Evaluation of the immune system
(Methods for evaluation of the immune system)

Department of Immunology
Faculty of Medicine
University of Belgrade

METHODS FOR EVALUATION OF THE IMMUNE SYSTEM

Evaluation of patient's immune system

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The immune system has a crucial role in host protection against various infections, but dysfunctions of the immune system may lead to pathological processes resulting in tissue injury and disease. Therefore, the equality sign is commonly placed between health and functional immune response. Patients frequently complain that their immune system is weakened causing them health problems (ranging from increased number of infections to malaise) and they often ask doctors to perform tests to "assess their immune status". **To date, there is no well-defined set of tests that could be performed in primary care institutions to meet this kind of patient requests.** Nevertheless, in these cases it is justified to use approach established for evaluation of patients with primary immune deficiencies. In this approach, it is necessary **to take anamnestic data from the patient, do a detailed physical examination as well as to perform various paraclinical tests.**

Anamnesis (medical history)

Anamnesis or "medical history" is a structured assessment conducted to create a comprehensive picture of a patient's health and health problems. While taking anamnesis, special attention has to be taken **for the type of infectious agent causing the disease and a number and/or duration of infections.** For example, the unusual sites of infections or uncommon infectious agents (so-called **oportunistic**) have to be carefully examined. Moreover, attention has to be taken when infections are prolonged and/or recurrent (i.e. repeated). In addition, antibiotic resistance has to be evaluated in the case of recurrent infections caused by the same microbe. It is also important to search for epidemiological characteristics of infection and define procedures that would prevent further disease transmission. Useful information can be obtained from applied therapy protocols, such as infections which require long-lasting parenteral therapy or administration of high drug doses. Furthermore, some chronic diseases, like diabetes mellitus, could be accompanied with increased susceptibility to infections. Finally, it is necessary to determine **presence of primary immunodeficiency or associated diseases in family history.**

Clinical examination

At clinical examination of newborns and infants, failure to thrive may reveal presence of inherited factors associated with defective immune response. However, this is uncommon, because primary immunodeficiencies are rare diseases. **Nutritional status** is important because **protein-calorie malnutrition** (or **protein-energy malnutrition**) may lead to qualitative and quantitative changes in the immune response and consequently immunodeficiency. On the other

hand, **obesity** may also be accompanied with higher susceptibility to infections. The **examination of skin and mucosa** may reveal signs of infection, hygiene habits and visualize the scars indicating **patient lifestyle** which could be relevant for the exposure to infectious agents and infections (e.g. overexposure to sunlight is frequently accompanied with reactivation of viral infections). Patient **psychological status** should not be neglected because depression, neurosis and exposure to psychological stress may change the ways in which immune system reacts to pathogens.

Other examination methods

Other examination methods are sometimes called **paraclinical** and they encompass **several imaging techniques for visualization of deep tissues and organs**, such as ultrasound, X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and various laboratory testing. For example, the imaging methods can detect **aplasia of thymus and tonsils,** bronchiectasis, infections of body cavities (e.g. sinus infections) and organs which may be associated with immune system defects. Likewise, the use of the imaging techniques made possible detection of morphological and functional defects accompanied with frequent infections, such as vesicoureteral reflux or sphincter dysfunction.

Laboratory testing include the assessment of the peripheral blood cells, humoral factors including complement system, evaluation of the humoral and T cell-mediated immune response as well as skin and provocative tests for allergies and other types of hypersensitivity diseases. Initially, it is necessary to determine complete blood count (CBC) and **white blood cell (WBC) differential** (relative percentage of each type of white blood cells). The blood smear may reveal presence of bands (i.e. immature neutrophils) as a consequence of response to an infection (so-called **neutrophil left shift**). Probably, the most important part of laboratory diagnosis is the determination of the number and phenotypic characteristics of different populations of immune cells (so-called **immunophenotyping**). The main lymphocyte populations and their subpopulations (such as $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, regulatory T cells, or Treg cells, etc.), or minor populations of lymphocytes with limited diversity (e.g. NKT cells and γδ T cells) can all be determined using antibodies labeled with fluorescent dyes by **flow cytometry**. In addition, **activation status** of those lymphocytes and other immune cells may be monitored through expression of activation markers (CD69, CD25, CD23, etc.) by flow cytometry. Finally, flow cytometry is also used for **MHC tetramer staining,** in which a **number of antigen-specific T cells** can be determined based on recognition of specific peptide in binding cleft of the multiple bound MHC molecules that are labeled).

Cell counting methods

Cell counting can be performed using **hemocytometer** (cells are manually counted under a microscope in specially designed chambers) or **automated counters** (hematology analyzers) that can distinguish each leukocyte population by laser light scatter. Automated scattered light also provides information about cell structure (granularity) and size. Sometimes, white blood cells differential can be easily determined by staining a blood smear. However, none of these methods can differentiate between various lymphocyte subpopulations, since they all share similar morphological characteristics. To this end, another approach should be used, based on the expression of specific antigens (surface markers) on various subsets of lymphocytes. Some commonly used markers for lymphocyte immunophenotyping are:

- for T cells: CD3, CD4, CD8, etc.
- for B cells: membrane immunoglobulins, CD19, CD20, etc.
- for NK cells: CD16, CD56, etc.

Monoclonal antibodies labeled with fluorescent dyes, which are specific for those cell markers are commonly used to identify various lymphocyte subpopulations using **flow cytometry** (For more detailed description of flow cytometry, please refer to section “Immunofluorescence” of the text “Antigen-antibody reactions with labeled reagents” from the previous week).

Separation of cell populations

Sometimes it is necessary to separate different cell populations according to their properties (so-called **cell sorting**). These properties can be various, but most commonly include size, shape (morphology), and surface protein expression. Cell sorting is especially important if the functional properties of cell populations are assessed. Currently there are several methods for cell sorting. Some are basic and do not require special equipment whereas others rely on sophisticated electronic appliances (see below).

Separation of peripheral blood mononuclear cells by isopycnic centrifugation

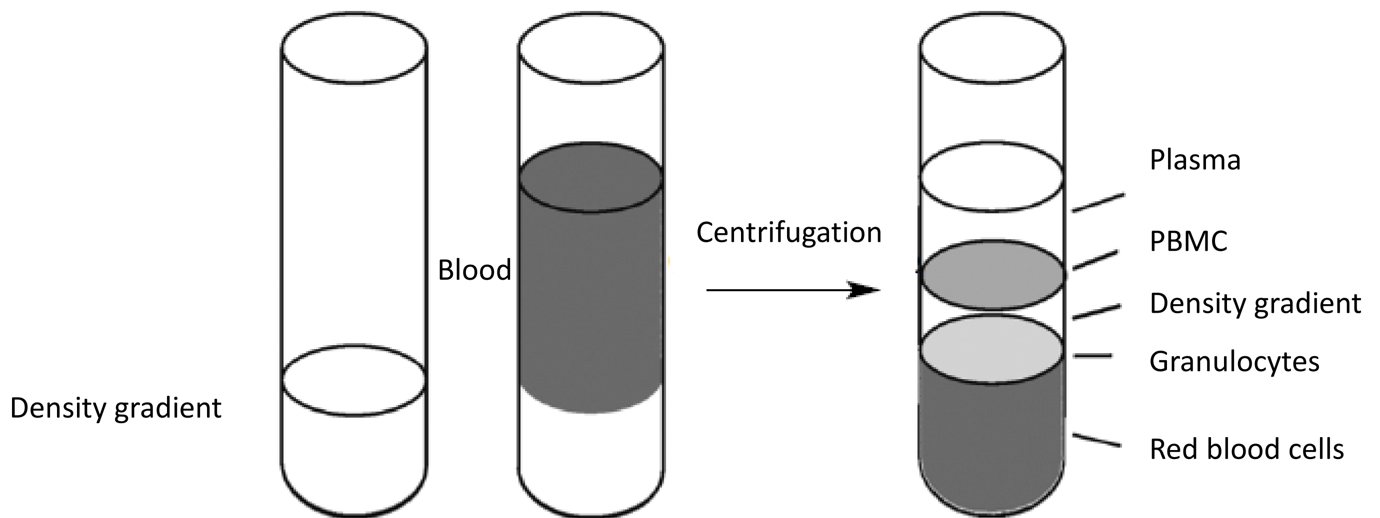
The **peripheral blood mononuclear cells (PBMC)** encompass cells with unsegmented nuclei (monocytes, T cells, B cells and NK cells) which have important role both in innate (monocytes and NK cells) and adaptive immunity (T and B cells). The separation of PBMC represents an important step in many diagnostic and experimental procedures. The PBMCs are separated from the whole blood using **isopycnic centrifugation** (the term isopycnic refers to a separation of cells based on their density). For this purpose, a **density gradient media** is used (density of media corresponds to density of PBMCs). During centrifugation, red blood cells and multi-lobed cells (e.g. neutrophils) will be pulled down through the density gradient while PMBC will float above the gradient media from where they can be easily collected and used for further analyses (Figure 1).

Fluorescent-activated and magnetic-activated cell sorting

As mentioned above, cell sorting is often performed using specially designed electronic devices. The most commonly used methods of cell sorting are those using antibodies labeled with fluorescent dyes and magnetic beads.

Fluorescent-Activated Cell Sorting, or **FACS**, utilizes flow cytometry and fluorescently labeled antibodies specific for various surface markers for sorting a cell population of interest (i.e. cells that express selected marker on the surface) from other cells (those that do not express the marker) in a heterogeneous mixture of cells. Similar principle is used in **Magnetic-Activated Cell Sorting** (**MACS**), but in this technique the cells are labeled with marker-specific antibodies conjugated to magnetic beads, and the separation is performed using columns in a magnetic field.

Figure 1. **Schematic representation of a density gradient separation of the peripheral blood mononuclear cells**



***In vitro* functional examination**

Innate immune system

The functional *in vitro* examination of the cells involved in the innate immunity comprises methods which assess phagocyte and NK cell functions. Phagocytes are most commonly tested for chemotaxis and phagocytosis (All phases of phagocytosis, from adherence to production of microbicidal molecules, may be tested). **Chemotaxis** is analyzed using special chamber with certain chemoattractant which initiates migration of the cells through the gel or filter. **Adherence** test is performed by adding phagocytes to a glass slide and by counting cells that adhere to it. **Ingestion** can be detected by microscopy or flow cytometry (e.g. by counting the phagocytes that ingested the fluorescent particle). **Intracellular killing** can be evaluated by tests in which phagocytes kill bacteria in the cell culture. Alternatively, respiratory burst test is often used to indirectly evaluate this phagocyte function, wherein generation of **reactive oxygen species and nitric oxide** is measured by colorimetry, chemiluminescence and flow cytometry.

In regard to **NK cells**, beside their number, **cytotoxic activity** is evaluated by methods using radioactive or non-radioactive markers released from killed target cells (usually some tumor cells that lack MHC class I molecules).

Adaptive immune system

In vitro examination of the adaptive immunity includes evaluation of **proliferative capacity** of the T and B cells upon stimulation with mitogens (molecules that induce cell activation and cell division of all lymphocytes regardless of their specificity), superantigens (between 5 and 20% of T lymphocytes may be activated to proliferate) or antigens (only antigen-specific lymphocytes are activated). Proliferative response can be measured by radioactive or non-radioactive colorimetric and fluorescent methods. The most commonly used test is

thymidine incorporation assay. In brief, cells (e.g. PBMCs or certain lymphocyte subpopulation) are incubated with mitogen (or superantigen or antigen) and after certain period of time, a radioactive nucleoside, ^{3}H-thymidine, is added to a culture, which then is incorporated into new strands of chromosomal DNA during mitotic cell division. A scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to a stimulus (the more the cells proliferate, the more ^{3}H-thymidine is incorporated).

CD8^{+} T cell cytotoxicity test is rarely performed and is usually used for the evaluation of post vaccination response. In comparison to NK cytotoxicity testing, CD8^{+} T cell cytotoxicity test requires targets cells expressing specific MHC class I molecules.

Cytokine production can be measured in serum or upon cell stimulation in supernatant using **ELISA** (please refer to "Enzyme immunoassay" in "Antigen-antibody reactions with labeled reagents" text). In addition, flow cytometry (intracellular staining with cytokine specific antibodies labeled with fluorescent dye) and **ELISPOT** is used to enumerate cytokine-secreting cells.

B cell function is most commonly investigated by measuring concentrations of each immunoglobulin isotype using nephelometry or radial immunodiffusion (please refer to "Immunoprecipitation" text). **Isohemagglutinin test** is one of the tests used for the evaluation of B cell function. Isohemagglutinins are antibodies against antigens (A and/or B) on red blood cells (ABO blood groups). Furthermore, antibody titer or avidity can be assessed upon vaccination or KLH (keyhole limpet hemocyanin, antigen that has not previously recognized by immune system) immunization.

Assessment of in vivo immune response

The assessment of *in vivo* immune system is performed by skin tests and provocative tests. Most common **skin tests** for **type I hypersensitivity** are prick test and intradermal test. It is used to confirm allergies that can be the cause of patient complaints.

Tuberculin skin test or **purified protein derivative (PPD) skin test** (PPD is obtained from filtrates of sterilized, concentrated cultures of *Mycobacterium tuberculosis*) is used to assess cellular immune response **(delayed** or **type IV hypersensitivity reaction)** in countries in which BCG vaccination is implemented, such as Serbia. Please note that, in the countries where BCG vaccination is not used on a regular basis (e.g. USA), the PPD skin test is a method used to diagnose tuberculosis. In those countries, cellular immune response is assessed by so-called **recall antigens** (i.e. antigens with whom the immune system has been previously in contact with), such as candidin (antigen derived from *Candida albicans*).

Provocative tests are used to determine excessive immune response against respiratory (spirometry) or food allergens (DBPCFC, Double Blind Placebo Controlled Food Challenge). The Specific Inhalation Challenge (SIC) is a diagnosis tool aimed to assess airway responsiveness to allergens as opposed to nonspecific stimuli, such as pharmacological agents histamine and methacholine. Briefly, tested subjects are exposed to a suspected antigen (allergen) in a controlled way under close supervision in a hospital laboratory. Spirometry is then used to measure airflow and results before and after the exposure to an allergen are compared (decrease in airflow is interpreted as an obstruction). To test for food allergens, samples are prepared in the capsules (those that contain allergen and those that do not, i.e. placebo). It is double blind testing in which neither patient nor physician knows whether patient is taking the allergen or placebo. In general, only one allergen can be tested during one day.