Immunological laboratory investigation

SEROLOGICAL REACTIONS

Course no. 1

BASIC DIVISION OF IMMUNOLOGICAL LABORATORY INVESTIGATION

- **Serological investigation**
  - *material for investigation*
    - **SERUM**

- **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 INACTIVATION

Heating serum at 56 degrees for 30 minutes is used to inactivate complement in several immunological assays.

Proteins, such as complement, can interfere with the immune response of cell lines. Functional inactivation of complement cascade but it is possible to measure concetration of complement factors.

Inactivation of HIV virus.

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)*

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
- ***antiinflammatory treatment***
  - cytokine neutralization (anti-TNFα, anti-IL-1, IL6, IL-17)
  - adhesion molecules blocade (anti-LFA-1, …)
- ***antitumor treatment***
  - anti-CD20, anti-EGF
- ***antiallergic treatment***
  - anti-IgE, anti-IL-15
- ***antiaggregation 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

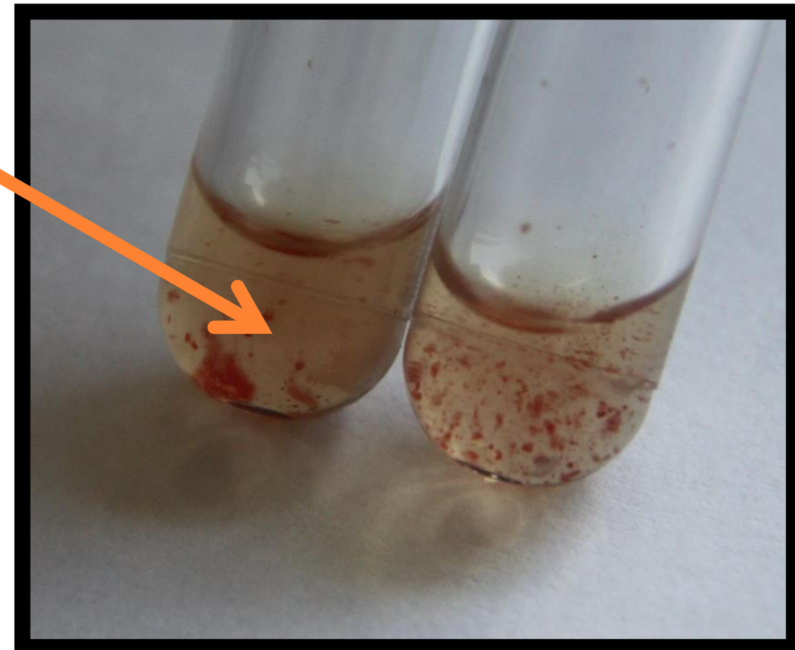
- ***visualization by secondary phase***
  - **AGGLUTINATION** *(direct, indirect)*
  - **PRECIPITATION** *(simple, in combination with electrophoresis, immunofixation)*
- ***visualization by following detection***
  - **IMUNOFLUORESCENCE**
  - **IMUNOANALYSIS** *(RIA, EIA, and modifications)*
  - **IMUNOBLOT,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

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

latex fixation test

detection of rheumatoid factor (RF)

autoantibody directed against Fc fragment of IgG

Principle of the method:

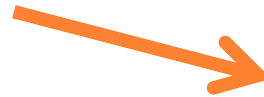
- *mix of investigated material (serum, urine or cerebrospinal fluid) with the coated latex particles in serial dilutions with normal saline (important to avoid the prozone effect) and observe for agglutination (clumping)*
- *agglutination of the beads in any of the dilutions is considered a positive result, confirming:*
  - *that the patient's body has produced the pathogen-specific antibody (if the test supplied the antigen)*
  - *that the specimen contains the pathogen's antigen (if the test supplied the antibody)*

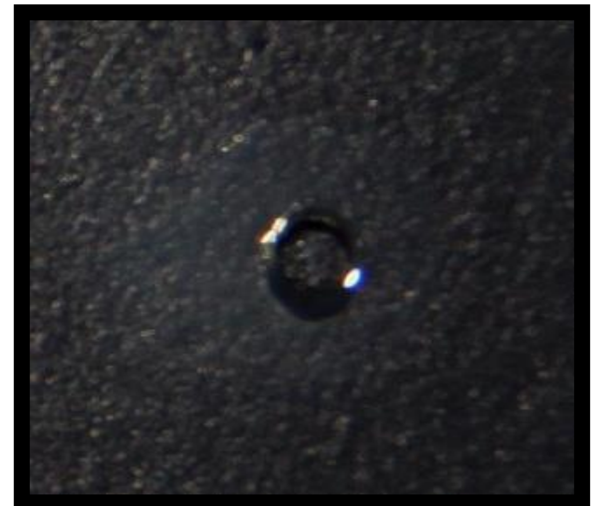
p r e c i p i t a t i o n

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.

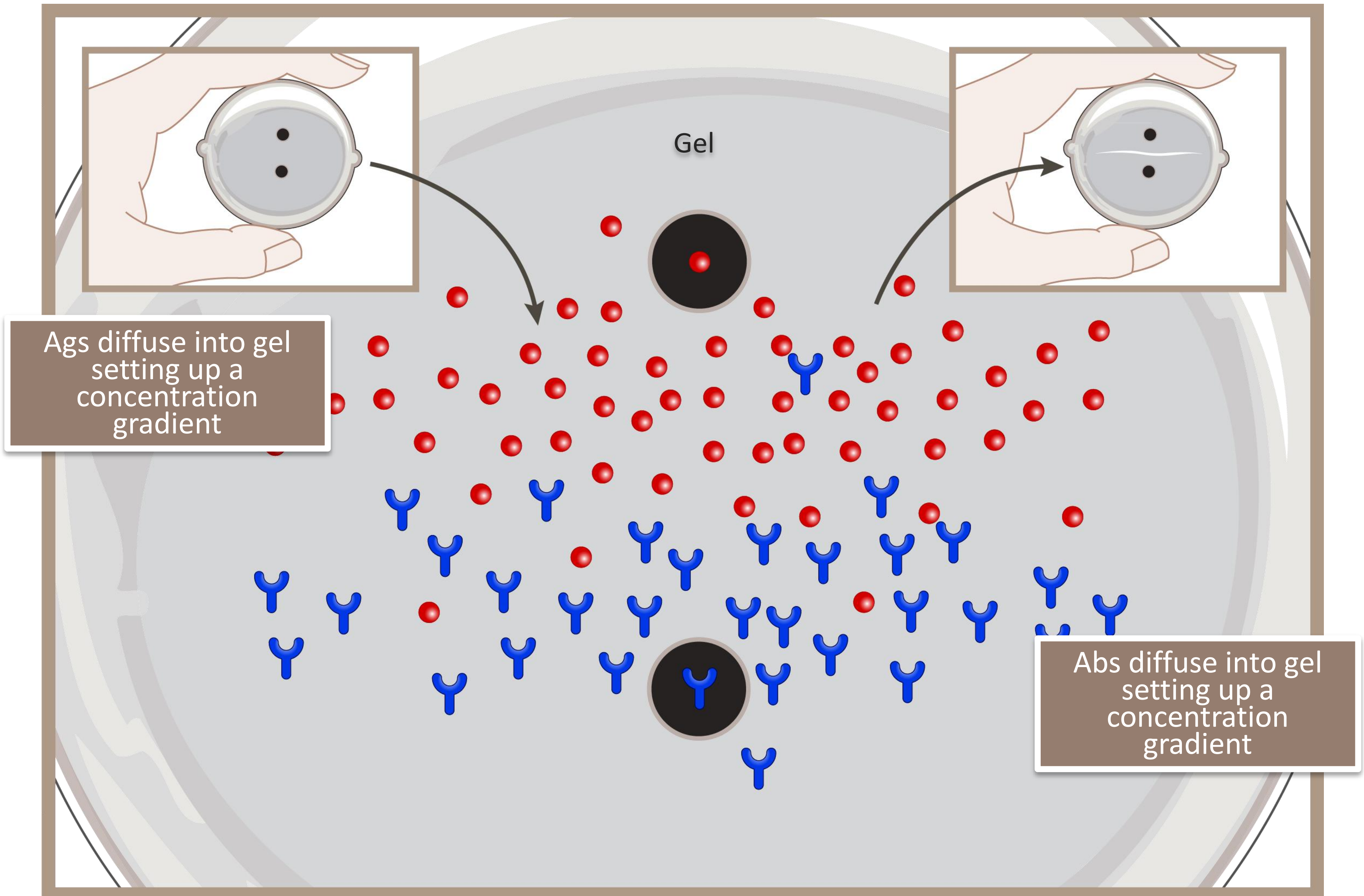




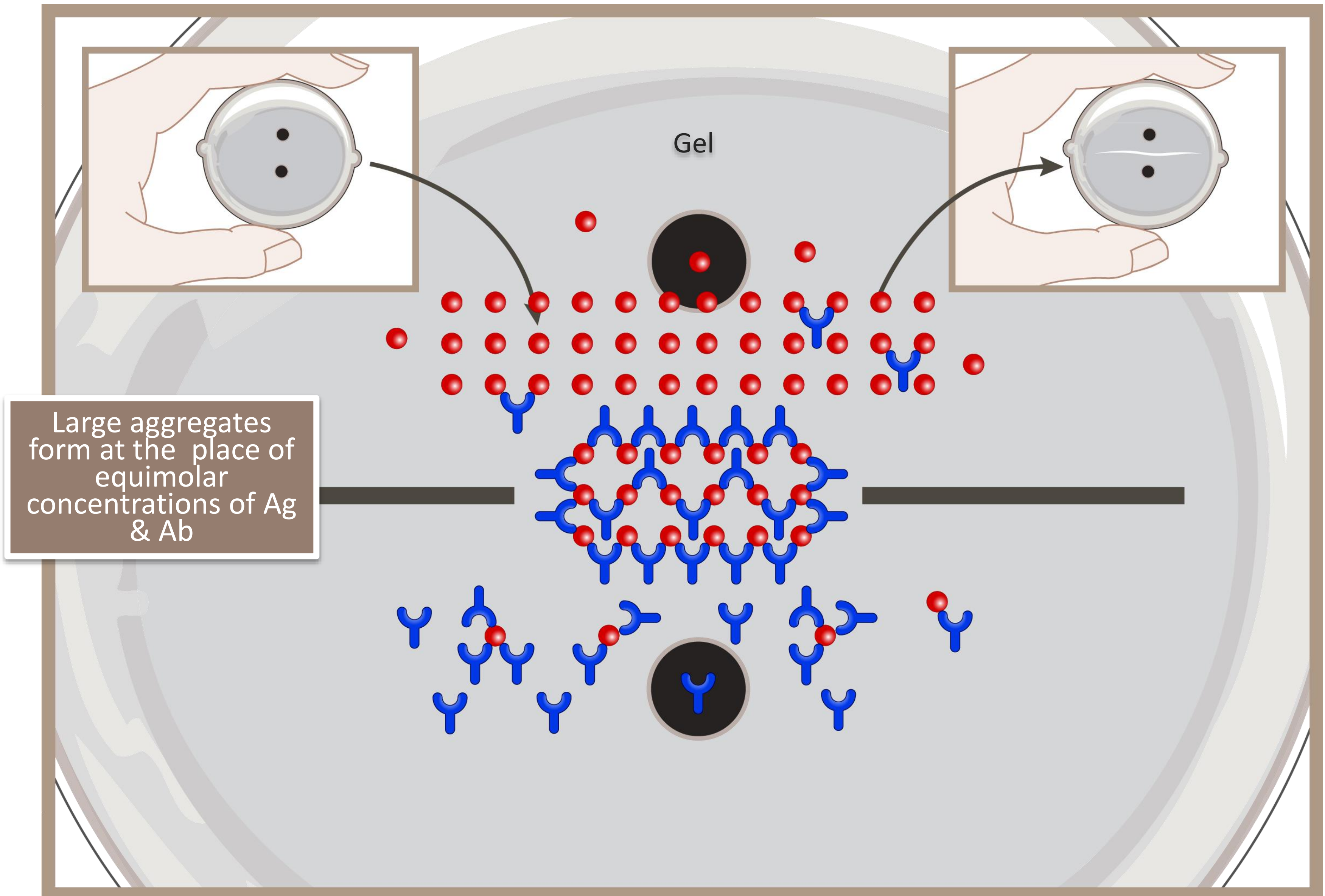
PRECIPITATION IN …

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

Immunodiffusion-I

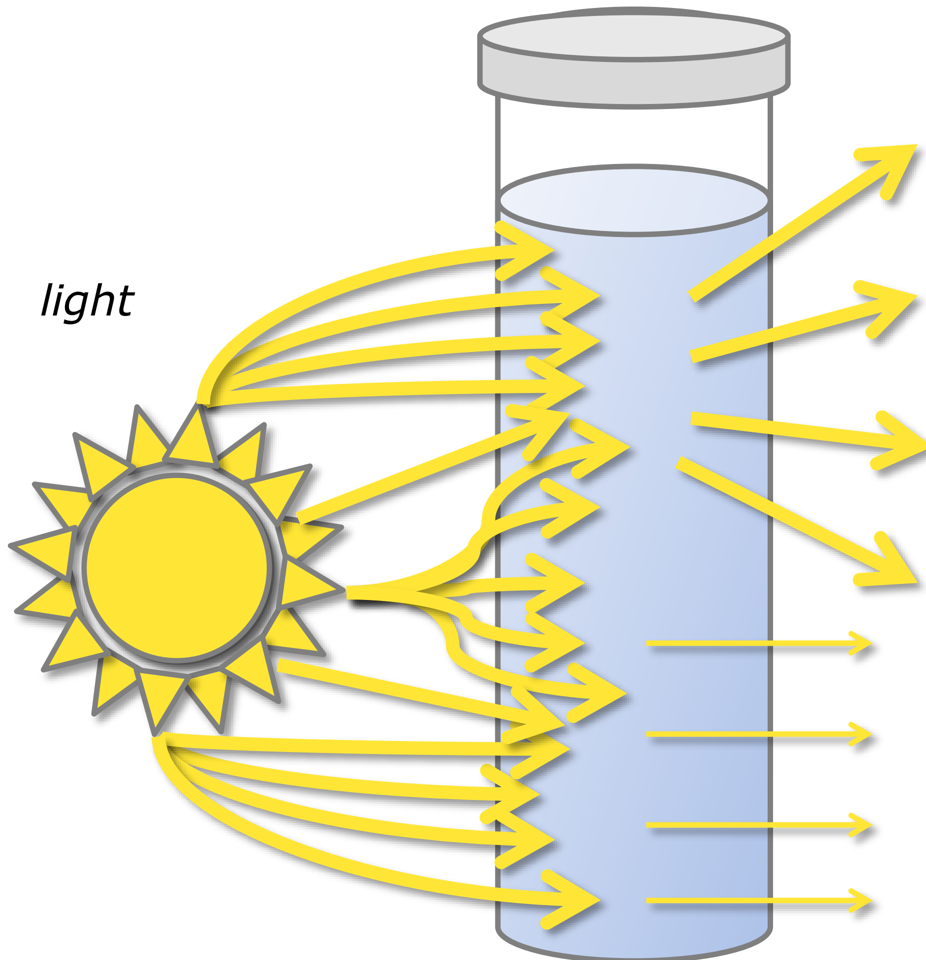


Immunodiffusion - II



precipitation

... in liquides



NEPHELOMETRY

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

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*

immunoelectrophoresis

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*

immunofixation

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)*

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*