Immunological laboratory investigation

SEROLOGICAL REACTIONS

Course no. 1

BASIC DIVISION OF IMMUNOLOGICAL LABORATORY INVESTIGATION

- **Serological investigation**
  - *material for investigation*
    - **SERUM or PLASMA**

- **Cellular investigation**
  - *material for investigation*
    - **PERIPHERAL VENOUS BLOOD**

- **Other material for immunological investigation**
  - Cerebrospinal fluid, lymph nodes, organ biopsy material, bone marrow, bronchoalveolar lavage fluid

SERUM AND PLASMA DEFINITION

SERUM

clear, yellowish fluid that remains after blood has been allowed to clot and the blood cells (including red blood cells, white blood cells, and platelets) have been removed

PLASMA

liquid component of blood, making up about 55% of total blood volume and contains clotting factors (fibrinogen and other proteins) that are essential for blood clotting

serum is essentially plasma without clotting factors

ANTIGEN – ANTIBODY REACTIONS IN VITRO

- **EPITOP (determinant)**
  - *the specific portion of the macromolecule (antigen), to which an antibody binds*
- **PARATOP**
  - *the specific portion of antibody binding site (area of N-terminal part of variable part of light and heavy chains)*
- **AFINITY**
  - *the strength of the binding between a single binding site of the molecule (antibody) and a ligand (antigen)*
- **AVIDITY**
  - *The overall strength of interaction between two molecules such as an antibody and antigen*

PRIMARY AND SECONDARY PHASE OF SEROLOGICAL REACTIONS

Primary phase of serological reaction

- *specific phase of the reaction, if specific antibody binds to specific antigen*
- ***It is not visible!***

Secondary phase of serological reaction

- *visualization of the fact of previously occurred primary reaction*

PRIMARY AND SECONDARY PHASE OF SEROLOGICAL REACTIONS

… resulting complexes are …

- ***visible***
  (AGGLUTINATION, PRECIPITATION)

- ***change of fluid character to colloidal solution***
  (TURBIDIMETRY, NEPHELOMETRY)

- *the course of the reaction enable only primary phase of reaction or only incomplete secondary phase and it is necessary to visualize the reaction by following imunohistochemical detection (IMMUNOASSAYS)*

ANTIGLOBULIN ANTIBODIES polyclonal antisera

- *obtained from animals (rabbits, goats, horses) by repeated immunization by antigen*

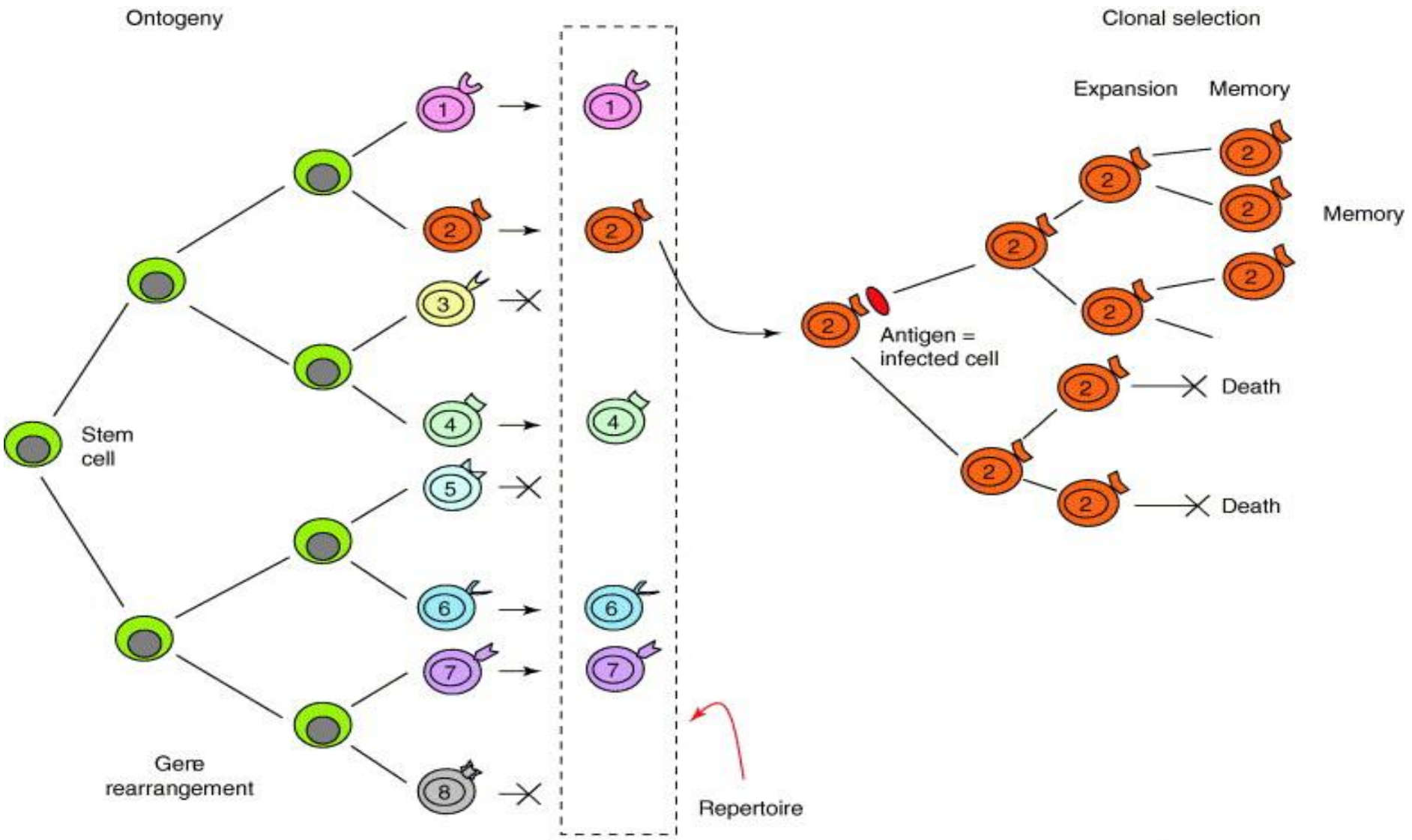
- *markedly polyreactive, because antibody binds to many epitopes of the antigen but also with other antigens*

This is advantageous in „classical" serological reactions (agglutination, precipitation)

Examples of secondary antisera:

- *RaHuIgG (rabbit anti-human IgG) reacts with human IgG of various specificities (anti-Rh, anti-microbial antigens)*

Clonal selection theory



SENSITIVITY OF THE METHODS FOR DETECTION OF ANTIBODIES

precipitation

30 μg/ml

agglutination

1 μg/ml

radioimunoassay and ELISA

1 pg/ml

INTERPRETATION OF LABORATORY TESTS

- ***SPECIFICITY***
  - *measures the proportion of **negatives that are correctly identified as such** (e.g. the percentage of healthy people who are correctly identified as not having the condition)*
  - ***TRUE NEGATIVE RATE***
- ***SENSITIVITY***
  - *measures the proportion of **positives that are correctly identified as such** (e.g. the percentage of sick people who are correctly identified as having the condition)*
  - ***TRUE POSITIVE RATE***

POLYCLONAL AND MONOCLONAL ANTIBODIES

- ***POLYCLONAL ANTIBODIES***
  - *collection of immunoglobulin molecules that react against a specific antigen, each identifying a different epitope*
  - *secreted by different B cell lineage within the body*
  - ***OBTAINED BY IMMUNIZATION OF ANIMALS***

- ***MONOCLONAL ANTIBODIES***
  - *Product of a single B lymphocyte with monovalent affinity, in that they bind to the same epitope*
  - *secreted by a single cell lineage*
  - ***OBTAINED BY IN VITRO METHODS***

MONOCLONAL ANTIBODIES

- ***PREPARATION***
- *prepared by immortalization of B-cells from immunized mouse*
- *hybridoma is composed of an antigen-specific B cell and mouse myeloma cell*
- *produced antibodies are strictly monospecific and therefore cannot be used in several „classical" serological reactions (agglutination, precipitation)*

LABORATORY USE OF MONOCLONAL ANTIBODIES

highly specific agent used for ELISAs, RIAs,

determination of cells surface antigens

Because they react only with a single epitope, number of „bridges“ is to low to overcome repulsive forces in classical reactions like agglutination or precipitation.

CLINICAL USE OF MONOCLONAL ANTIBODIES

- **Immunosuppressive treatment**
  - anti CD3, CD54, CD20
- **Antinflammatory treatment**
  - Cytokine neutralization (anti-TNFa, anti-IL1, IL6, IL-17)
  - Adhesion molecules blocade (anti-LFA-1, …)
- **Anti-tumor treatment**
  - anti-CD20, anti EGF
- **Anti allergic treatment**
  - anti-IgE, anti-IL15
- **Anti aggregation treatment**
  - anti- gpIIb-IIIa – blocks activation of thrombocytes)

COMPLETE AND INCOMPLETE ANTIBODIES

- ***COMPLETE ANTIBODIES***
  - *visible agglutination or precipitation reaction after reaction with antigen*
- ***INCOMPLETE ANTIBODIES***
  - *despite the fact that the reaction between epitope and antibody occurred, the agglutinate or precipitate cannot be detected*

CAUSES

- **because of antigen**
  - *low antigenicity (low numbers of epitope, bad accessibility of epitopes for antibody binding)*
  - *low number of bridges between antigens, to intense repulsive forces between antigens*
- **because of antibody**
  - *monovalent antibodies (IgM x IgG)*

SURVEY OF METHOD FOR DETECTION OF ANTIGEN OR ANTIBODY

- ***visualization by secondary phase***
  - **AGGLUTINATION** *(direct, indirect)*
  - **PRECIPITATION** *(simple, in combination with electrophoresis, immunofixation)*
- ***visualization by following detection***
  - **IMUNOFLUORESCENCE**
  - **IMUNOANALYSIS** *(RIA, EIA, and modifications)*
  - **IMUNOBLOT, IMUNODOT**

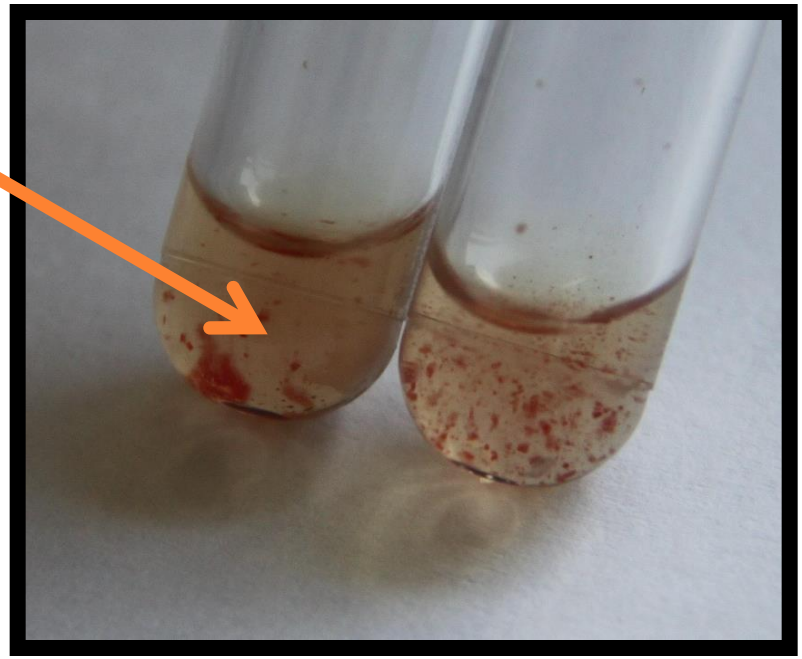
a g g l u t i n a t i o n

principle of reaction

antigen: INSOLUBLE PARTICULATE ANTIGEN

the action of an antibody when it cross-links multiple antigens producing clumps of antigen

… ***AGGLUTINATE***



- ***easy visualization of occurred reaction***
  - due to antigen size
  - due to reaction in liquid

a g g l u t i n a t i o n

factors influencing quality of agglutination

- ***enough antibodies***
  - low concentration of antibodies → no agglutination
- ***antibodies directed to various epitopes***
  - difference in agglutination between monoclonal and polyclonal antibodies
- ***distance between particles***
  - the force of attraction or repulsion between two electrically charged particles, in addition to being directly proportional to the product of the electric charges, is inversely proportional to the square of the distance between them; this is known as Coulomb´s law

a g g l u t i n a t i o n

diretc and indirect

direct agglutination

antigen is present on the particle surface

blood groups, direct Coomb´s test, bacterial agglutination tests for sero-typing and sero-grouping (e.g. Vibrio cholerae, Salmonella spp)

indirect agglutination

antigen is bind to appropriate macromolecular particle (red blood cells, polystyrene latex, ...)

Latex fixation test, indirect Coomb´s test, detecting cholera toxins, etc.

a g g l u t i n a t i o n

blood group detection

ANTIGENS OF ERYTHROCYTE SURFACE

polysaccharides

blood group system AB0 (antigen A, antigen B)

blood group system Lewis, P a Ii

glycoproteins

blood group system Rh (antigen D)

blood group system MNSs, Lutheran, Kell, Duffy, Diego

a g g l u t i n a t i o n

blood group system Rh

COOMB´S TEST

detection of incomplete antibodies against Rh antigen

DIRECT Coomb´s test

*detection of in vivo **bound antibodies** against erythrocytes*

INDIRECT Coomb´s test

*detection of **circulating antibodies** against erythrocytes*

Coomb´s antiserum

ANTIBODIES AGAINST HUMAN SERUM GLOBULINS

(polyspecific antiserum containing antibodies directed against IgG, complement, light and heavy chains of immunoglobulins)

a g g l u t i n a t i o n

Coomb´s test

THE PRINCIPLE OF THE TEST

if human serum or whole blood is added to anti-human globulin serum (as used in the Coombs test) the latter will be deprived of its power to agglutinate red cells sensitized with incomplete Rh antibody

The procedures used with the reagent are based on the principle of heteroagglutinins directed against components of human serum. Normal human red blood cells, in the presence of antibody directed toward an antigen they possess, may become sensitize but fail to agglutinate due to the particular nature of the antigen and antibody involved.

Anti-human serum will react with red cells sensitized with gamma globulin (red blood cell antibody) or components of human complement and cause agglutination of the red blood cells.

p r e c i p i t a t i o n

principle of the reaction

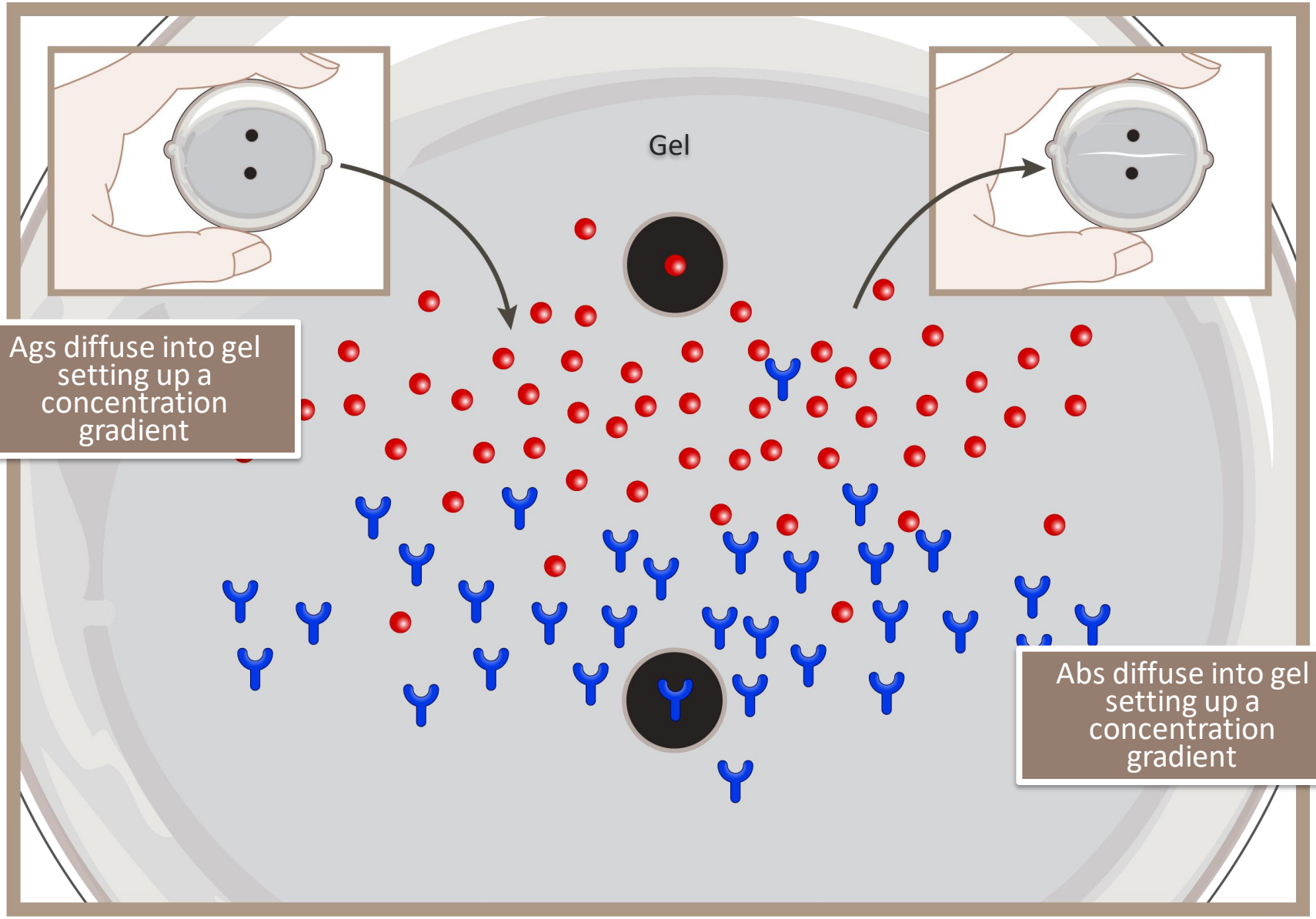
antigen: SOLUBLE ANTIGEN OF LOW MOLECULAR WEIGHT

Reaction between polyclonal antiserum and soluble (molecular) antigen. A complex lattice of interlocking aggregates is formed. If performed in a solution the precipitate falls out of the solution.

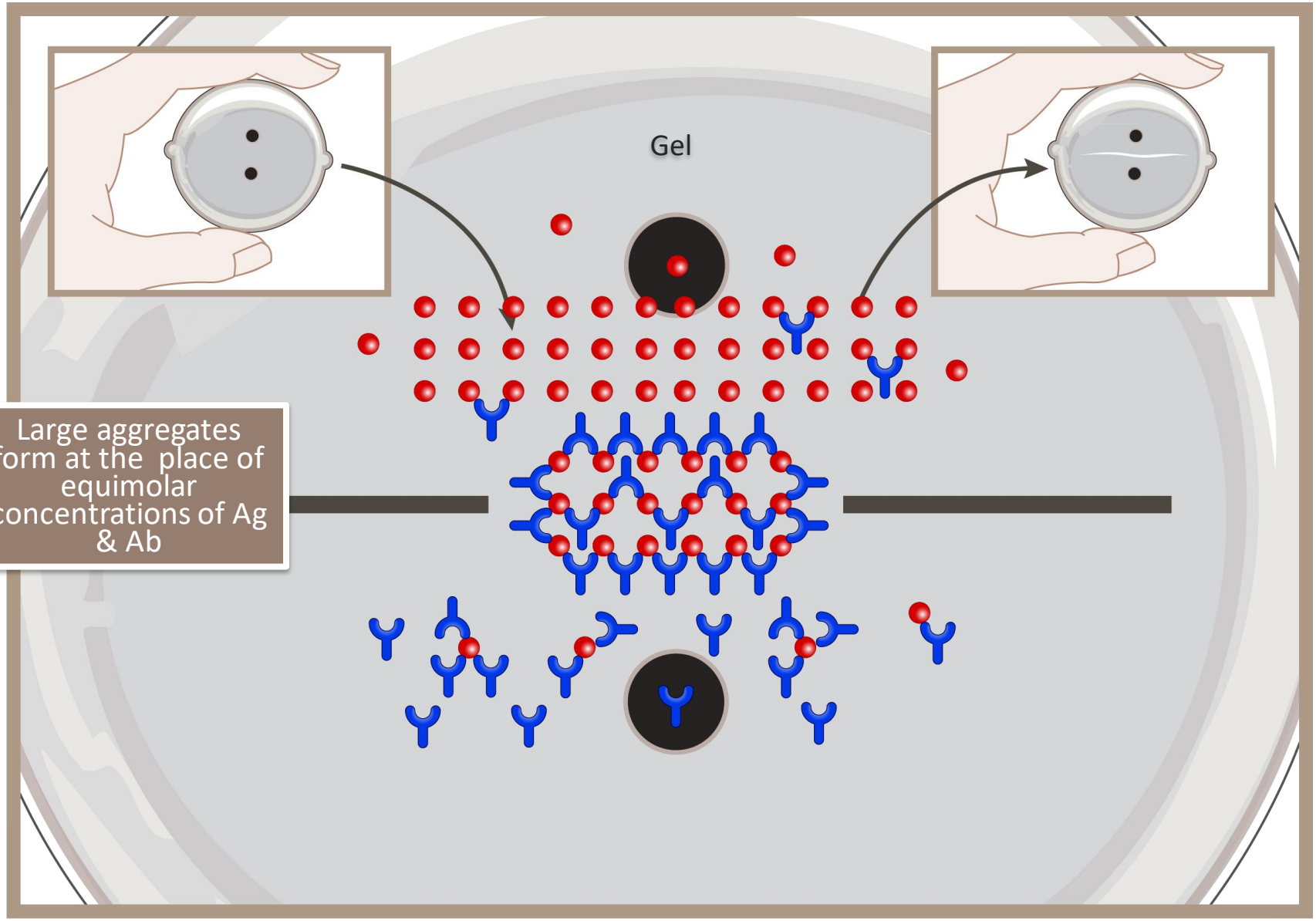
PRECIPITATION IN …

- *in liquids (nephelometry, turbidimetry)*
- *v gels (immunodiffusion)*

Immunodiffusion-I

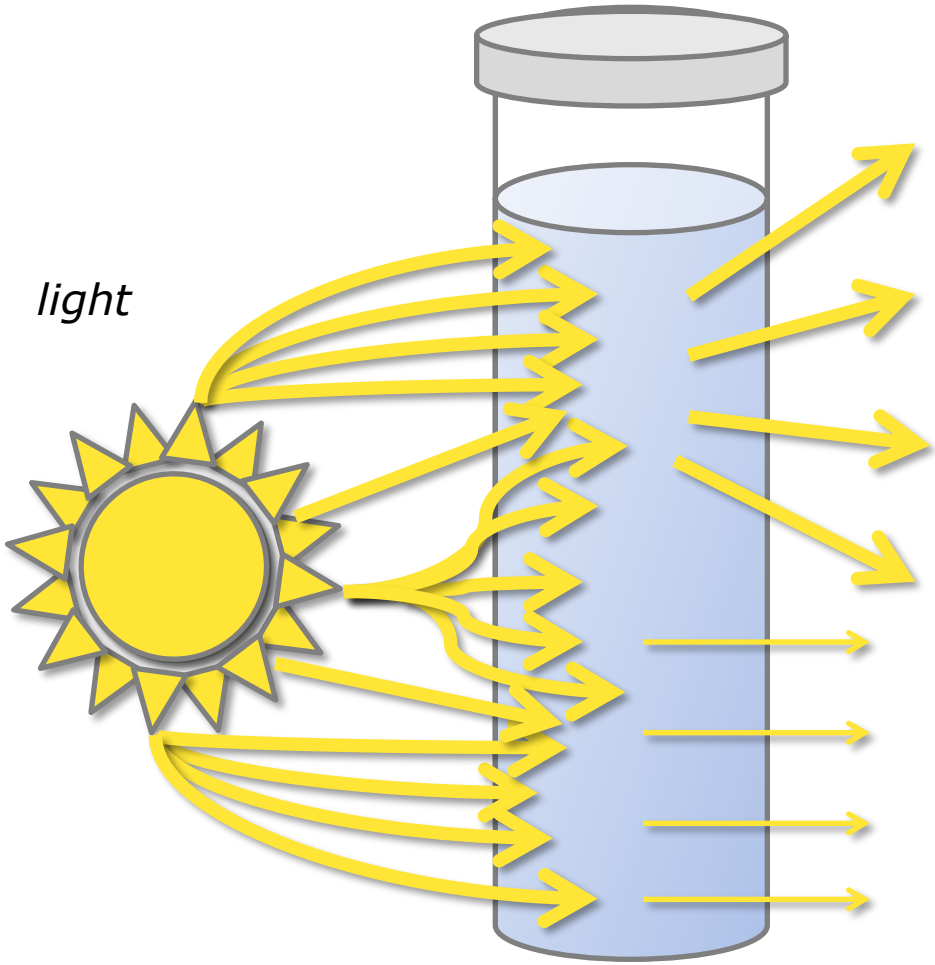


Immunodiffusion - II



p r e c i p i t a t a t i o n

... in liquides



PASSED LIGHT

NEPHELOMETRY *measurement is made by measuring the light passed through a sample at an angle*

LOSS OF LIGHT INTENSITY

TURBIDIMETRY

loss of intensity of transmitted light due to the scattering effect of particles suspended in it

E L I S A

enzyme-linked immunosorbent assay

principle of reaction

detection of antigen or antibody concentration

***enzyme** is used for visualization of reaction between antigen and antibody (anti-human Ig conjgated with enzyme)*

Use in clinical practice:

- *currently the most widely used laboratory method in immunological and clinical laboratories*
- *detection of antibodies (antibacterial, antiviral, autoantibody) or antigens*
- ***the high sensitivity of the assay allows detection of low concentration analytes***

- *ELISA is not suitable for detection od analytes with higher concetration*

E L I S A

enzyme-linked immunosorbent assay

principle of reaction

detection of antigen or antibody concentration

- *coating of the ELISA plate with diluted capture antibody or antigen*
  - *incubation and washing of the microtitre plate*
- *adding of investigated serum with or without antibodies against coated antigen (creation of immunocomplexes)*
  - *incubation and washing of the microtitre plate*
- *adding of appropriate dilution of the secondary antibody conjugated with enzyme (horse radish peroxidase)*
  - *incubation and washing of the microtitre plate*
- *adding of substrate to well*
  - *incubation and washing of the microtitre plate*
- *stopping of the enzymatic reaction*
- *reading of plates on an ELISA microplate reader*

I M M U N O F L U O R E S C E N C E

principle of the method

detection of antigen or antibody presence

flourochrome *is used for detection of antigen or antibodies (conjugate of animal antibody against antigen or human antibody in IgG, IgA or IgM class with fluorochrome)*

DIRECT IMMUNOFLOURESCENCE

- *detection of antigens or antibodies in tissues due to second antibodies conjugated with flourochrome*
- *diagnostic approach in SLE, vasculitis, glomerulonephritis, etc.*

INDIRECT IMMUNOFLOURESCENCE

- *detection of specific antibodies in serum of the patient (antibodies present in serum bind to antigen in tissue, they are visualized by animal anti-human antibodies conjugated with flourochrome)*
- *detection of antibody positivity*

e l e c t r o p h o r e s i s

principle of the method

- *the migration of charged colloidal particles or molecules through a stationary medium under the influence of applied electric field usually provided by immersed electrodes*
- *a method of separating substances, especially proteins, and a nalyzing molecular structure based on the rate of movement of each component in a colloidal suspension while under the influence of an electric field*

Application of electrophoresis in clinical practice:

- *Analysis and separation of protein mixture, charakterzation of bacterial or viral surface, diagnosis of monogenic diseases*

i m m u n o e l e c t r o p h o r e s i s

principle of the method

general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies

1. step

- *immunoglobulins migrate through the gel according to the difference in their individual electric charges*

2. step

- *antiserum is placed alongside the slide to identify the specific type of immunoglobulin present*

Application of immunoelectrophoresis in clinical practice:

- *the results are used to identify different disease entities, and to aid in monitoring the course of the disease and the therapeutic re sponse of the patient to such conditions as immunodeficiencies, autoimmune disease, chronic infections, chronic viral infections, and intrauterine fetal infections*

i m m u n o f i x a t i o n

principle of the method

electrophoretic separation of proteins in geles and following immunoprecipitazion with monospecific antisera

1. step

- *protein electrophoresis separates proteins based on their size and electrical charge in 6 lines*

2. step

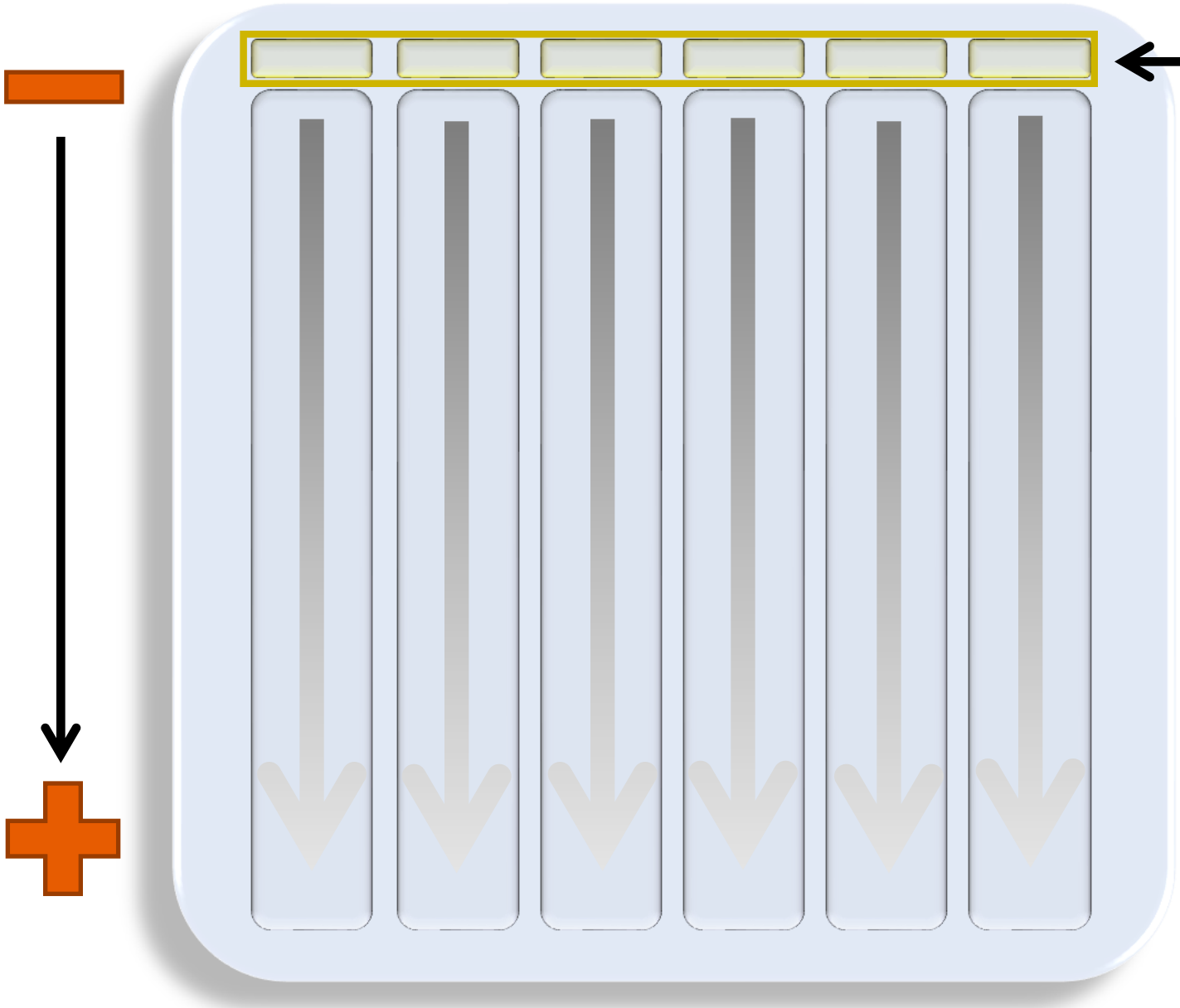
- *adding of monospecific antiserum (anti- IgG, IgA, IgM, kappa, lambda) one to each line*
- *diffusion of antigen and antibodies in gel – forming of immunocomplexes – precipitation in gel*

Application in clinical practice:

- *immunofixation of serum proteins (typing of paraprotein)*
- *imunofixation of urine proteins – detection and typing of Bence-Jones protein)*

immunofixation

Application of serum of one patient into 6 electrophoretic lines …



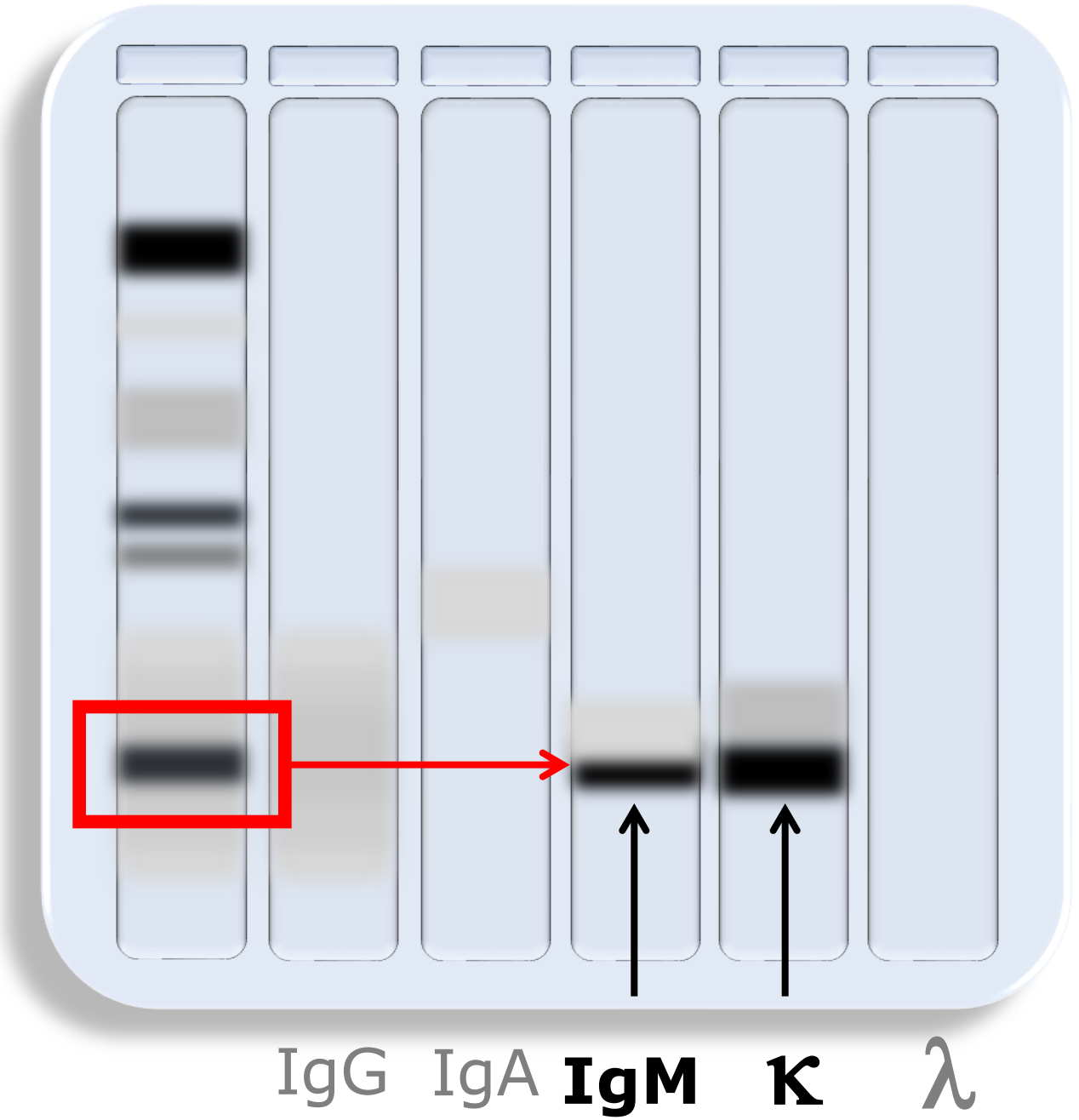
… separation of serum proteins according to the size and charge …

… electrophoretic separation of serum of 1 patient in 6 lines

… application of monospecific antisera against IgG, IgA, IgM and light and heavy κ a λ chains …

monoclonal immunoglobulin detection and typing

monoclonal band



Western blot
immunoblot

principle of the method

electrophoretical separation of proteins and their blot to membrane with following detection with specific antibodies

- *load and separate protein samples on SDS-PAGE*
- *electrophoretically transfer fractionated proteins onto PVDF membrane*
- *block the membrane with neutral protein (BSA or milk casein)*
- *incubate the membrane with primary antibody specific to target protein*
- *incubate the membrane with HRP-labeled secondary antibody specific to primary antibody*
- *incubate the blot with HRP substrate and expose to film*

Application in clinical practice:

- *tests for confirmation of HIV positivity, diagnostic of Borrelia infections, confirmation of hepatitis B positivity*

MOMOCLONAL GAMMOPATHIES

presence of abnormal proteins called monoclonal proteins or M proteins in the blood

these proteins are produced by a single clone (a group of identical cells) of plasma cells
(B lymphocytes producing antibodies)

paraprotein = M protein = monoclonal protein

MOMOCLONAL GAMMOPATHY

BIOCHEMICAL POINT OF VIEW

presence of monoclonal immunoglobulin in the blood or urine
(electrophoresis of serum or urine proteins)

CLINICAL POINT OF VIEW

presence of monoclonal immunoglobulin in the blood or urine
(electrophoresis of serum or urine proteins)

MOMOCLONAL GAMMOPATHIES

monoclonal gammopathies can be associated with various underlying diseases and conditions, and they are typically identified through blood tests and further diagnostic evaluations

<u>examples of the key monoclonal gammopathies</u>

monoclonal gammopathy of undetermined significance (MGUS)

multiple myeloma

Waldenström macroglobulinemia

AL amyloidosis

other less common monoclonal gammopathies

MOMOCLONAL GAMMOPATHIES

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS)

is an asymptomatic, premalignant clonal plasma cell proliferative disorder characterized by the presence of a serum M protein in persons who lack evidence of multiple myeloma, macroglobulinemia, amyloidosis, or other related diseases

it is defined by the presence of a serum M protein < 3 g/dL, bone marrow plasma < 10%, plus absence of anemia, hypercalcemia, lytic bone lesions, or renal failure that can be attributed to the plasma cell proliferative disorder

MOMOCLONAL GAMMOPATHIES

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS)

it is the most common plasma cell dyscrasia and is prevalent in approximately **3% of the general population 50 years of age** and older

the prevalence increases with age

the incidence has been reported as 1.7% in those 50 to 59 years of age and as over 5% in those older than the age of 70

age-specific incidence is higher in males than females

*the main clinical significance of MGUS is its **lifelong risk of transformation to myeloma or related malignancy** at a fixed but unrelenting rate of **1% per year***

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM)

accounts for about 10% of all hematologic malignancies

the diagnosis of MM requires the presence of one or more myeloma defining events (MDE) in addition to evidence of either 10% or more clonal plasma cells on bone marrow examination or a biopsy-proven plasmacytoma

MDE consists of established CRAB features (hypercalcemia, renal failure, anemia, or lytic bone lesions) and 3 specific biomarkers: clonal bone marrow plasma cells ≥60%, serum free light chain (FLC) ratio ≥100 (provided involved FLC level is ≥100 mg/L), and more than one focal lesion on magnetic resonance imaging (MRI)

Each of the new biomarkers is associated with an approximately 80% risk of progression to symptomatic end-organ damage in two or more independent studies

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM)

clinical manifestation

the clinical presentation of multiple myeloma can vary from person to person, and the disease can be asymptomatic (without noticeable symptoms) in its early stages, however as the disease progresses, various symptoms and complications may develop

bone pain and pathological fractures: *One of the hallmark symptoms of multiple myeloma is bone pain, which is often described as a deep, aching pain in the bones. This pain can occur in any bone but is most commonly felt in the back, ribs, hips, and skull. The bone pain is due to the weakening of bones caused by the growth of myeloma cells. Weakened bones in multiple myeloma can lead to fractures that occur with minimal or no trauma, known as pathological fractures.*

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM)

clinical manifestation

fatigue: *Many people with multiple myeloma experience significant fatigue and weakness. This can be related to anemia, a condition in which there is a shortage of red blood cells, which carry oxygen throughout the body.*

recurrent infections: *Multiple myeloma weakens the immune system, making individuals more susceptible to infections. This can lead to frequent or severe infections, such as pneumonia, urinary tract infections, and skin infections.*

unexplained weight loss: *Some people with multiple myeloma experience unexplained weight loss, which can be a result of the cancer's impact on metabolism and appetite.*

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM)

clinical manifestation

renal problems: *Myeloma proteins can damage the kidneys, leading to symptoms such as increased thirst, frequent urination, and swelling in the legs and ankles. This can progress to kidney dysfunction and failure if left untreated.*

hypercalcemia: *High levels of calcium in the blood (hypercalcemia) can occur in multiple myeloma and lead to symptoms like excessive thirst, frequent urination, constipation, nausea, vomiting, and confusion.*

neurological symptoms: *In some cases, myeloma-related proteins can affect nerves, leading to symptoms like numbness, tingling, weakness, and problems with coordination.*

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM)

clinical manifestation

bleeding and bruising: *Multiple myeloma can disrupt the normal functioning of blood clotting factors, leading to an increased risk of bleeding and easy bruising.*

anemia: *Anemia, which is a deficiency of red blood cells, can result in symptoms such as fatigue, weakness, pale skin, and shortness of breath.*

elevated blood protein levels: *Blood tests may reveal elevated levels of certain proteins, such as monoclonal (M) proteins or light chains, which are produced by myeloma cells and can be detected in the blood and urine.*

MOMOCLONAL GAMMOPATHIES

MULTIPLE MYELOMA (MM) TREATMENT

patients eligible for transplantation

typically, patients are treated with approximately **3–4 cycles of induction therapy** with bortezomib, lenalidomide, dexamethasone (VRd) prior to stem cell harvest

stem cell transplantation

patients eligible for transplantation

initial therapy is with VRd is administered for approximately 8–12 cycles, followed by maintenance therapy with lenalidomide

Thank you for your attention

SEROLOGICAL REACTIONS

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