smaller numbers of binding sites.

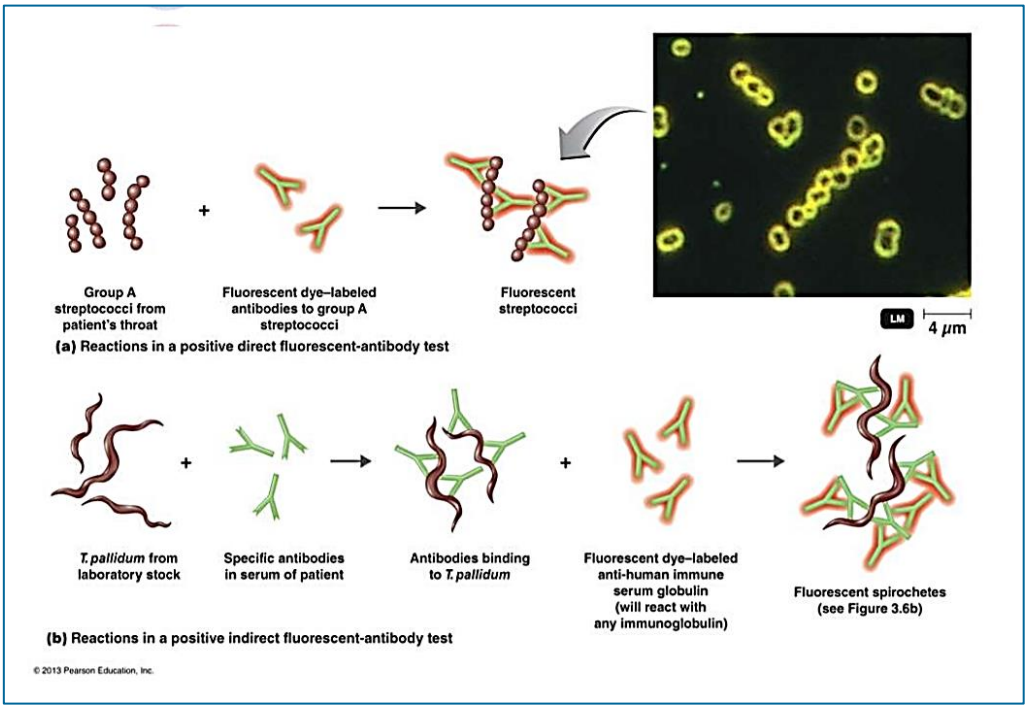


Fig. 62 Direct immunofluorescence for the detection of antigens in sample (a)
Indirect immunofluorescence for the detection of antibodies in serum (b)
(Source: *https://encrypted-tbn0.gstatic.com/images?q=tbn:ANd9GcTYEmjUn2be0G6vLTd0p2gjNMARLOjLatYnLw&usqp=CAU*)

4.2 IMMUNOANALYTICAL METHODS

Immunoanalytical methods are modern bioanalytical methods in which quantitation of analyte depends on the reaction of an antigen (analyte) with a specific antibody. They make it possible **to determine low concentrations of antigens or antibodies,** which usually cannot be determined by classical serological methods. They can also be used **to determine small amounts of various hormones, cytokines, tumor markers,** etc. Immunoassay methods have been widely used in many important areas of analysis such as diagnosis of diseases, therapeutic drug monitoring, clinical pharmacokinetics and bioequivalence studies in drug discovery and pharmaceutical industries.

Principle: when these immunoanalytical reagents are mixed and incubated, the analyte (antigen) is bound to the antibody, forming an immune complex. This complex is separated from the unbound reagent fraction by the physical or chemical separation technique. The analysis is achieved by measuring the label activity (e.g. radiation, fluorescence, or enzyme) in either the bound or free fraction. A standard curve, which represents the measured signal as a function of the concentration of the unlabelled analyte in the sample is constructed. The unknown analyte concentration is determined from this calibration curve.

To visualize the reaction between the antigen and the antibody, a 'secondary' antibody must be used, which is labelled with different labels:

- enzymes (enzyme immunoassay),
- radioactive atoms (radioimmunoassay),
- chemiluminescent substances (chemiluminescent immunoassay), or
- fluorescent probes (fluorescence immunoassay).

Immunoassays are divided into homogeneous and heterogeneous. For heterogeneous, it is necessary to separate the bound reactants from the unbound components of the reaction. This is ensured by washing with a washing solution between the individual reaction steps.

4.2.1 Enzyme immunoassay – EIA

Enzyme immunoassay (EIA) can be used for the detection of either antigens or antibodies in serum and other body fluids of the patient. In EIA techniques, antigens or antibodies labelled with enzymes are used. The enzymes used in the EIA tests are alkaline phosphatase, horseradish peroxidase, and β-galactosidase.

ELISA (Enzyme-Linked ImmunoSorbent Assay)

ELISA is a type of enzyme immunoassay that is commonly used to quantify the level of a specific target within a sample. Samples routinely used in ELISA include serum, plasma, cell culture supernates, cell lysates, saliva, tissue lysates, and urine. The antigen or antibody is coated on a solid surface such as in plastic tube or well of 96-well microtiter plate. Thus, after the antigen and antibody have combined and antigen-antibody complex is formed, they remain firmly attached to a solid surface during subsequent washing stages. The enzyme system of ELISA consists enzyme which is labelled to a specific antibody or antigen and a chromogenic substrate that is added after the antigen-antibody reaction. The chromogenic substrate is hydrolysed by the enzyme attached to antigen-antibody complexes to give color change. The color in the reaction can be read visually or the reaction is detected by reading the optical density (colorimetrically) using a microassay plate reader – ELISA reader. Usually, a standard curve based on known concentrations of antigen or antibody is prepared from which the unknown quantities of the substance to be determined, are calculated.

ELISA is currently one of the most common immune assays used in clinical and experimental procedures. There are different types of ELISAs available for the detection and quantitation of either the antigen or antibodies in serum and other body fluids (Figure 63). There are four main types of ELISA tests: direct, indirect, sandwich, and competitive. Each type has its own advantages and disadvantages.

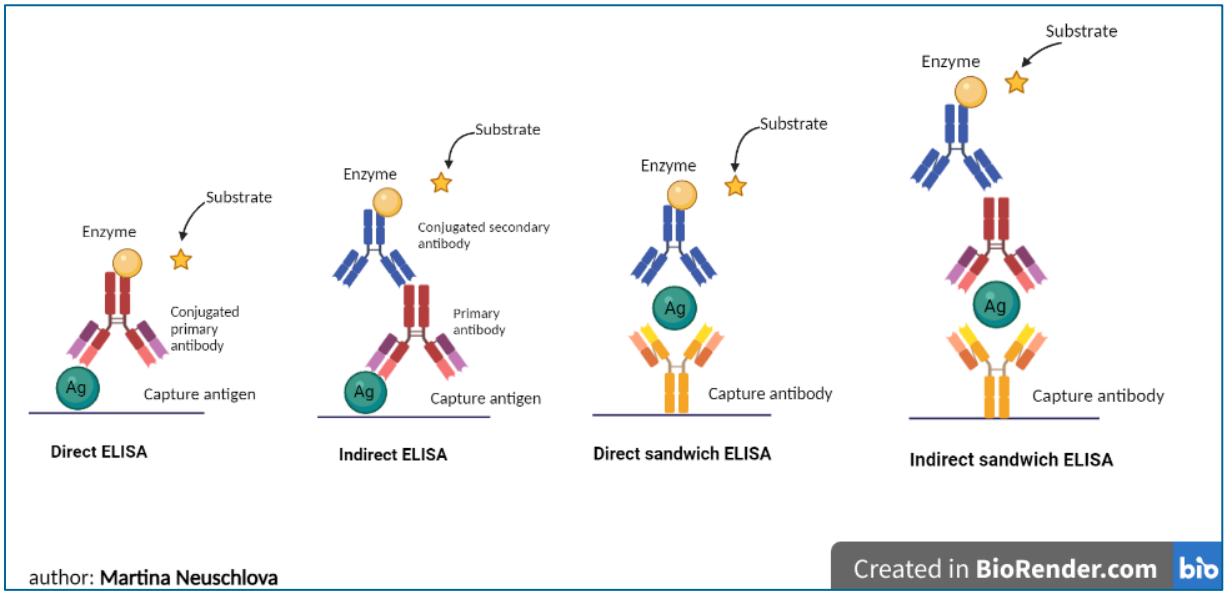
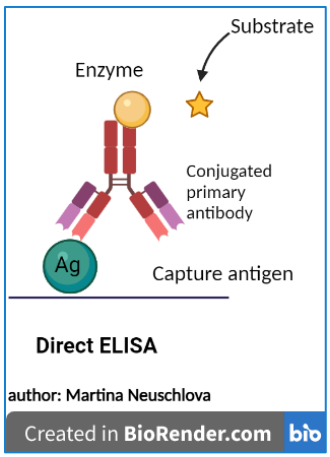


Fig. 63 Types of ELISA (Source: *Neuschlova, 2021; created in BioRender*)

Direct ELISA

Direct ELISA is used mostly for antigen detection.



- An antigen is attached to the plate and
- enzyme-labelled antibody (primary antibody conjugate) is added that can react with the antigen.
- A substrate is then added, producing a signal (color change) that is proportional to the amount of analyte in the sample (Figure 64, 65).

Fig. 64 Direct ELISA - principle (Source: *Neuschlova, 2021; created in BioRender*)

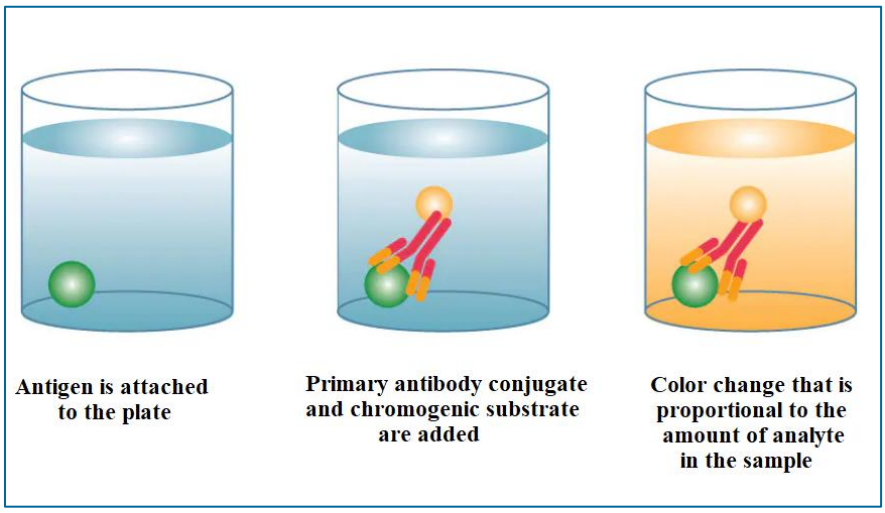


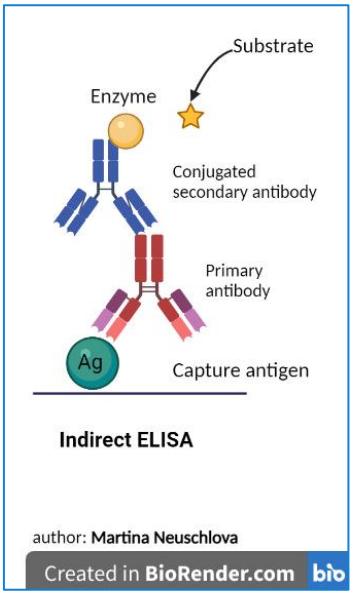
Fig. 65 Direct ELISA

(Source according to: *https://www.rndsystems.com/resources/what-is-an-elisa-and-elisa-types*)

Advantages: fast and simple protocol, no cross-reactivity from secondary antibody.
Disadvantages: low flexibility – the primary antibody must be conjugated, no signal amplification.

Indirect ELISA

Indirect ELISA is used mainly for antibodies detection. Indirect ELISA is a technique that uses a two-step process for detection.



- An indirect ELISA is similar to a direct ELISA in that an antigen is adsorbed on a plate, but it includes an additional amplification detection step.
- An unconjugated primary detection antibody is added and binds to the specific antigen.
- A conjugated secondary antibody (enzyme-labelled antibody) directed against the host species of the primary antibody is then added (amplification).
- A chromogenic substrate is added that produces a signal (color change) proportional to the amount of determined antibody bound in the well (Figure 66, 67).

Fig. 66 Indirect ELISA principle (Source: *Neuschlova, 2021; created in BioRender*)

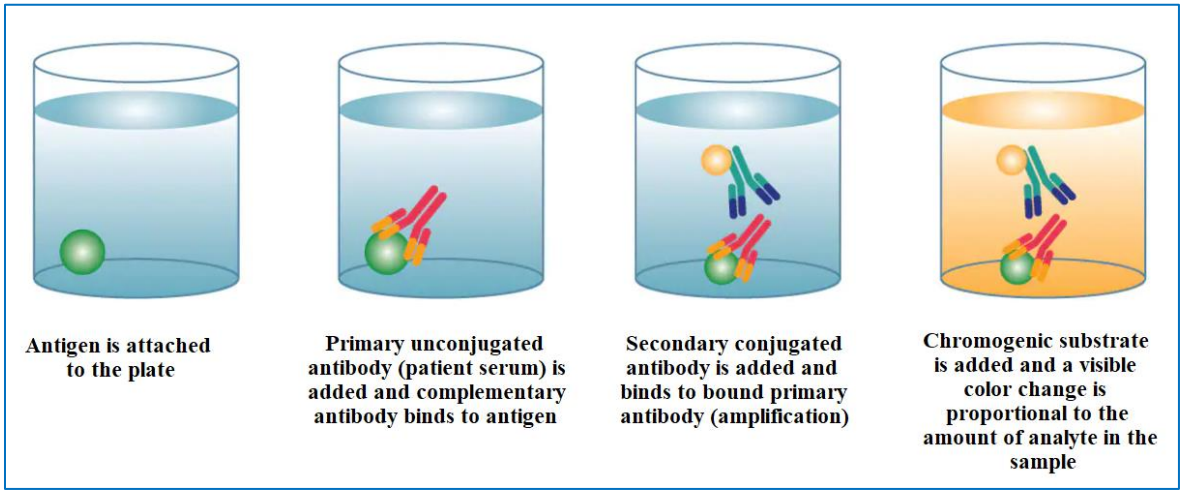


Fig.67 Indirect ELISA

(Source according to: *https://www.rndsystems.com/resources/what-is-an-elisa-and-elisa-types*)

Advantages: amplification using a secondary antibody, several secondary antibodies will bind to the primary, high flexibility: the same secondary antibody may be used for several primary antibodies.

Disadvantages: longer protocol if compared to direct ELISA, potential for cross-reactivity caused by secondary antibody.

Application of indirect ELISA

Indirect ELISA used for anti-SARS-CoV-2 antibodies detection. A coated SARS-CoV-2 antigen onto wells on a plate interacts with the primary antibody (anti-SARS-CoV-2 antibody) that can be in a patient's sample. After adding a conjugated secondary antibody (enzyme-labelled antibody), it recognizes and interacts with the primary antibodies (amplification). A chromogenic substrate is then added and is cleaved by the enzyme and results in color change after incubation Result is read by ELISA plate reader (Figure 68).

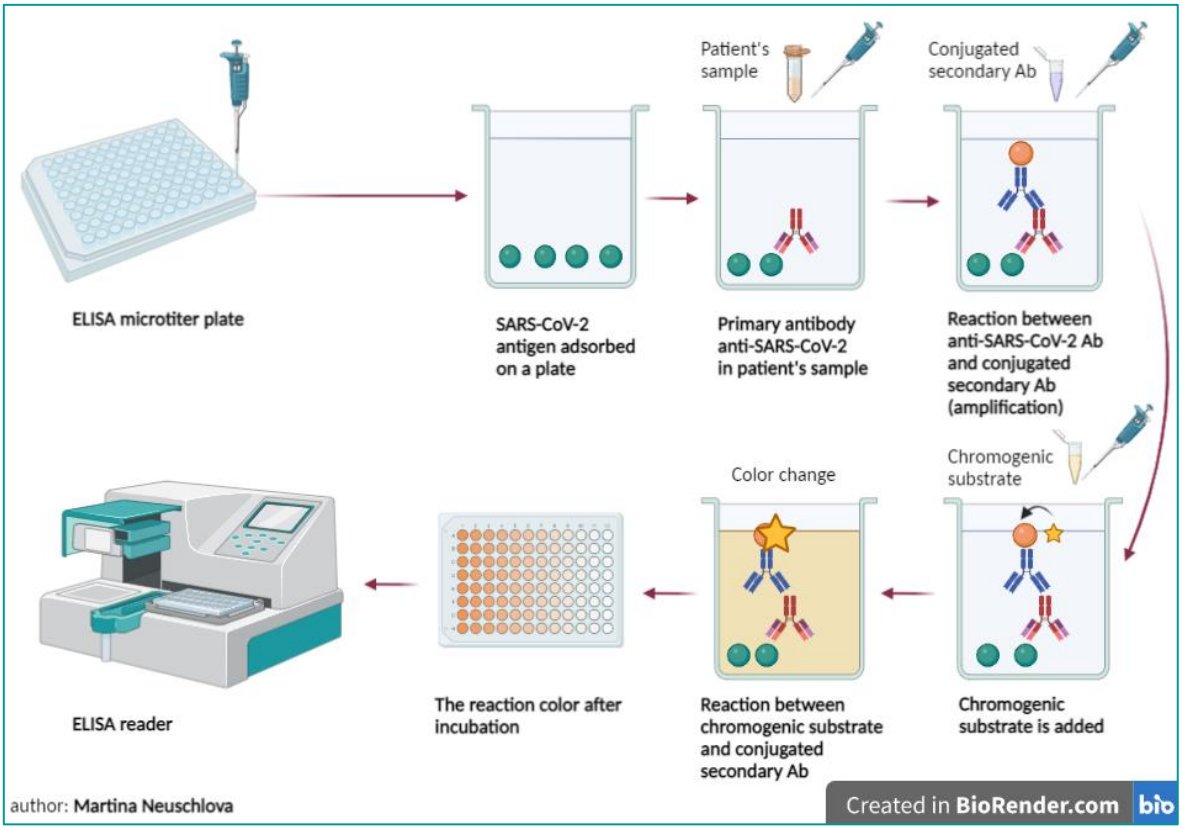


Fig. 68 Indirect ELISA to detect anti-SARS-CoV-2 antibodies – schematic flowchart

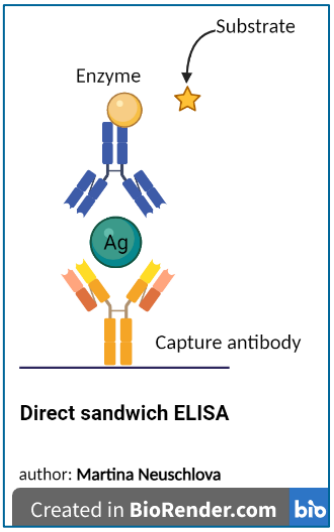
(Source: *Neuschlova, 2021; created in BioRender.com*)

Sandwich ELISA

Sandwich ELISA is the most common type of ELISA. The sandwich ELISA quantify antigens between two layers of antibodies (i.e. capture and detection antibody).

This assay uses two different antibodies that are reactive with different epitopes on the antigen. The antigen to be measured must contain at least two antigenic epitopes capable of binding to antibody since at least two antibodies act in the sandwich. Monoclonal or polyclonal antibodies can be used as the capture and detection antibodies. The procedure for a sandwich ELISA requires the well of an ELISA plate to be coated with a capture antibody. The analyte or sample is then added, followed by the detection antibody. According to the detection principle and whether to use the enzyme-labelled capture antibody, sandwich ELISA could be divided into direct sandwich ELISA and indirect sandwich ELISA.

Direct sandwich ELISA



- The capture antibody is coated on a plate,
- test solutions containing antigen at an unknown concentration are added to the wells on the plate and allowed to bind.
- A conjugated-detection antibody (enzyme-labelled antibody) is then added and binds to an additional epitope on the target antigen.
- A chromogenic substrate is then added and produces a signal (color change) that is proportional to the amount of analyte present in the sample (Figure 69, 70).

Direct sandwich ELISA – the detection antibody is enzyme-conjugated.

Fig. 69 Direct sandwich ELISA – principle (Source: *Neuschlova, 2021; created in BioRender*)

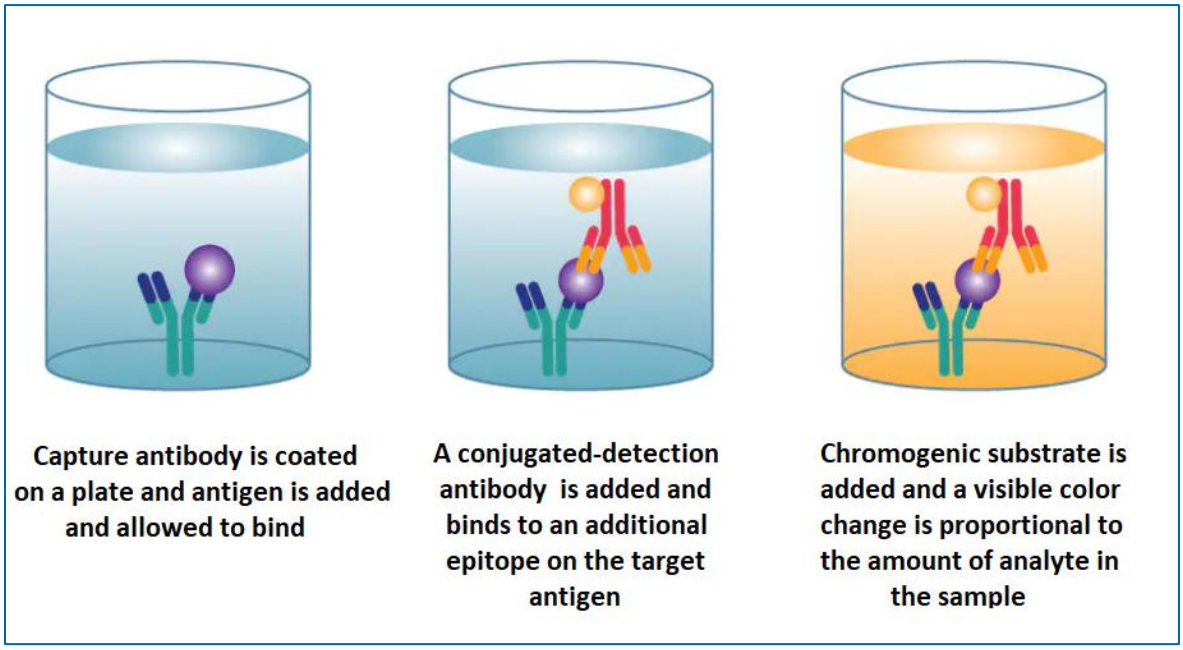
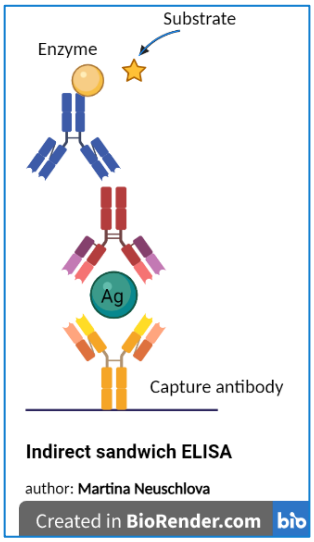


Fig.70 Direct sandwich ELISA

(Source according to: *https://www.rndsystems.com/resources/what-is-an-elisa-and-elisa-types*)

Indirect sandwich ELISA

The primary antibody used is unlabeled and a secondary detection antibody is enzyme-conjugated (Figure 71).



Sandwich ELISAs are highly specific since two antibodies are required to bind to the protein of interest.

Advantages: high specificity: the antigen is specifically captured and detected, high flexibility: both direct and indirect methods can be used and high sensitivity: more sensitive than direct or indirect ELISA.
Disadvantages: longer protocol, challenging to develop two antibodies against the same antigen that recognize different epitopes and work together.

Fig. 71 Indirect sandwich ELISA – principle (Source: *Neuschlova, 2021; created in BioRender*)

Application of sandwich ELISA

Home pregnancy test utilizes the principle of sandwich enzyme immunoassay, with a unique monoclonal antibody combination specific against hCG present in urine. The patient's urine specimen is allowed to react with the monoclonal antibody (capture antibody) directed against hCG, coated on the microtiter wells and the monoclonal conjugated-detection antibody (enzyme-labelled antibody). If hCG is present in the test specimen, an antibody-hCG-antibody enzyme complex will be formed on the surface of the microtiter well which results in the development of color change. The intensity of the color is proportional to the concentration of hCG present in the urine specimen (Figure 72).

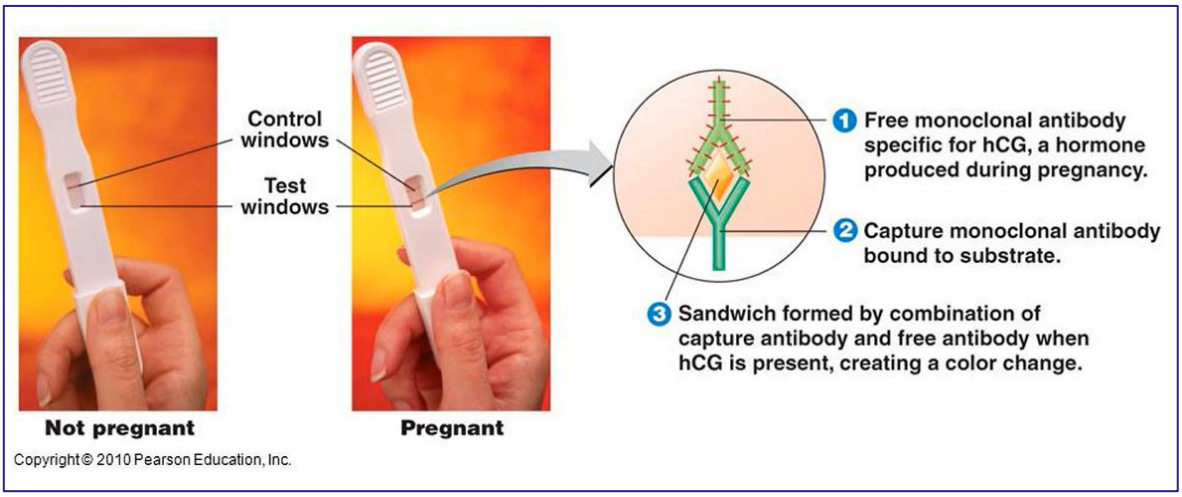


Fig. 72 Pregnancy test – principle
(Source: *https://slideplayer.com/slide/4937003/63/video/Practical+Applications+of+Immunology.mp4*)

Competitive ELISA

Competitive ELISA assays, also known as inhibition ELISA measure the concentration of an antigen by detection of signal interference. Each of the previous formats can be adapted to the competitive format.

The sample antigen competes with a reference antigen for binding to a specific amount of labelled antibody. The reference antigen is pre-coated on a plate and the sample is pre-incubated with labelled antibody and added to the wells on the plate. Depending on the amount of antigen in the sample, more or less free antibodies will be available to bind the reference antigen in the wells. This means the more antigen is in the sample, the less reference antigen will be detected and the weaker the signal.

Advantages: ability to quantitate small molecules (eg. small antigens, hormones) that cannot be bound by two different antibodies such as in the sandwich ELISA technique, higher flexibility.

4.2.2 Radioimmunoassay – RIA

RIA (Radio Immuno Assay) is an *in vitro* immunoassay that measures usually the presence of an antigen present in the sample (for example, hormone levels in the blood) by antibodies directed against this antigen with very high sensitivity. Radioisotopes (usually ^{125}I, ^{131}I) are used instead of enzymes as labels to be conjugated with antigens, primary or secondary antibody.

The classical radioimmunoassay methods are based on competitive binding. An unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity. A known amount of antigen is labelled with a radioactive element (radioisotope) and mixed with a known amount of antibody directed against that antigen. When the sample containing the antigen of interest is added, the unlabeled antigens are substituted for the radiolabeled antigens. After removal of the non-fixed antigens by washing, it is possible to measure the amount of free radiolabeled antigen (that is not bound to antibody), which is proportional to the quantity of unlabeled antigen in the mixture. The radioactivity of the free antigens is measured.

Direct RIA uses radionuclide labelling of the primary antibodies which are incubated with the antigen. The unbound antibodies are washed off and the radioactivity is recorded by a gamma counter.

Indirect RIA method first applies unlabelled primary antibodies that bind to the antigen and then a secondary radionuclide-labelled antibody that binds to immunoglobulin. A gamma counter measures the bound radioactivity.

RIA is characterized by high sensitivity and specificity. It also allows **determining** hormones, vitamins, their metabolites, some autoantibodies and other **biologically important substances in nano- and pictogram concentrations.** However, as they require work with radioisotopes, special measurement techniques, trained personnel and generate isotope waste, the legislation is very strict and they are usually part of the nuclear medicine departments. Nowadays most RIA assays have been replaced by non-isotopic immunoassays.

4.2.3 Chemiluminescent immunoassay

Chemiluminescent immunoassay uses luminophore markers as a label - substances that emit light during oxidation. Light is emitted during the chemical reaction and is detected by a luminometer.

4.2.4 Fluorescence immunoassay

Fluorescence immunoassay uses fluorochrome as a label and signal detection is done fluorimetrically. The principles of both methods are identical to the methods in enzyme-linked immunosorbent assays and are described above.

Procedure: The test is conducted in well on a polystyrene microtiter plate capable of adsorbing protein. A soluble antigen is added and covalently bound to the artificial surface of the well. Unbound material is removed by washing. Serum containing antibodies is added to the well. Specific antibodies bind strongly to the antigen. Unbound antibodies are removed by washing. A fluorochrome-labelled antibody that binds to human antibody molecules is added to the well. The unbound labelled antibodies are removed by washing. Fluorescence indicates the presence of the epitope.

4.3 IMMUNOBLOTING TECHNIQUES - IMMUNOBLOT

Blotting methods **combine electrophoresis and immunoassay**. They take place in various modifications.
They can be used for:

- DNA analysis (Southern blot),
- RNA analysis (Northern blot),
- protein analysis (Western blot),
- Eastern blot analysis (used to analyze protein post-translational modifications).

Western blot and immunodot are most commonly used.

4.3.1 Western blot

Principle: A western blot is a laboratory method used to detect specific protein molecules from a complex mixture of proteins. This technique uses three steps to accomplish this task:

- separation by size,
- transfer to a solid support,
- and marking target protein using a proper primary and secondary antibody to visualize.

Procedure: In this technique, a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis (Figure 73). These results are then transferred to a blotting membrane producing a band for each protein. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to an enzyme that produces color or light, which allows it to be easily detected and imaged.

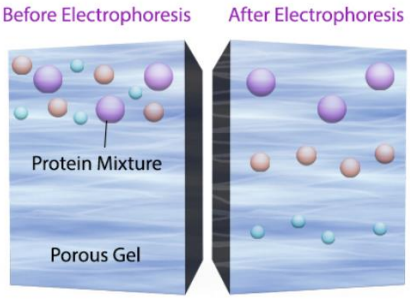


Fig. 73 Gel electrophoresis (Source: *https://www.biolegend.com/fr-ch/western-blot*)
Negatively charged small proteins move through the gel matrix, toward the positive electrode, more quickly than larger negatively charged proteins

Result: As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present (Figure 74).

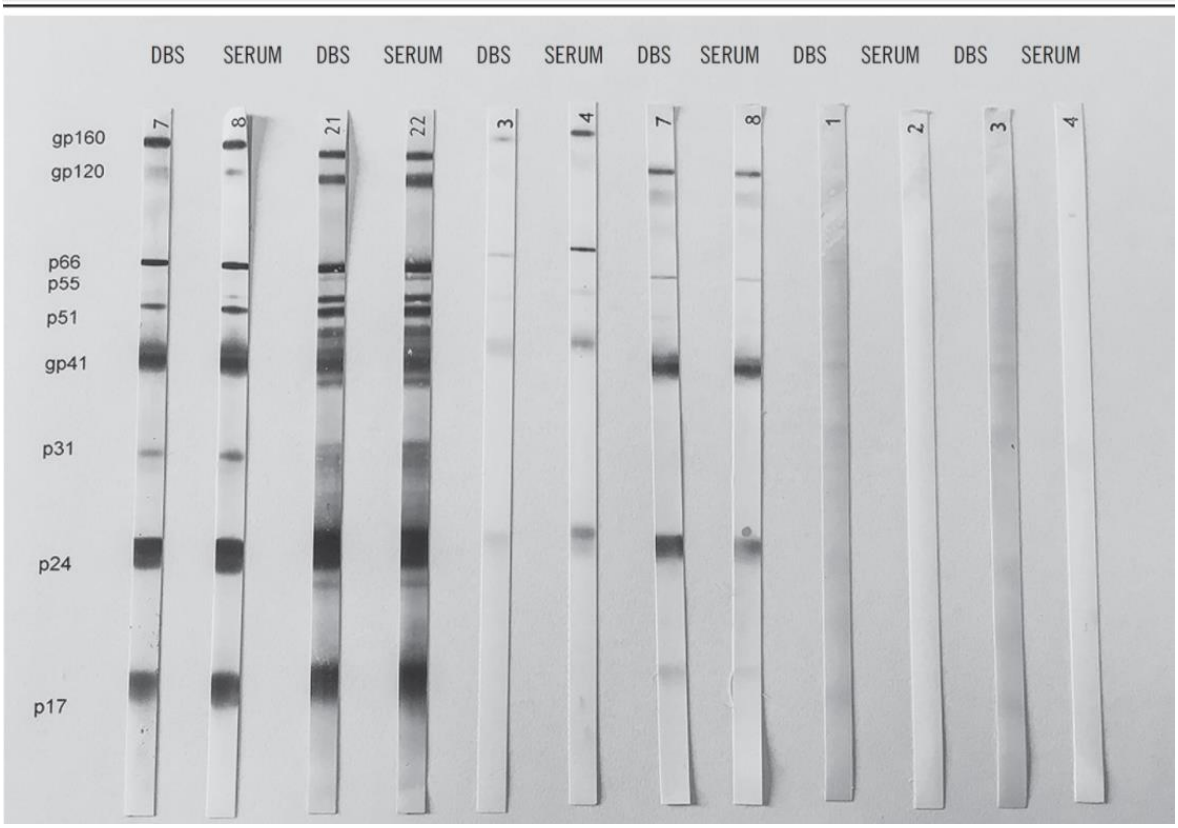


Fig. 74 Western blot results
(Source: *https://www.scielo.br/j/jbpml/a/LmqFQqSms3CXGkWPbBGCBwD/?lang=en*)

4.3.2 Immunodot

Immunodot is a simple and rapid assay that utilizes an enzyme-linked immunoassay (EIA) dot technique for the detection of antibodies. The antigens are dispensed as discrete dots onto a solid membrane. Reading immunodotes is easier, but tests are more expensive (Figure 75).

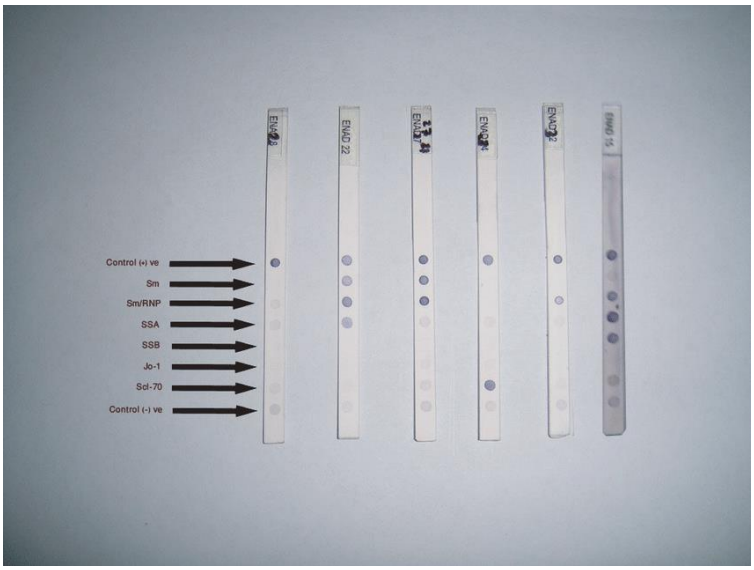


Fig. 75 Immunodot – analysis of antibodies (Source: *Sharim et al., 2011*)

4.3.3 Application of immunoblotting techniques

The advantage of blotting techniques is the ability to detect several specific antibodies against individual antigens at once. Blotting methods are currently widely used due to their high sensitivity and specificity. They make it possible to detect a specific antibody response against various proteins in mixtures of antigens. They are used in the analysis of microorganisms antigens (e.g. against individual antigens of HIV, HCV, against antigens of *Helicobacter pylori*, *Treponema pallidum*, *Borrelia*, etc.), in the analysis of allergens, enzymes, proteins, hormones, antibodies, autoantibodies and many others.

These assays are used as **confirmatory assays to determine specific antibodies against infectious agents**. They are used as another possibility of specific evidence in cases where other methods have not given a clear result.

4.4 ELISpot (Enzyme-Linked ImmunoSpot assay)

The Enzyme-Linked Immunospot (ELISpot) assay is a sensitive method for quantification of the number of cytokines secreting cells. ELISpot is a highly sensitive immunoassay that measures the frequency of cytokine-secreting cells at the single-cell level. In this assay, cells are cultured on a surface coated with a specific capture antibody in the presence or absence of stimuli. Proteins, such as cytokines, that are secreted by the cells will be captured by the specific antibodies on the surface. After an appropriate incubation time, cells are removed and the secreted molecule is detected using a detection antibody in a similar procedure to that employed by the ELISA. The detection antibody is either biotinylated and followed by a streptavidin-enzyme conjugate or the antibody is directly conjugated to an enzyme. By using a substrate with a precipitating rather than

a soluble product, the result is visible spots on the surface. Each spot corresponds to an individual cytokine-secreting cell (Figure 76, 77).

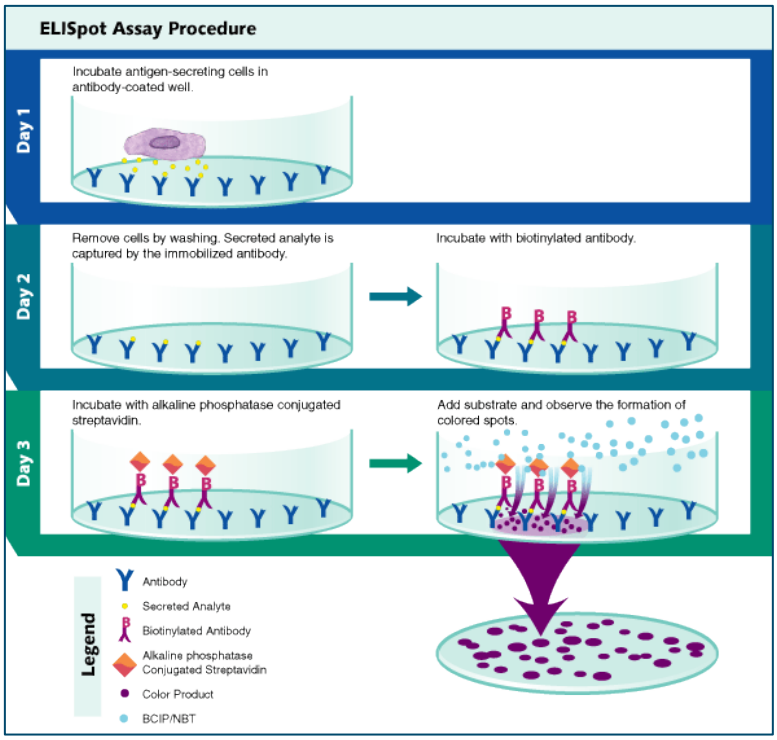


Fig. 76 ELISpot assay procedure (Source: *https://www.rndsystems.com/products/elispot-assay-principle*)

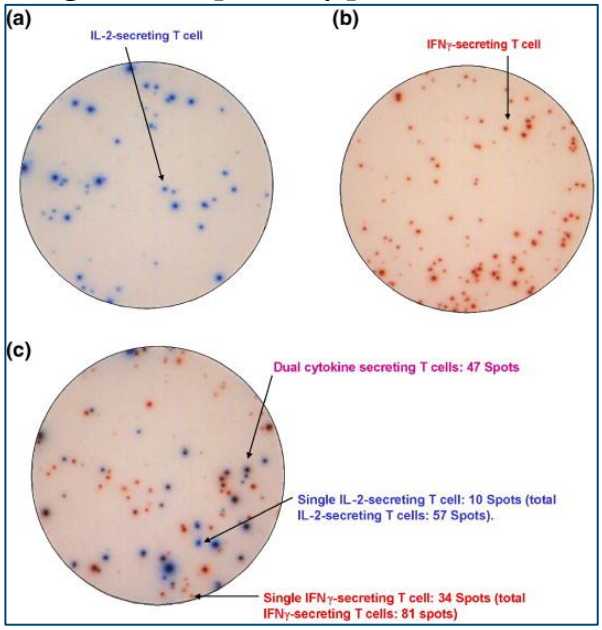


Fig. 77 ELISpot – spot morphology of spot forming cells:
(a) cells secreting only IL-2,
(b) only IFN-γ and
(c) both cytokines.
Arrows point to
red (IFN-γ),
blue (IL-2) and
purple (IL-2 and IFN-γ) spots distinguished by the ELISpot reader instrument.
(Source: *Boulet et al. J Immunol Methods, 2007*)

The assay is considered one of the most sensitive cellular assays available. The high sensitivity of the assay makes it particularly useful for studies of the small population of cells found in specific immune responses. ELISpot can discriminate between subsets of activated T cells. Th1 cells produce cytokines IFN-gamma, IL-2 and TNF-alfa, whereas Th2 cells produce cytokines IL-4, IL-5, IL-13.

The ELISpot assay is carried out in a 96-well plate. An automated ELISpot reader is used for analysis. The assay is easy to perform and allows rapid analysis of a large number of samples.

Result: A spot is created at the bottom of the well at the site where the cell (e.g T cell) produced the cytokine.

Analysis: intensity and size of spots depends on the amount of cytokine released by the cell

- each spot = corresponds to an individual cytokine-secreting cell
- number of spots = number of cytokine-secreting cells

Application: ELISpot is a sensitive method for investigating specific immune responses. Standardization of the ELISpot assay is well described and the method is the basis of a diagnostic test, the T-spot test for tuberculosis by measuring IFN-gamma secretion from T cells responding to antigens from *Mycobacterium tuberculosis* (Figure 78). The ELISpot technique is not limited to the measurement of cytokines; it is also suitable for almost any secreted protein where single-cell analysis is of interest. The method was originally developed to quantify immunoglobulin-secreting cells. ELISpot is used to directly assess immunoglobulin-secreting cells, to evaluate the number of memory B cells in the blood and to detection of B-cell responses to infections and responses elicited by vaccination.

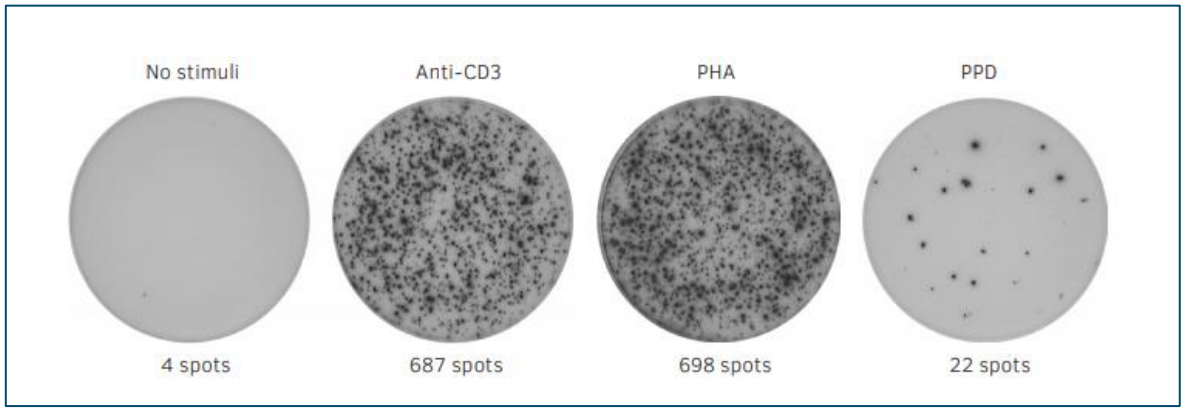


Fig. 78 Human IFN-gamma ELISpot

IFN-gamma secretion by peripheral blood mononuclear cells incubated overnight without stimuli or with anti-CD3, phyto-haemagglutinin (PHA) or purified protein derivative (PPD) (Source: *https://www.mabtech.com/sites/default/files/elispot-brochure.pdf*)

4.5 PCR – Polymerase Chain Reaction

The polymerase chain reaction, or **PCR**, is one of the most well-known techniques in molecular biology, with applications ranging from basic research to disease diagnostics. PCR was invented by **Kary Mullis** in 1983 (awarded the Nobel Prize in Chemistry in 1993). Over the years, the fundamental principles of PCR have remained the same, but methods have evolved with vast performance improvements to DNA polymerases and reagents, as well as innovations in instrumentation and the plastic vessels that hold the reactions.

PCR is **a biochemical process capable of amplifying a single DNA molecule into millions of copies *in vitro*** in a short time. Amplification is achieved by a series of three steps: denaturation, annealing and extension.

Principle of PCR

The polymerase chain reaction is carried out in a reaction mixture which comprises the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in a buffer solution. The tubes containing the mixture reaction are subjected to repetitive temperature cycles several tens of times in the heating block of a thermal cycler. The thermal cycler is an apparatus in which the temperature can vary, very quickly and precisely, from 0 to 100 °C. The apparatus allows the programming of the duration and the succession of the cyles of temperature steps. Each cycle includes three periods of a few tens of seconds. PCR is divided into three stages:

1. **Denaturation** – the double-stranded DNA templates are heated to separate the strands. The first stage is carried out at a temperature of 94 to 98 °C, called the denaturation temperature. The matrix DNA is denatured into single-stranded DNA.
2. **Hybridization (annealing)** – short DNA molecules called primers bind to flanking regions of the target DNA. This second stage is carried out at a temperature generally between 40 and 70 °C, called primer hybridization temperature. The primers, short single-strand sequences complementary to regions that flank the DNA to be amplified, hybridize more easily than long strand matrix DNA.
3. **Extension** – DNA polymerase extends the 3' end of each primer along the template strands. This third stage is carried out at a temperature of 68 to 72 °C, called elongation temperature. Taq polymerase binds to primed single-stranded DNAs and catalyzes replication using the deoxyribonucleoside triphosphates present in the reaction mixture.

These steps are repeated ("cycled") in a **thermal cycler** 25–35 times to exponentially produce exact copies of the target DNA (Figure 79). The detection and analysis of the products can be very quickly carried out by agarose gel electrophoresis or acrylamide gel electrophoresis, or by fluorescence.

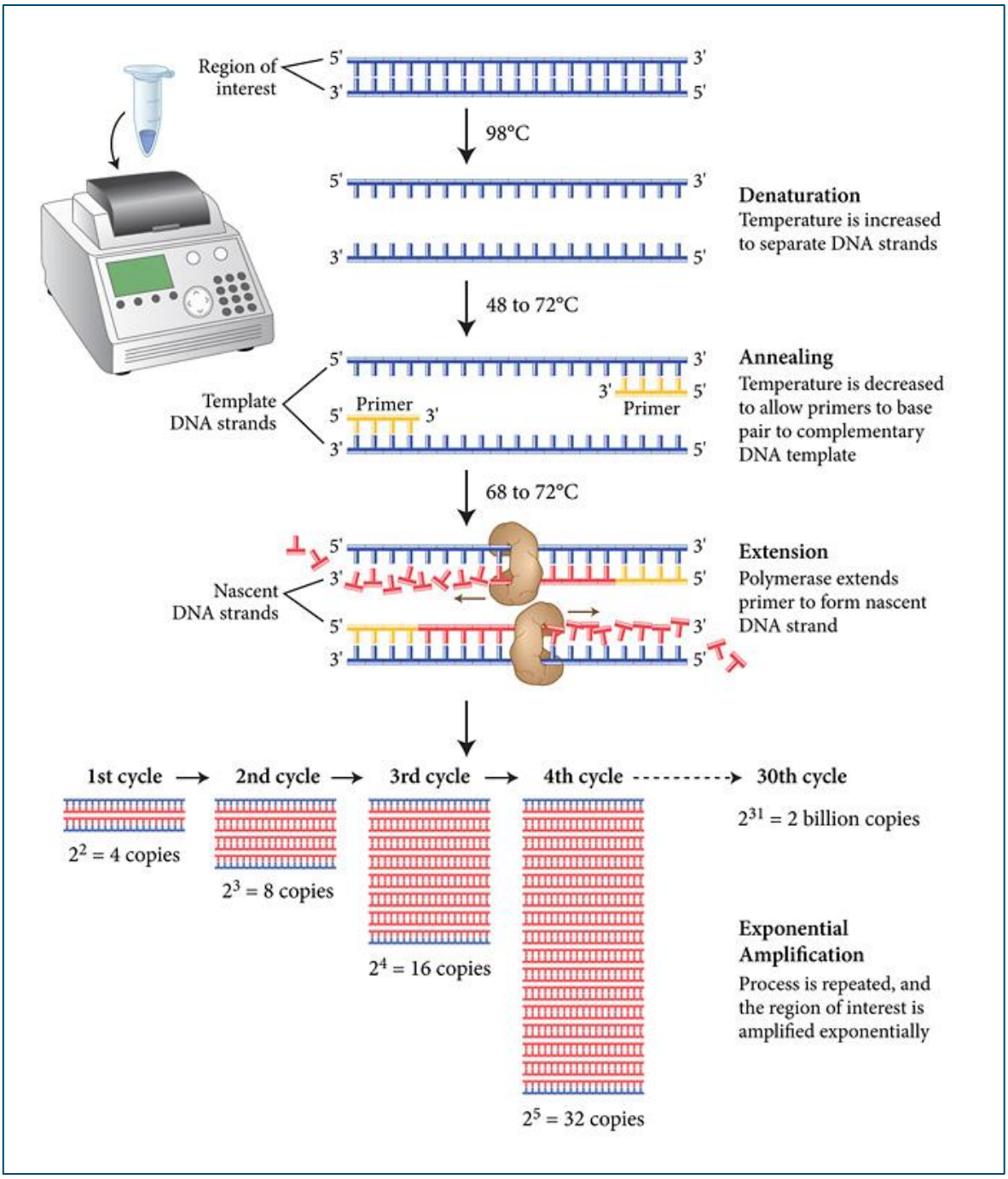


Fig. 79 PCR and DNA amplification (Source: *https://www.neb.com/~/media/NebUs/Page%20Images/Applications/DNA%20Amplification%20and%20PCR/pcr.jpg*)

Applications: PCR is an important diagnostic tool. It is already widely used in the detection of genetic diseases, infectious diseases (viral, bacterial, parasitic, etc.), as is the case for *Mycobacterium tuberculosis, Chlamydia trachomatis, Borrelia burgdorferi, Legionella.*spp., *Mycoplasma pneumoniae, Bartonella henselae*, hepatitis C, AIDS, etc. PCR has the advantage to produce very reliable and rapid results from biological samples in which the presence of the pathogen is not always detectable with other standard techniques.

PCR – Six Critical Components are (Figure 80):

- **Template DNA** – PCR template for replication can be of any DNA source, such as genomic DNA (gDNA), complementary DNA (cDNA), and plasmid DNA
- **DNA polymerase** – Taq Polymerase
- **Primers** – synthetic DNA oligonucleotides of approximately 15–30 bases
- **Deoxynucleoside triphosphates** (dNTPs) – four basic nucleotides (dATP, dCTP, dGTP, dTTP) as building blocks of new DNA strand
- **Required cofactor: Mg^{2+}** – Magnesium ion (Mg2+) functions as a cofactor for the activity of DNA polymerases by enabling the incorporation of dNTPs during polymerization.
- **Buffer** – PCR is carried out in a buffer that provides a suitable chemical environment for the activity of DNA polymerase.

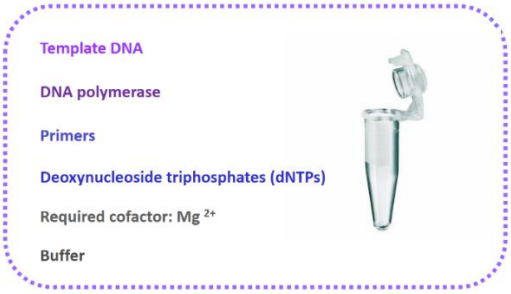


Fig. 80 PCR – Six Critical Components (Source: Authors)

Questions for self-assessment

1. What is immunofluorescence? Direct immunofluorescence is used to prove what? Is indirect immunofluorescence more often used to detect antigens or antibodies?
2. What are the common features of immunoassay methods and how are they divided?
3. What reaction is used in the enzyme immunoassay in the visualization phase? What is the principle of the ELISA test? Why is washing between ELISA steps important? Which component of the reaction must be bound to the wall of the ELISA microtiter plate for antibody detection and which for antigen detection?
4. What are immunoblotting techniques? Explain the principle of Western blot and immunodot. When and why are blotting techniques used? Explain what does confirmatory tests mean?
5. What is ELISPOT? What is measured by ELISPOT? Give examples where this method can be used.
6. What is the polymerase chain reaction? What is the principle of PCR? Which steps do consist of one PCR cycle? In the diagnosis of which diseases is PCR used?

New methods in Immunology https://portal.jfmed.uniba.sk/articles.php?aid=449

The interactive presentation contains study material about modern laboratory methods in immunological and microbiological laboratories and a set of test questions. Through these questions, the students can verify the acquired knowledge with feedback.

METHODS FOR ANALYSIS OF CELL-MEDIATED IMMUNITY

5

LABORATORY ASSESSMENT OF SPECIFIC CELL-MEDIATED IMMUNITY IN VIVO AND IN VITRO METHODS USEFUL IN ESTABLISHING A DIAGNOSIS OF TUBERCULOSIS

- Tuberculin Skin Test (TST)
  - Interpretation of the tuberculin skin test
  - Criteria for a positive tuberculin skin test reactions
  - What does a positive/negative tuberculin skin test mean?
  - Limitations of the test
- IGRA tests (Interferon Gamma Release Assay)
  - Interpretation of IGRA tests
  - Advantages of IGRA tests
  - Disadvantages and limitations of IGRA tests
  - QuantiFERON test

Questions for self-assessment

5.1 LABORATORY ASSESSMENT OF SPECIFIC CELL-MEDIATED IMMUNITY, IN VIVO AND IN VITRO METHODS USEFUL IN ESTABLISHING A DIAGNOSIS OF TUBERCULOSIS

Tuberculosis is a chronic infectious disease caused mainly (in up to 95% of cases) by acid-resistant bacteria ***Mycobacterium tuberculosis*** (*M. tuberculosis*). The target organs are most often the lungs (pulmonary tuberculosis). However, they can also cause disease in the extrapulmonary localization (extrapulmonary tuberculosis).

Latent tuberculosis infection (LTBI) means the establishment of tuberculous mycobacteria in the body without clinical manifestation. Only about 10-15% of latently infected people become ill with active tuberculosis. The emergence of the active form of tuberculosis from the latent form can take several decades under conditions where the function of defense mechanisms is weakened for various reasons.

Latent tuberculosis infection is a reservoir for new tuberculosis infections in countries with a low incidence of tuberculosis. It affects approximately 1/3 of the world's population and is a source of tuberculosis disease, especially in people with weakened immunity. Although people with latent *M. tuberculosis* infection do not manifest overt symptoms of active tuberculosis and are not infectious, they are at increased risk for developing active tuberculosis disease and becoming infectious. Identification and preventive treatment can reduce the number of tuberculosis diseases and thus prevent the further spread of tuberculosis in the population. Treatment of people with active tuberculosis is the priority for tuberculosis control. The second priority in countries with a low incidence of tuberculosis is the identification and treatment of patients with latent tuberculosis infection. In most people, infection with *M. tuberculosis* remains latent. However, latent tuberculosis infection has the potential to develop into active tuberculosis at any time, and people with active tuberculosis become a source of new infections. Treatment of latent infection reduces the likelihood that active tuberculosis will develop. It has the potential to protect the health of an individual as well as the public health by reducing the number of tuberculosis sources.

To identify latent tuberculosis infection immunological tests are used, in addition to standard diagnostic procedures. *In vivo* Tuberculin Skin Test and *in vitro* Interferon Gamma Release Assay test (IGRA). The standard diagnostic procedures include:

- clinical assessment, symptoms (e.g. fever, cough, night sweats, weight loss)
- chest radiography
- bacterial identification in the microbiology laboratories

The main goal is to identify people who are at risk for the development of tuberculosis (see Table 5) and will benefit from the treatment. Candidates for testing for latent tuberculosis infection are:

- people who are likely to have been infected recently and
- people who are at increased risk for tuberculosis because of certain clinical conditions.

Tab. 5 People at increased risk who should be tested for latent tuberculosis infection
(Source: *Jasmer et al., 2002*)

Risk	**Example of people with risk**
increased risk of exposure to infectious cases	○ people with recent close contact with people known to have active tuberculosis [a] ○ health care workers who work at facilities where patients with tuberculosis are treated
increased risk of tuberculosis infection	○ foreign-born people from countries with a high prevalence of tuberculosis ○ homeless people ○ people living or working in facilities providing long-term care
increased risk of active tuberculosis once infection has occurred	○ HIV-infected people ○ people with recent tuberculosis infection [b] ○ injection-drug users ○ patients with end-stage renal disease, silicosis, diabetes mellitus, hematological cancers ○ patients receiving immunosuppressive therapy ○ malnourished people or those with a recent weight loss of more than 10 % of their ideal body weight ○ people who have undergone gastrectomy or jejunoileal bypass

[a] Close contact was defined as at least 12 hours of contact with a people with infectious tuberculosis, but there are no well-established criteria for such contact.

[b] People with recent infection include children less than four years of age and people found to have tuberculin conversion, defined as an increase in induration of at least 10 mm on a tuberculin skin test within a two-year period (Jasmer et al., 2002).

Tuberculin skin test (TST), also known as a Mantoux test, is *in vivo* test for latent tuberculosis infection that involves a small injection of tuberculin just under the skin.

IGRA tests are i*n vitro* immunoassays of whole blood for cell-mediated immunity used to see whether a person has been exposed to *M. tuberculosis*. It is a modern alternative to the tuberculin skin test.

5.1.1 Tuberculin Skin Test (TST)

The tuberculin test (Mantoux test) is a worldwide accepted standard method of determining a latent form of tuberculosis infection. It was developed by Robert Koch based on the observation that infection with *M. tuberculosis* caused cutaneous reactivity to tuberculin. Tuberculin is a Purified Protein Derivate (PPD). It is a mixture of antigens derived from tuberculous bacteria. The standard PPD dose is 5 tuberculin units (5 TU) per 0.1 ml.

Principle: Tuberculin skin test is the clinical demonstration of the **delayed-type hypersensitivity** response, which belongs to **IV. type of hypersensitivity**. Robert Koch

described this form of hypersensitivity in patients with tuberculosis. Following skin injection of tuberculin, patients experienced a general feeling of disease with fever and edema and hardening of the skin at the site of the injection. It is this skin reaction that is used today in the tuberculin skin test. T-lymphocytes, upon contact with PPD presented by antigen-presenting cells, release numerous Th1-type cytokines (mainly interferon-gamma - IFNγ, and interleukin 2) into the environment, which induce changes typical of these reactions. They attract macrophages to the site of tuberculin application, where macrophages accumulate and are activated. Hardening, edema, and erythema have the greatest intensity in 48 to 72 hours, hence the "delayed" type of hypersensitivity (Figure 81).

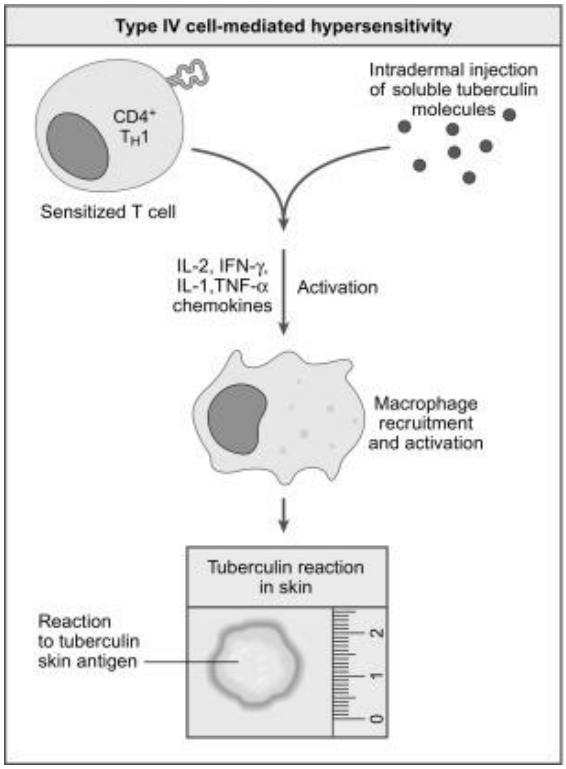


Fig. 81 Principle of tuberculin reaction (Source: *https://www.sciencedirect.com/science/article/pii/B9780128165720000085*)

Procedure: Tuberculin skin test is an intradermal injection 0.1 ml of PPD = 5 tuberculin units into the upper layer skin of the forearm without abnormalities. It is applied strictly intradermally (Figure 83). Proper application leads to a discreet pale elevation of the skin at the application site 6 to 10 mm in diameter, which is rapidly absorbed (Figure 82).

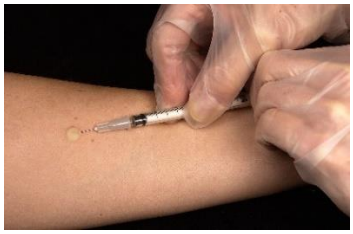


Fig. 82 Application of tuberculin into the upper layer skin of the forearm without abnormalities

(Source: *http://laboratorytests.org/mantoux-test/*)

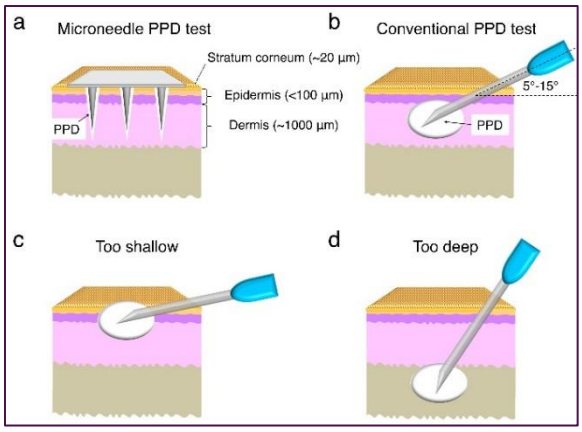


Fig. 83 Comparison of a microneedle tuberculosis test with a traditional test administered with a hypodermic needle. The lower images show needle-depth problems that can occur with the conventional test

(Source: Marco Rolandi *https://www.washington.edu/news/2013/08/26/microneedle-patch-could-replace-standard-tuberculosis-skin-test/*)

Reading: The skin test reaction should be read between 48 and 72 hours after tuberculin administration. The reaction to tuberculin is the presence of a palpable, raised hardened area around the injection site, called **induration**. The reaction should be measured in millimetres of the induration. The diameter of the indurated area is measured across the long axis of the forearm (Figures 84, 85). The application site may itch, blisters filled with fluid, or other disruption of skin integrity at the injection site. Redness (erythema) or bruising is not measured. The erythema has no diagnostic significance and should be ignored. Although positive reaction persists for several days following the 3-day period, after more than 72 hours, the result is already distorted.

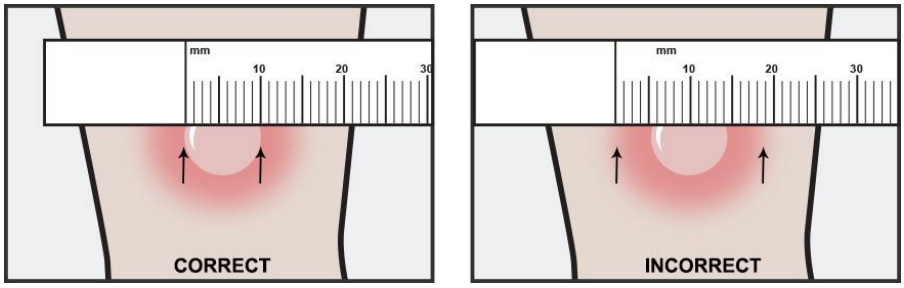


Fig. 84 Reading the tuberculin skin test (correct vs. incorrect)
(Source: *https://tbskintesting.weebly.com/reading-the-test.html*)

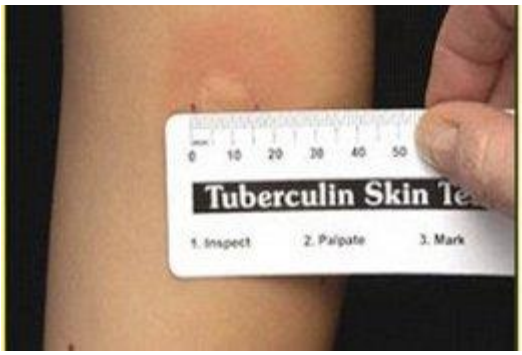


Fig. 85 Procedure for reading the skin test (the diameter of the indurated area is measured across the long axis of the forearm)
(Source: *https://tbskintesting.weebly.com/reading-the-test.html*)

Interpretation of the tuberculin skin test

Interpretation of tuberculin skin test always remains difficult. The criteria for interpreting the reaction as positive (indicating the presence of tuberculosis infection) depend on certain characteristics of the person being tested. Sensitization to tuberculin can also be induced by infection with nontuberculous mycobacteria, and BCG (Bacille Calmette-Guérin) vaccine, which is used in many parts of the world as a vaccine against tuberculosis. Previous vaccination with BCG may cause a "false positive" reading. The specificity and sensitivity of the test are low due to post-vaccination immunity. The specificity (the probability that the test will be negative in disease-free individuals) is 46-73% in the BCG vaccinated population. The sensitivity (the probability that the test will be positive in patients) in the BCG vaccinated population is 77% (*Pai et al., 2010*). According to the Center for Disease Control (CDC), skin test interpretation depends on measurement in millimetres of the induration and person's risk of being infected with tuberculosis and of progression to disease if infected.

Criteria for positive tuberculin skin test reactions:

An **induration of 5 or more millimetres** is considered positive in

- HIV-infected people
- a recent contact of a person with tuberculosis disease
- people with fibrotic changes on chest radiograph consistent with prior tuberculosis
- patients with organ transplants
- people who are immunosuppressed for other reasons

An **induration of 10 or more millimetres** is considered positive in

- recent immigrants (< 5 years) from high-prevalence countries
- injection drug users
- residents and employees of high-risk congregate settings
- mycobacteriology laboratory personnel
- people with clinical conditions that place them at high risk
- children < 4 years of age
- infants, children, and adolescents exposed to adults in high-risk categories

An **induration of 15 or more millimetres** is considered positive in

- any person, including people with no known risk factors for tuberculosis. However, targeted skin testing programs should only be conducted among high-risk groups.

What does a positive/negative tuberculin skin test mean?

- **Positive result**: person's body was infected with tuberculosis bacteria. Additional tests are needed to determine if the person has latent tuberculosis infection or tuberculosis disease. A health care worker will then provide treatment as needed.
- **Negative result**: person's body did not react to the test, and that latent tuberculosis infection or tuberculosis is not likely.

Limitations of the test:

The tuberculin skin test has several limitations. A positive reaction may be observed in both latent and active tuberculosis infections. It is unreliable in differentiating whether the patient is currently having tuberculosis or had been infected in the past or at the carrier stage.

False-positive result: In some people, the BCG vaccination may cause a positive tuberculin skin test even they are not infected with *M. tuberculosis*. If the test is positive in vaccinated people, additional confirmatory tests are needed. False-positive tuberculin skin test results may occur not only after BCG vaccination but also in infections with atypical nontuberculosis mycobacteria, incorrect method of tuberculin administration, incorrect interpretation of the reaction or when tuberculin is applied to a site of pathologically altered skin, etc.

False-negative result: there are various factors that may cause a false-negative result even if the person is infected with *M. tuberculosis*. It may occur after a recent viral illness (e.g. measles and smallpox), vaccination with a live-virus vaccine, HIV patients, patients who are taking immunosuppressive/corticosteroid treatment, patients with cutaneous anergy (inability to react to skin tests due to weakened immune system), children aged months or less or elderly patients (immature or waning immunity), etc.

5.1.2 IGRA tests (Interferon Gamma Release Assay)

IGRA tests are *in vitro* immunoassays of whole blood for cell-mediated immunity, based on the release of interferon-gamma (IFN-gamma) from sensitized T-lymphocytes. They are approved internationally for the detection of latent tuberculosis infection. However, they do not help differentiate latent tuberculosis infection from tuberculosis disease. The IGRA tests work by measuring the immune response of the body to the tuberculosis bacteria. They are not used to test for active tuberculosis disease. IGRA tests may be used to diagnose latent tuberculosis infection. There is a risk that latent tuberculosis infection can progress to active tuberculosis disease. The risk is increased in young children, old people, people with weak immunity. Tuberculosis disease can develop if *M. tuberculosis* starts to multiply and causes clinical symptoms (e.g. fever, weight loss, cough, tiredness). Latent tuberculosis infection can be treated with special therapy to prevent it from progressing to active tuberculosis disease in the future. Two IGRA tests have been approved: QuantiFERON®-TB Gold In-Tube test (QFT-GIT) and T-SPOT®.TB test (T-Spot).

Principle: IGRA tests are based on the quantification of interferon-gamma (INF-γ) released from sensitized plasma T-lymphocytes in response to *M. tuberculosis* antigens using an ELISA test (Chapter 4.2.1) or an ELISPOT (Chapter 4.4). To conduct the tests, fresh blood samples are mixed with antigens and controls. T-lymphocytes from persons that have been infected with *M. tuberculosis* will release interferon-gamma when mixed with antigens derived from this bacteria (ESAT-6, CFP-10, TB7.7). These antigens are not present in BCG. QFT-GIT and T-Spot are expected to be more specific than the tuberculin skin test because the antigens used in these tests are relatively specific to *M. tuberculosis* and should produce fewer false-positive tests. The antigens, testing methods, and interpretation criteria for IGRA tests differ (see Table 6).

Tab. 6 Differences in currently available IGRA tests

(Source: *https://www.cdc.gov/tb/publications/factsheets/testing/igra.htm*)

	QFT-GIT	T-Spot
Initial process	Process whole blood within 16 hours	Process peripheral blood mononuclear cells (PBMCs) within 8 hours, or if T-Cell Xtend® is used, within 30 hours
M. tuberculosis antigen	Single mixture of synthetic peptides representing ESAT-6, CFP-10 & TB7.7	Separate mixtures of synthetic peptides representing ESAT-6 & CFP-10
Measurement	IFN-gamma concentration	Number of IFN-gamma producing cells (spots)
Possible results	positive, negative, indeterminate	positive, negative, borderline, invalid

The use of IGRA tests is preferred in contact investigations (mainly health care workers exposed to patients with active tuberculosis disease), testing during pregnancy, testing in patients before starting biological treatment with TNF-α antagonists, screening of health care workers and others undergoing serial evaluation for *M. tuberculosis* infection, as well as in risk groups (recent immigrants, homeless people, drug-users, soldiers in missions in countries with a high prevalence of tuberculosis, prisoners). Generally, the selection of the most suitable test for detection of *M. tuberculosis* infection should be made based on the reasons and the context for testing, test availability, and overall cost-effectiveness of testing.

Interpretation of IGRA tests

IGRA interpretations are based on the amount of released IFN-gamma or on the number of cells that release IFN-gamma[1]. As with the tuberculin skin test, IGRA tests should be used as an aid in diagnosing infection with *M. tuberculosis*.

- **Positive result** suggests that *M. tuberculosis* infection is likely.
- **Negative result** suggests that *M. tuberculosis* infection is unlikely.
- **Indeterminate result** indicates an uncertain likelihood of *M. tuberculosis* infection.
- **Borderline result** (T-Spot only) also indicates an uncertain likelihood of *M. tuberculosis* infection.

[1] Interpretation criteria for IGRA tests (Tables 1 to 3) available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5905a1.htm?s_cid=rr5905a1_e#tab2

A diagnosis of latent tuberculosis infection requires that TB disease be excluded by medical evaluation. This should include checking for signs and symptoms suggestive of TB disease, a chest radiograph, and, when indicated, the examination of sputum or other clinical samples for the presence of *M. tuberculosis*. Decisions about a diagnosis of *M. tuberculosis* infection should also include epidemiological and historical information.

Advantages of IGRA tests:

- Requires a single patient visit to conduct the test.
- Results can be available within 24 hours.
- Does not boost responses measured by subsequent tests.
- Prior BCG vaccination does not cause a false-positive IGRA test result.

Disadvantages and limitations of IGRA tests:

- Blood samples must be processed within 8-30 hours after collection while white blood cells are still viable.
- Errors in collecting or transporting blood specimens or in running and interpreting the assay can decrease the accuracy of IGRA tests.
- Limited data on the use of IGRA tests to predict who will progress to tuberculosis disease in the future.
- Limited data on the use of IGRA tests for:
    - Children younger than 5 years of age;
    - People recently exposed to *M. tuberculosis*;
    - Immunocompromised persons;
    - Serial testing.
- Tests may be expensive.

QuantiFERON test

QuantiFeron test (QFT) is a simple blood test that aids in the detection of *M. tuberculosis*. It is a controlled laboratory test that requires only one patient visit and is unaffected by previous BCG vaccination. Prior BCG vaccination does not cause a false-positive IGRA test result. This test is highly specific and sensitive: a positive result is strongly predictive of true infection with *M. tuberculosis*. However, like the tuberculin skin test and other IGRA tests, QFT cannot distinguish between active tuberculosis disease and latent tuberculosis infection. Radiography and other medical and diagnostic evaluations are needed for risk assessment. Like any diagnostic aid, QFT cannot replace clinical judgment.

Procedure: QFT uses unique blood collection tubes that enable immediate exposure of viable blood lymphocytes to highly specific tuberculosis (TB) antigens and test controls coated on the inner surface of the tubes. Antigen exposure produces a quantifiable immune response. There are used three tubes. One tube contains test antigens that consist of ESAT-6, CFP-10, TB-7.7. The two accompanying tubes serve as negative and positive controls: the negative-control tube contains heparin alone, and the positive-control tube contains heparin, dextrose, and phytohemagglutinin :

- Nil tube: negative control to adjust for background IFN-gamma (Figure 86)
- TB Antigen tube: to detect the CD4+ T cell responses to TB antigens
- Mitogen tube: positive control; low response may indicate inability to generate IFN-gamma

Fig. 86 QFT – three tubes (Source: *https://www.quantiferon.com/products/quantiferon-tb-gold/*)

Blood (1 ml) is collected into each of the three tubes, mixed with the reagents already in the tubes, and incubated for 16 to 24 hours, which can occur on-site or at the testing laboratory. Plasma is separated, and the IFN-gamma concentration in the plasma is determined using the ELISA (Figure 87, 88). The result is calculated as the difference in IFN-gamma concentration in plasma from blood stimulated with TB antigens minus the IFN-gamma concentration in plasma from blood incubated without antigen (Nil tube).

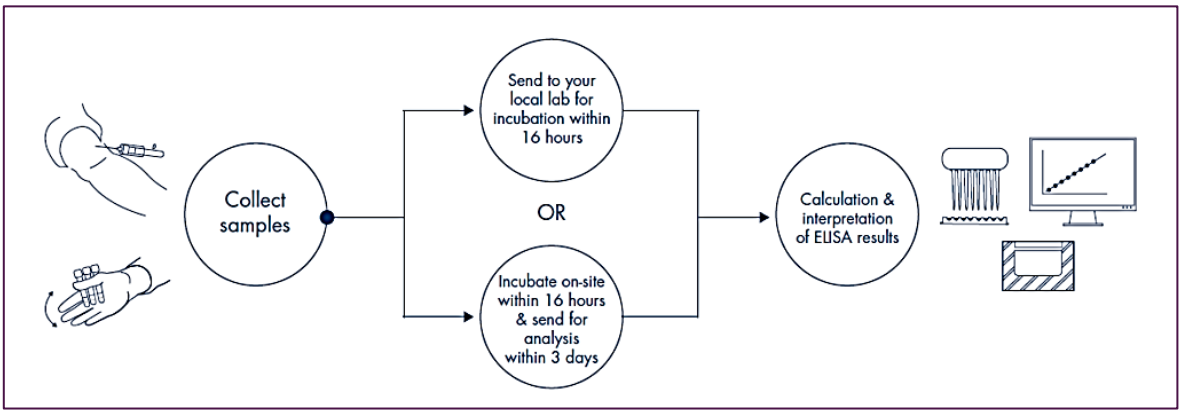


Fig. 87 QFT – procedure (Source: *https://www.quantiferon.com/products/quantiferon-tb-gold/*)

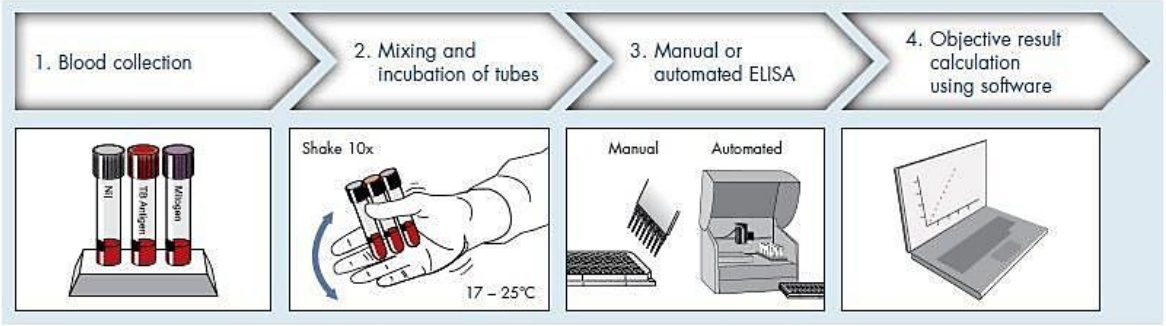


Fig. 88 The workflow for QFT

(Source: *https://www.quantiferon.com/wp-content/uploads/2016/11/The-workflow-for-QFT.jpeg*)

Benefits of the QuantiFERON technology

- The use of whole blood in this test makes T cell incubation simple and fast and reduces labor
- No tedious lymphocyte isolation, washing, counting, diluting or culturing
- Technology designed for clinical screening of large sample numbers, providing the standards, controls, and assay reproducibility needed for clinical diagnosis
- Convenient and objective ELISA technology
- Single-visit testing
- Prior BCG vaccination does not cause a false-positive test result

Both types of tests *in vivo* (tuberculin skin test) and *in vitro* (IGRA tests) are shown schematically in Figure 89.

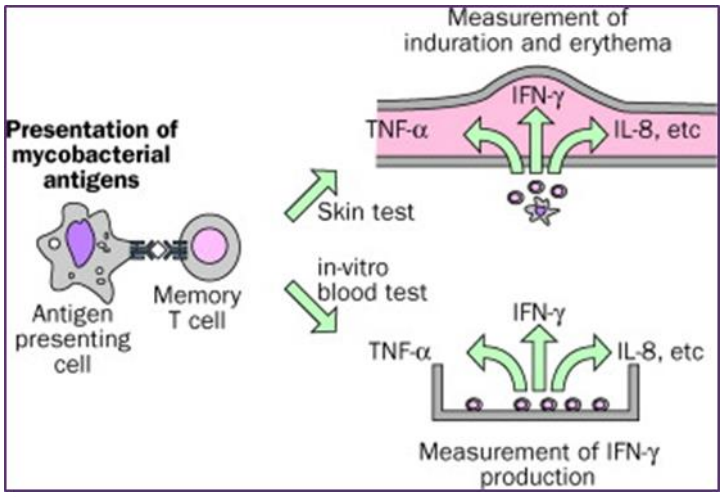


Fig. 89 Tests in vivo and in vitro to detect latent tuberculosis infection
(Source: *http://www.thelancet.com/cms/attachment/2002735434/2009762277/gr1.jpg*)

Questions for self-assessment

1. Which diagnostic tests are intended to identify latent tuberculosis infection?
2. Which methods are used to demonstrate specific cellular immunity *in vivo* and *in vitro*?
3. What is the principle of a tuberculin skin test? What is tuberculin?
4. What type of hypersensitivity is responsible for tuberculin skin test reaction? Which cytokines are released from T-lymphocytes upon contact with tuberculin?
5. How does the tuberculin skin test work?
6. How do you read tuberculin skin test result? What does a positive tuberculin skin test mean? What does a negative tuberculin skin test mean? When can it be a false positive and false negative?
7. What are the IGRA tests? How are IGRA tests done? What if IGRA test is positive? On what principle are IGRA tests based?
8. What are the benefits of IGRA testing in the population vaccinated with BCG vaccine?

IMMUNE DEFICIENCY

6

Immunodeficiency is an immune deficiency, a disorder of the immune system manifested by reduced defence. It is a condition of the organism in which a component (one or more components) of the immune system is disrupted, which means lacking, reduced or damaged. The suspicion of an immunodeficiency condition usually arises with increased susceptibility to infections; recurrent infections are common.

Immunodeficiencies can be inherited (primary, congenital, genetic disorders) or acquired (secondary). Congenital immunodeficiency conditions are caused by defects of genes that encode individual components necessary for the functions of the immune system.

- **Primary immunodeficiencies** – their severe forms are less common than secondary immunodeficiencies. Most primary immune deficiencies become at about six months of age when the maternally derived antibodies begin to disappear, and the children become dependent upon their immune system.
- **Secondary immunodeficiencies** are acquired during life, may occur at any time of life. They occur more frequently than primary immunodeficiencies and can be found in many medical fields. The causes of their origin are diverse (e.g. medical treatment, iatrogenic induction, infections, stress, malnutrition, cancer, etc.).

Immunodeficiencies can be induced artificially or spontaneously, they may relate to any functions or components of the immune system - recognition, regulation, defence function, cellular or humoral immunity, specific or non-specific immunity. They can be selective or combined.

6.1 PRIMARY IMMUNODEFICIENCIES

Primary immune deficiencies are **congenital disorders caused by genes defects for individual components of the immune system**; they usually are serious, but rare. They can be detected by a wide range of clinical symptoms from easier to severe infections leading to the death of the affected individual at young age (severe combined immune deficiency disease SCID). Usually, positive family history can be found.

6.1.1 Clinical manifestation

Clinical manifestation is diverse. Some features are found in most forms of immunodeficiencies, and some occur only in limited forms of deficiencies. Characteristics are failure to thrive, which is the failure of infants to gain weight naturally, chronic diarrhoea, atypical skin rashes, recurrent or chronic infections caused by opportunistic pathogens, hepatosplenomegaly can be observed. Recurrent skin or organic abscesses, repeated severe infections with an inadequate response to the use of the standard antibiotic treatment can be present. The most widespread immunodeficiencies are defects in B cells responsible for the majority of immunodeficiency diseases (more than 70%). They are characterized by abnormal production of immunoglobulins. Then cellular and combined defects (20%), defects in phagocytic cells (9%) and defects in the complement system (1%). The fundamental division of primary immunodeficiencies is summarized in Table 7.

Tab. 7 Primary immunodeficiencies and their characteristics (Source: Authors)

	Antibody deficiency	Cellular and combined deficiency	Defects in phagocytosis	Defects in complement
Incidence of immunodeficiencies	70 %	20 %	9 %	1 %
Symptoms usually begin	Since 6th month to adulthood	After birth until approx. 2nd year of life	After birth, in childhood and in adulthood	in childhood and in adulthood
Symptoms	Respiratory tract infections, otitis, arthritis	Failure to thrive, respiratory infections, diarrhoea, dermatitis	Omphalitis, pyoderma, adenitis, otitis	Pyogenic infections, oedema
Most commonly isolated pathogens	Encapsulated microorganisms, echoviruses	Viruses, pneumocysts, fungi, Mycobacterium	Staphylococci, fungi, Enterobacteriaceae	Neisseria
Complications	Echovirus infections, tumors, autoimmune disease	Tumors, autoimmune disease	Infections, various complications	Various complications
Some examples of immunodeficiencies	- Selective IgA deficiency (sIgAD) - Bruton's X-linked agamaglobulinemia (XLA) - Common variable immunodeficiency (CVID)	- Severe combined immune deficiency (SCID)	- Chronic granulomatous disease (CGD) - Leukocyte adhesion defect 1 (LAD)	- Hereditary angio-edema (HAE)

Warning signs indicating possible primary immunodeficiency are listed in Table 8.

Tab. 8 Warning signs for finding at-risk patients with immunodeficiencies (Source: *Čižnár, 2006*).

1.	A child with 8 or more otitis during the year, or an adult with recurrent ear complications
2.	5 or more confirmed sinus infections per year
3.	2 cases of pneumonia per year or 1 pneumonia per year for two consecutive years
4.	2 or more infectious bronchitis per year
5.	2 or more deep tissue infections or skin abscesses
6.	Need longer-term or intravenous antibiotic treatment
7.	Sepsis or meningitis
8.	Atypical infections (e.g. yeast infection in the mouth or on the skin after the first year of life)
9.	Persistent diarrhoea, malabsorption, not gaining weight or growth retardation in children
10.	Presence of chronic infections
11.	Primary immunodeficiency in families

Characteristic infections associated with immune deficiency syndromes are in Table 9.

Tab. 9 Infections associated with immunodeficiency syndromes (Source: *Rich et al., 2019*)

Deficiencies of T cell-mediated immunity ○ Mucocutaneous fungal infections (especially *Candida albicans*) ○ Systemic fungal infections ○ Systemic infections with attenuated viruses (e.g. live viral vaccines), with viruses of usually low pathogenicity ○ *Pneumocystis jiroveci* pneumonia
Antibody deficiencies ○ Infections by encapsulated bacteria (e.g. *Streptococcus* spp., *Haemophilus influenzae*) ○ Recurrent pneumonia, bronchitis, sinusitis, otitis media ○ Enteritis caused by *Giardia lamblia*
Phagocyte deficiencies ○ Infection by gram-positive bacteria (e.g. staphylococci, streptococci) ○ Gram-negative sepsis ○ Systemic fungal infections (e.g. *Candida* spp., *Aspergilus* spp.)
Complement deficiencies ○ C3 deficiency – infections with encapsulated bacteria ○ Deficiency of terminal components: infections with gram-negative bacteria (especially *Neisseria* spp.)

6.1.2 Treatment of primary immunodeficiencies

Patients with primary immunodeficiencies are highly susceptible to recurrent and opportunistic infections. As a preventative measure, isolation and the provision of an aseptic environment are recommended and have been shown to protect against infections for extended periods. Treatment of congenital immunodeficiencies varies **depending on the type and severity of the immunodeficiency.**

- In antibody immunodeficiencies, **supplementation of deficient immunoglobulins** and **antibiotic supportive therapy** is applied.
- In severe combined immunodeficiencies, severe forms of phagocytosis defects, and in many cases of combined immunodeficiency, **hematopoietic stem cell transplantation** is performed. In SCID patients should be kept in a protective isolation environment, receive prophylactic antibiotics, immunoglobulin replacement, and enhanced nutrition. Patients with SCID usually die of overwhelming infections or suffer associated complications once maternally transferred antibodies have been metabolized, usually within 3-6 months after

birth. If diagnosed early and before the onset of complications, most of these patients can be treated successfully with hematopoietic stem cell transplantation. Success depends on the suitability of the donor and the timeliness of the diagnosis.

- If transplantation cannot be performed for some reason, **symptomatic treatment** is used, e.g. intravenous application of immunoglobulins, broad-spectrum antibiotics, antifungal drugs, virostatics.

6.2 SECONDARY IMMUNODEFICIENCIES

These are immune disorders accompanying the patient's underlying disease, treatment, malnutrition, infections, stress, etc. They are more common than primary immunodeficiencies. Acquired immunodeficiencies can be encountered in many medical disciplines.

6.2.1 Causes of secondary immunodeficiencies

Many factors can induce secondary immunodeficiencies:

- metabolic disorders (uremia, diabetes, hypothyroidism, long-term reduction diets, malnutrition),
- iatrogenic effects (cytostatics, immunosuppressive therapy, irradiation),
- cancer,
- viral diseases (AIDS, measles, cytomegalovirus infection, infectious mononucleosis),
- chronic infections,
- in case of liver failure, liver cirrhosis,
- alcoholism,
- after splenectomy,
- chronic stress,
- serious injuries,
- extensive burns,
- general anaesthesia,
- concerning age (premature babies, the elderly).

Temporary immunodeficiencies have been reported after infection with some herpes viruses and also after infection with measles virus.

The causes can be combined (patient with malignancy, used treatment and secondary malnutrition). However, **HIV infection and malnutrition dominate worldwide as the cause of secondary immunodeficiency** conditions. According to the predominant damage component, the secondary immunodeficiencies may be antibody, cellular, phagocytic, and complement, or combinations thereof.

6.2.2 Treatment of secondary immunodeficiencies

Treatment of secondary immunodeficiencies consists in identifying and treating the root cause of secondary immunodeficiencies (or alleviating them), as part of supportive treatment, therapy with preparations that individually modulate the functions of the immune system is appropriate.

6.3 DIAGNOSTICS OF IMMUNODEFICIENCIES

The medical history and physical examination of the patient provide the first information important for the use of laboratory tests. Specific tests have been designed to screen for the four basic mechanisms of host defence: antibody, T cell, phagocyte, and complement. The clinical immunology laboratory is a powerful adjunct to the clinician in the initial evaluation of immunodeficiency disorders and in the design of more sophisticated testing for selected patients.

6.3.1 Familial and Personal History

The basis of the diagnosis of immune deficiency is a history. The incidence of recurrent infections or deaths in the family is sought, even further into the past (in previous generations). The search is thorough, especially in maternal ancestors, because many primary immunodeficiencies are bound to the X chromosome. The personal history examines the course of pregnancy and childbirth, psychomotor and physical development in the first year of life, the incidence of infections, their severity and recurrence, what were the reactions after vaccination. If secondary immunodeficiency is suspected, especially in adults, the underlying cause which could have led to its occurrence is sought. The aim is to identify both the underlying disease and immunodeficiency.

6.3.2 Clinical manifestation

The next step is a clinical manifestation that may be diverse but is **dominated by recurrent infections**. When primary immunodeficiencies are suspected, they are most often respiratory infections.

- **By immune deficiency caused by defects in B cells and immunoglobulins**, these are mostly recurrent respiratory infections that begin to appear suddenly after the child is 6 months old, as the protective effect of maternally transplanted antibodies is lost. The most common causative agents are extracellular bacteria (*Haemophilus influenzae, Streptococcus pneumoniae*, etc.) and viruses.
- **By immune deficiency caused by defects in T cells,** these are recurrent respiratory infections; in the most severe conditions, when the combined immunodeficiency is present, the infections tend to be very serious and occur in early childhood. The most common causes are intracellular bacteria, various atypical strains of microorganisms, viral and fungal infections.
- **By immune deficiency caused by defects in phagocytosis,** these are infections caused by pyogenic bacteria, most often staphylococci.

- **By immune deficiency caused by defects in complement,** these are infections caused mainly by *Neisseria.*

Depending on the nature of the immunodeficiency, a complex clinical manifestation typical of a given immune system disorder may also occur (e.g. Bruton's agammaglobulinemia, DiGeorg's syndrome, hereditary angioedema, etc.). In secondary immunodeficiencies, the spectrum of clinical signs is very diverse, and the typical picture (as in primary immunodeficiencies) does not occur here, although they may be divided according to the predominant damaged component (similar to primary).

6.3.3 The spectrum of immunological examinations in the diagnosis of immunodeficiencies

The basis is to examine immunoglobulins and specific antibodies. As a guide, serum electrophoresis can be used to capture the region of gamma globulins in which the immunoglobulins move (see Figure 53). Tests are needed to confirm a diagnosis of immunodeficiency. Initial screening tests should include:

- Complete blood count (CBC) with manual differential
- Quantitative immunoglobulin (Ig) measurements
- Antibody titers
- Skin testing for delayed hypersensitivity

If results are abnormal, further tests in specialized laboratories are needed to identify specific deficiencies. If chronic infections are objectively documented, initial and specific tests may be done simultaneously. If clinicians suspect that immunodeficiency may be still developing, tests may need to be repeated, with monitoring over time, before a definitive diagnosis is made.

In order to examine serum, blood is collected in a dry tube without anticoagulants. After visible separation of the blood cake, after centrifugation of the tube, the serum is aspirated. The serum should be processed immediately after collection so that degradation does not alter some parameters. Suppose the serum cannot be processed immediately. In that case, it can be stored in the freezer for a long time at -20 ° C. Immunoglobulin levels, and their subclasses and levels of specific antibodies after vaccination are determined (to demonstrate the ability to respond by antibody production after application of specific protein or polysaccharide antigens).

Uncoagulated blood is required for cellular immunity analysis, and the addition of an anticoagulant prevents clotting. The blood thus prepared is used for further processing; various separation methods can be used. Polymorphonuclear leukocytes, lymphocytes, can be isolated, counted, and T and B cell subpopulations determined.

Initial and Additional Laboratory Tests for Immunodeficiency are shown in Table 10.

Tab. 10 Initial and Additional Laboratory Tests for Immunodeficiency (Source: *https://www.msdmanuals.com/professional/immunology-allergic-disorders/immunodeficiency-disorders/approach-to-the-patient-with-suspected-immunodeficiency*)

Type	Initial tests	Additional tests
Humoral immunity deficiency	- IgG, IgM, IgA, and IgE levels - Isohemagglutinin titers - Antibody response to vaccine antigens (e.g. *Haemophilus influenzae* type b, tetanus, diphtheria, conjugated and nonconjugated pneumococcal, and meningococcal antigens)	- B-cell phenotyping and count using flow cytometry and monoclonal antibodies to B cells - Flow cytometry for CD40 and CD40 ligand - Evaluation for mutations in genes that encode BTK and NEMO - Sweat test
Cellular immunity deficiency	- Absolute lymphocyte count - Delayed hypersensitivity skin tests (e.g. using *Candida*) - HIV testing - Chest x-ray for size of thymus in infants only	- T-cell phenotyping and count using flow cytometry and monoclonal antibodies to T cells and subsets - T-cell proliferative response to mitogens - TREC test (a genetic test that identifies infants with abnormal T cells or a low T-cell count due to SCID or other disorders)
Phagocytic cell defects	- Phagocytic cell count and morphology	- Flow cytometric oxidative burst measurement using dihydrorhodamine 123 (DHR) or nitroblue tetrazolium (NBT) - Flow cytometry for CD18 and CD15 - Neutrophil chemotaxis
Complement deficiency	- C3 level - C4 level - CH50 activity (for total activity of the classical pathway) and AH50 activity (for total activity of the alternate complement pathways) - C1 inhibitor level and function	- Specific component assays

AH50 = alternate complement hemolytic assay; BTK = Bruton tyrosine kinase; C = complement; CH = hemolytic complement; Ig = immunoglobulin; NEMO = nuclear factor–kappa-B essential modulator; SCID = severe combined immunodeficiency; TREC = T-cell receptor excision circle.

6.3.4 Methods used in laboratory diagnosis of immunodeficient conditions

Examination of humoral immunity

- determination of IgG, IgM, IgA immunoglobulin levels - turbidimetry, radial immunodiffusion (RID)
- IgE – ELISA
- Determination of immunoglobulin's subpopulations – ELISA
- Levels of specific antibodies after vaccination - ELISA
- Determination of cryoglobulins
- Determination of immunocomplexes - turbidimetry
- Evidence of acute phase inflammation proteins and mucosal and serum proteins (CRP, alpha1antitrypsin, macroglobulin, transferrin, ceruloplasmin, orosomucoid, lysozyme) - RID, turbidimetry
- Evidence of autoantibodies – immunofluorescence
- Detection of complement cascade function and levels - turbidimetry, RID, CH50, determination of C3, C4 components of the complement

Examination of cellular immunity

- Blood count and differential leukogram
- T and B-cell counts – the test of active rosettes, ELISPOT, lymphokine levels (spontaneous and after antigen stimulation)
- Skin tests
- Determination of T-cell subpopulations - flow cytometry, ELISA
- Determination of NK cells
- Determination of neutrophil count and function - phagocytosis, phagocytic activity, phagocytic index, nitrotetrazolium blue test
- Typing of HLA antigens - serotyping, PCR

In addition to screening tests, accurate diagnostics of immunodeficient conditions is performed in specialized laboratory workplaces. Detailed differential diagnosis is in the hands of a clinical immunologist.

Early recognition of the diagnosis is essential for the patient. Delaying the diagnosis leads to low quality of life for the patient, especially due to frequent morbidity, leads to irreversible tissue damage and increases the cost of long-term treatment.

Questions for self-assessment

1. What is immunodeficiency?
2. What causes primary immunodeficiencies?
3. What causes secondary immunodeficiencies? What dominates as the most common cause of secondary immunodeficiencies?
4. What groups of primary immunodeficiencies can occur and which of them are most common? What are the warning signs for finding patients at risk?
5. What procedures are used to diagnose immunodeficient conditions?

AUTOIMMUNE DISEASES AND THEIR DIAGNOSTICS

7

7.1 AUTOIMMUNITY

Autoimmunity can be **defined as the disruption of the mechanisms responsible for tolerance to own antigens and the induction of an immune response against own components in the body.** This immune response may not be harmful in all cases. However, in many cases of autoimmune diseases, it is clear that the products of the immune system damage the own body. Both antibodies and T-lymphocytes can act as powerful tools in the development of autoimmune diseases. The incidence of autoimmune diseases is at the level of 5-7% of the population.

Autoimmune diseases are due to an inappropriate immune response against the organism's own antigens (called autoantigens), leading to chronic inflammation, tissue destruction and dysfunction. Generally unknown by the public and perceived as uncommon, they represent nevertheless the important cause of clinical mortality in industrialized countries. There is epidemiological evidence of increasing prevalence of certain autoimmune diseases, which cannot be attributed to focus on or improvement of the diagnosis alone.

7.2 AUTOANTIBODIES

Autoantibodies are **immunoglobulins that are directed against the body's own antigens** (e.g., proteins, phospholipids, nucleic acids, etc.) and are found in serum and other body fluids.

Autoreactivity is to some degree necessary for the normal function of the human body. Thus, autoreactive processes also take place in the body of a healthy person. These autoantibodies are referred to as **natural (physiological) autoantibodies**, have a low affinity, and are usually of the IgM isotype. As their serum concentration is low, their presence is often not detected by commonly available laboratory methods. Their incidence increases with age. The finding of autoantibodies is common in older age, but their role in autoimmune disease is not significant at this age. Monitoring is required in children if elevated autoantibodies are detected.

However, it is also true that **autoimmune disease occurs when the number of autoimmune processes exceeds the regulatory** (adaptive and homeostatic) **abilities of the organism and leads to damage.** For this reason, the finding of autoantibodies alone is not evidence of an autoimmune disease, the clinical manifestation is necessary. There are healthy individuals with the finding of autoantibodies. Autoantibodies may be present in the body a long time before the onset of the disease.

Autoantibodies probably are not the immediate cause of autoimmune disease, but rather the marker of autoimmune disease. Their finding can precede clinical manifestations or can be part of an autoimmune disease. Autoantibodies can persist after the disease, but they can also be in the body without the disease.

7.3 CLASSIFICATION OF AUTOIMMUNE DISEASES

Autoimmune diseases are divided **according to the organs and tissues that they affect**. Doctors generally distinguish systemic and organ-specific autoimmune diseases, depending on if they affect multiple organs or only one organ specifically.

- **Organ-specific** - the immune response is directed against antigens that are associated with a specific target organ, the pathological process affects a particular organ
- **Organ-non-specific (systemic)** - the immune response is directed against antigens that are not related to the target organ, and several organs and tissues are affected

The etiology of autoimmune diseases is clearly multifactorial. Both intrinsic factors (e.g. hormones, age, genetics) and environmental factors (e.g. infections, diet, environmental chemicals, drugs) may contribute to the induction, development and progression of autoimmune diseases (Figure 90). **The environment can trigger autoimmunity in genetically predisposed individuals under conditions of immune dysregulation.**

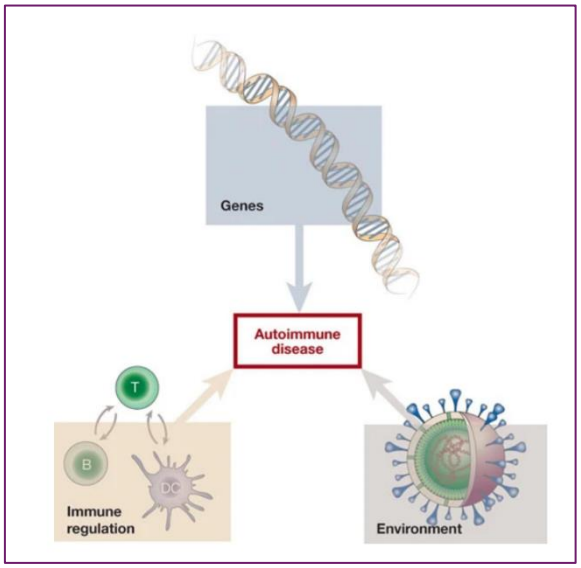


Fig. 90 Conditions for the development of autoimmune disease (Source: *Fathman et al., 2005*)

The clinical manifestation of autoimmune diseases depends on many factors and is very diverse. According to the involvement of organs or tissues by the autoimmune process, they are divided into systemic and organ-specific autoimmune diseases.

7.4 SYSTEMIC AUTOIMMUNE DISEASES

Systemic autoimmune diseases include the following:

- Systemic lupus erythematosus – a multiorgan disease with painful involvement of the joints, skin, kidneys, CNS, oral or nasopharyngeal mucosa, pleuritis, pericarditis, neurologic disorder, etc.
- Rheumatoid arthritis – inflammatory involvement of the joints with pain and morning stiffness of the joints.

- Dermatomyositis/polymyositis – a disease of muscles and joints with muscle weakness, eczema above the joints, on the face, oedema of the eyelids with a hint of purple. Severe forms can lead to various complications with poor diagnosis, like the development of interstitial pulmonary disease.
- Sjögren's disease – affects the glands with external secretions, as well as internal organs, dry mouth syndrome, dry eyes.
- Systemic scleroderma – progressive fibrosis of the skin and organs, from the beginning of fading of the fingers, which is repeated.
- Sharp syndrome – mixed connective tissue disease is similar to systemic scleroderma, but with a milder course.
- Antiphospholipid syndrome – vascular thrombosis, premature births or miscarriages, myocardial infarction or stroke in young people.
- Primary vasculitis – necrotizing inflammation of blood vessels in various organs and tissues
- Sarcoidosis – systemic granulomatous inflammation of many body parts, especially in the lungs

Systemic autoimmune diseases require comprehensive patient care.

7.5 ORGAN-SPECIFIC AUTOIMMUNE DISEASES

Organ-specific autoimmune diseases include the following:

- **Autoimmune endocrinopathy:**
  - Hashimoto's thyroiditis – thyroid destruction and hypofunction
  - Graves-Basedow disease – thyroid stimulation and hyperfunction
  - Diabetes mellitus type I (juvenile) – damage to the beta cells of the pancreas, which produce insulin, origin in childhood and young adulthood
  - Addison's disease – damage to the adrenal cortex and adrenal insufficiency, impaired production of glucocorticoids and mineralocorticoids
  - Autoimmune reproductive disorders and premature ovarian failure syndrome – fertility disorders
- **Autoimmune diseases of the hematopoietic system**
  - Hemolytic anaemia – the breakdown of erythrocytes
  - Thrombocytopenia – the breakdown of platelets
  - Neutropenia –leukocyte breakdown
- **Autoimmune skin diseases**
  - Pemphigus – blistering skin disease, antibodies against the basal layer of the skin
  - Psoriasis – a skin condition with typical joint disorder
- **Autoimmune diseases of the nervous system**
  - Myasthenia gravis – progressive muscle weakness, antibodies against acetylcholine receptor
  - Sclerosis multiplex – a disorder of brain tissue with serious consequences for the sensitivity and motor skills of various parts of the body
  - Peripheral demyelinating neuropathy - peripheral nerve disorder, antibodies against peripheral nerve sheaths and destruction of the fatty protective covering (myelin sheath) over the nerves

- **Autoimmune kidney diseases**
  - Goodpasture syndrome – glomerulonephritis with or without pulmonary hemorrhage, and the presence of circulating anti-glomerular basement membrane antibodies (anti-GBM antibodies). Anti-GBM antibodies are targeted to collagen IV, a component of the glomerular basement membrane

- **Autoimmune diseases of the digestive tract**
  - Primary biliary cirrhosis - inflammation of the liver. Slow, progressive destruction of the small bile ducts of the liver can result in cirrhosis, which begins in middle age with itching of the body, fatigue and increased liver enzymes
  - Autoimmune hepatitis - inflammation of the liver that can lead to scarring of the liver (cirrhosis), starts non-specifically (fatigue), an increase in liver enzymes
  - Celiac disease - a consequence of hypersensitivity to cereal gluten (gluten), most often in childhood. When people with Celiac disease eat gluten, their immune system attacks their own small intestine lining, and the patients suffer symptoms like diarrhoea, stomach cramps, bloating, vomiting.
  - Crohn's disease - together with colitis ulcerosa belongs to **I**nflammatory **B**owel **D**iseases (IBD) that are non-specific intestinal inflammations, Crohn's disease affects both the small bowel and the colon and occurs in childhood or young adults (between 15 and 25 years)
  - Colitis ulcerosa – ulcerative colitis belongs to Inflammatory Bowel Diseases (IBD) that are non-specific intestinal inflammations of the large intestine (colon and rectum) with ulcerations
  - Chronic atrophic gastritis and pernicious anemia – characterized by circulating antibodies to parietal cells and intrinsic factors. The end-stage of the disease is pernicious anemia, which is considered to be the most common cause of vitamin B12 deficiency.

- **Autoimmune diseases of the eyes**
  - Uveitis - inflammation of the middle layer of the eye, swelling and irritation of the uvea

Organ-specific autoimmune diseases are treated by specialists according to the organs and tissues where the autoimmune process is clinically manifested. Treatment of autoimmune diseases is usually symptomatic and includes anti-inflammatory and immunosuppressive agents.

7.6 DIAGNOSTICS OF AUTOIMMUNE DISEASES

Autoimmune diseases are generally incurable and require lifelong treatment. They have a strong impact on public health costs. Their accurate diagnosis has become of major importance. All the diagnosis techniques available on the market are based on the ability to detect the presence of autoantibodies in the patient's serum. Autoantibodies can be detected by immune serological methods. Immunofluorescence, ELISA, immunoblots and immunodot tests, agglutination, precipitation and biochips are most commonly used for laboratory detection of autoantibodies. The presence of antibodies (qualitative) and their amount (quantitative) is demonstrated.

7.6.1 Indirect immunofluorescence

Indirect immunofluorescence is widely considered the gold standard in the examination of autoantibodies. **It is used to detect autoantibodies reacting with antigens that are localized in tissues or are located inside the cell.**

Principle: For the detection of autoantibodies, it is necessary to have a substrate on which the test is performed. It can be a microtome section from the appropriate tissue, a cell suspension or tissue culture to which a serum sample from the patient being examined is added. If the serum contained specific autoantibodies, they would bind to the target substrate structure. In the next step, a secondary fluorochrome-labelled antibody against the Fc fragment of a human autoantibody is added. Between steps, the preparation is washed to remove unbound antibodies. This method is referred to as indirect immunofluorescence (Chapter 4.1.2) (Figure 91, 92).

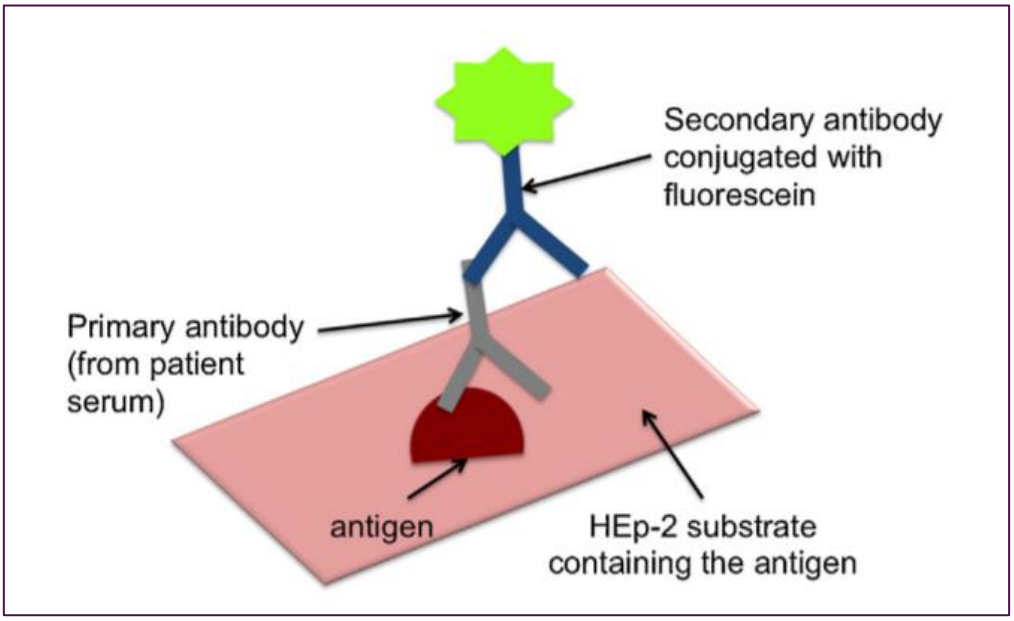


Fig. 91 Principle of indirect immunofluorescence using Hep-2 cell substrate (Source: *https://daneshyari.com/article/preview/469107.pdf*)

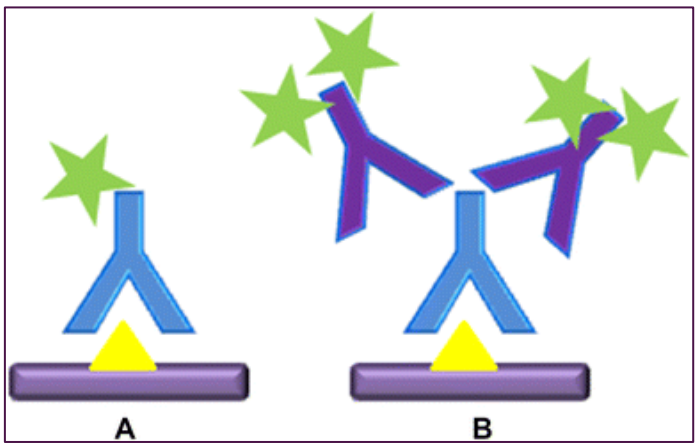


Fig. 92 Comparison between direct (A) and indirect (B) immunofluorescence (Source: *https://media.springernature.com/lw685/springer-static/image/art%3A10.1007%2Fs11696-018-0445-3/MediaObjects/11696_2018_445_Fig4_HTML.gif*)

Indirect immunofluorescence is a method that requires technical equipment on the one hand (the basis is a fluorescence microscope), professional experience and financial security on the other hand. In most cases, commercially produced sections or preparations are used in laboratories to determine autoantibodies. Hep-2 cell lines of human laryngeal carcinoma, human neutrophil leukocytes, monkey and rat kidneys, liver, or stomach, monkey oesophagus, monkey skeletal muscle and others can currently be used as the substrate used to detect autoantibodies.

Each bound autoantibody shows typical fluorescence depending on the location of the antigen (Figure 93). Individual types of autoantibodies are associated with various autoimmune diseases. A particular group of autoantibodies usually occurs with a high frequency at a particular diagnosis, but at the same time may be detectable in other autoimmune diseases (usually with a lower percentage here). For example, the finding of antinuclear (ANA) autoantibodies to ds-DNA is most commonly associated with the occurrence of SLE (systemic lupus erythematosus), but may also be associated with autoimmune hepatitis, thyroiditis, Sjögren's syndrome and others. However, it appears that some patterns are quite confusing and notable for the less experimented clinician. A secondary technique is generally needed to confirm the result.

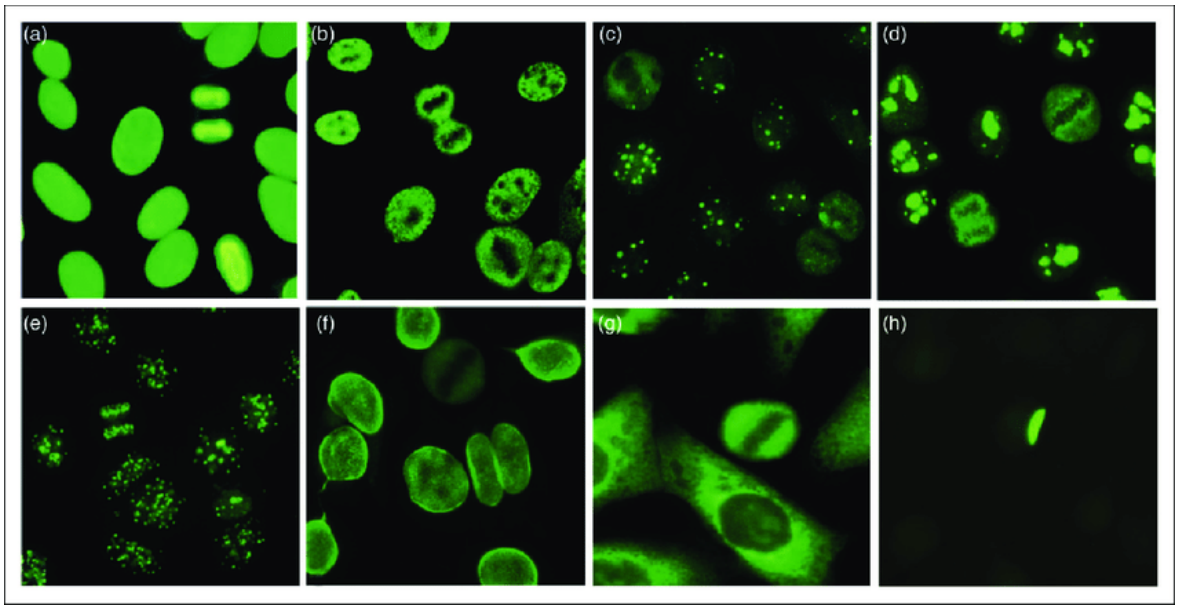


Fig. 93 Different types of indirect immunofluorescence – ANA immunofluorescence patterns (a) homogeneous, (b) speckled, (c) nuclear dots, (d) nucleolar (e) centromeres. Additionally, the following patterns of related antibodies can be recognized: (f) nuclear membranes and (g) cytoplasmic. Finally, negative and unspecific results (h)
(Source: *Krause C et al., 2015*)

7.6.2 Other methods in the diagnosis of autoimmune diseases

Indirect immunofluorescence on organ and tissue sections and cytological preparations make it possible to determine the basic spectrum of autoantibodies and thus determine the further direction in the diagnostic process.

To determine the specificity of autoantibodies, if the target antigen is known, **ELISA**, **immunoblots** (e.g. Western blot), **immunodot tests**, or **RIA** are used. However, there are situations where the target antigen is not yet known and therefore the specificity of detected autoantibodies in the patient may be impossible to determine with available tests.

ELISA, RIA

The principle of these methods is described above (Chapters 4.2.1 and 4.2.2). They serve to accurately demonstrate the specificity of autoantibodies. They use an enzyme – or radionuclide – labelled secondary antibody conjugate, and allow visualization of the reaction site of a human antibody with an antigen under *in vitro* conditions.

Blotting techniques

Blotting techniques (immunoblot, immunodot) are essentially a combination of electrophoresis and immunodetection and are based on the sandwich principle (Chapter 4.3).

Biochips

The trend in the detection of autoantibodies is miniaturization in the form of so-called biochips. A biochip is a collection of miniaturized test sites arranged on a solid substrate that permits many tests to be performed at the same time in order to achieve higher throughput and speed. A biochip's surface area is no larger than a fingernail. In this area are microparticles with bound antigens. The biochip is incubated in vitro with tested serum, and bound autoantibodies are visualized using a colour probe. The result is a matrix of colour dots that are scanned by an optical system, and the data obtained are analyzed using computer technology. The biochip thus makes it possible to examine several autoantibodies simultaneously (Figure 94).

Fig. 94 Biochip

(Source: *http://www.phadia.com/Global/A%20Document%20Library/Product%20Catalogues/Product-Catalog-2015.pdf*)

Flow cytometry

Autoantibodies can also be determined by **flow cytometry**, where the autoantigen is bound to special carriers - beads. The autoantibody from the tested serum binds to it, and the antigen-antibody complex is visualized using a secondary antibody labelled with a fluorescent substance. A suspension of the labelled cells is excited by the laser to emit light at varying wavelengths. Several detectors detect light signals. The individual light signals are converted into electrical signals that are processed by a computer using special software, and the results are available in graphical and numerical form. Carriers (beads) are coated with one type of antigen, but a mixture of several beads with different antigens is defined in one reaction well. Thus, multiple autoantibodies can be determined from a single serum sample (Figure 95). Flow cytometry represents the gold standard for the determination of cell populations. It is a fast, sensitive, automated method for statistical processing.

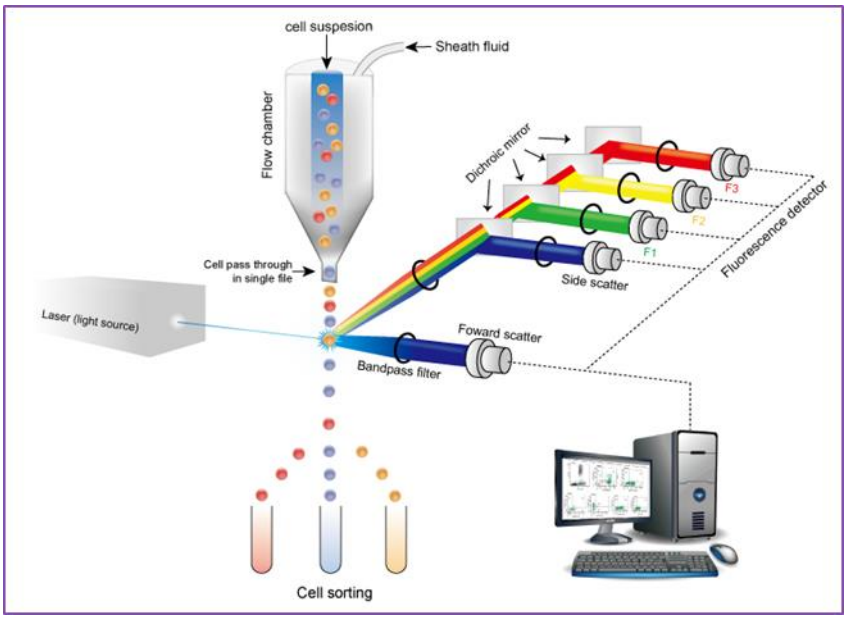


Fig. 95 Flow cytometer scheme (Source: according to*: https://www.creative-diagnostics.com/images/Flow-cytometry-guide-1.jpg*)

Interpretation

The finding of autoantibodies should be evaluated concerning the individual's clinical condition and the results of other laboratory testing methods. Examination and monitoring of autoantibody dynamics are beneficial for early diagnosis of autoimmune disease, but also for treatment and comprehensive patient care.

Questions for self-assessment

1. How is autoimmunity defined? What are autoantibodies? Are autoantibodies present physiologically?
2. How are autoimmune diseases divided according to the organs and tissues they affect? What factors apply to the etiology of autoimmune diseases?
3. Give examples of some systemic and organ-specific autoimmune diseases.
4. What is used as a standard method in the diagnosis of autoimmune diseases? What other methods are used in their diagnosis?
5. How is the finding of autoantibodies interpreted?

VACCINE IMMUNOLOGY

8

Vaccines are considered to be the most effective interventions of modern medicine. Ever since **Edward Jenner first used the smallpox vaccine in 1796**, the use of vaccines has become indispensable for eradication of disease, reducing the number of deaths, or reducing the incidence of complications in many diseases. Today, more than 70 vaccines have been licensed for use against approximately 30 microorganisms, saving countless lives. The beneficial effects of vaccination on public health result not only from the development of effective vaccines, but also from adequate infrastructure for vaccine production, for the regulation and supervision of safety, organized approaches to delivery and from the determination of the most effective route of vaccine administration. Vaccines are the cheapest and most effective way to protect against devastating epidemics. They represent the most effective way to prevent the loss of human lives, to maintain the health of the individual and the team and high quality of life. Society derives economic benefits by preventing hospitalization, avoiding long-term disability, and reducing absence from work. It is always better to prevent the disease than to treat it.

Critical to the modulation of the immune response is the presentation of specific antigens to the immune system. Dendritic cells play a central role in this process. They modulate the response to specific antigens and adjuvants. Traditional vaccines have relied on live-attenuated or inactivated organisms, attenuated bacteria or capsules, or inactivated toxins. Progress has been made recently in enhancing immunity through an understanding of the biology of dendritic cells and their response to adjuvants. Alternative delivery, including virus-like particles or structured arrays with the use of phage or nanoparticles, also stimulate effective immunity and provide powerful tools to confer protection for a specific pathogen (Figure 96).

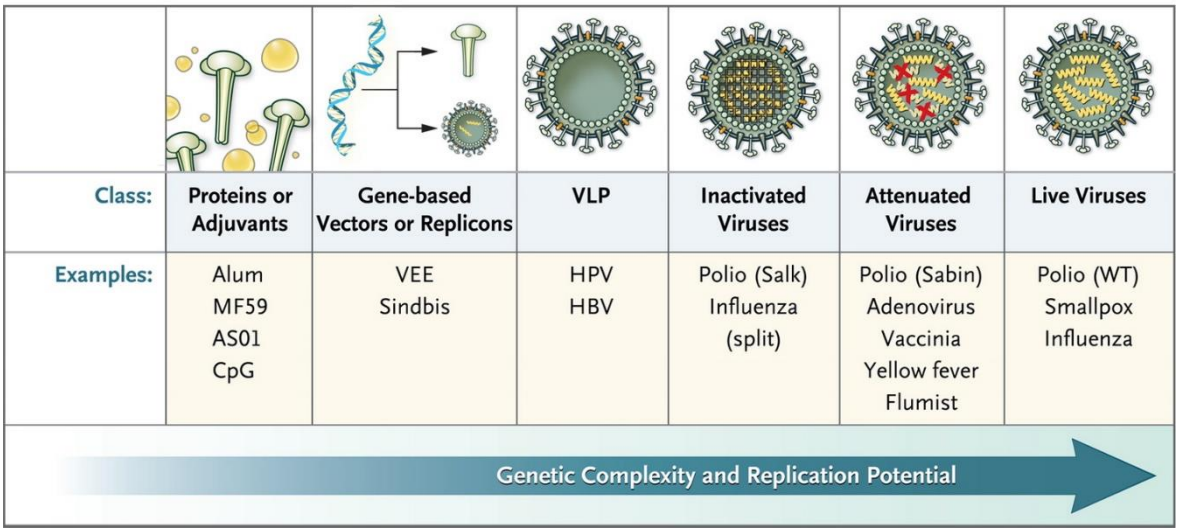


Fig. 96 Spectrum of costimulation from adjuvants to viruses (Source: *Nabel, 2013*)
Rather than generating responses through infection, immune stimulation can be achieved by increasingly complex modes of antigen presentation that range from the introduction of selected proteins, with or without adjuvants, to gene-delivered immunogens, virus-like particles (VLP), structured arrays, or attenuated viruses (HBV – hepatitis B virus, HPV – human papillomavirus, VEE – Venezuelan equine encephalitis, WT – wild type).

8.1 IMMUNIZATION

Immunization is the deliberate administration of an antigen or immunogen to induce active immunity. It is a **way of inducing specific protection against certain microbial agents, which can cause dangerous diseases.** The aim of vaccination is to induce protection sufficient to prevent clinical manifestations of natural infection.

How do vaccines mediate protection?

Vaccines protect by inducing effector mechanisms (cells or molecules) **capable of rapidly controlling replicating pathogens or inactivating their toxic components.** Vaccine-induced immune effectors are essentially **antibodies** (immunoglobulins produced by B lymphocytes) capable of binding specifically to the pathogens or their toxins. Other potential effectors are **cytotoxic CD8+ T lymphocytes** that may limit the spread of infectious agents by recognizing and killing infected cells or secreting specific antiviral cytokines. The next potential effectors are **CD4+ T-helper (Th) lymphocytes**. These Th cells may contribute to protection through cytokine production and provide support to the generation and maintenance of B and CD8+ T-cell responses (see Table 11).

Tab. 11 Effector mechanisms triggered by vaccines

(Source: *https://www.who.int/immunization/documents/Elsevier_Vaccine_immunology.pdf*)

Antibodies prevent or reduce infections by clearing extracellular pathogens through:	– Binding to the enzymatic active sites of toxins or preventing their diffusion – Neutralizing viral replication (e.g., preventing viral binding and entry into cells) – Promoting opsonization and phagocytosis of extracellular bacteria (i.e., enhancing their clearance by macrophages and neutrophils) – Activating the complement cascade
CD8+ T cells do not prevent infection but reduce, control, and clear intracellular pathogens by:	– Directly killing infected cells (release of perforin, granzyme, etc.) – Indirectly killing infected cells through antimicrobial cytokine release
CD4+ T cells do not prevent infection but participate in the reduction, control, and clearance of extracellular and intracellular pathogens by their homing and cytokine production. Their main subsets include:	– Th1 effector cells producing interferon IFN-γ, TNF-α/TNF-β, IL-2, and mainly involved in protection against intracellular pathogens (viruses, *Mycobacterium tuberculosis*) – Th2 effector cells producing IL-4, IL-5, IL-13, and responding to extracellular pathogens (bacteria and helminths) – Th9 effector cells producing IL-9 and also responding to extracellular pathogens – Th17 effector cells producing IL-17, IL-22, and IL-26 and contributing to mucosal defence (*Streptococcus pneumoniae, Bordetella pertussis, M. tuberculosis*) – Follicular T-helper (Tfh) cells producing mainly interleukin (IL)-21 and providing B-cell help

The induction of antigen-specific B- and T-cell responses requires their activation in the draining lymph nodes by specific antigen-presenting cells (APCs), essentially dendritic cells (DCs) that must be recruited into the reaction. Immature DCs patrol throughout the body. When exposed to pathogens in the tissues or at the site of injection, they undergo maturation, modulate specific surface receptors, and migrate toward secondary lymph nodes (for example, the axillary and inguinal area following deltoid and quadriceps injection, respectively), where the induction of T- and B-cell responses occurs (Figure 97).

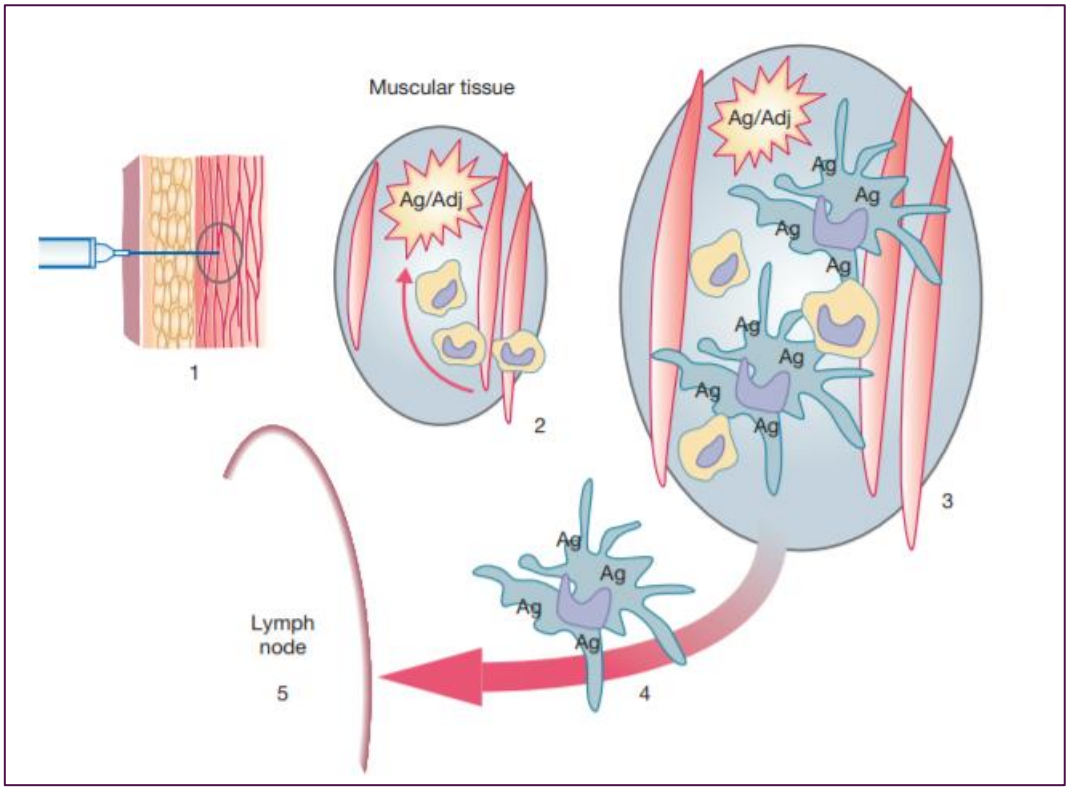


Fig. 97 Initiation of a vaccine response (Source: World Health Organization, Vaccine Immunology, *https://www.who.int/immunization/documents/Elsevier_Vaccine_immunology.pdf*)

Following injection (1), the antigens in vaccine attract dendritic cells, monocytes, and neutrophils (2). "Danger signals" activate monocytes and dendritic cells (3); the activation changes their surface receptors and induces their migration along lymphatic vessels (4), to the draining lymph nodes (5) where the activation of T and B lymphocytes will take place.

The aim of vaccination is to induce **protection sufficient to prevent clinical manifestations of natural infection.** Live or nonlive antigenic substance such as protein or polysaccharide, induces an immune response. The protection can happen in two ways passively or actively. Both passive and active immunity can be either natural or artificial (Figure 98).

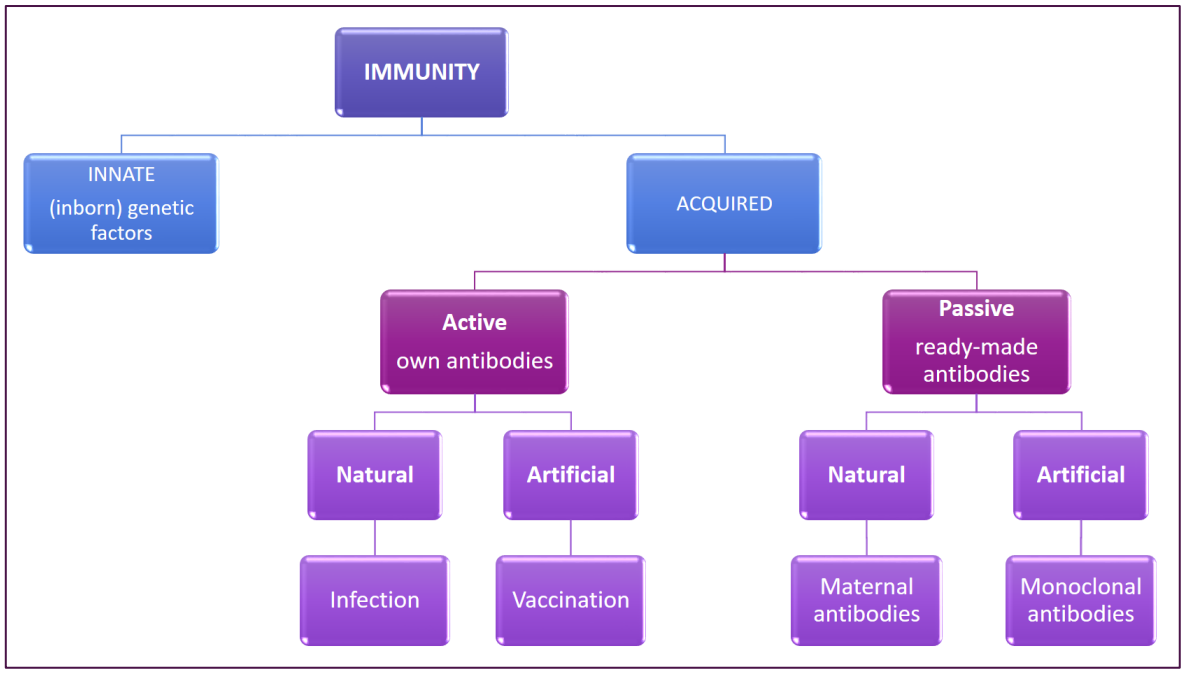


Fig. 98 Types of immunity and mechanisms of induction of acquired immunity
(Source: *authors*)

8.2 PASSIVE IMMUNITY

8.2.1 Advantages and disadvantages of passive immunity

The immunity, in which a person receives antibodies or lymphocytes that have been produced by another individual's immune system, is known as passive immunity. **Passive immunity** (Figure 99) **is induced by the transfer of antibodies produced by humans (homologous antibodies) or animals (heterologous antibodies).** It does not require exposure to an infectious agent or its antigen. The immune system of the individual is not actively involved but rather passive. The immunity is conferred only by readymade antibodies. Antibodies are not produced, but directly transferred. Memory cells are not formed. Passive immunization only protects the recipient from infectious diseases because the preformed antibodies are naturally degraded.

Advantages: artificial passive immunity is effective as a post-exposure remedy, characteristic is the **immediate onset of protection without lag period**. Passive immunity is useful in cases of immuno-compromised, immuno-deficient or severe combined immunodeficiency.

Disadvantages: only transient protection, the absence of a secondary response, the risk of blood-borne diseases or allergic reactions. A condition called serum sickness can result from exposure to antisera. Protection rendered is less effective and may not be complete. Frequent re-administration is needed for renewed protection. Subsequent doses can be less effective due to immune elimination.

8.2.2 Ways to gain passive immunity

Passive immunity is when a person is given antibodies produced by someone else. This could happen naturally or artificially. This form of immunity is short-lived as no memory cells are produced.

- **Naturally** when a **mother passes her own antibodies to her baby either through her placenta or her breast milk.** A fetus receives **IgG isotype antibodies from the mother across the placenta.** These transplacental maternal antibodies break down gradually and around the 2nd to 3rd month are already at the level of about 50% of the value compared to the period after birth. **Through breast milk, the baby receives IgA** isotype antibodies.
- **Artificially** when **a person is given an injection of preformed antibodies** if it is suspected that he has been exposed to a disease such as tetanus, diphtheria, rabies etc. The goal of artificial passive immunization is prophylaxis or treatment. In cases of prophylaxis, preparations are administered before infection begins or in the initial stages of the incubation period (in events of accidental exposure to certain pathogens such as hepatitis B). In cases of treatment, preparations are administered for protection of immuno-compromised or immuno-deficient individuals, in persons with severe combined immunodeficiency, who are unable to make the appropriate antibody response, as well as in the treatment of serious infectious diseases and certain autoimmune diseases.

Homologous antibodies obtained from healthy blood donors or **heterologous antibodies obtained after immunization of animals** (e.g. horses) are used. **Currently, the use of homologous sera that contain antibodies obtained from humans is preferred**. They are prepared from the blood of healthy donors who have high titres of antibodies to particular microbes. Antibodies given to immune-deficient patients are usually IgG-derived from pooled normal plasma or purified blood products of immune people. Heterologous sera are used against some diseases Antibodies are performed in animals the most common being that of the horses. However, the danger of immune complex formation and conditions like serum sickness with repeated administration must be checked for.

Preformed antibodies are administered through intravenous or intramuscular routes. Preformed antibodies have to be given on a continuous basis, since they are continuously catabolized and only effective for a short period.

8.3 ACTIVE IMMUNITY

8.3.1 Advantages and disadvantages of active immunity

Active immunity (Figure 99) is the immunity conferred by recovery from an infectious disease. Depending on the response by the person's own immune system, active immunity **may be acquired naturally from infection to the body or can be induced artificially by immunization, also known as vaccination.** The individual's own immune system is stimulated to produce antibodies and lymphocytes. It requires exposure to a pathogen or to an antigen of the

pathogen. The immune system of the individual is stimulated to produce antibodies and memory cells against an infectious agent and thus is actively involved in this process. The response involves both humoral and cell-mediated immunity. There are involved B cells (plasma cells and memory B cells), T cells (cytotoxic, helper, suppressor and memory T cells), antigen-presenting cells (dendritic cells, macrophages, B cells). Active immunity results in the formation of protective antibodies and long-lasting memory cells. The first exposure to an antigen leads to a primary response and in the case of subsequent exposure to the same pathogen later in the future, the secondary response is much faster and stronger.

Advantages: the protection is long-lived since it leads to the formation of long-lasting memory cells. Subsequent doses with antigens cause booster effects. It is very effective for prophylaxis of infectious contagious diseases. The advantage of vaccination is to ensure that a large enough number of antibodies and lymphocytes capable of reacting against a specific pathogen or toxin are available still before exposure to this pathogen occurs.

Disadvantages: The protective response takes time to establish ranging from a few days to weeks, which makes it inefficient as a post-exposure remedy. Active immunization can be not suitable for the protection of immuno-compromised or immuno-deficient individuals.

8.3.2 Ways to gain active immunity

Active immunity can be acquired naturally or artificially.

- ✓ **Naturally – from an infection of the body.** Antibodies and memory cells are produced in response to exposure to pathogenic microorganisms and infection. For example, a person who recovers from rubella is immune to further rubella infection, because the rubella virus stimulates the immune system to produce protective antibodies that specifically recognize and neutralize the pathogen the next time it is encountered.
- ✓ **Artificially – by immunization, also known vaccination**, via an injection of weakened (attenuated) or dead antigens. In this case, an immune response is activated resulting in the production of antibodies and memory cells. For immunization, vaccines may be inactivated i.e. bacterial toxins, killed microbes, parts of microbes, or viable but weakened microbes. A person vaccinated against an infectious agent who encounters the pathogen will show the same rapid, memory-based secondary response as someone who has had the disease.

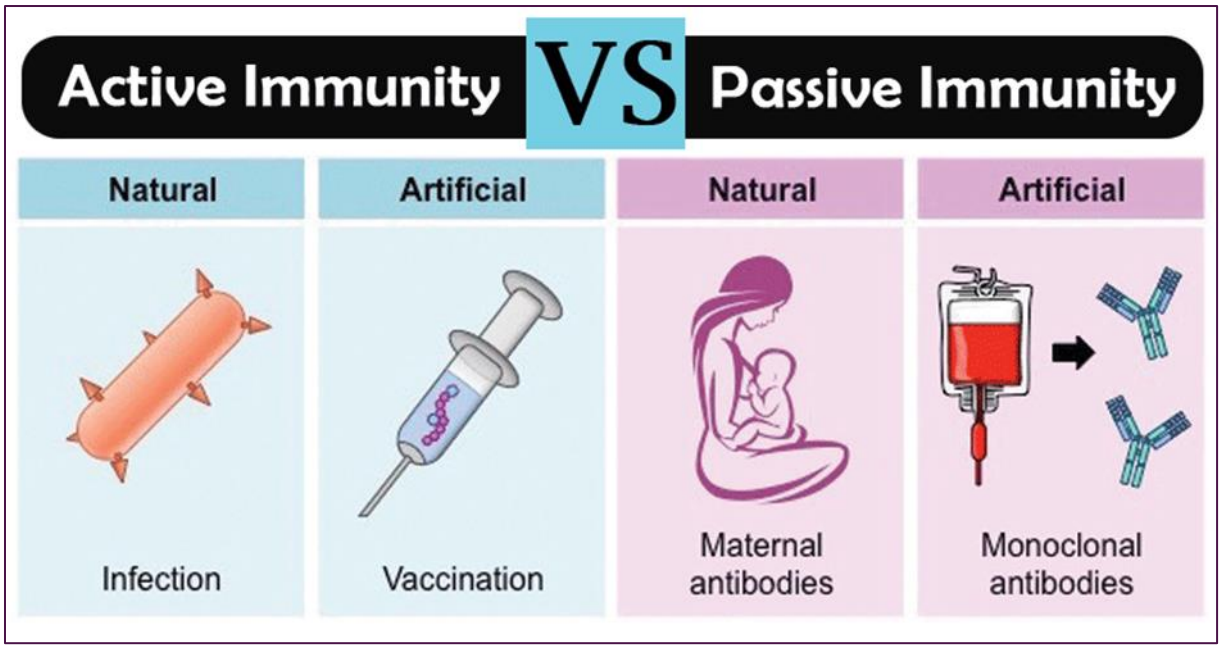


Fig. 99 Ways to gain active and passive immunity (Source: *https://microbenotes.com/differences-between-active-immunity-and-passive-immunity/*)

8.4 VACCINE TYPES

Vaccine trains the body's immune system so that it can fight disease it has not come into contact with before. Vaccines are designed to prevent disease, rather than treat a disease.

Primary vaccination – is the administration of the vaccine (one or several primary doses of the vaccine) so that a long-lasting specific immunity develops.

Booster vaccination – revaccination means the administration of a single booster dose of a vaccine of a similar composition to that used in the primary vaccination course. The booster vaccination may be beneficial to maintain longer-term protection levels and decelerate the decline of herd immunity in the population.

Most countries in the developed countries recommend the same kinds of vaccines for babies, children and adults. However, vaccination schedules are not exact and can be different in individual countries. Differences may be in: the type of vaccines, the ages at which vaccines and boosters are recommended, the number of vaccine doses for each vaccine, the types of vaccines recommended for the whole population and for special groups (such as immuno-compromised persons).

Vaccine is used for active immunization, which means the administration of live attenuated or killed infectious microorganisms, their surface proteins, or their inactivated toxins into the body. The vaccine is able to elicit an active immune response in the vaccinated individual, which thus becomes resistant to the infection. There are several different types of vaccines. Each type is designed to teach the immune system how to fight off certain kinds of microorganisms and the serious diseases they cause. Scientists around the world together focus their efforts on developing vaccines to prevent Covid-19. Some vaccines utilise similar technologies to existing

vaccines in use, but others involve newer approaches (genetic code). Newer vaccines such as mRNA vaccines and viral vector vaccines differ from traditional vaccines in the way they activate the immune system.[2] Most traditional vaccines use the administration of the antigen directly into the body.

Live attenuated vaccines

Live attenuated vaccines contain a living virus that has been weakened so that it does not cause serious disease in persons with a healthy immune system. Live attenuated vaccines can be prepared in several different ways, e.g. by the methods that involve passing the virus through a series of cell cultures or animal embryos (chick embryo). With each passage, the viruses become better at replicating in new cells but lose their ability to replicate in humans. The attenuated viruses will be less able to live in human cells and they can be used in a vaccine. Attenuated viruses in a vaccine are not able to replicate enough to cause illness in vaccinated persons (like they can naturally) but will stimulate immune responses that can protect against infection in the future.

Live attenuated vaccines are: measles, mumps, rubella (MMR combined) vaccine, rotavirus vaccine, oral polio vaccine, influenza vaccine (nasal spray), varicella (chickenpox) vaccine, zoster (shingles) vaccine, yellow fever vaccine, adenovirus oral vaccine, BCG vaccine against tuberculosis, oral typhoid vaccine.

Live attenuated vaccines have **advantages and disadvantages**. These vaccines stimulate immune responses that are similar to the natural infection that they help prevent, they create a strong and long-lasting immune response. They often require only a single immunization, often without repeated booster doses. Live attenuated vaccines are relatively easy to prepare for certain viruses but difficult for bacteria, which have thousands of genes. Therefore there are only a few live attenuated bacterial vaccines (BCG vaccine and oral typhoid vaccine). Live attenuated vaccines cannot be administered to individuals with weakened immunity (such as people with cancer, HIV, immuno-suppressed people). Live attenuated viruses may revert to a virulent form and can cause disease in these people. Attenuated vaccines also may be associated with complications similar to those seen in natural diseases. These vaccines need to be kept cool, it can be difficult to use these vaccines in countries with limited access to refrigerators.

Inactivated (killed) vaccines

Inactivated vaccines contain killed or altered bacteria or viruses so that they cannot replicate. A common method is the inactivation of the pathogen by heat or by chemical treatment. Inactivated vaccines do not contain any live bacteria or viruses. They cannot revert to a more virulent form, thus they cannot cause disease against which they protect, even in individuals with weakened immunity. Inactivated vaccines provide a shorter length of protection than live

[2] Nucleic acid and viral vectored vaccines explained. Available from: https://vk.ovg.ox.ac.uk/vk/covid-19-vaccines

attenuated vaccines. Several doses over time (booster doses) are required to create long-lasting immunity.

Examples of whole killed vaccines: hepatitis A vaccine, influenza (injectable) vaccine, polio (injectable) vaccine, rabies vaccine, Japanese encephalitis vaccine.

Whole-cell (whole-virion) inactivated vaccines are composed of killed whole pathogen (bacteria or virus). In the case of bacteria, vaccines are called whole-cell vaccines (e.g. whole-cell pertussis vaccine). In the case of viruses, vaccines are called whole-virion vaccines (e.g. influenza vaccine, Salk injectable polio vaccine, hepatitis A vaccine, rabies vaccine).

Subunit, polysaccharide, conjugate and recombinant vaccines

Subunit, polysaccharide, conjugate and recombinant vaccines contain specific pieces of the microorganisms (e.g. proteins, sugars, capsid). These vaccines are used to protect against diseases caused by Haemophilus influenzae type b (Hib), hepatitis B, human papillomavirus (HPV), as well as against whooping cough (acellular vaccine), pneumococcal, meningococcal disease, shingles.

Subunit vaccines do not contain whole bacteria or virus but contain only one or more specific antigens from the surface of the pathogens. A specific protein from the pathogen is isolated and presented separately as an antigen. Because these vaccines use only specific pieces of the pathogen, they induce a strong immune response that is targeted to key parts of the pathogen. Specific molecules are highly purified and can avoid some of the risks associated with live attenuated or whole killed vaccines. They can be used by everyone, including people with weakened immunity. They require repeated doses initially and subsequent booster doses to get sufficient protection. Adjuvants are often added to subunit vaccines. Adjuvants help to strengthen and lengthen the immune response to the subunit vaccine.

Polysaccharide vaccines are used against the microorganisms that have polysaccharide capsules such as *Streptococcus pneumoniae*, *Haemophilus influenzae* group b, *Neisseria meningitidis*. Pure polysaccharide vaccines are not effective in young children; their immune system is not able to recognize the polysaccharide antigens. In general, polysaccharide antigens elicit a T-independent immune response. The capsular polysaccharide is presented directly to the B cell surface and not through the antigen-presenting cells, the CD4+ T helper cells are not stimulated, resulting in a weak proliferation of B cells to produce IgM and IgG without formation of memory cells. The immune response is characterized by lack of memory and poor immunogenicity in young children below 2 years of age and certain immunocompromised individuals.

Conjugate vaccines are prepared using a combination of capsular polysaccharide and protein carriers. Normally, polysaccharides cannot be loaded onto MHC of antigen-presenting cells because MHC can only bind peptides. If capsular polysaccharide is attached (conjugated) to diphtheria or tetanus toxoid protein, then is able to be presented on the MHC molecule and T cells can be activated. The immune system recognises these proteins very easily a helps to generate a more powerful immune response to the polysaccharide. By conjugating capsular polysaccharides to carrier proteins it is possible to induce a T-dependent immune response. T cells allow antibody class switching from IgM to IgG producing memory cells and longer response. This process leads to stronger immunogenic and protective effects in young infants. By combining the bacterial

capsular polysaccharide with protein antigen, the immune system of young children is able to respond.

Recombinant protein vaccines are biosynthetic vaccines. Yeasts or bacterial cells are used to manufacture vaccines. A small piece of DNA from the pathogenic virus or bacterium is inserted into the manufacturing yeast or bacterium. These cells then produce encoded protein, which is purified and used in the vaccine as the major substance. The immune system will recognize the expressed protein and provide protection against the target pathogen in the future. Example: hepatitis B vaccine (part of DNA from hepatitis B virus is inserted into the DNA of yeast, which then produces surface proteins of this virus, which is used in the vaccine).

Toxoid vaccines

Some bacteria release toxins when they attack the body. The immune system recognises these toxins in the same way that it recognises other antigens on the surface of the bacteria. It can trigger a strong immune response to them. Vaccines with inactivated versions of these toxins are **toxoid vaccines** (**toxoid = anatoxin**) because they look like toxins but are not poisonous. Toxoid vaccines are made from certain toxins that have been sufficiently attenuated and can induce a humoral immune response. These vaccines (for example, vaccines against infections caused by *Corynebacterium diphtheriae* and *Clostridium tetani*) can be prepared by purifying bacterial toxins and their inactivation with formaldehyde to form a toxoid. Inoculation of a **toxoid vaccine** to the body **induces the formation of anti-toxin antibodies that are capable of binding toxins and neutralizing their effects.** Toxoid vaccines need booster doses to get ongoing protection against diseases. Toxoid vaccines are used to protect against **diphtheria** and **tetanus**. Pertussis vaccine against *Bordetella pertussis* contains pertussis toxoid, together with proteins from the surface of this bacteria. Pertussis vaccine is also called an **acellular pertussis vaccine.**

Nucleic acid vaccines

Nucleic acid vaccines are the newer technology different to other vaccines. They do not supply the antigen (protein) to the body but they provide the genetic information of protein antigen to the body cells. Subsequently, the cells produce the antigen, which stimulates an immune response (Figure 100).

DNA vaccine is a type of vaccine that transfects a specific sequence of DNA onto the cells of a vaccinated person. They work by injecting a genetically engineered plasmid that contains the DNA sequence encoding the antigen from a pathogen against which an immune response is induced. DNA must be translated to mRNA within the cell nucleus before it can subsequently be translated to protein antigens which stimulate an immune response. Many DNA vaccines are in development.

RNA vaccines use messenger RNA (mRNA) in a lipid membrane, which protects mRNA when it enters the body and helps by fusing with the cell membrane. mRNA is translated inside the cell into the antigen protein. This mRNA lasts a few days only, but in that time sufficient antigen

is made to stimulate an immune response. After few days it is naturally broken down and removed by the body. Two RNA vaccines were authorized for emergency use against COVID-19 Pfizer BioNTech and Moderna.

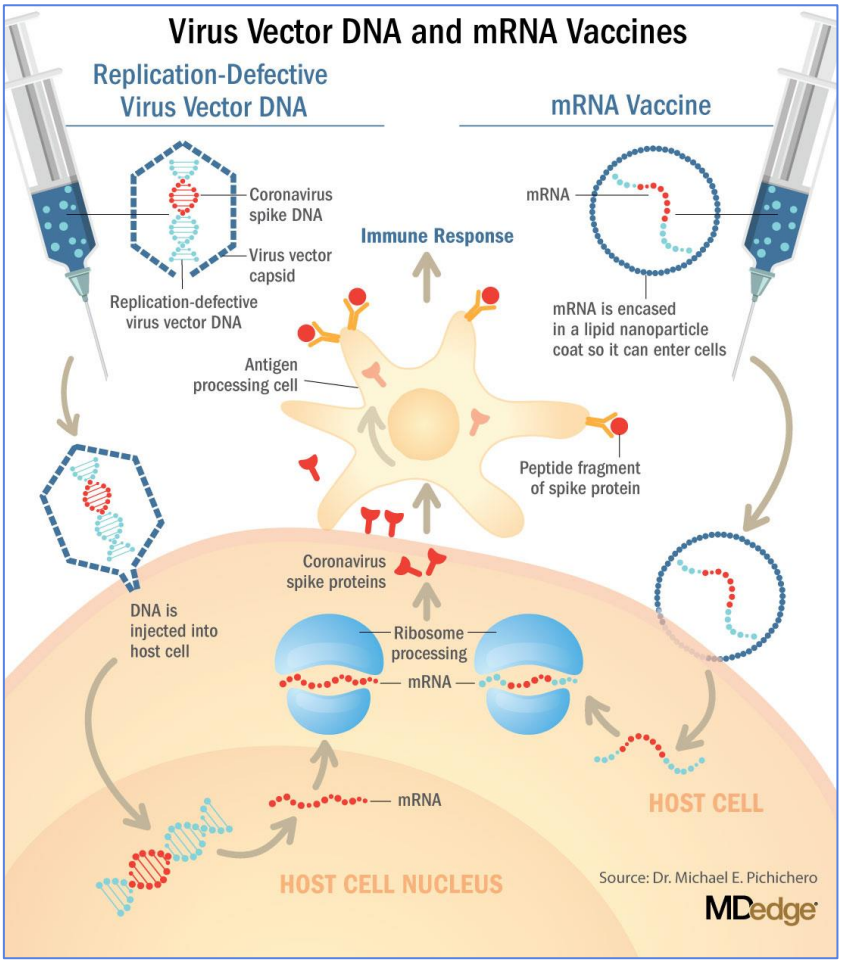


Fig. 100 Virus vector DNA versus mRNA vaccines (Source: *https://www.mdedge.com/hematology-oncology/article/233491/coronavirus-updates/understanding-messenger-rna-and-other-sars?sso=true*)

Viral vectored vaccines

Viral vectored vaccines are a new technology that uses harmless viruses (called viral vectors) to deliver the genetic code of target antigens to the cells of the body. A gene encoding a major antigen of a virulent pathogen is introduced into a harmless or attenuated virus. These viruses expressed the antigen (protein) of the pathogen in the vaccinated person, which induce the humoral and cell-mediated immune responses. Harmless or attenuated viruses are used as viral vectors.

Replicating viral vectors can provide a continuous source of vaccine antigen over a long period of time compared to non-replicating vaccines, and can produce a stronger immune response. A single dose of vaccine may be enough to give protection. Due to viral replication, there is an increased risk of mild adverse reactions. An example is vaccine to prevent Ebola called Ervebo (approved for use in 2019).

Non-replicating viral vectors are not able to make new viral particles because viral genes responsible for replication have been removed in the laboratory. These vaccines cannot cause

disease. Adverse reactions associated with viral vector replication are reduced when compared to replicating viral vectors. Vaccine antigen can be produced in only few days. Generally, the immune answer is weaker than with replicating viral vectors. Booster doses are usually required. Examples are non-replicating viral vectored vaccines to prevent Ebola (approved in July 2020), the Oxford-AstraZeneca against COVID-19 approved for emergency use in December 2020.

8.5 INDIVIDUAL AND HERD IMMUNITY

Individual protection against certain viruses or bacteria protects a particular individual (immunity developed through vaccination or previous infection).

'Herd immunity', also known as 'population immunity' or 'community immunity' is the indirect protection from an infectious disease. When a high percentage of the population is vaccinated, it is difficult for infectious diseases to spread, because there are not many people who can be infected. Herd immunity gives protection to vulnerable groups of people who cannot get vaccinated such as newborn babies, elderly people or people who are not be vaccinated due to serious health conditions like weakened immune system, dysfunctional spleen or allergic reactions to the vaccine. For example, if someone with measles is surrounded by people who are vaccinated against measles, the disease cannot easily be passed on to anyone, and it will quickly disappear again. The percentage of people who need to be immune in order to achieve herd immunity varies with each disease. For example, herd immunity against measles requires about 95% of the population to be vaccinated. The remaining 5% of non-vaccinated people will be protected by the fact that measles will not spread among vaccinated people. For polio, the threshold is about 80%.[3] Herd immunity with safe and effective vaccines makes diseases rarer and saves lives.

Herd immunity does not protect against all vaccine-preventable diseases. The best example of this is tetanus, which is caught from bacteria in the environment, not from other people who have the disease. No matter how many people around you are vaccinated against tetanus, it will not protect you from tetanus.

The basic principles of proper vaccination must be followed during vaccination. These include following the manufacturer's instructions, following absolute and relative contraindications, following the correct vaccination technique, and taking an individual approach to the individual being vaccinated.

Contraindications to vaccination are situations where the vaccine should not be given under any circumstances, as there is an increased risk of a serious adverse reaction to the vaccine (severe allergic reaction after a previous dose or allergy to vaccine components, severe acute illness, specific contraindications for each vaccine).

History and physical examination data should be considered in **the individual approach to the vaccinated individual**. In particular, these are serious diseases (autoimmune diseases, immunodeficiencies, hemoblastosis and other malignancies), reactions after previous vaccinations,

[3] https://www.who.int/news-room/q-a-detail/herd-immunity-lockdowns-and-covid-19 cit.27.02.2021

use of immunosuppressive drugs, allergies to vaccine components, ongoing acute illness, pregnancy. It is necessary to teach about possible reactions and their treatment.

Questions for self-assessment

1. What are antigens? What are antibodies?
2. Discuss the advantages and disadvantages of immunization.
3. Compare passive natural immunity and passive artificial immunity.
4. Compare active natural and active artificial immunity.
5. Compare active and passive immunity.
6. Why does breastfeeding may confer passive immunity to a child?

REFERENCES

Abbas AK, Lichtman AH, Pillai S (2007). *Cellular and Molecular Immunology*. 6th Saunders/Elsevier, Philadelphia. pp. 329-347.

Actor JK. Immune Hypersensitivities. In: Introductory Immunology 2nd edition, 2019; 103-110.

Ahmad H, Chapnick EK. Conjugated Polysaccharide Vaccines. Infectious Disease Clinics of North America 1999; 13 (1): 113–133.

Alhabbab RY. Treponema pallidum Hemagglutination (TPHA) Test. In: Basic Serological Testing 2018; 35-39. ISBN: 978-3-319-77693-4.

Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet 2000; 356(9235):1099-104.

Aryal S. Rocket Immunoelectrophoresis- Objectives, Principle, Procedure, Results, Applications, Advantages and Limitations. 2018. [online]. [cit. 03.12.2021]. Available on: https://microbenotes.com/rocket-immunoelectrophoresis/

Bain JM, Gow NAR, Erwig LP. Novel insights into host-fungal pathogen interactions derived from live-cell imaging. Seminars in Immunopathology 2014; 37(2).

Barrett KE, Barman SM, Boitano S, Brooks HL. Ganong's Review of Medical Physiology. 24th ed. New York: The McGraw Hill Companies; 2012. ISBN 978-0071780032.

Bartůňková J, Paulik M a kol. Vyšetřovací metody v imunologii. Praha: Grada publishing 2011; s. 164. ISBN 978-80-247-3533-7.

Becheva ZR, Gabrovska KI, Godjevargova TI. Comparison between direct and indirect immunofluorescence method for determination of somatic cell count. Chemical Papers 2018; 72: 1861-1867.

Boulet S, Ndongala ML, Peretz Y, Boisvert MP, Boulassel MR, Tremblay C, Routy JP, Sekaly RP, N Bernard NF. A dual color ELISPOT method for the simultaneous detection of IL-2 and IFN-gamma HIV-specific immune responses. J Immunol Methods 2007; 30: 320 (1-2): 18-29.

Boyce JA. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. Immunol Rev 2007; 217:168.

Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macroghages: key players in the innate defence of the airways. Thorax 2015; 70: 1189-96.

Castejon MJ, Yamashiro R, Oliveira CAF, Veras MASM. Performance validation of western blot for anti-HIV antibody detection in blood samples collected on filter paper (DBS). J Bras Patol Med Lab 2017; 53 (1). [online]. [cit. 19.11.2021]. Available on: https://www.scielo.br/j/jbpml/a/LmqFQqSms3CXGkWPbBGCBwD/?lang=en

Costabile M. Measuring the 50% Haemolytic Complement (CH50) Activity of Serum. J Vis Exp 2010; 37: 1923.

Čižnár P. Primárne poruchy imunitného systému – ich diagnostika a liečba. Via pract 2006; 3 (3): 120-4.

Dai X, Xiong Y, Li N, Jian C. Vaccine Types. IntechOpen 2019, [online]. [cit. 19.11.2021]. Available on: https://www.researchgate.net/publication/331726890_Vaccine_Types

Di Cataldo S, Tonti S, Bottino A, Ficarra E. ANAlyte: A modular image analysis tool for ANA testing with indirect immunofluorescence. Elsevier: Computer methods and programs in biomedicine 2016; 86-99.

D-tek We Apply Science. 2011; 11: 1-32.

Elektronická verzia prednášok z imunológie pre Jesseniovu lekársku fakultu v Martine Univerzity Komenského v Bratislave 2021, [online]. 2021. Dostupné na internete: www.jfmed.uniba.sk

Elektronická verzia praktických cvičení z imunológie pre Jesseniovu lekársku fakultu v Martine Univerzity Komenského v Bratislave 2021, [online]. 2021. Dostupné na internete: www.jfmed.uniba.sk

Elektronická verzia video – mikrobiológia praktické cvičenia pre Jesseniovu lekársku fakultu v Martine Univerzity Komenského v Bratislave 2021, [online]. 2021. Dostupné na internete: www.jfmed.uniba.sk

Fathman CG, Soares L, Chan SM, Utz PJ. An array of possibilities for the study of autoimunity. Nature 2005; 435: 605-611.

Folds JD, Schmitz JL. Clinical and laboratory assessment of immunity. J Allergy Clin Immunol 2003;111:S702-11.

Goding JW. Immunofluorescence. In: Monoclonal antibodies 3rd ed. Academic Press 1996; 352-399. ISBN 9780122870231.

Gupta-Wright A, Tembo D, Jambo KC, Chimbayo E, Mvaya L, Caldwell S, Russell DG, Mwandumba HC. Functional Analysis of Phagocyte Activity in Whole Blood from HIV/Tuberculosis-Infected Individuals Using a Novel Flow Cytometry-Based Assay. Front Immunol 2017; 8: 1222.

Gurish M, Castells MC. Mast cell derived mediators. Wolters Kluwer 2016, [online]. 2021. [cit. 16.04.2021]. Dostupné na internete: http://www.uptodate.com/contents/mast-cell-derived-mediators

Horváthová M. Celulárna imunológia. Laboratórne metódy. Bratislava: SZU 2015, [online]. 2021. [cit. 14.2.2021]. Dostupné na internete: http://www.szu.sk/userfiles/file/Katedry/kat_149/BUNKOV%C3%81%20IMUNOL%C3%93GIA%20-%20laborat%C3%B3rne%20met%C3%B3dy.pdf

Hořejší V, Bartuňková J, Brdička T, Špíšek R. Základy imunologie (5. vyd.). Praha: Triton 2013; s. 330. ISBN 978-80-7387-713-2.

Janeway CA, Travers P, Walport M, Shlomchik MJ. Immunobiology the immune system in health and disease 6th ed. New York: Garland Science Taylor and Francis Group 2005; pp. 823. . ISBN 0-8153-4101-6.

Jasmer RM, Nahid P, Hopewell PC. Latent Tuberculosis Infection. N Engl J Med 2002; 347 (23): 1860-1866.

Jeseňák M, Rennerová Z, Bánovčin P a kol. Recidivujúce infekcie dýchacích ciest a imunomodulácia u detí. Praha: Mladá fronta 2012; s. 631. ISBN 978-80-204-2618-5.

Jin J, Reese V, Coler R, Carter D, Rolandi M. Chitin Microneedles for an Easy-to-Use Tuberculosis Skin Test. Advanced healthcare material. Wiley Online Library 2013; 3 (3): 349-353.

Joshi S, Yu D. Immunofluorescence. Basic Science Methods for Clinical Researchers. 2017; 135-150.

Kadri K. Polymerase Chain Reaction (PCR): Principle and Applications, Synthetic Biology - New Interdisciplinary Science, Nagpal ML, Boldura OM, Baltă C, Enany S, IntechOpen 2019 [online]. 2021. [cit. 2021.09.16]. Available from: https://www.intechopen.com/books/synthetic-biology-new-interdisciplinary-science/polymerase-chain-reaction-pcr-principle-and-applications

Kanagarajan S, Tolf C, Lundgren A, Waldenstrom J, Brodelius PE. Transient Expression of Hemagglutinin Antigen from Low Pathogenic Avian Influenza A (H7N7) in *Nicotiana benthamiana*. PLoS ONE 2012; 7 (3): e33010, 1-10.

Karki G. Widal test: Introduction, Principle, Procedure, Result interpretation, Applications and limitations. 2018. [online]. [cit. 02.12.2021]. Available on: https://www.onlinebiologynotes.com/widal-test-introduction-principle-procedure-result-interpretation-applications-and-limitations/

Khan S. Conjugate vaccines and polysaccharide response. CMAJ 2006; 174 (7): 976-977.

Kindt TJ, Goldsby RA, Osborne BA, Kuby J (2007). Kuby Immunology. 6th W.H. Freeman, New York. pp. 168-188.

Klement C a spol. Vybraná terminológia I. Banská Bystrica: SZU Bratislava 2014; 220s. ISBN 978-80-89057-48-1.

Kompaníková J, Nováková E, Neuschlová M. Mikrobiológia nielen pre medikov. Žilina EDIS 2013; s. 209. ISBN 978-80-554-0827-9.

Kompaníková J, Nováková E, Neuschlová M. Mikrobiológia nielen pre medikov - 2. upravené a doplnené vydanie. Multimediálna podpora výučby klinických a zdravotníckych disciplín :: Portál Jesseniovej lekárskej fakulty Univerzity Komenského 2020. [online]. [cit. 25. 11. 2021]. Dostupný z www: https://portal.jfmed.uniba.sk//clanky.php?aid=398. ISSN 1337-7396.

Krause C, Ens K, Fechner K et al., Voigt J, Fraune J, Rohwäder E, Hahn M, Danckwardt M, Feirer C, Barth E, Martinetz T, Stocker W. EUROPatterb Suite technology for computer-aided immunofluorescence microscopy in autoantibody diagnostics. Lupus 2015; 24: 516-529.

Kumar V, Abbas AK, Aster JC. Robbins Basic Pathology. 8th ed. Elsevier Saunders 2013; pp.603. ISBN 978-1-4377-1781-5.

Limaye S. Tests for cell-mediated immunity. Aust Prescr 2010; 33: 84-7.

Litzman J a kol. Základy vyšetření v klinické imunologii. Brno: Masarykova Univerzita Lékařská fakulta 2011; s. 53. ISBN 978-80-210-4227-8.

Locke BA, Dasu T, Verbsky JW. Laboratory Diagnosis of Primary Immunodeficiencies. Clinical Rev Allerg Immunol 2014; 46: 154-168.

Marshall JS, Jawdat DM. Mast cells in innate immunity. J Allergy Clin Immunol 2004; 114:21.

Mazurek GH, Jereb J, Vernon A, LoBue P, Goldberg S, Castro K. Updated Guidelines for Using Interferon Gamma Release Assays to Detect Mycobacterium tuberculosis Infection --- United States, 2010. Recommendations and Reports 2010; 59 (RR05): 1-25.

Moticka EJ. Techniques to detect and quantify host defenses. In: A historical perspective on evidence-based immunology, Elsevier 2016; pp. 353-364.

Nabel GJ, Fauci AS. Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine. Nat Med 2010; 16: 1389-91.

Nabel GJ. Designing Tomorrow's Vaccines. N Engl J Med 2013; 368: 551-60.

Nair M. Protein Conjugate Polysaccharide Vaccines: Challenges in Development and Global Implementation. Indian J Community Med 2012; 37 (2): 79-82.

Neuschlová M. Cells of the Immune System. Multimedia support in the education of clinical and health care disciplines :: Portal of Jessenius Faculty of Medicine Comenius University [online] , [cit. 25. 11. 2021]. Available from www: https://portal.jfmed.uniba.sk//articles.php?aid=395. ISSN 1337-7396.

Neuschlová M. Phagocytosis. Multimedia support in the education of clinical and health care disciplines :: Portal of Jessenius Faculty of Medicine Comenius University [online] , [cit. 25. 11. 2021]. Available from www: https://portal.jfmed.uniba.sk//articles.php?aid=403. ISSN 1337-7396.

Neuschlová M. Complement. Multimedia support in the education of clinical and health care disciplines :: Portal of Jessenius Faculty of Medicine Comenius University [online] , [cit. 25. 11. 2021]. Available from www: https://portal.jfmed.uniba.sk//articles.php?aid=408. ISSN 1337-7396.

Neuschlová M. Specific immunity Immunoglobulins. Multimedia support in the education of clinical and health care disciplines :: Portal of Jessenius Faculty of Medicine Comenius University [online] , [cit. 25. 11. 2021]. Available from www: https://portal.jfmed.uniba.sk//articles.php?aid=431. ISSN 1337-7396.

Neuschlová M, Nováková E, Kompaníková J. Imunológia – ako pracuje imunitný systém. Jesseniova lekárska fakulta v Martine Univerzity Komenského v Bratislave 2017; s. 189. ISBN 978-80-8187-031-6.

Neuschlová M, Nováková E, Kompaníková J. Návody na praktické cvičenia z imunológie. Jesseniova lekárska fakulta v Martine Univerzity Komenského v Bratislave 2016; s. 114. ISBN 978-80-8187-017-0.

Neuschlová M, Nováková E, Kompaníková J. Abeceda imunológie – terminologický slovník. Jesseniova lekárska fakulta v Martine Univerzity Komenského v Bratislave 2016; s. 60. ISBN 978-80-8187-016-3.

Neuschlová M, Nováková E, Kompaníková J, Sadloňová V. A to Z glossary of immunological terms. Comenius University in Bratislava Jessenius Faculty of Medicine in Martin 2020; pp. 80. ISBN 978-80-8187-088-0.

Nováková E, Oleár V, Klement C. Lekárska vakcinológia nielen pre medikov. PRO Banská Bystrica 2007; 141 s. ISBN 978-80-89057-18-4.

Nováková E, Sadloňová J, Kompaníková J, Neuschlová M, Sadloňová V. Microbiology – principal and interpretation of laboratory examination Part 1. Multimedia support in the education of clinical and health care disciplines :: Portal of Jessenius Faculty of Medicine Comenius University [online] , [cit. 04. 12. 2021]. Available from www: https://portal.jfmed.uniba.sk//articles.php?aid=376.

O'Connel TX, Horita TJ, Kasravi B. Understanding and Interpreting Serum Protein Electrophoresis. Am Fam Physician 2005;71(1):105-112. [online]. [cit. 28. 11. 2021]. Available on: https://www.aafp.org/afp/2005/0101/p105.html

Pai M, Zwerling A, Menzies D. Systemic Review: T-cell–based Assays for the Diagnosis of Latent Tuberculosis Infection: An Update. Ann Intern Med. 2008;149:177-184.

Rich R, Fleisher T, Shearer W, Schroeder H, Frew A, Weyand C. Clinical Immunology 5th Edition: Principles and Practice. Elsevier 2019 pp. 1392. ISBN 9780702070396.

Salazar, A. et al. Allergen-Based Diagnostic: Novel and Old Methodologies with New Approaches. 2017; 10.5772/intechopen.69276. [online]. [cit. 22. 07. 2021]. Available on: https://www.researchgate.net/publication/320265684

Sharmin S. et al. Evaluation of Dot Immunoblot for identification of antibodies to Extractable nuclear antigen. 2011. [online]. [cit. 19.11.2021]. Available on: https://www.researchgate.net/figure/Dot-Blot-strips-Strip1-negative-Strip-2-Anti-Sm-RNP-SSA-positive-Strip-3-Anti-Sm-RNP_fig1_273263071

Siegrist CA. Vaccine Immunology. pp.16-26. [online]. [cit. 09.02.2021]. Available on: https://www.who.int/immunization/documents/Elsevier_Vaccine_immunology.pdf

Stratton CW. Serum bactericidal test. Clin Microbiol Rev 1988; 1 (1): 19-26.

Suto H, Nakae S, Kakurai M, et al. Mast cell-associated TNF promotes dendritic cell migration. J Immunol 2006; 176:4102.

Šterzl I a kol. Základy imunologie pro zubní a všeobecní lékaře. Praha: Univerzita Karlova v Praze 2007; s.207. ISBN 978-80-246-0972-0.

The University of Quesland Australia. Bacground of Immunofluorescence. [online]. [cit. 07.10.2021]. Available on: https://di.uq.edu.au/community-and-alumni/sparq-ed/cell-and-molecular-biology-experiences/immunofluorescence/background-immunofluorescence

Vollmer W, Blanot D, De Pedro MA. Peptidoglycan structure and architecture. FEMS Microbiology Reviews 2008; 32 (2): 149-167.

Votava M. Lékařská mikrobiologie obecná. Brno: Neptun 2005; s. 351. ISBN 80-86850-00-5.

Young B, Lowe SJ, Alan S, Heath JW. Wheater's Functional Histology. 5th ed. Elsevier Limited 2006; pp.448. ISBN 978-0-443-06850-8.

Internet sources

Agglutination Assays. [online]. [cit. 14.01.2021]. Available on: https://opentextbc.ca/microbiologyopenstax/chapter/agglutination-assays/

Approach to the Patient With Suspected Immunodeficiency. [online]. [cit. 28.01.2021]. Available on: https://www.msdmanuals.com/professional/immunology-allergic-disorders/immunodeficiency-disorders/approach-to-the-patient-with-suspected-immunodeficiency

Bacterial serotyping guide for Salmonella. [online]. [cit. 28.11.2021]. Available on: https://www.bio-rad.com/webroot/web/pdf/fsd/literature/FSD_14-0699.pdf

Blood Specimens: Chemistry and Hematology. [online]. [cit. 03.12.2021]. Available on: https://www.labcorp.com/resource/blood-specimens-chemistry-and-hematology

Cold Agglutinins Test. University of Michigan Health. [online]. [cit. 03.12.2021]. Available on: https://www.uofmhealth.org/health-library/hw4158

Competition (Inhibition) ELISA. [online]. [cit. 15.11.2021]. Available on: https://www.antibody-creativebiolabs.com/competition-inhibition-elisa.htm

Complement Activation Pathways. [online]. [cit. 27.03.2020]. Available on: https://www.sinobiological.com/complement-activation-pathways-a-1511.html

Complement Fixation Test: Principle, Procedure, Results. [online]. [cit. 14.01.2021]. Available on: https://microbeonline.com/complement-fixation-test-principle-procedure-results/

Complement fixation test (CFT). [online]. [cit. 14.01.2021]. Available on: https://www.clinisciences.com/en/read/serological-tests-in-mycology-1190/complement-fixation-test-cft-2094.html

Complement Pathways: Types, Functions, Regulation. [online]. [cit. 28.03.2020]. Available on: https://microbeonline.com/complement-system-pathways-functions-regulation/

Complement total (ch50) blood test. [online]. [cit. 26.03.2020]. Available on: https://www.walkinlab.com/products/view/complement-total-ch50-blood-test

Coronavirus (COVID-19) data. [online]. [cit. 27.02.2021]. Available on: https://www.who.int/news-room/q-a-detail/herd-immunity-lockdowns-and-covid-19

Counter Current Immunoelectrophoresis. [online]. [cit. 05.12.2021]. Available on: https://microbenotes.com/counter-current-immunoelectrophoresis/

C-reactive protein. [online]. [cit. 09.04.2020]. Available on: http://www.ilexmedical.com/files/PDF/CRP_ARC_CHEM.pdf

C-Reactive Protein (CRP) Test- Principle, Uses, Procedure and Result Interpretation. [online]. [cit. 08.04.2020]. Available on: https://microbiologyinfo.com/c-reactive-protein-crp-test-principle-uses-procedure-and-result-interpretation/

Detecting Antigen-Antibody Complexes in vitro. [online]. [cit. 26.03.2020]. Available on: https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(OpenStax)/20%3A_Laboratory_Analysis_of_the_Immune_Response/20.2%3A_Detecting_Antigen-Antibody_Complexes_in_vitro

Detect single-cell secretion with ELISpot. [online]. [cit. 08.02.2021]. Available on: https://www.mabtech.com/sites/default/files/elispot-brochure.pdf

ELISA principles and types. [online]. [cit. 14.11.2021]. Available on: https://www.abcam.com/kits/elisa-principle#What%20is%20an%20ELISA

ELISpot Assay Principle. [online]. [cit. 08.02.2021]. Available on: https://www.rndsystems.com/products/elispot-assay-principle

ELISpot Assay Principle. [online]. [cit. 08.02.2021]. Available on: https://www.mabtech.com/knowledge-center/assay-principles/elispot-assay-principle

Guidelines for Preventing the Transmission of Mycobacterium tuberculosis in Health-Care Settings, 2005. [online]. [cit. 22.01.2021]. Available on: https://www.cdc.gov/mmwr/pdf/rr/rr5417.pdf

Herd immunity (Herd protection). [online]. [cit. 27.02.2021]. Available on: https://vk.ovg.ox.ac.uk/vk/herd-immunity

Hemagglutination Inhibition Test: Principle, Procedure, Uses. [online]. [cit. 05.12.2021]. Available on: https://microbeonline.com/hemagglutination-inhibition-test-hai-principle-procedure-result-interpretations/

How do vaccines work? [online]. [cit. 11.02.2021]. Available on: https://vk.ovg.ox.ac.uk/vk/sites/default/files/ScriptforanimationPDFforwebsite.pdf

Immune system. [online]. [cit. 10.02.2021]. Available on: http://www.ethiopianteachers.org/EATOnlineResource/Subjects/Biology/EBook/LessonFourteen.html

Immunoelectrophoresis. [online]. [cit. 30.11.2021]. Available on: https://www.edvotek.com/272

Interferon gamma release assay test (IGRA test). [online]. [cit. 27.01.2021]. Available on: https://www.health.nsw.gov.au/Infectious/tuberculosis/Pages/interferon-gamma-release-assay-test.aspx

Laboratory Procedure Manual: C-reactive protein. [online]. [cit. 09.04.2020]. Available on: https://www.cdc.gov/nchs/data/nhanes/nhanes_07_08/crp_e_met.pdf

Laboratory Tests. [online]. [cit. 14.11.2021]. Available on: https://microbenotes.com/category/laboratory-tests/

Lysozyme Detection Kit. Product information. [online]. [cit. 26.03.2020]. Available on: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/1/ly0100bul.pdf

Mantoux Test : Principle, Procedure, Interpretation and Limitations. [online]. [cit. 22.01.2021]. Available on: http://laboratorytests.org/mantoux-test/

Microneedle patch could replace standard tuberculosis skin test. [online]. [cit. 22.01.2021]. Available on: https://www.washington.edu/news/2013/08/26/microneedle-patch-could-replace-standard-tuberculosis-skin-test/

Molecular explorations through biology and medicine. [online]. [cit. 26.03.2020]. Available on: https://pdb101.rcsb.org/motm/9

Ouchterlony Double Immunodiffusion technique. [online]. [cit. 05.12.2021]. Available on: https://microbenotes.com/ouchterlony-double-immunodiffusion-technique/

PCR Setup—Six Critical Components to Consider. [online]. [cit. 28.01.2021]. Available on: https://www.thermofisher.com/sk/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-component-considerations.html#dNTPs

Phagocytosis Assays. [online]. [cit. 29.07.2020]. Available on: https://www.cellbiolabs.com/phagocytosis-assays

Phagocytosis- definition, mechanism, steps with example. [online]. [cit. 16.04.2020]. Available on: https://microbenotes.com/phagocytosis-introduction-mechanism-steps-and-example/

Polyacrylamide Gel Electrophoresis (PAGE). [online]. [cit. 05.12.2021]. Available on: https://microbenotes.com/polyacrylamide-gel-electrophoresis-page/

Precipitation test. [online]. [cit. 28.11.2021]. Available on: https://www.biosciencenotes.com/precipitation-test/

Radial Immunodiffusion- Objectives, Principle, Procedure, Results, Applications, Advantages and Limitations. [online]. [cit. 02.12.2021]. Available on: https://microbenotes.com/radial-immunodiffusion/

Rapid Typhoid Detection. [online]. [cit. 02.12.2021]. Available on:https://idlbiotech.com/products/tubex/

Sandwich ELISA with Direct Detection. [online]. [cit. 15.11.2021]. Available on: https://www.antibody-creativebiolabs.com/sandwich-elisa-with-direct-detection.htm

The Connection Between the Digestive System and Autoimmune Diseases. [online]. [cit. 12.01.2021]. Available on: https://www.sfadvancedhealth.com/blog/the-connection-between-the-digestive-system-and-autoimmune-diseases

Tuberculin skin test (TST). [online]. [cit. 22.01.2021]. Available on: https://tbskintesting.weebly.com/reading-the-test.html

Tuberculin Skin Test (Mantoux test): Principle, Procedure, Results. [online]. [cit. 22.01.2021]. Available on: https://microbeonline.com/tuberculin-skin-test-mauntoux-testprinciple-procedure-results-limitations/#google_vignette

Type IV hypersensitivity. [online]. [cit. 11.02.2021]. Available on: https://www.osmosis.org/learn/Type_IV_hypersensitivity?from=/foundational-sciences/pathology/immune-system/hypersensitivity-reactions/type-i-hypersensitivity-reactions

Typhidot Rapid IgG/IgM (Combo). [online]. [cit. 02.12.2021]. Available on: https://www.reszonics.com/wp-content/uploads/2017/04/Reszon-IFU-TYPHIDOT-Rapid-IgG_IgM-combo-2014-04.pdf

Updated Guidelines for Using Interferon Gamma Release Assays to Detect Mycobacterium tuberculosis Infection --- United States, 2010. [online]. [cit. 27.01.2021]. Available on: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5905a1.htm?s_cid=rr5905a1_e

Vaccine Types. [online]. [cit. 12.02.2021]. Available on: https://www.vaccines.gov/basics/types

Western blot. [online]. [cit. 19.11.2021]. Available on: https://www.nature.com/scitable/definition/western-blot-288/

Western blotting. [online]. [cit. 19.11.2021]. Available on: https://www.biolegend.com/fr-ch/western-blot

What is a vaccine, and how do vaccines work? [online]. [cit. 11.02.2021]. Available on: https://vk.ovg.ox.ac.uk/vk/how-do-vaccines-work

What is an ELISA? [online]. [cit. 14.11.2021]. Available on: https://www.rndsystems.com/resources/what-is-an-elisa-and-elisa-types

WHO Global Foodborne Infections Network. Laboratory Protocol: "Serotyping of Shigella spp." [online]. [cit. 28.11.2021]. Available on: https://antimicrobialresistance.dk/CustomerData/Files/Folders/6-pdf-protocols/58_23-10-gfn-shigellaserotypification-final-29-06-10.pdf

19 Differences between Active Immunity and Passive Immunity. [online]. [cit. 09.02.2021]. Available on: https://microbenotes.com/differences-between-active-immunity-and-passive-immunity/

Notices

Knowledge and best practice in this field are constantly changing. As new research and experience broaden an understanding, changes in research methods, professional practices, or medical treatment may become necessary.

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