Antigen-antibody reactions
Immunoagglutination
Immunoprecipitation

Department of Immunology
Faculty of Medicine
University of Belgrade

ANTIGEN – ANTIBODY REACTIONS

Basic characteristics of antigen-antibody reactions
Immunoagglutination
Immunoprecipitation

The antigen-antibody reactions are reactions between specific antigens and matching antibodies that occur in a laboratory under controlled conditions (*in vitro*). These reactions form the basis of immunological tests or **immunoassays** used for determining the presence of a variety of antigens or antibodies in samples. Since antibodies can bind with high specificity many different antigens, such as structural molecules of microbes and their secreted products or products of immune and other cells (e.g. cytokines, hormones, etc.), these methods have a wide range of application in laboratory diagnostics in different fields of medicine (biochemistry, immunology, microbiology, endocrinology, hematology, etc.), as well as in research. Antigen-antibody reactions are sometimes referred to as **serological reactions**, based on the fact that serum is commonly used sample in these reactions.

Antigen-antibody reactions can be seen as if they were equations with one unknown variable. In other words, based on the known variable (antigen or antibody) and the final result of the reaction (presence or absence of antigen-antibody complex), it is possible to determine the presence and the quantity of the other component in reaction (antibody or antigen, respectively). Thus, in order to detect an antigen of interest in a given sample, an antibody specific for that antigen should be used. However, if the goal is to detect antibodies in the sample, in addition to a known antigen, another antibody must be added as a reagent in the reaction. This antibody is specific for the epitopes of Fc fragment (constant region of heavy immunoglobulin chain) of an antibody of a particular isotype (class), such as γ-chain in the case of IgG detection, and, therefore, it is called **anti-immunoglobulin antibody** or **anti-antibody** (because they are specific for other antibodies). Anti-immunoglobulin antibodies are sometimes also referred to as the secondary antibodies, considering the fact that the antibodies that bind directly to an antigen are, so called, primary antibodies (Figure 1). These anti-immunoglobulin antibodies are usually obtained from animals that are immunized with immunoglobulins of another species (typically human) and they not only enable detection of specific antibodies in a sample, but also allow determining their class, which can help in diagnosis of many diseases.

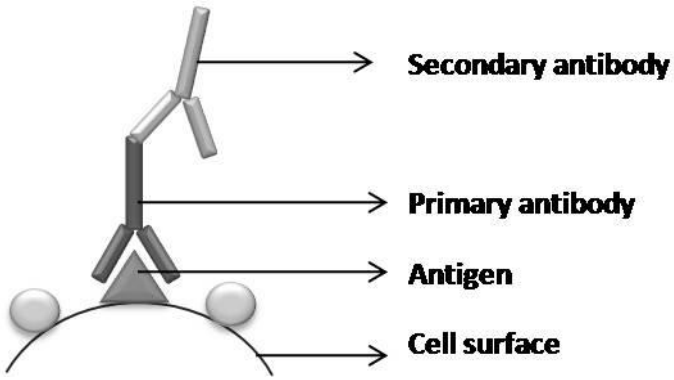

Figure 1. **Primary and secondary antibody**

In order to obtain valid results of immunoassays, it is necessary to detect or visualize antigen-antibody complexes (**immune complexes**) that are formed in reaction between antigens and antibodies. In general, there are two ways how the immune complexes may be detected in these tests:

1. Antigens and antibodies, under some conditions, tend to to form large complexes that can be detected by bare eye or using photometers (The extent of complex formation depends on the antigen characteristics, such as number of epitopes and size and solubility of an antigen, as well as antibody characteristics, primarily a number of binding sites for an antigen, which can vary from 2, in IgG, to 10 in IgM).
2. It is also possible to label one of the reagents, usually antibodies, with an appropriate marker (e.g. fluorescent dye), that can be detected upon completion of reaction (regardless of the size of the formed complex) by bare eye or using a specific equipment.

All assays based on the antigen-antibody reactions can be qualitative, semi-quantitative or quantitative. Results of the **qualitative tests** can be either **positive** or **negative**. Results of the **semi-quantitative test** are usually **graded** (for example, as borderline positive, weakly positive, moderately positive and strongly positive or often through the use of symbols, such as +, ++, +++ or Ø for negative result) or expressed as a **titer** of the antibody (see below). Results of the **quantitative tests** are presented mostly as **concentrations** using different units (e.g. μg/ml, nM/l or, sometimes, international units, IU/ml etc.). The concentrations of analytes present in a sample are calculated by comparison with **standards** (samples with known properties, i.e. concentrations) using **standard curve** (or **calibration curve**) that is formed by means of a serial dilution of the standard (see below).

In some cases (e.g. when a deficiency of humoral immunity is suspected), it is necessary to measure the total amount of particular class of immunoglobulins in a serum of a patient (IgG, IgM or others), irrelevant of their specificity, and this amount is expressed as a concentration. In contrast, when the quantity of antibodies specific for a particular antigen is measured (e.g. amount of the antibody specific for an antigen of a microorganism in suspected infectious disease in a patient), in most cases it is not possible to determine the concentration accurately due to a small number of specific antibodies relative to the total amount of antibodies in the sample. Therefore, the amount of specific antibody is expressed as **titer**. For determination of the antibody titer, it is necessary to make a serial dilution of the sample, most commonly two-fold serial dilutions (e.g., 1/2, 1/4, 1/8, 1/16 and so on). Each of these dilutions reacts with standard (conventional, prescribed) amount of specific antibody and the result is determined (positive or negative). **The titer** represents the **reciprocal value** of the **highest dilution** of the sample resulting in a **positive reaction**. For example, if the positive reaction is obtained with 1/200 dilution, and the negative with dilution 1/400, then the titer is 200. In practice, the results are often presented as the highest dilution that gives a positive reaction (e.g. 1/8, 1/200, etc.). Sometimes, a change in antibody titer over time is used for a diagnosis of infectious diseases. To this end, two samples are taken from the same patient at two different time points (usually two weeks apart) and the amount of the antibodies specific for a microorganism that causes disease that is suspected in a patient is measured in both sample using the same method. A four-fold or higher increase in antibody titer between the two samples is considered significant.

Every time the immunoassay is performed, it is necessary to use negative and positive controls. The negative control perfectly mimics the sample but it does not contain the tested substance, whereas the positive control must contain the substance that gives positive result in reaction. The test is considered to be correctly performed and the results are regarded as reliable, if the negative control is clearly negative and the positive control clearly positive. The reliability of the results of each test based on antigen-antibody reactions can be expressed through its sensitivity and specificity. Briefly, **sensitivity** (also called the **true positive rate**) measures the proportion of positives which are correctly identified as such (e.g. the percentage of sick people who are correctly identified as having the condition), and is complementary to the **false negative rate** (e.g. in the assay with the sensitivity of 97%, we can expect 3% of false negative). **Specificity** (also called the **true negative rate**) 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) and is complementary to the **false positive rate** (in the assay with the specificity of 95%, we can expect 5% of false positive). (For more detailed explanation of these statistical measures please consult statistics books). Monoclonal antibodies are used in the majority of modern tests based on the principle of antigen-antibody reactions, which is why these tests generally have high specificity and sensitivity and give reliable results.

Finally, it is also important to point out that a two-way communication between doctors who require an analysis and the doctors and laboratories where analysis is performed is necessary for the proper interpretation of the test results. For this purpose, laboratory should receive all relevant information on the patient whose sample has been sent to laboratory, and, conversely, laboratory should provide a reference values and specify the method used in testing together with the results of analysis. Results of two analyzes can be compared only if they were performed using the same method.

The basic principles of the most commonly used tests based on antigen-antibody reactions will be described, with some examples of their use in everyday clinical practice.

Immunoagglutination

Immunoagglutination (often simply called **agglutination**) is a laboratory diagnostic test based on the reaction between a particular antigen and the matching specific antibody, wherein the **antigen is insoluble**, or represent an integral **part of a large insoluble particle** (e.g. red blood cells, bacteria or inert particles). In this reaction, a large number of antibody and antigen molecules cross-link (agglomerate) in a big branched immune complexes and, as a result, aggregate called **agglutinate** is formed that can be detected by the naked eye (or optionally with the magnifying glass). **Hemagglutination** is a specific form of agglutination that involves red blood cells. All antibody isotypes can agglutinate antigens, but IgM antibodies have the most prominent capacity for agglutination, since they are pentamers and have 10 binding sites for antigen. In addition to the class of antibody, the effectiveness of agglutination is influenced by the amount of antigen and antibodies in the reaction. Optimal agglutinate formation occurs when the amounts of antigen and antibody are approximately equal (so called **zone of equivalence**). If either the antigen or the antibody is in excess, they will react, but very small complexes will form that do not clump to form visible agglutination resulting in false negative result. The lack of agglutination at high concentrations of antibodies is traditionally called **prozone effect** (or **prozone**

phenomenon). This problem can be solved by diluting the tested sample, which reduces the amount of antibodies, so that the equivalence between the antigen and the antibody can be achieved.

Immunoagglutination can be **direct** or **indirect** (indirect is sometimes termed **passive**). In direct immunoagglutination, the antigen (antigenic determinant) is **an integral part of a large particle** (e.g. bacterial surface molecule) that is directly agglutinated by antibodies, while in **indirect** immunoagglutination **the antigen is a soluble molecule** passively adsorbed or chemically attached to the surface of **a large insoluble particle** (erythrocytes, polystyrene inert particles or latex microbeads) that becomes passive carrier of this antigen.

Immunoagglutination methods are generally **qualitative tests** (results are reported as positive or negative) but may also be **semi-quantitative** (e.g. detection of the antibody titer). They are usually performed on the slide glass or in a test tube within 10 minutes. These tests have **satisfying reproducibility**, **specificity** and **sensitivity** and they are **inexpensive** and **easy to perform**. Therefore, immunoagglutination tests are often used in laboratory diagnostics in medicine. One of the most important applications of direct immunoagglutination is in **determination of blood types**. In addition, immunoagglutination (both direct and indirect) has been used in microbiology for the **diagnosis of many infectious diseases**, on the basis of detection of specific antibodies or antigens in the patient's samples (e.g. serum or cerebrospinal fluid). Indirect immunoagglutination is also used for **detection of various autoantibodies** that may occur in many autoimmune diseases. A typical example is the **rheumatoid factor** (RF) that represents an autoantibody specific for another antibody (typically IgM that is specific for Fc fragment of IgG). RF occurs in serum of patients with rheumatoid arthritis, as well as some other rheumatic diseases, and its identification is important in the diagnosis and/or prognosis of these diseases.

A **Coombs test** (or **antiglobulin test**) is a special form of immunoagglutination used for the detection of anti-erythrocyte antibodies that bind to red blood cells but do not lead to their agglutination. Such antibodies occur in a variety of diseases such as hemolytic anemia (autoimmune or caused by medication) or hemolytic disease of the newborn. The latter disease can be caused by transplacental passage of maternal anti-RhD IgG antibodies from Rh negative mother's bloodstream that is sensitized to the Rh positive fetus (today, sensitization can be prevented by using appropriate therapy). There are two types of Coombs tests, the **direct** and **indirect Coombs test**. The **direct Coombs test** (**DCT**, also known as **direct antiglobulin test** or **DAT**) examines whether the red blood cells of a patient (e.g. the one with hemolytic anemia) have already been coated with sub-agglutinating amount of an anti-erythrocyte antibodies. Test is done by ensuring that an antibody specific for human immunoglobulins (anti-immunoglobulin antibody) is added to the patient sample, which leads to agglutination of red blood cells. The **indirect Coombs test** (also known as **indirect antiglobulin test** or **IAT**) is used for detection of anti-erythrocyte antibodies in the person's serum, (e.g. the presence of anti-Rh antibodies in a serum of Rh negative mothers) and is done in two phases. In the first phase, patient serum is incubated with human erythrocytes (commercially available), and then, in the second phase, anti-immunoglobulin antibody is added, which leads to agglutination of red blood cells.

Immunoprecipitation

Immunoprecipitation (also called **precipitin reaction** or **precipitin test**) is a laboratory diagnostic test based on the **precipitation** (deposition) of the **soluble antigen** by the matching specific antibody (often called **precipitin**) due to the formation of large insoluble immune complexes**.** The result of the reaction is a **precipitate**, which can be easily detected (usually with the bare eye) and its formation only occurs when optimal **concentration ratio** of antigen and antibody (approximately equal amounts) is achieved (**the zone of equivalence**). In the case of antigen or the antibody excess, the reaction between them will occur but the small soluble complexes form (so called microprecipitates) that remain in the solution. Although invisible for naked eye, these complexes can still be detected using the appropriate device, such as nephelometer (see below).

Immunoprecipitation assays can be performed in solution (**liquid medium**) and in gel made of agar, agarose or polyacrylamide (**semisolid medium**). Both types of methods are based on the diffusion of the soluble antigens and/or antibodies in appropriate medium, whereby the diffusion in the gel occurs more slowly. The precipitate in the **liquid** media is formed **relatively quickly** (often within 10 minutes), while the precipitation in the **gel** takes much **longer time** (sometimes up to 72 hours). Therefore, the latter method is sometimes performed by using an electric current, with the aim to accelerate the diffusion and formation of precipitates (electroimmunodiffusion method). Precipitate that form in liquid media is unstable and it is often invisible (but can be detected using nephelometer). On the other hand, precipitation in gel is characterized by formation of clearly visible **precipitation line** at the site where concentration ratio of antigen and antibody reach equivalence and it is stable for longer period of time.

Immunoprecipitation is most commonly used as **quantitative test** to determine the amount of antigen or antibody in **liquid samples**, such as serum, plasma or cerebrospinal fluid. It is primarily used for **determining the amount of certain classes of immunoglobulins** in the blood which is an important diagnostic procedure in clinical immunology, given that the concentration of immunoglobulin is often changed in some disorders, like immunodeficiencies (e.g. hypogammaglobulinemia). This method is also important for **quantification** of other **plasma proteins** (in addition to immunoglobulins), such as complement components (C3 or C4), C-reactive protein (CRP), etc. Immunoprecipitation is used as **qualitative** and **semi-quantitative test** for detection of a certain antigen and/or specific antibodies in the serum, too. Apart from clinical immunology, precipitin test is sometimes used in microbiology (for detection of some microbial products) and in criminology for determining the human or other source of a bloodstain. Finally, in biochemistry, immunoprecipitation can also be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins.

Quantitative methods of immunoprecipitation

Quantitative methods of immunoprecipitation most commonly used for measuring the amount of immunoglobulins and other proteins in the serum or other biological fluids are **nephelometry** performed in liquid medium and **radial immunodiffusion** performed in gel. **Nephelometry** is a modern **quantitative** immunoprecipitation method that is used for rapid and accurate **measurement of the concentration of many proteins** in liquid samples (e.g. classes of immunoglobulin, components of the complement system, C-reactive protein,

etc. in sera). It is based on turbidity measurement of the **microprecipitates** (made of antigen-antibody complexes) in a sample. In other words, antibody and the antigen are mixed in concentrations such that only small aggregates are formed that do not quickly settle to the bottom. The turbidity is measured using specific device called **nephelometer** by passing light through the sample at an angle, which is than scattered by those aggregates. Concentration of the protein of interest is determined by comparing the obtained values for the tested protein with the corresponding values of a standard (samples of known concentration). Nephelometry is a relatively expensive method (expensive equipment and reagents), but it is also a **very sensitive**, **accurate** and **reliable** technique that is used on a regular basis in clinical practice.

The radial immunodiffusion (**RID test**) is also **quantitative** method, which is based on a diffusion of the soluble antigen through a gel that contains incorporated uniformly distributed antibody (given that only the antigen diffuses, this technique is sometimes called **single radial immunodiffusion assay**). In the zone of equivalence an insoluble **precipitate in the shape of a circle** is formed (antigen is placed in a well that is punched out of the gel medium and diffuses in all directions). When such a circular precipitation line is formed, the diameter of the circle and the amount of applied antigen are in direct proportion, which can be used for determining the unknown concentration of antigen in the sample, by comparison with the standards (i.e. standard curve). RID test is used to measure the **concentration** of a large number of **plasma proteins** (e.g. IgG, IgM, IgA, C3, C4, etc.). Compared to nephelometery, this technique is **less sensitive and precise** and more cumbersome to perform, but it is considerably **less costly**. Therefore, this technique is usually used in small diagnostic laboratories that have a limited number of samples, and do not have nephelometer. However, RID test cannot be used for the measurement of proteins that are present in low concentrations in the blood such as immunoglobulin E (IgE).

Qualitative methods of immunoprecipitation

Double gel immunodiffusion is often used for qualitative detection of an antigen or specific antibody in a tested sample. This assay is based on the migration of both soluble antigen and antibody (hence the name “double“). In this technique, the formation of the precipitation line in the zone of equivalence indicates the presence of tested antigen (or antibody) in the sample and the result is considered positive. If the sample does not contain tested antigen/antibody, no complex formation will occur and the absence of precipitin line is reported as negative. Although this assay is a **qualitative**, under certain conditions it can be **semi-quantitative** (the quantity of the tested analyte is inferred from the position of the line in a gel)**.** This **relatively reliable** and **inexpensive** technique is still often used, usually to determine the presence of specific **autoantibodies** in sera from the patients with various rheumatic diseases.