Immunological laboratory investigation

CELLULAR METHODS

- **Course no. 2**

DIFFERENTIAL BLOOD COUNT

Differential blood count gives relative percentage of each type of white blood cell and also helps reveal abnormal white blood cell populations

	infant	***childs***	***adults***
LEUKOCYTES	**9 – 15 x 10^9/l**	**8 – 12 x 10^9/l**	**4 – 9 x 10^9/l**
GRANULOCYTES	%	%	%
neutrophil granulocyte	**25 - 65**	**35 - 70**	**55 - 70**
• segmented cells („segs“)	**22 - 65**	**25 - 65**	**50 - 70**
• bands	**0 - 10**	**0 - 10**	**3 - 5**
eosinophil granulocyte	**1 - 7**	**1 - 5**	**2 - 4**
basophil granulocyte	**0 - 2**	**0 - 1**	**0 - 1**
MONONUCLEAR LEUKOCYTES	%	%	%
lymphocytes	**20 - 70**	**25 - 50**	**25 – 40**
monocytes	**7 - 20**	**1 - 6**	**2 - 6**

CD classification system (Paris 1982)

CD markers (CD = cluster of differentiation or cluster of designation)

- *nomenclature system of cell surface molecules expressed on leukocytes and other cells relevant for the immune system*
- **CD for humans is numbered more than 400**

  (CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology)

- **Usage in clinical practice:** investigation of absolute number and percentage of cell subpopulations by flow cytometry (T cells, B cells and their subpopulation, NK cells)
- **Blood sample:** *anticoagulant-treated blood* (EDTA)

LYMPHOCYTE SUBPOPULATIONS	CD MARKERS	PERCENTAGE FROM LYMPHOCYTES
T lymphocytes	**$CD3^+$**	**58 – 85 %**
Th lymfocyty	$CD3^+CD4^+$	**30 – 60 % /$CD3^+$**
Tc lymfocyty	$CD3^+CD8^+$	**15 – 35 % /$CD3^+$**
B lymphocytes	**$CD19^+$**	**7 – 23 %**
NK cells	**$CD16^+/56^+$**	**6 – 20 %**

SURVEY OF CELLULAR IMMUNOLOGICAL INVESTIGATION

We can performed following immunological cellular investigation:

- **percentage and absolute counts of cells of immune system**
  - *The first step is to find out number of leukocytes and differential blood counts (percentage and absolute counts of lymphocytes, monocytes and granulocytes)*
- **immune cell function**

FLOW CYTOMETRY

Investigation of percentage and absolute counts of lymphocyte subpopulation

FLOW CYTOMETRY

- principle: ***direct immunofluorescence***
- Cells are incubated with secondary antibody conjugated with fluorescent dye and they are directed against CD markers of cell surface
- suspending cells in a stream of fluid and passing them through an electronic detection apparatus

Investigation of percentage and absolute counts of lymphocyte subpopulation

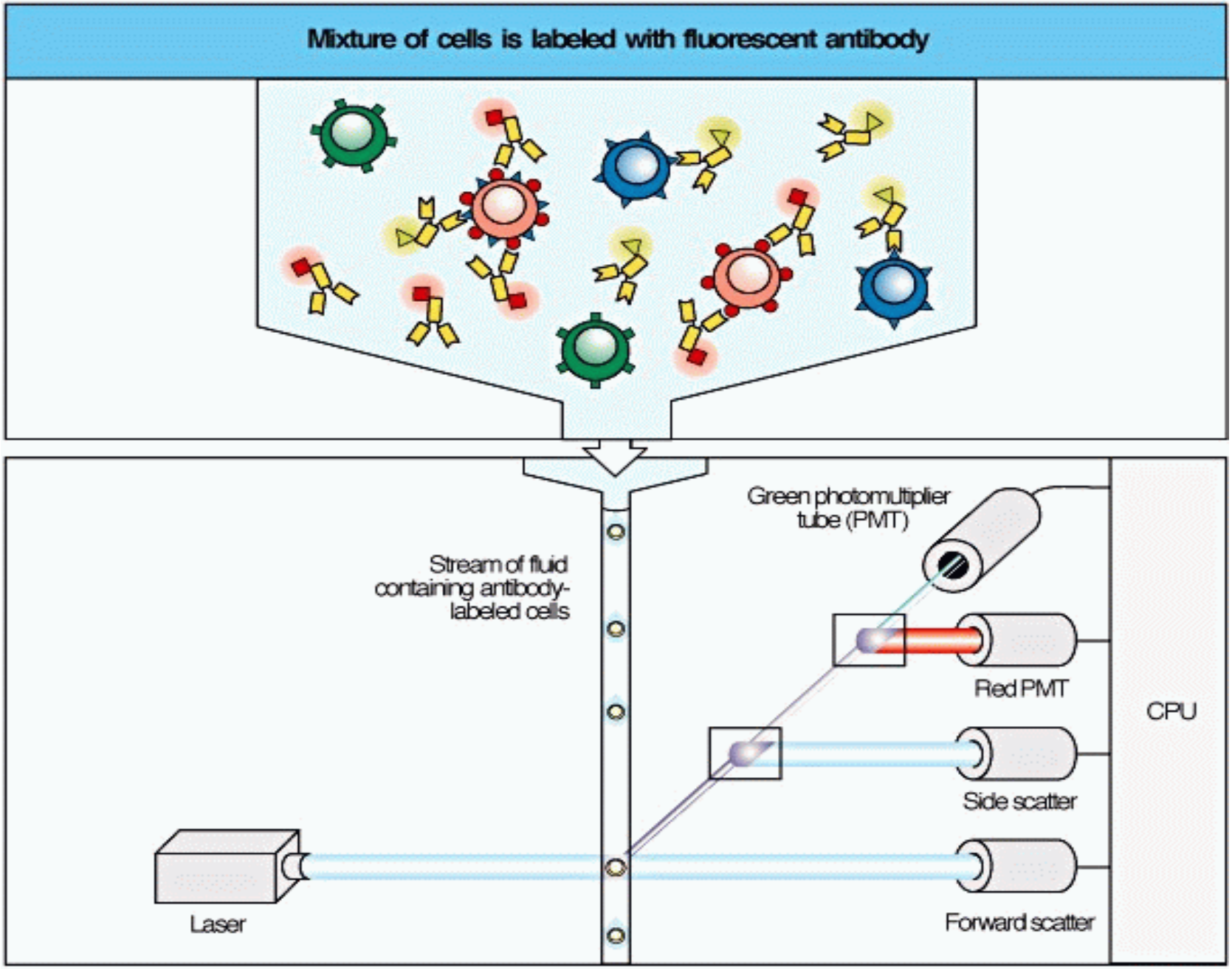
FLOW CYTOMETRY

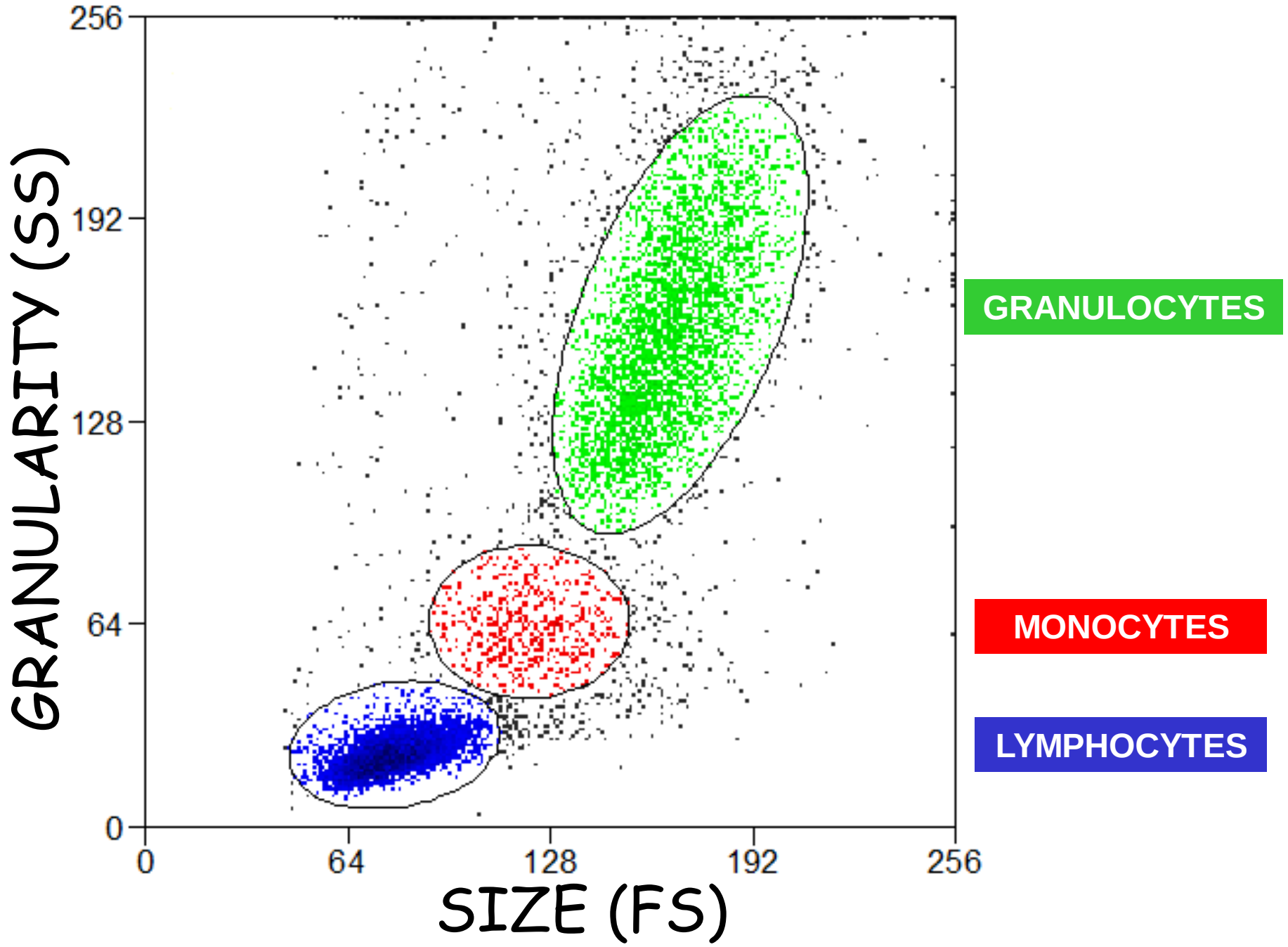
- *cells are differentiated according to size and granularity:*
  - **LYMPHOCYTE**, **MONOCYTES** and **GRANULOCYTES**

- *Cells are differentiate according to cell surface markers:*
  - Subpopulations of **T-LYMPHOCYTES, B-LYMPHOCYTES** and **NK CELLS**

Routinely usage of flow cytometry:

- diagnostic of primary and secondary immunodeficiencies
- diagnostic of hematological malignancies


Mixture of cells is labeled with fluorescent antibody
Green photomultiplier tube (PMT)
Stream of fluid containing antibody-labeled cells
Red PMT
CPU
Side scatter
Laser
Forward scatter


256
192
128
64
0
GRANULARITY (SS)
0
64
128
192
256
SIZE (FS)
GRANULOCYTES
MONOCYTES
LYMPHOCYTES

LYMPHOCYTE PROLIFERATION

Lymphocyte cultivation in vitro

cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment

the stimulation of lymphocytes by antigens or mitogens, rendering them metabolically active and causing them to differentiate into effector cells

evaluation of function of the cells

^{3}H Thymidine Proliferation Assay

- widely used proliferation assay is the ^{3}H thymidine uptake assay
- cells incorporate the radiolabeled nucleotide into newly synthesized DNA
- in this way, the level of radioactivity as measured by liquid scintillation gives a relative measure of cellular proliferation

Functional investigation of lymphocyte proliferation

PROLIFERATION OF LYMPHOCYTES

physiological proces during cell activation

- Lymphocytes activated by
  - **polyclonal mitogens (non-specific stimuli)**
    - for B-lymphocytes
      - pokeweed mitogen (PWM)
    - for T-lymphocytes
      - phytohemagglutinin (PHA)
      - concanavalin A (ConA)
  - **Antigens (specific stimuli)**
    - tuberculin
    - tetanic toxoid

Functional investigation of lymphocyte proliferation in vitro

PROLIFERATION OF LYMPHOCYTES

one of the physiological signs of cellular activation

- **isolation of lymphocytes from peripheral blood**
- **cultivation of lymphocytes with mitogens**
  tetanic toxoid – 7 days
  PHA, PWM – 3 days
  - **Adding of ^{3}H-thimidin day before end of proliferation** (2^{nd} day or 6^{th} day) – incorporation of thymidine into DNA of proliferating cells)
- **stop of proliferation by freezing**
- **detection of radioactivity by beta-counter**
- **result – stimulation index (SI)**
  - cpm (counts per minute) experimental/ cpm background unstimulated
  - Positivity: SI $\geq$ 5 for antigens, SI $\geq$ 100 for mitogens

Functional investigation of lymphocyte proliferation in vitro

PROLIFERATION OF LYMPHOCYTES

one of the physiological signs of cellular activation

When investigate proliferation of lymphocytes?

diagnostic of severe immunodeficiencies

(SCID)

Functional investigation of lymphocyte subpopulations in vitro

further specialized functional investigation

- measurement of production and release of important cytokines and therefore functional investigation od T-cell subpopulations (Th1 and Th2)
    - ***ELISA, ELISPOT, PCR***
- measurement of cell surface molecule expression, which are necessary for cell synapsis, or activation markers of cells
    - ***Flow cytometry***
- measurement of B lymphocyte immunoglobulin production
    - ***ELISA, ELISPOT***

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

is done to see if you have ever been exposed to tuberculosis
(IV. type of hypersenzitivity reactions)

it is called also ***Mantoux tuberculin test***

- the test is done by putting a small amount of TB protein (antigens) under the top layer of skin on your inner forearm
- if the patient has ever been exposed to the TB bacteria (Mycobacterium tuberculosis), his skin will react to the antigens by developing a firm red bump at the site within 2 days (24–48 hours)

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

is done to see if you have ever been exposed to tuberculosis
(IV. type of hypersenzitivity reactions)
it is called also ***Mantoux tuberculin test***

Test is positive

induration 6–15 mm

- *normal response in sensitized person*

induration > 15 mm (in children < 5 years < 10 mm)

- *indication to chest X-ray*

Test is negative

Induration < 6 mm

- *patient was not sensitized before*
- *impaired responsiveness of patient T cells*

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

Why is it done?

A tuberculin skin test is done to find people who have tuberculosis (TB), including:

- people who have been in close contact with someone known to have TB
- health care workers who are likely to be exposed to TB
- people with TB symptoms, such as an ongoing cough, night sweats, and unexplained weight loss
- people who have had an abnormal chest X-ray
- people who have had a recent organ transplant or have an impaired immune system, such as those with human immunodeficiency virus (HIV).

Functional investigation of T cells in vivo

CELL MEDIATED IMMUNITY TEST

(CMI test)

- Intradermal application of anamnestic antigens (tuberculin, candidin, toxoplasmin, tetanus toxoid, antigens of staphylococci, streptococci, etc.)
    - No induration after application of antigen after 48 hours → impaired T-lymphocyte responsiveness (patient is anergic)

Investigation of phagocyte functions

number of phagocyte cells

Determination of number of cells capable of phagocytosis

- **number of neutrophil granulocytes**

  differential blood count
- **Determination of specific cell surface markers of granulocytes and monocytes**

  CD15 for neutrophil granulocytes

  CD14 for monocytes

markedly or repeated descrease is indication to investigate phagocyte functions

Investigation of phagocyte functions

function of phagocyte cells

Investigation of phagocytosis steps

- **defects of adhesion**
- **defects of chemotaxis**
- **defects of ingestion**
- **defects of respiratory burst**

material for investigation

peripheral blood

Investigation of phagocyte functions

Investigation of adhesion

expression of markers CD11/CD18

flow cytometry

LAD1 syndrom

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

NBT test

chemiluminiscence

burst test

Defects in chronic granulomatous disease

Transient defects in infections, traumas and malnutrition

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

NBT test
(nitro blue tetrazolium chloride test)

Nitro blue tetrazolium is a chemical compound composed of two tetrazole moieties. It is used in immunology for sensitive detection of alkaline phosphatase (with BCIP). NBT serves as the oxidant and BCIP is the AP-substrate (and gives also dark blue dye)

reduction of colorless nitro blue tetrazolium into colorful formazan

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

BURST TEST

- heparinized whole blood is incubated at 37 °C with phorbol myristate acetate (PMA), a compound known to stimulate oxidative burst aktivity, each flow cytometry pattern is referenced to the patients non-stimulated cells; in addition, a control blood is included in each run
- upon stimulation, granulocytes and monocytes produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid) which destroy bacteria inside the phagosome

formation of the reactive oxidants during the oxidative burst can be monitored by the addition and enzymatic oxidation of a fluorogenic substrate, DHR 123

the level of reactive oxygen radicals is determined by flow cytometry

INVESTIGATION OF ATOPY DISEASES

ATOPY DISEASES

atopy is the tendency to produce an exaggerated immunoglobulin E (IgE) immune response to otherwise harmless substances in the environment

allergic diseases are **clinical manifestations of such inappropriate, atopic responses**

(allergic rhinoconjunctivitis, asthma bronchiale, food allergy, urticaria and angioedema, atopic eczema and anaphylactic shock)

DIAGNOSTIC APPROACH IN ATOPY DISEASES

patient history

skin prick tests

eosinophilia

serum levels of IgE and specific IgE

provocation and elimination tests

functional cellular tests

DIAGNOSTIC APPROACH IN ATOPY DISEASES

skin prick test (SPT)

skin testing can confirm many common types of allergies

in some cases, skin tests can be the most accurate and least expensive way to confirm allergens

placement of a small drop of the possible allergen on the skin

pricking or scratching skin with a needle through the drop

if the patient is sensitive to the substance →

development of redness, swelling and itching at the test site within 15 minutes

DIAGNOSTIC APPROACH IN ATOPY DISEASES

skin prick test (SPT)

WHAT IS IMPORTANT TO KNOW?

a positive skin test result does not by itself diagnose an allergy

a positive skin test does not predict the severity of an allergic reaction

a negative skin test usually means you are not allergic

DIAGNOSTIC APPROACH IN ATOPY DISEASES

serum concentration of IgE immunoglobulins

reference values approximately 0– 100 kU/l

(mean total serum IgE concentration is remarkably constant in normal non-allergic Caucasians)

serum concentration of IgE correlate with presence of atopic disease but more than one third of allergic patients have total IgE concentration within the normal range

specificity of IgE: **90 %**

senzitivity of IgE: **30–40 %**

DIAGNOSTIC APPROACH IN ATOPY DISEASES

serum concentration of IgE immunoglobulins

insufficient to establish an atopy diagnosis

INCREASED LEVELS OF IgE

except atopy diseases also allergic bronchopulmonary aspergillosis, nasal polyposis, parasite infection, AIDS, Wiskott-Aldrich syndrome, hyper IgE syndrome, myeloma, …

DECREASED LEVELS OF IgE

up to 60–70 % of allergic patients may not have elevated IgE levels

DIAGNOSTIC APPROACH IN ATOPY DISEASES

serum concentration of specific IgE antibodies

NATIVE ALLERGENS

an allergenic compound or protein derived from natural sources; its molecular linkage and three-dimensional structure remain unchanged after the extraction and purification process

RECOMBINANT ALLERGENS

most crystal and solution structures of allergens have been obtained using recombinant allergens; structural information on allergens allows insights into their evolutionary biology, illustrates clinically observed cross-reactivities, and makes the design of hypoallergenic derivatives for allergy vaccines possible

DIAGNOSTIC APPROACH IN ATOPY DISEASES

serum concentration of specific IgE antibodies

an allergen-specific immunoglobulin E (IgE) test is a blood test that measures the levels of different IgE antibodies in a person's blood

allergen-specific IgE tests are sometimes used to diagnose and better manage food allergies

they can also be helpful for environmental allergy diagnosis in some cases

SPECIFICITY → 95 %

SENSITIVITY → 75–85 %

DIAGNOSTIC APPROACH IN ATOPY DISEASES

number of eosinophils

EOSINOPHILS ARE IMPORTANT FOR ALLERGY INFLAMMATION

eosinophils are one form of terminally differentiated granulocytes; they function to neutralize invading microbes, primarily parasites and helminthes but also certain types of fungi and viruses

eosinophils produce and release on demand a range of toxic reactive oxygen species (hypobromite, hypobromous acid, superoxide, and peroxide) and they also release on demand a preformed armamentarium of cytokines, chemokines, growth factors, lipid mediators (leukotrienes, prostaglandins, platelet activating factor), and toxic proteins (metalloproteinases, major basic protein, **eosinophil cationic protein**, eosinophil peroxidase, and eosinophil-derived neurotoxin).

DIAGNOSTIC APPROACH IN ATOPY DISEASES

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DIAGNOSTIC APPROACH IN ATOPY DISEASES

eosinophil cationic protein (ECP)

basic protein located in the eosinophil primary matrix

ECP is released during degranulation of eosinophils

this protein is related to inflammation and asthma because in these cases, there are increased levels of ECP in the body

ECP > 24 ng/ml

(aktivity of eosinophil inflammation)

DIAGNOSTIC APPROACH IN ATOPY DISEASES

tryptase

tryptase is the most abundant secretory granule-derived serine proteinase contained in mast cells and has been used as a marker for mast cell activation

elevated levels of serum tryptase occur in both anaphylactic and anaphylactoid reactions, but a negative test does not exclude anaphylaxis

the rise in tryptase levels starts to be detected in serum within minutes of anaphylaxis but the level will gradually revert to normal over the next 6–24 hours depending on the height of the increase and often correlates with the severity of the anaphylaxis

DIAGNOSTIC APPROACH IN ATOPY DISEASES

provocation tests

compared to skin or laboratory tests, they have a number of disadvantages

only one allergen can be tested during one visit to the doctor greater burden and risk for the patient higher time requirement the need for hospitalization

it is the only diagnostic procedure that proves not only sensitization (atopy), but also causality (allergy)

DIAGNOSTIC APPROACH IN ATOPY DISEASES

provocation tests

Nasal provocation tests

specific provocation test (considered causal allergen)

Bronchial provocation tests

specific bronchoprovocation test (considered causal allergen), nonspecific bronchoprovocation test (histamine, methacholine, mannitol, cold air, physical exertion)

Gastrointestinal provocation tests

double-blind placebo-controlled food challenge test

DIAGNOSTIC APPROACH IN ATOPY DISEASES

elimination tests

especially in suspision of food allergy

elimination of possible causative foods from the menu and evaluation of the clinical effect

to confirm the correctness of the result → performing of re-exposure

BASOPHIL ACTIVATION TEST

principle of the investigation

- **PRESTIMULATION** by IL-3
- **ACTIVATION**
  - positive control – fMLP
  - negative control – negative control from prick tests
  - investigated sample – investigated allergen
- **STOP OF DEGRANULATION** on ice
- **LABELING** by anti-CD45, anti-IgE, anti-CD63, anti-CD203c
- **LYSIS**
- **ANALYSIS** by flow cytometry

Thank you for your attention

CELLULAR METHODS

Course no. 2