CHAPTER

Introduction to immunological techniques in the clinical laboratory

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1 Introduction

The field of immunology has exponentially expanded in the last few decades. This has resulted in the rise of several powerful immunodiagnostic techniques that have revolutionized clinical analysis laboratory medicine including the study of microbial detection. In this chapter series we describe several of these techniques including an overview of their immunological basis.

2 Basics of the immune response

The immune system includes multiple components such as organs, cells, and the chemical messengers used for communication. The function of the immune system is to respond to the myriad of foreign substances that invade the body while ensuring that self-antigens remain unharmed. The immune response is traditionally divided into innate and adaptive components. The innate immune response is swift but does not display memory of an encounter with a pathogen while the adaptive immune response is slower, but through the formation of long-living memory lymphocytes, has acquired memory (Danilova, 2006; Male, Brostoff, Roth, & Roitt, 2013). Lymphocytes are mainly divided into three main types based on specific receptors on their surface: T-cells (display T-cell receptor or TCR), B-cells (display B-cell receptor or BCR) and Natural Killer cells (reviewed in Helbert, 2017). There are several subtypes of T-cells such as T-helper, T-cytotoxic, and T-regulatory and those are also differentiated based on specific membrane receptors. The innate immune response comprises the first and second lines of defence, while the adaptive response comprises the third. The first line of defence constitutes structures like the skin, mucus membranes, tears, defensins, etc. These are further divided into physical, chemical

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and biological barriers. The second line, involves physiological responses such as inflammation, fever, phagocytosis, complement activation, interferon (IFN) production, etc. (Helbert, 2017). Pattern recognition receptors, such as Toll-like receptors, play a vital role in detecting pathogen-associated molecular patterns, such as bacterial peptidoglycan or double-stranded RNA, and hence alert the body to the presence of the foreign substance (Akira, Uematsu, & Takeuchi, 2006; Beutler, 2009; Hoffmann, 2003; Medzhitov, 2007).

2.1 Interferon and the QuantiFERON-TB Gold assay

The interferon (IFN) protein family consists of two main types, type I and II. Type I IFNs include IFN alpha and beta, while IFN-gamma belongs to the type II family (Green, Cooperband, & Kibrick, 1969; Isaacs & Lindenmann, 1957; Wheelock, 1965; reviewed in Helbert, 2017). Type I IFNs are antiviral molecules and promote viral resistance by preventing the spread of viral infection (Wheelock, 1965; reviewed in Male et al., 2013). On the other hand, IFN-gamma is a powerful immune modulator that mainly activates T-helper I cells, which are the principal regulators of cell-mediated immunity (Wheelock, 1965; Gray & Goeddel, 1982). In this regard in the early stages of infection, IFN-gamma is produced primarily by activated natural killer (NK) cells (a subset of T cells) which stimulates macrophages to perform multiple functions, including to further activate certain T-cell subsets (Parham, 2014). IFN-gamma also mediates immunoglobulin class-switching by B-cells, stimulates T-cytotoxic cells (CD8+), and inhibits T-helper 2 cells (Helbert, 2017). This illustrates the important role of IFN-gamma in cell-mediated immunity and chronic diseases caused by intracellular bacteria that require a cell-mediated immune response such as tuberculosis. The concomitant release of IFN-gamma in response to a pathogen such as *Mycobacterium tuberculosis* is the basis for the QuantiFERON-TB Gold test (QFT) which will be further discussed later in this chapter.

2.2 Adaptive immune response

If the innate immune response is insufficient to deal with a foreign substance, the adaptive immune response intervenes. This is done through interaction between several effector lymphocytes, mainly T-helper, T-cytotoxic, and B-cells, depending on the pathogen (Mosier & Coppleson, 1968). The interaction between the aforementioned cells of the adaptive immune response is mediated via soluble paracrine/autocrine chemical messengers called cytokines (Cohen, Bigazzi, & Yoshida, 1974). If the pathogen is extracellular, the humoral immune response predominates, whereas intracellular pathogens trigger primarily a cell-mediated immune response. It is important to note that both arms of the adaptive immune response are activated with one of them being the predominant component, depending on pathogen cellular localization.

The humoral immune response, first described by Hans Buchner in 1890, exacts its effect through differentiated B-cells called plasma cells (Metchnikoff, 1905).

In most cases antibody production occurs initially after the processing of antigen by antigen presenting cells such as macrophages or dendritic cells. This is followed by the interaction of the processed peptide antigen with MHC molecules and the T-cell receptor located on the surface of helper T cells. Upon completion of the preceding events, B-cells differentiate into plasma cells that secrete soluble effector molecules known as antibodies or immunoglobulins (Ig). Igs are large proteins made up of four polypeptides, two heavy and two light chains (Edelman & Gally, 1962), with high specificity to an antigenic determinant similar to a receptor-ligand interaction, that is to say non-covalently. In the Ig molecule, the two heavy and two light chains are joined together by intermolecular disulfide bonds. The heavy chains determine the five antibody isotypes: alpha for IgA, mu for IgM, gamma for IgG, delta for IgD, and epsilon for IgE. Antibodies are typically monomeric, such as IgG, IgD, and IgE, while IgM is a pentamer and IgA is either a monomer or dimer. The different Ig isotypes have distinct properties. IgM is typically the first Ig produced during a primary immune response partly due to its effective activation of the complement system. This is followed by IgG which is also considered the protective antibody due to its exclusive release by memory B-cells. IgD is the B-cell receptor (Preud'homme et al., 2000) while IgA is typically found in bodily secretions and plays a pivotal role in mucosal immunity (Hochman, Inbar, & Givol, 1973). IgE is usually associated with type I hypersensitivity (allergy) as well as immunity to parasitic worm infections.

Plasma cells use genetic recombination to randomly shuffle a few thousand genes to generate the billion combinations of antibodies that enable us to respond to the multitude of antigenic possibilities (Hozumi & Tonegawa, 1976; Litman et al., 1993). Antigens bind to antibodies on the surface of B-cells and thus determine the clone of cells which will be propagated from the repertoire of existing clones (Parker, 1993). Both light and heavy chains of an antibody contain both a variable and constant region. The variable region of the immunoglobulin binds specifically to the antigenic determinant called the epitope. B-cells can also undergo a state of hyper-mutagenesis, thereby generating antibodies that bind known epitopes with an even higher affinity. The collective heterogenous antibodies produced by different clones of plasma cells that respond to multiple epitopes are termed polyclonal. Monoclonal antibodies that respond to only one epitope are the products of recombinant DNA technology and are not normally generated in the body (B-cell myelomas are an exception). The inherent specificity and affinity of an antibody to an epitope is the basis for many serological methods used in clinical laboratory diagnostics.

2.3 Antibodies and immune detection

While antibodies are highly specific for an epitope on an antigen, cross-reactivity exists because of epitope similarities between different antigens. This can be consequential in the interpretation of antibody-based diagnostic assays and is often the reason for false-positive results which may require additional testing. The remainder of

this chapter will describe selected examples of how different qualitative and quantitative assays are used and the general principle of each test. In this introductory chapter we briefly describe select immunological techniques that are in use in the clinical laboratory.

3 Hypersensitivities

Originally proposed by Gell and Coombs (1963), there are four types of hypersensitivity reactions (types I–IV). Type I hypersensitivity, commonly referred to as allergy, involves IgE release from sensitized B-cells which results in the release of histamine from basophils and mast cells through degranulation. Type II hypersensitivity is mediated by IgG or IgM binding to antigens on cell surfaces or extracellular matrix which triggers cell death, also referred to as antibody-mediated cytotoxicity and, in certain cases, is associated with autoimmune disease. Type III hypersensitivity involves circulating antigen-antibodies (mainly IgG) that deposit into tissues, resulting in damage and vasculitis. This hypersensitivity is also referred to as immune complex disease (Kumar, Abbas, & Aster, 2014). While types I–III hypersensitivities are antibody-mediated, Type IV is cell-mediated. In this scenario, both T-helper 1 cells and macrophages are involved and react with the release of cytokines—typically 48–72 h after exposure (Black, 1999). The Mantoux (PPD) TB skin test is a type IV hypersensitivity.

4 Survey of current immunological techniques

4.1 Serology

Serology is the analysis of bodily fluids such as blood, semen, saliva and cerebrospinal fluid. Serologic studies are common in the Forensic Sciences and Medicine. In medical analytics, serology most commonly refers to the diagnostic identification of serum antibodies directed against pathogens, such as bacteria and viruses, relevant to the patient's clinical condition for which culture or some other diagnostic procedure is not helpful, but also other macromolecules recognized as non-self can be detected by this detection system. Related techniques are therefore used in diagnostic microbiology, ABO and Rhesus blood typing, auto-immune and more recently allergy diagnostics.

4.1.1 Agglutination

4.1.1.1 Introduction

Agglutination (clumping) is the oldest antibody-based reaction in medical diagnostics and is currently used in haematology and diagnostic microbiology. Specific agglutination was first described by the British physician Herbert Edward Durham and Austrian bacteriologist Max Von Gruber in 1896 and is known as the Gruber-Durham reaction. The first example of serum diagnosis underlying this principle

was applied in the diagnosis of typhoid fever the same year by the French physician Fernand Widal, the inventor of the Widal test (Mochmann & Köhler, 1989). In 1900, the Austrian physician Karl Landsteiner reformed transfusion medicine by introducing hemagglutination based on ABO blood typing (Landsteiner, 1900).

4.1.1.2 Working principle

Agglutination occurs when an antigen of interest is mixed with a matching antibody, referred to as isoagglutinin. Visual clumping occurs because one antibody simultaneously binds multiple antigens thereby forming larger complexes. Within the body, IgM is particularly well suited for this reaction because of its pentameric structure and high avidity. It is therefore also one of the first antibody isotypes expressed after antigen exposure during a primary immune response.

4.1.1.3 Different types of agglutination tests

4.1.1.3.1 Hemagglutination. Immune hemagglutination, used for cross-matching donor red blood cells (RBCs), refers to coagulation of RBCs, with antibodies either directed to the RBC themselves or specific carbohydrates coating the cells (Gyenes & Sehon, 1962). The method is highly sensitive, and the degree of agglutination is directly proportional to the concentration of free antibody against RBC antigenic determinants and inversely proportional to the amount of antigen. A modification of this technique, for the purpose of detecting an infection, is the indirect or passive hemagglutination assay. Examples of this type of test procedure, that has been used for many years by clinical diagnostic laboratories, are well described in one of the other chapters (Pavia, 2020) included in this volume.

4.1.1.3.2 Hemagglutination inhibition. Non-immune hemagglutination can be measured and quantified in response to blood-borne pathogens, such as viruses or bacteria (Bricout, 1962; Neter, 1956). Associated tests measure exposure to a hemagglutinating pathogen via plasma concentrations of corresponding antibodies—typically IgM (early response) or IgG (immune status). Because serum antibody concentration and pathogen exposure are directly proportional, the highest serum dilution that blocks hemagglutination can be referred to as the hemagglutination titre.

4.1.1.3.3 Coombs test. The Coombs test, named after the British Immunologist Robin R. Coombs, together with his colleagues, first described it in 1945 (Coombs, Mourant, & Race, 1945). There are two types of Coombs test, the direct and indirect antiglobulin test (DAT, IAT). Both utilize rabbit anti-human globulin that is also referred to as Coombs reagent. Common uses of this test include suspicion of autoimmune hemolytic anaemia, prenatal compatibility testing, and cross-typing for blood transfusions. The direct Coombs test assesses antibodies adherent to the patient's own RBCs, while the indirect test measures free serum antibodies against RBCs of a fetus during pregnancy or recipient of a blood transfusion.

4.1.1.3.4 Antibody detection agglutination. This immune-PCR detection method is a recent addition to the clinical laboratory medicine agglutination repertoire and is exceptionally sensitive (Tsai, Robinson, Spencer, & Bertozzi, 2016). It combines

the specificity of antibody-antigen interaction with the sensitivity of PCR amplification. The capture and identification of autoantibodies, for example, is much more sensitive than with ELISA or radioimmunoassay (RIA). For this purpose, specific capture antigens are conjugated with short synthetic DNA strands which by themselves cannot be amplified. If antibodies of interest are present in an analytical sample, they will agglutinate several antigen-DNA conjugates. The proximity of the synthetic DNA strands facilitates ligation and PCR amplification for quantitation.

4.1.1.3.5 Latex fixation/agglutination test (LA test). The LA Test is clinically used for identification and typing of microorganisms in patient samples such as serum, urine or cerebrospinal fluid (Leinonen, 1980). It is based on the principle that a pathogenic encounter will result in a chronic pathogenic presence and/or the formation of pathogen-specific antibodies. Contrary to the other agglutination techniques, pathogen-specific antigens or antibodies are immobilized to latex microbeads acting as agglutination matrix once positive patient samples are added in serial dilution. This technique is popular for use with suspected bacterial, viral or autoantibody presence.

4.1.2 Immunodiffusion/precipitation reactions

While methods in this category are less specific than agglutination reactions, some have remained the gold standard among serological techniques in the clinical laboratory. Their underlying principle is based on the diffusion of an antibody (called precipitin) and antigen through a matrix, such as a semi-solid gel, and the formation of a visible precipitate upon encounter. One of those tests is the Ouchterlony passive double immunodiffusion test which was first described in 1948 and named after the Swedish physician, Ouchterlony (1948). Here, the two reactants (i.e., cell extract and purified precipitin or serum) are loaded into adjacent wells of an agar/agarose matrix and allowed to diffuse toward each other. A positive reaction will be visible as a white line in the matrix which is most intense at the site of perfect proportion between antigen and precipitin. The Mancini method, also known as radial immunodiffusion and used for antigen quantitation, was developed by the physician scientist Giuliana Mancini in 1965 in Turin, Italy (Mancini, Carbonara, & Heremans, 1965). In this method, precipitin is evenly embedded into the gel matrix (either on a microscopic slide or in a dish) with the antigen loaded centrally and allowed to diffuse radially. The diameter of the precipitation circle is then interpreted in comparison to a quantitation standard.

4.1.3 Immunoassays

Clinical immunoassays are a group of biochemical methods using specific antibodies and occasionally antigens, to determine the presence and concentration of macromolecules of interest against an internal standard of the same molecule of known concentration (calibrator). These antigens are referred to as analytes and they can differ in size or type as long as they are compatible with a water-soluble workflow for antibody detection. Rosalyn Sussman Yalow, an American medical

physicist pioneered the immunoassay technology in the 1950s. Together with her collaborator, the American physician scientist Solomon A. Berson, she developed the first radioimmunoassay (RIA) in the 1950s (Berson & Yalow, 1968).

4.1.3.1 Enzyme linked immunosorbent assay (ELISA)

4.1.3.1.1 Introduction. One of the most widespread methods in medical diagnostics is the enzyme-linked immunosorbent assay (ELISA), also known as enzyme immunoassay (EIA) which was conceptualized and developed by the Swedish scientists Engvall and Perlmann at the University of Stockholm (Engvall & Perlmann, 1971). Since its creation, the method has evolved alongside the greater availability of commercially available antibodies and increasingly efficient automation platforms reducing manual input and human error. The most advanced systems are fully automated with turnaround times as fast as 2–3 h that include data collection, analysis, and report. Most ELISA experiments are carried out on multi-well microtiter plates encompassing small reaction vessels (wells) made of a plastic (polystyrene, polyvinyl chloride) that are chemically resistant and inert to nonspecific antibody interaction. Common plate formats are multiples of 96 and up to 384 individual sample wells. ELISAs are popular because they are fast, sensitive and highly reproducible. Sample sizes can be easily scaled up and the same automation platform can be used for many different types of experiments, rendering ELISAs extremely cost effective and versatile. Due to this, ELISAs are commonly utilized in medical diagnostics, biomedical research, and industries charged with the diagnosis and quality control of food and other biological samples. In addition, many of them are commercially available from reliable manufacturers of antibody-detection systems for use by clinical microbiology and immunology diagnostic laboratories.

4.1.3.1.2 Working principle. The principle behind this technique is the detection of an antigen of interest of unknown concentration in a complex liquid sample. In medical diagnostics, hormones, peptides, nucleic acids, lipids and other metabolites are considered analytes and commonly assessed in liquid biological samples such as blood, cerebrospinal fluid (CSF), secretions, excretions or cell- and tissue lysates. Analytes must be attachable to a stationary phase (i.e., interior wall of reaction well), antibody-detectable and compatible with a water-soluble workflow, which may require pre-treatment.

In its simplest version, biological samples are filled into reaction wells and their entire molecular contents, including the analyte of interest, immobilized to the stationary phase. Using an analyte-specific capture antibody in conjunction with the stationary phase, will increase sensitivity, add specificity and reduce false-positive background signals. To detect and quantify immobilized analyte, a detection antibody is used that recognizes another epitope not masked by the capture antibody (if used). Visualization of analyte-detection antibody binding is most facilitated by one of two enzymes conjugated with the detection antibody: alkaline phosphatase (AP) or horseradish peroxidase (HRP). These enzymes act as catalytic amplifiers and will turn a colorless substrate into a chromogenic product which is then detected and quantified via internal standard dilution analysis of known analyte concentrations.

It is important to realize that specificity of this method is largely dependent on the quality of the detection antibody used. However, false positive results can never fully be ruled out with this method and may require confirmatory testing with independent techniques (Tighe, Ryder, Todd, & Fairclough, 2015).

4.1.3.1.3 ELISA types *(Aydin, 2015; Lequin, 2005)*

4.1.3.1.3.1 Direct ELISA This method is the simplest form of this technique and omits use of a capture antibody. Here, the entire molecular content, including the analyte, is introduced to the stationary phase for immobilization. If the detection antibody is the same that carries the conjugated enzyme involved in signal amplification, the method is considered direct.

4.1.3.1.3.2 Indirect ELISA This method uses two antibodies for analyte detection. The so-called primary detection antibody will bind to an epitope on the analyte while several enzyme-conjugated secondary detection antibodies will bind to one primary antibody at its Fc portion. This generally causes signal amplification but also increases nonspecific background. Therefore, the overall benefit of signal amplification may not be universal and needs to be evaluated for each analyte. Indirect ELISAs make it unnecessary to provide dedicated, enzyme-couple primary antibodies for each analyte. This increases flexibility and provides cost savings.

4.1.3.1.3.3 Sandwich ELISA This method uses a capture antibody immobilized to the stationary phase inside the reaction wells thereby enhancing specificity as well as sensitivity. This feature prevents binding competition and enriches the analyte of interest at the stationary phase. Following capture, the analyte is sandwiched between the capture antibody and the detection antibody. It is important to note, that while both antibodies are raised against the same analyte, they cannot compete for binding and must therefore recognize different epitopes. With this method, detection can be either direct or indirect.

4.1.3.1.3.4 Competition/inhibition ELISA This method follows the same principle as the hemagglutination inhibition method discussed above. The technique is used to detect and quantify an analyte or antibody of interest in a clinical sample. The sample analyte or antibody will compete for binding to the capture molecule (analyte or antibody immobilized to the stationary phase) with an enzyme-linked or otherwise labelled reference analog. The higher the concentration of the sample analyte or antibody, the more competition for the labelled reference analog and the weaker the signal at the stationary phase. Therefore, sample analyte or antibody concentration correlates inversely with the strength of the output signal.

4.1.3.2 Interferon gamma release assays (IGRA)

This method is also ELISA-based and assesses the cytokine IFN-gamma which is an important activator of macrophages and critical for innate and adaptive immune responses against viral, and some bacterial and protozoal infections. While suitable for a wide range of infections, an IFN-gamma status is primarily considered in the diagnosis of tuberculosis caused by *Mycobacterium tuberculosis*. There are currently two competing assays on the market:

4.1.3.2.1 The QuantiFERON-TB Gold In-Tube test (QFT-G). This is the latest version of its kind and is currently in use in the clinical laboratory (Theel et al., 2018). Fresh blood is drawn and the cells must stay viable throughout the duration of the assay which naturally shortens preanalytical time. A person that has sensitized lymphocytes to *M. tuberculosis* is going to release IFN-gamma in response to synthetic peptides derived from the bacterium. (https://www.cdc.gov/tb/publications/factsheets/testing/igra.htm). Furthermore, this assay is not affected by prior vaccination with BCG (Bacille Calmette-Guérin) which makes it superior to the Mantoux skin test by reducing false positives. Another advantage of this assay is the ability to derive both qualitative and quantitative analysis. Nevertheless, the results of this test need to be combined with the clinical picture and other assays for determination of diagnosis (Center for Disease Control Fact Sheets, n.d.; Pai, Riley Lee, & Colford, 2004).

4.1.3.2.2 T-SPOT.TB test. The T-SPOT.TB test works in a similar way and also uses synthetic peptides derived from *M. tuberculosis* with fresh blood samples (8–30 h after collection). In contrast to the QFT-G, this test measures the number of IFN-gamma producing cells (spots).

4.1.3.3 Radioimmunoassay (RIA)

4.1.3.3.1 Introduction. The radioimmunoassay pioneered the immunoassay methodology in the 1950s and earned Rosalyn Yalow a shared Nobel Prize for Physiology or Medicine in 1977, 5 years after the death of Solomon Berson who was instrumental in its development (Glick, 2011). While RIAs are highly specific, sensitive and cost-effective in high throughput, the use of gamma-radiation is controversial, requires licensing, specific equipment and training.

4.1.3.3.2 Working principle. In its most common clinical application, this technique resembles the Competition ELISA method. In an RIA, a labelled test-analyte of known concentration competes with its natural analog from a patient sample for antibody interaction. Any biological macromolecule, that can be radiolabeled and is able to interact with an antibody, is suitable for this technique. Traditionally, most analytes are peptides or proteins and labelled with radioactive iodine (^{125}I) attached to tyrosine. In this case, measurements are usually indirect and defined by the amount of test-analyte in the supernatant. If a test-analyte is not suitable for radiolabeling, a direct approach using a labelled detection antibody like a sandwich ELISA can be pursued. The radioallergosorbent test (RAST) is a common blood test in which a radiolabeled antibody is used against a patient's serum IgE (Johansson, Bennich, & Foucard, 1973; Wide, 1973). For this test, the suspected allergen is immobilized on a stationary phase and the patient's serum added. The test is positive if the radiolabeled anti-IgE co-localizes with the antigen/IgE complex. This test is direct, and the radioactive signal intensity is directly proportional to the IgE bound to the antigen.

4.1.3.4 Lateral flow immunochromatographic assay

4.1.3.4.1 Introduction. This method is also known as the Lateral Flow Test and provides a simple and fast way to test a liquid patient sample, such as blood, urine, serum, saliva, sweat or other fluids, for a specific analyte (Koczula & Gallotta, 2016). The simplicity lies in the disposable test-strip design which incorporates all functions of an ELISA without the labor or equipment. These tests are very versatile and used for point of care testing, at home, or in a laboratory with results typically within 30 min (Jiang et al., 2019). While the home pregnancy test is a more traditional example, current use for infectious diseases as well as the incorporation of smartphone technology, indicate the enormous potential of this technology—especially for underdeveloped nations.

4.1.3.4.2 Working principle. The simple design includes a series of pre-loaded capillary beds designed to move a liquid sample from one end (sample pad) to the other (wicking pad). The self-contained housing for these elements may be larger or arranged on a dipstick which simplifies the sample loading. In a standard sandwich assay, the sample to be tested for the analyte, is applied to a sample pad from which it flows into a conjugate pad pre-loaded with analyte-specific detection antibody conjugated to colored particles for detection (reporters). Typically, these are latex (blue) or gold particles (red) but other reporters are also used. The analyte-antibody complex then moves into the detection pad across a test line which contains immobilized capture antibody—similar in fashion to a sandwich ELISA. The test line will appear colored, if the patient sample contained measurable quantities of analyte. Subsequently, the sample moves across the control line of the detection pad which contains affinity ligands for the reporter. This ensures that the liquid has moved across the entire assay thereby validating the test. While most lateral flow tests are designed to be qualitative, quantitative and competitive assays do exist and may require additional detection equipment.

4.2 Immunohistochemistry

4.2.1 Introduction

Clinical laboratory applications utilizing immunofluorescent antibodies were first reported by Coons, Creech, and Jones (1941) and have since then revolutionized histological specimen analysis in clinical laboratory medicine and research (Ramos-Vara, 2005). In most cases, fluorescent antibodies are used to label antigens of interest in fixed (paraformaldehyde or formalin) and paraffin-embedded or frozen sections and then microscopically studied. Common clinical applications target bacterial surface protein to determine infection, the presence of certain Ig isotypes associated with cutaneous conditions, or the presence of specific cell-surface markers indicating malignancy. Immunofluorescence techniques can be either direct or indirect.

4.2.2 Working principle

Whether fixed or frozen sections are studied, the preservation of cell integrity, tissue architecture and epitope antigenicity are key determinants to successful clinical immunohistochemistry. While preparation and workflow involving frozen sections (typically 6–30 μm thick) is more straightforward, fixed and paraffin/wax embedded sections tend to be thinner (1–6 μm) and are more readily archived. Preservation and detection of small soluble molecules, lipophilic substances or nucleic acids in subcellular compartments can be challenging and may require additional steps during sample preparation and throughout the workflow. Depending on the application, tissue sections are prepared on a cryostat, microtome or vibratome which nowadays provide various levels of automation. Non-specific immunostaining and autofluorescence are common hurdles in immunohistochemistry and may require blocking or specific signal quenching. To observe targets of interest into their proper histological context, fluorescent counterstains are used, such as 4′,6-diamidino-2-phenylindole (DAPI) for nuclear DNA and lipophilic carbocyanine dyes or lectin conjugates for cell membranes and associated glycoproteins, respectively. Specimen analysis is carried out with standard epifluorescence, confocal or photon excitation microscopy.

4.3 Flow cytometry

4.3.1 Introduction

This technique determines and quantifies the genotypic, phenotypic and functional characteristics of a cell sample in suspension. Standard diagnostics are carried out with blood, bone marrow and lymphatic fluid for diseases, and conditions such as acute leukaemia, immunodeficiency, myeloproliferative and lymphoproliferative disorders and platelet status. The Canadian bacteriologist, Andrew Moldovan, is generally credited with the prototype design in 1934 which, however, was never built (Moldavan, 1934). The first functional cell sorter was invented by the engineer Mack Fulwyler at the Los Alamos National Laboratory, New Mexico in 1965 (Fulwyler, 1965). He recognized the combined value of automated cell size analysis with the newly developed inkjet technology for fast cell sorting.

4.3.2 Working principle

This technique sorts cells based on distinguishable physical characteristics such as cell size, morphology, genotype, and protein expression. These characteristics are determined by specific light-scattering profiles via labels, dyes, and stains. Immunophenotyping in flow cytometry refers to the use of fluorescence-conjugated antibodies to highlight physical attributes of interest, such as a cell surface marker for cancer, which can be sorted.

4.4 Immunoblotting

Immunoblotting refers to a process by which complex mixtures of peptides and proteins are added to a polyacrylamide matrix, subjected to an electrical field (electrophoresis) for molecular weight resolution, and transfixed onto nitrocellulose sheets for detection with antigen-specific labelled antibodies.

4.4.1 Western blot

4.4.1.1 Introduction

Western blotting is a popular analytical technique used in clinical analysis laboratories as well as in research (Gavini & Parameshwaran, 2019). The method was first described in 1979 by a postdoctoral fellow, Harry Towbin, in the laboratory of Julian Gordon at the Friedrich Miescher Institute for Biomedical Research (FMI) in Basel, Switzerland (Towbin, Staehelin, & Gordon, 1979). The method was named Western blot as a play on the Southern blot technique for DNA invented by the British biologist Edwin Southern. Western blotting is frequently used for the confirmatory medical diagnosis of infectious diseases such as Lyme disease, HIV infection, bovine spongiform encephalopathy (BSE), hepatitis C infection, syphilis, inflammatory muscle conditions such as myositis, and certain autoimmune disorders (e.g., paraneoplastic disease). For Lyme disease and HIV infection, these are the only two microbial diseases for which an initial borderline or positive ELISA must be followed by a confirmatory Western blot.

4.4.1.2 Working principle

Prior to electrophoresis, protein samples are subjected to denaturation through heat and a reducing agent [beta-mercaptoethanol (bME) or dithiothreitol (DTT)] for the resolution of disulfide bonds. The addition of an anionic surfactant [sodium dodecyl sulfate (SDS)] ensures that all proteins are coated with negative charges, facilitating separation by molecular weight in an electrical field. Protein bands are then transferred to a nitrocellulose membrane via electro blotting and visualized with protein-specific antibodies linked to chromogenic stains, fluorescence or radioactivity. In most cases, staining is indirect with the labelled antibody being secondary. In its present form, the sensitivity of the Western blot technique is limited and an analysis semi-quantitative at best. Current research efforts are directed at this issue and further promote the principle behind this technique for single cell applications (Mishra, Tiwari, & Gomes, 2017; Taylor & Posch, 2014).

5 Conclusion

In conclusion, here we have discussed the basic immunological principles, especially as they relate to clinical laboratory diagnostics and then provided a comprehensive survey of current basic clinical immunological techniques. The rest of the chapters in this book volume will cover in-depth assays as they specifically relate to the immunodiagnosis of select infectious diseases, such as Lyme disease, Zika virus disease and brucellosis.

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