* Serological Reaction-Continue
*Pus Preparation

TOXIN-ANTYTOXIN REACTIONS

Exotoxin

- An **exotoxin** is a toxin secreted by bacteria. An exotoxin can cause damage to the host by destroying cells or disrupting normal cellular metabolism.
- They are highly potent and can cause major damage to the host.

Toxoid or Anatoxin

- This part of the exotoxin, which has lost its toxin character but has its antigenic character as a result of treatment with limited temperature (40 ℃) and formol, is called **toxoid or anatoxin**.

- Specific antibodies that form when exotoxin or toxoid enter the organism are called **antitoxin**, immune sera containing them are also called **antitoxic serum**.

In vivo experiments,

I. When toxin alone was given to sensitive experimental animals,
   - the animal was found to have died of certain pathological findings.

II. When the animal was given a mixture of toxin + antitoxin and waiting for a while,
   - no pathological lesion appeared. For this reason, antitoxins are used for the treatment

Danysz phenomenon

- When an equivalent amount of diphteria toxin and an anti-toxin serum is mixed
  - the mixture is non-toxic;
- When the same amount of toxins is added to the same amount of antiserum at intervals (with a 30 minute break)
  - the mixture is toxic.

In this case,

- the first part of the toxin added fractionally is combined with **a relatively large proportion of the antitoxin** in the medium.
- Then, the added toxin will remain a free form. (it can not find the antitoxin can be combined in the environment). And it will be effective.
- This is an experiment showing that the formation of the antigen - antibody complex is slow.

- The toxin-antitoxin binding is similar to the antigen-antibody binding principle.
- However, exotoxins effects decrease over time and antigenic properties do not change.
- Therefore, they must be stored in the form of lyophilized powder and the test toxin should be titrated before each experiment.
- This requires a standard test toxin.

- E.g.: Standard Dried Antitoxin is used for the diphtheria in Copenhagen Serum Institute. The amount of antitoxin present at 0.0628 mg is called 1 International Antitoxin Unit (IU).
- Starting from this standard dry antioxidant, test toxin is first titrated. Then, using this toxin, the amount of antitoxin in a desired serum is determined.

Lt (Lethal Dose)

- In vivo studies for diphtheria toxin use Lt (Lethal Dose) as a unit of measurement.
- Lt
- The amount of toxin that leads to death in 4 days when it is given to a 250 g of guinea pig after it has been mixed with an international antitoxin unit (IU) for a while is called Lt (Lethal dose).
- The test toxin is titrated against standard dry antioxidant and the amount of antitoxin in the desired serum is determined.

Lf (Floculation Dose)

- The amount of toxin that gives the fastest and most pronounced flocculation (precipitation) when mixed with an international antitoxin unit (IU).

MLD (Minimal Lethal Dose)

- It is the lowest amount of toxin that causes death in 4 days with significant findings when placed under the skin of a 250 g guinea pig.

Lo (Zero Limit Dose)

- It is the highest amount of toxin that has no effect when mixed with 1 international antitoxin unit (IU) and given under guinea pig skin.

Lr (Reaction Limits Dose)

- It is amount of toxin that causes redness, edema, stiffness, and necrosis, resulting in the least reaction when mixed with an international antitoxin unit (IU) and given under guinea pig skin.

Obtaining Antitoxic Serums and Areas of Usage

- Why Important !!!
- Antitoxic serums are successfully used in the treatment and protection of Diphtheria, Tetanus, Botilusmus etc. (resulting directly from the effects of exotoxins). These serums are obtained by immunization of animals.
- The most appropriate animal is horse because of quick, abundant and high in serum levels.
- It can also be used in cattle, goats, sheep and rabbits.

I. Healthy animals are immunized by increasing doses (every other day) of toxoid
II. when the antitoxin titre is measured from time to time
III. the highest level of titre is found
IV. all the blood is taken from the animal
V. the serum is separated
VI. protective materials are added.
VII. it is stored in the dark and cold for a while. When the antitoxin is stabilized, it is bottled and used.

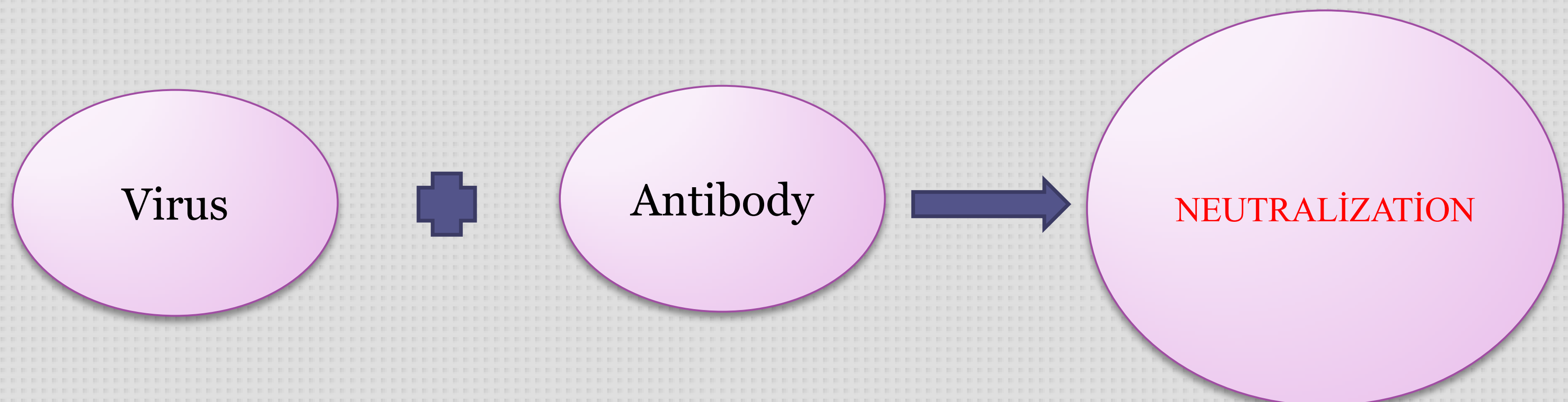
Neutralization Tests

- It is usually the experiments used with the aim of detecting viral and less specific bacterial antigens and antibodies.
- The experiment can be used for 2 purposes:

1. Investigate antibodies against known live viruses and diagnose disease
2. To identify viruses by comparing unknown viruses with known serums

Principle of the experiment

- Virus inoculation media;
  - Inoculation into embryonated egg
  - Animal Inoculation
  - Cell Culture
- These viruses are mixed with immune sera obtained against them and given to these living systems.
- After for a while, pathological events will not occur due to neutralization.



- The experiment is quantitative. For this, the activity of the live virus used as the antigen must be titrated firstly. Experimental animals (in groups) susceptible to the virus are injected with different dilutions of the virus.
- Later, according to the number of dead and sick animals;

- **LD50:** The dose killing 50% of the animals (Lethal dose)
- **PD50:** The dose that paralyzing 50% of the animals (Paralytic dose)
- **ID50:** The dose infecting 50% of the animals (Infective dose)

Values are determined.

- These doses are used as a measure;
  1. Group: Normal serum + virus
  2. Group: Patient serum + virus be injected to animals.
- The results are compared and the antibody titre in the patient's serum is determined. Diagnosis of the disease is meaningful the level of antibody to 4 fold increase in two serum samples taken at the beginning of one's disease and close to healing.

Fluorescent Antibody Assay (Immuno-fluorescence)

- ✓ Antibodies marked with stains containing fluorescent,
- ✓ after combining with the antigens on the lames,
- ✓ give fluorescence when examined under fluorescence microscope.

- For this purpose, fluorescein isothiocyanate (FITC) is used mostly

- Fluorescent antibody assays are used in the detection of A group hemolytic streptococci, *Treponema pallidum*, meningococci, intestinal pathogens and some other pathogenic bacteria and antigens or antibodies of many viruses.

2 basic methods can be used:

Direct Fluorescence Antibody Test

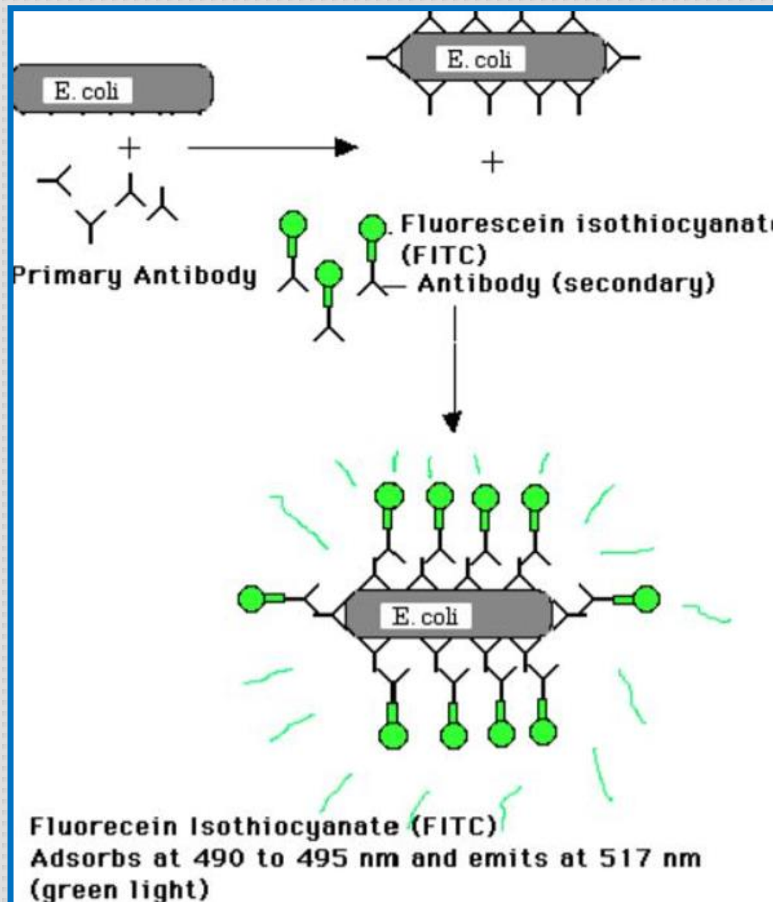
- It is used for various purposes such as detection of viral antigen (Eg demonstration of rabies virus antigens in infected brain tissue)in tissue sections.
- The labeled antibody added onto the tissue section or smear sample is washed after waiting for a while. When examined under a Uv-ray microscope, green-colored radiation appears in the areas of attachment.

Indirect Fluorescence Antibody Test

- It is mostly used in the search whether antibodies to specific antigens or not in human sera. Ex. Serological diagnosis of syphilis.

- Antigen specific primary antibody and secondary antibody (labeled with FITC capable of binding to the primary antibody) are used.

Indirect Fluorescence Antibody Test



- Antibody 1 bound by specific antigen in the tissue sample
- It is detected with the labeled antibody 2.
- Places where they are connected are observed as green-colored radiation fields on a fluorescence microscope after washing.

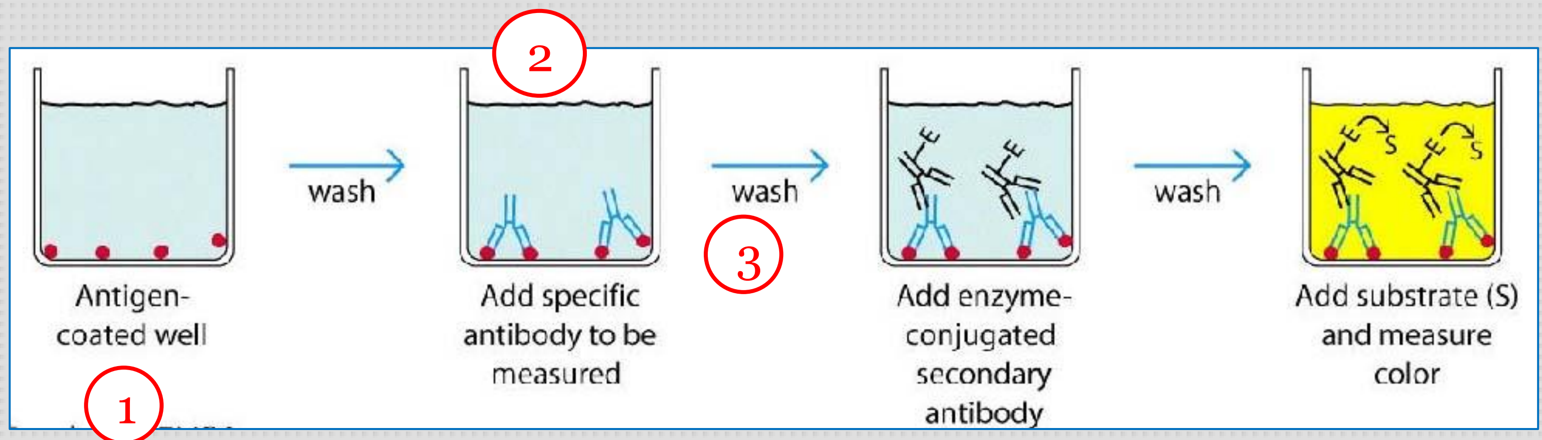
ELISA (Enzyme-Linked Immuno Sorbent Assay)

- It is based on conjugate labeled with enzyme and coloration using the enzyme substrate in order to demonstrate specific antigen-antibody binding. (Eg, hepatitis, AIDS)
- If there is a known antigen / antibody, we can determine the presence, type and amount of the antibody / antigen in question.

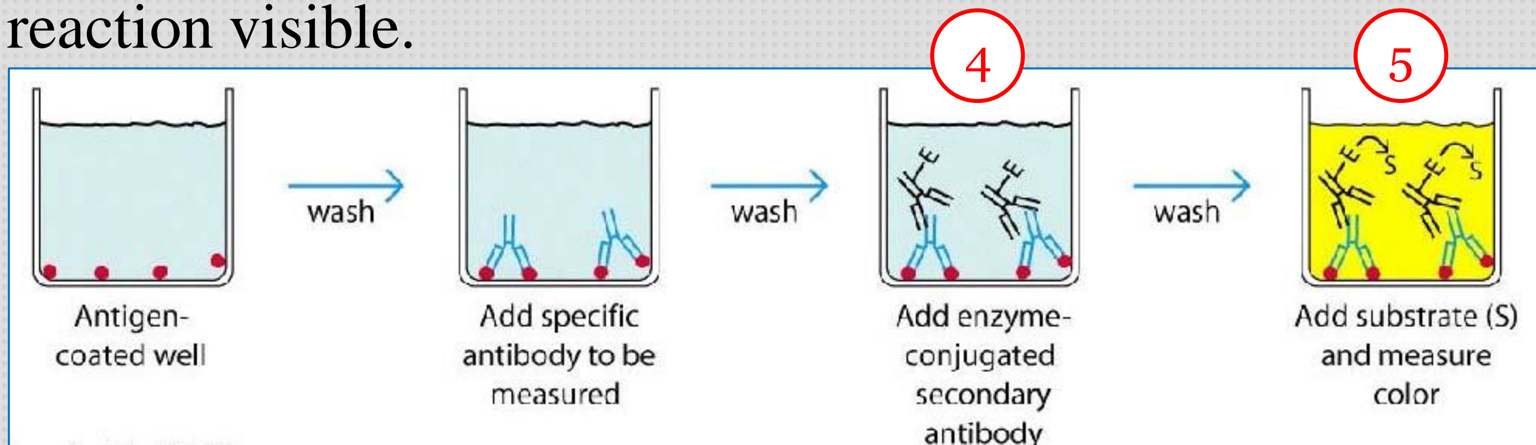
Search for antibodies (Indirect ELISA)

1. 96-well flat-bottomed polystyrene plates can be used as the solid phase. The solid phase binds to known antigens.
2. Serum samples are added to antigen-bound wells and incubated at room temperature for a certain period of time.
3. At the end of the incubation, the serum samples added to the wells are poured and the wells are washed with buffered liquid.

(If there is a specific antibody in the serum added to the pool, it can not be removed from the medium by the washing process, because the antigen in the solid phase is bound.)



4. An enzyme-labeled anti-Ig antibody is added to the wells to detect the antigen-bound antibody in the solid phase. The enzyme added to the conjugate structure is usually **peroxidase**. In addition, other enzymes such as alkaline phosphatase, glucose oxidase, beta D-galactosidase can be used. At the end of the incubation, washing is carried out several times with buffered water.
5. In order to show the bound conjugate in the medium, the **enzyme-compatible substrate** is added to the wells and the **chromogen-containing mixture** is added to make the reaction visible.



- ELISA methods; Competitive and noncompetitive ELISA, indirect ELISA, sandwich ELISA, macro and micro-ELISA, avidin-biotin attached ELISA method

RIA (Radio Immuno Assay)

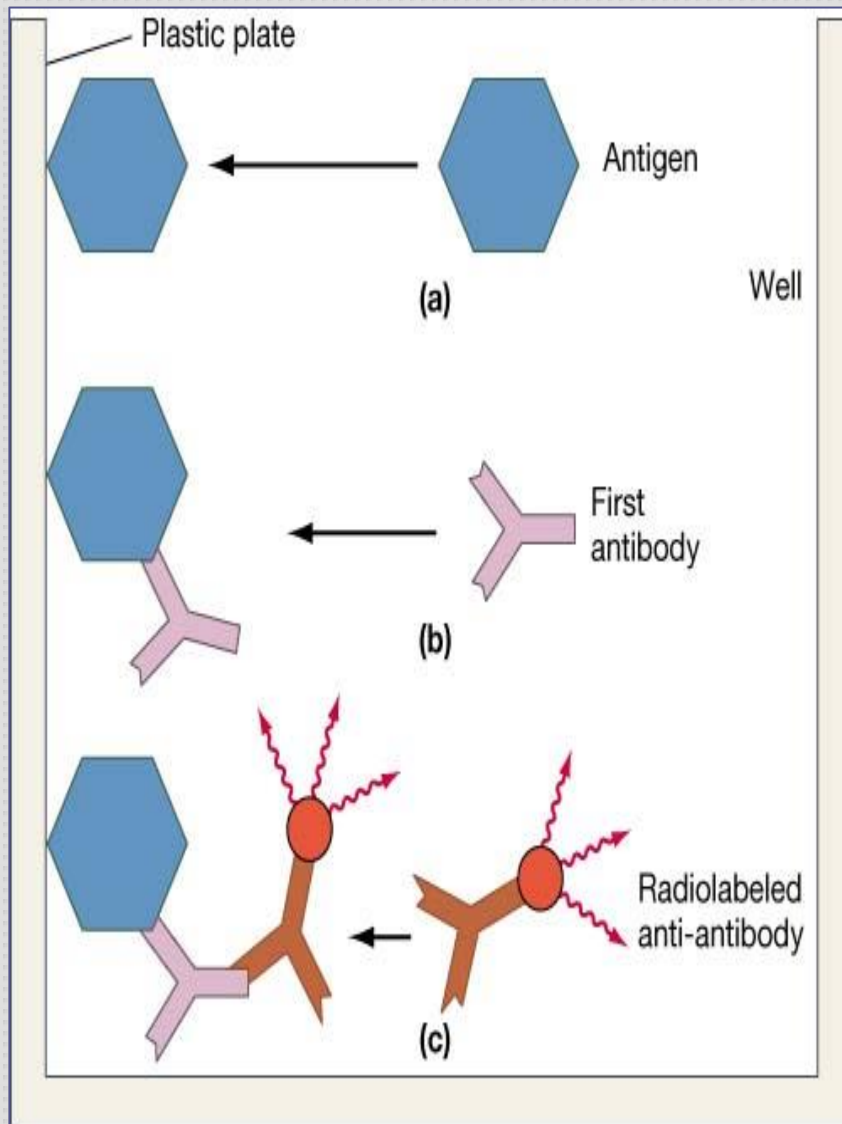
- The radioimmunoassay method has a very high specificity, especially in the detection of low levels of antigen.
- This method is used for the quantitation of antigens or antibody that can be radioactively labeled.
- Radioisotopes such as tritium, carbon 14, iodine 125 are commonly used in RIA techniques.
- It is mostly used for the search of hormones, enzymes, drugs and other biological molecules in body fluids.

- In the sandwich method,
- antigen-specific and unlabeled antibody is bound to the solid phase (tube).
- The antigen sample added to the tube is kept for a certain period of time.
- If the antigen is present in the sample, it will be binded to the antibody in the solid phase.
- After washing, the tube is incubated with antibody specific to the same antigen and labeled with radioisotope.
- The labeled antibody will bind to the entrapped antigen via the antibody in the solid phase and will not be removed by washing.
- The value given by the tube in the post-processing gamma counter (detector) is related to the amount of antigen in the serum sample.

- In the competing mechanism,
- the unknown antigen specific antibody is bound to the solid phase (tube).
- Antigen sample and radioisotope labeled antigen are added into the tube.

I. If there is no suitable antigen in the sample, the antibody in the solid phase will bind to the labeled antigen
   - we obtained a high level reading in the post-test gamma counter.

II. If there is a suitable antigen in the sample, unlabelled antigen in the serum will bind antibody on the solid phase instead of labeled antigen.
   - The post-processing gamma counter provides much lower readings than the previous case.



- To search for antibodies, the process is monitored using plastic tubes or plates coated with the appropriate antigen.

Opsono Cytophagic Test (Opsonic index)

- Phagocytosis is a specific form of endocytosis by which cells internalise solid matter, including microbial pathogens.
- While most cells are capable of phagocytosis, it is the professional phagocytes of the immune system, including macrophages, leukocytes, neutrophils and immature dendritic cells, that truly excel in this process.

- In the experiment, citrated blood from normal subjects and leukocytes separated by centrifugation is used as a phagocyte.

- ✓1 drop of leucocyte suspension + 1 drop of bacterial susp. + 1 drop of physiological saline
- ✓1 drop of leucocyte suspension + 1 drop of bacterial susp. + 1 drop of normal serum
- ✓1 drop of leucocyte suspension + 1 drop of bacterial susp. + 1 drop of immune serum
- ➢ After waiting for a while at 37 ° C, the preparation is stained.

Sporadic bacteria in the leucocytes

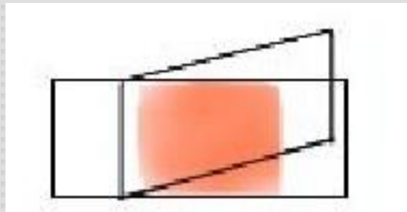
Several bacteria in the leukocytes

The bacteria are phagocytized in abundance and in clusters within leukocytes.

- The agent that increases phagocytosis has been found to be antibodies in immune serum.
- Antibodies that increase phagocytosis are called **opsonin**.
- Opsonins prepare phagocytose by enclosing phagocytes, this event is called **opsonization** (preparing food).
- We measure quantitatively opsonins in immune sera is called **the Opsonic Index.**
- For this purpose;

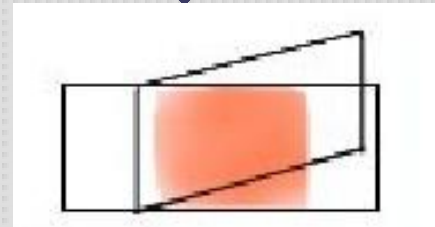
Normal serum+
Normal leukocyte+
Bacteria

Hold for 20 minutes at 37 °C and spread on clean lames and dried and painted with blood stain.



Immune serum+
Normal leukocyte+
Bacteria

Hold for 20 minutes at 37 °C and spread on clean lames and dried and painted with blood stain



*50 - 100 leukocytes and bacteria in them are counted in each preparation. The number of bacteria is divided by the number of leukocytes. There is a phagocytosis index.

- The sum of the bacteria counted separately in each preparation and the sum of the leukocytes counted is calculated.

- **Phagocytosis index** = number of bacteria / number of leukocytes

(Calculated separately for immunized serum and normal serum.)

- **Opsonic Index** = Immune serum phagocytosis index / Normal serum phagocytosis index

- The size of the opsonical index is directly proportional to the antibody titre of the immune serum.

- Increases the rate and quantity of phagocytosis in immune serum;
  - ✓ Ig G antibodies against bacterial antigens etc.
  - ✓ C_3 pieces of the complement

PUS-WRIGHT STAINING

- The preparation is initially **fixated in the alcohol tank for 2-3 min.**
- Add 8 drops Wright stain. Wait 3-5 min.
- Add 8 drops buffered water and wait completed in 10 minutes.
- Washed with water, dried and examined with the immersion objective.

PUS-GRAM STAINING

-The preparation is initially **fixated in the alcohol tank for 2-3 min** and then Gram staining is done.

According to the granules in the nuclei of leukocytes;

Granulocytes (Particulate leukocytes)

- Neutrophil % 40 - 75
- Eosinophil % 5
- Bazofil % 0.5

Agranulocyte leukocytes (Mononuclear leukocytes)

- Lymphocyte % 20 - 50
- Monosit % 1 - 5