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A Short Review of Immunochemistry

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Abstract

Immunochemistry involves the study of the molecular mechanisms underlying the function of the immune system with specificity on the nature of antibodies, antigens and their interactions. Immunochemistry also entails the use of antibodies in the labeling of epitopes of interest in cells (immunocytochemistry) or tissues (immunohistochemistry). This technique has application in the diagnosis as well as in the prognosis of diseases and tumors through the development of locus-specific monoclonal antibodies. Also, immunochemistry has prognostic applications in tumors and this has paved way for the implementation of preventive measures against these tumors development. In addition, disease diagnoses have undergone improvement through the use of immunochemistry the evolution of diagnostic markers is significantly changing the clinical practices of the surgical pathologists.

Keywords: Immunochemistry; Immunohistochemistry; Immunocytochemistry; Diagnosis; Prognosis

Introduction

Immunochemistry (IC) provides the basis of molecular mechanisms associated with immune system functions as it relates to the nature of antibodies, antigens and their interactions [1]. The various methods in immunochemistry have been developed and refined and been used in scientific study, from virology to molecular evolution [2]. The application of the methods of physical chemistry to the study of the theory of toxins and antitoxins has been published by Svante Arrhenius in 1907 which described immunochemistry [3].

Immunochemistry has also been studied from the aspect of using antibodies to label epitopes of interest in cells (immunocytochemistry) or tissues (immunohistochemistry) and these have been used in the diagnosis of diseases [4]. The detection of Syphilis by Wasserman test provided the diagnostic use of IC in infectious diseases [1]. In immunochemistry, antibody titer and dilutions as well as incubation time, temperature and pre-treatment of tissue samples are tightly interwoven in their effect on staining quality [5]. These factors can be changed independently, or as is more often the case, in complementary fashion to bring about positive differences [6]. The predominant goal of an immunochemical staining is to achieve optimal specific staining accompanied by minimal interference from background staining [7]. Hence, this paper provides a short review of the basic immunochemistry techniques and their applications in the diagnosis and prognosis of diseases and tumor.

Immunohistochemistry

Immunohistochemistry (IHC) is the process of detecting antigens (e.g. proteins) in cells of a tissue section by utilizing monoclonal and polyclonal antibodies [1]. IHC is derived from "immuno" (meaning antibodies used in the procedure) and "histo" (meaning tissue). The procedure was first demonstrated by Albert Coons in 1941 but has existed in the 1930s [3]. In IHC, the distribution and localization of biomarkers and proteins expressed differently in various parts of a biological tissue in health and diseases are determined using monoclonal and polyclonal antibodies [4]. IHC also plays an important role in various areas of pathology including oncologic pathology, neuropathology, and haematopathology [7]. It is widely used for diagnosis of cancers as a result of the increased uncontrolled expression of specific tumor antigens in certain cancers [8]. IHC also provide a greater comprehension of autopsy pathology when combined with basic histologic examination of tissues [6,9]. Also IHC has been employed as a diagnostic tool in surgical pathology [2,10].

Principle of immunohistochemistry

The aim of IHC is performance of the IHC staining using the least amount of antibody with adequate care to cause least damage on the cell or tissue [1]. The principle of IHC is based on specific antigen-antibody reactions in biological tissues [2]. In IHC, available biopsies are processed into sections using a microtome followed by incubation of the sections with an appropriate antibody [8]. The antibody binding site is then visualized by a marker such as fluorescent dye, enzyme, radioactive element, or colloidal gold, using an ordinary or fluorescent microscope [11]. The marker is directly linked to the primary antibody or to an appropriate secondary antibody [7]. Fluorescein isothiocyanate (FITC)-labeled antibodies with a fluorescent dye were used by Coons and his colleagues for the localization of pneumococcal antigens in infected tissues [3]. Following advancement in IHC technique, the use of enzyme labels (antibody-enzyme conjugation) such as peroxidase [12,13] and alkaline phosphatase [14] have been introduced. Also the discovery of colloidal gold [11], label has led to the identification of immunohistochemical reactions at both light and electron microscopy levels. Other labels include radioactive elements, and the immunoreaction can be visualized by autoradiography [4].

Immunohistochemistry Staining Procedure

Sample preparation

Tissue sample preparation plays a vital role in the outcome of IHC [4]. This requires proper tissue collection, fixation, and sectioning [4]. The tissue may be fixed in a solution of paraformaldehyde or other methods [5]. The purpose of the experiment and nature of the tissue itself determine whether the tissue is to be sliced or the whole tissue is to be utilized [3]. Following fixation, embedding of the tissue sample in paraffin wax or a cryomedia is done and the embedded tissue is sliced using a microtome or cryostat at 4-40 μm thickness. The slices are then mounted on glass slides and later dehydrated in increasing concentrations of alcohol washes (e.g., 70%, 80%, 90%, 95%, absolute). The dehydrated slices on the slides are then cleared using xylene and later imaged under a microscope [15].

The sample may require additional steps such as deparaffinization and antigen retrieval to reveal epitopes for antibody binding and this depends on the method of fixation and tissue preservation [1]. Antigen retrieval is a prerequisite for formalin-fixed paraffin-embedded tissues, and this involves pre-treatment of the sections with heat or protease [1]. Prior to staining, endogenous biotin or enzymes may be blocked or quenched depending on the type of tissue and method of antigen detection [4].

Samples are incubated with a buffer that blocks the nonspecific reactive sites of antibodies to reduce background staining that would otherwise mask target antigen detection [3]. The common blocking buffers include normal serum, non-fat dry milk, BSA, or gelatin. The occurrences of background stainings are can be minimized through the dilution of the primary or secondary antibodies [4]. However, changes in the incubation time or temperature as well as using a different system of detection or different primary antibody have also been reported to reduce background staining [1].

Sample labeling

Antibody types: Polyclonal or monoclonal antibodies can be utilized for specific detection in IHC. The polyclonal antibodies are made up of dissimilar antibodies with specificities for several epitopes whereas monoclonal antibodies show specificity for a single epitope [4]. Polyclonal antibodies are produced by injecting animals with the protein of interest, or a peptide fragment and, followed by isolation of the antibodies from whole serum after a secondary immune response is stimulated [1].

For IHC detection strategies, antibodies are classified as primary or secondary reagents [1]. The primary antibodies are specific for the target antigen, whereas the secondary antibodies target the immunoglobulins produced by the primary antibodies [4]. The secondary antibody is usually conjugated to a linker molecule, such as biotin, which then recruits reporter molecules, or the secondary antibody itself is directly bound to the reporter molecule [1].

Immunohistochemistry reporters

The most popular reporter molecules are chromogenic reporters (enzyme-mediated) and fluorescence reporters (fluorophore-mediated) based on the nature of the detection method [16].

Target antigen detection

The detection of target antigens can be direct or indirect [4]. The direct method is a one-step staining method and involves a direct reaction of the labeled antibody (e.g. FITC-conjugated antiserum) with the antigen in tissue sections [4]. Although this method is simple and rapid as it utilizes one antibody, it has a very lower sensitivity than the indirect method due to little signal amplification [16]. Hence, the direct target antigen detection method is less frequently used.

On the other hand in the indirect method, an unlabeled primary antibody (first layer) binds to the target antigen in the tissue and this is followed by reaction of a labeled secondary antibody (second layer) with the primary antibody. As mentioned above, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised [4]. The signal amplification produced as a result of binding of several secondary antibodies to each primary antibody makes the indirect method more sensitive than the direct detection strategies because of as the secondary antibody [16].

Counterstaining

This is the application of a second stain after immunohistochemical staining of the target antigen [4]. The aim is to provide contrast that enables the primary stain to stand out. Many of these counterstains show specificity for specific classes of biomolecules, while others will stain the whole cell [1]. Hematoxylin, Hoechst stain, and DAPI are commonly used counterstains in IHC [4].

Immunocytochemistry

Immunocytochemistry (ICC) is another aspect of IC whereby antigens are detected by using labeled antibodies on cells (in contrast to IHC that uses stained tissues) [1]. ICC has been used as biochemical tools to detect and quantify molecules in cell extracts and to identify specific proteins after they have been fractionated by electrophoresis in polyacrylamide gels. When coupled to an inert matrix to produce an affinity column, the labeled antibodies can be used either to purify a specific molecule from a crude cell extract or if the molecule is on the cell surface, to pick out specific types of living cells from a heterogeneous population [17]. However, ICC has also been employed in the diagnosis of diseases.

Protocol for immunocytochemistry

- The adherent cells are seeded on 6-well tissue culture plate in a sterile tissue culture hood. The cells grow at 37°C in a humidified CO_2 incubator until they are 50%-75% confluent cells.
- The cells are then fixed by incubation in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature.
- This is followed by heating of cells in antigen retrieval buffer (if necessary).
- The cells are then permeabilized by incubation in 0.1% Triton 100X for 15 min at room temperature.
- The cells are blocked by incubation in 10% goat serum in PBS for 1 h at room temperature.
- Cells are then incubated in the primary antibody/antibodies.
- This is followed by incubation in the secondary antibody/antibodies.
- This is followed by mounting of coverslips and visualization under a microscope.

Applications of immunochemistry

Prognostic markers in cancer: Immunochemistry is been used as a prognostic tool in cases of tumors through the identification of enzymes, tumor-specific antigens, oncogenes, tumor suppressor genes, and tumor cell proliferation markers [7]. The analysis of tumors by IHC has provided a significant improvement over the conventional prognostic considerations by clinical staging and histologic grading [18]. The use of IHC with specific tumor markers has aided in cancer diagnosis (as benign or malignant), tumor stage and grade determinations and identification of cell types and origin (in case of metastasis) to locate the site of the primary tumor [18]. The selection of antibodies being made is based on clinical history, morphological features, and results of other relevant investigations [7]. The expression of IHC stains for intermediate filaments (such as keratin, desmin, vimentin, neurofilaments, and glial fibrillary acidic proteins) by tumor cells, has also been used to identify tumor cells [18].

Infections: Immunochemistry has been utilized in the detection and identification of the aetiology in viral, bacterial, fungal, and parasitic infections. Thus, IHC methods have been utilized in the confirmation of infectious agents in "fresh"/unfixed tissues through the use of specific antibodies against microbial DNA or RNA [19]. The optimum visualizing expression of the target in the affected tissue enhances the application of IHC in the routine validation of disease targets during the disease process [3]. IHC can also be used to detect organisms in cytological preparations such as fluids, sputum samples, and material obtained from fine needle aspiration procedures [4]. This has been utilized in detection of Pneumocystis from the sputum of an immunocompromised patient whereby rapid and precise confirmation of infection is required to begin immediate and appropriate therapy [3].

Viral infections: The traditional diagnosis of viral infections that relied on cytopathic changes observed on routine histopathology are often subtle and sparse [20] as only 50% of the known viral diseases are associated with characteristic intracellular inclusions [21]. This use of immunohistochemistry has been necessitated especially where the viral inclusions present cannot be identified with confidence and differentiated from those of other viral diseases [22]. Viruses diagnosed using ICC include herpesvirus [23-25], Kaposi sarcoma virus [26,27], cytomegalovirus [28,29], Epstein-Barr virus [30], adenoviruses [22,31], Parvovirus B19 [32,33], influenza A virus and respiratory syncytial virus infections in the absence of cultures [34]; BK virus infection in renal transplant specimens [35]; Ebola virus infection [36], yellow fever [37], and dengue hemorrhagic fever [38]; and specifically diagnostic for rabies when the characteristic Negri bodies are inconspicuous [39].

Bacterial infections: Bacterial infections diagnosed by ICC include Helicobacter pylori infection [40,41], Whipple disease [42], Rocky Mountain Spotted Fever [43], Bartonella Infections [44,45], *Coxiella burnetii* infection [46,47], leptospirosis [48], spirochetes in patients with syphilis [49] and soft tissue infections associated with group A *Streptococcus*, *Staphylococcus aureus*, and *Clostridium* sp. [50].

Fungal infections: The great majority of fungi are readily identified by hematoxylin-eosin staining alone or in combination with special stains such as Gomori methenamine silver stain and periodic acid–Schiff stain used in routine histopathology [51]. IHC has been used to identify various fungal elements in formalin-fixed tissue providing rapid and specific identification of several fungi, allowing the identification of unusual filamentous hyphal and yeast infections and distinguishing true infection from harmless colonization [52]. IHC using dual immunostaining techniques can also be helpful when more than one fungus is present to highlight the different fungal species present in the tissue [53].

The fungal infections that have been diagnosed by IHC in include candidiasis [54], aspergillosis [55,56], pneumocystosis cysts and trophozoites of *Pneumocystis carinii* [57], *Penicillium marneffei* [58,59].

Parasitic infections: Diagnosis is difficult in protozoal infections due to distortion of the parasite morphology as a result of tissue necrosis, autolysis or in unusual disease presentation, hence the use of IHC has been adopted in such cases [60]. The diagnosis of leishmaniasis by IHC has been attempted with limitations resulting from small numbers of parasites, the presence of the parasite in unusual locations, or distortion of the disease morphology due to necrosis [61].

Immunohistochemistry has been used in the identification of helminthes such as *Fasciola gigantica* [62-64] and *Paramphistomum gracile* [64] *via* the detection of 28.5 kDa and 16 kDa antigens respectively.

Immunohistochemistry, emerging infections, and bioterrorism: The roles of pathologists in the diagnosis and characterization of emerging infectious agents as well as in the investigation of bioterrorism-related cases cannot be ruled out. The identification of agents of biologic terrorism has been made possible using immunohistochemical methods and such agents include the causative agents of anthrax [65], tularaemia [66], plague [67], brucellosis [68] viral encephalitides (Eastern equine encephalitis) [69] and rickettsioses [70].

Furthermore, the vitality of IHC can be appreciated in the identification and study of several emerging infectious diseases such as West Nile virus encephalitis [71], enterovirus 71 encepahlomyelitis [72], Nipah virus infection [73], Ebola hemorrhagic fever [36], hendra virus encephalitis [74] and in identification of a new coronavirus associated with severe acute respiratory syndrome [19].

Neurodegenerative disorders: Neurodegenerative disorders are characterized by the dysfunction and death of specific, selectively vulnerable populations of nerve cells. IHC has played an increasingly important role in the subclassification of neurodegenerative disorders and the development of consensus criteria for their diagnosis [4,75].

Brain trauma: Head injury has been reported to liberate beta-amyloid precursor protein. This protein has been stained by IHC to detect axonal injury within 2-3 hours following head injury [76]. Hence, IHC has been used in the establishment of the timing of a traumatic insult in medico-legal settings through the detection of axonal injury [4].

Muscle diseases: Muscular dystrophies are associated with abnormalities in several muscle proteins located in the sarcolemma, extracellular matrix, cytosol, nucleus, and other sites within muscle fibers. However, skeletal muscle biopsy can play the main role in differentiating vascular dystrophy from non-dystrophic disorders while IHC can assist in establishing a specific diagnosis of the dystrophies for which specific protein abnormalities are known [77].

Research: Immunohistochemistry is an important tool in diagnostic and research laboratories. Much of the research into the causes of neurodegenerative diseases is directed at identifying the factors that result in the formation of paired helical filaments, the deposition of beta-amyloid and cytoplasmic accumulations of alpha-synuclein [76].

Consequently, studies to localize and quantify the abnormal proteins that constitute reasons of neurodegenerative diseases are of central importance. IHC using antibodies to beta-amyloid, alpha-synuclein, ubiquitin, huntingtin, polyglutamine, and others has become a routine tool for a sensitive detection and quantification of these abnormal proteins in both human tissues and in experimental animals that are used to model some of the features of these diseases [4]. It has also been employed in the understanding the glycation behaviour of the casein and β-lactoglobulin fractions of flavored drinks under UHT processing conditions [78].

Novel uses: Immunohistochemistry has been used in the establishment of new diagnostic markers, which are significantly changing clinical practice for surgical pathologists. These include lineage-restricted transcription factors, protein correlates of molecular alterations and diagnostic markers identified by gene expression profiling [17]. It has also being used in liver tissue to detect the degree of liver injury, liver apoptosis, autophagy, and the expression of microtubule associated protein 1 light chain 3 alpha (Map1lc3, or LC3), Beclin 1, phospho-mTOR, mTOR, phospho-Akt (P-Akt), and Akt [79].

Diagnostic immunohistochemistry markers: Immunohistochemical technique can be used to demonstrate the exact location of a given protein within the tissue examined [1]. This has led to the development of some diagnostic, prognostic and predictive biomarkers including;

- Cytokeratins used for identification of carcinomas but may also be expressed in some sarcomas [80].
- CD15 and CD30 used for Hodgkin's disease [1].
- Alpha-fetoprotein for yolk sac tumors and hepatocellular carcinoma [81].
- CD117 (KIT) for gastrointestinal stromal tumors (GIST) and mast cell tumors [82].
- CD10 (CALLA) for renal cell carcinoma and acute lymphoblastic leukemia [1].
- Prostate specific antigen (PSA) for prostate cancer [1].
- Estrogens and progesterone receptor (ER and PR) staining are used in diagnosis (breast and gyn tumors), prognosis (breast cancer) and prediction of response to therapy (estrogen receptor) [83].
- CD20 identification of B-cell lymphomas [1].
- CD3 identification of T-cell lymphomas [1].

Conclusion

Immunochemistry has been utilized in the diagnosis as well as in the prognosis of diseases and tumors through the development of locus-specific monoclonal antibodies. These have enabled the adequate diagnosis of diseases as compared to the traditional diseases diagnostic techniques. However, the prognostic applications of immunochemistry in certain tumors have also paved way for the utilization of preventive measures against these tumors development. Hence, the evolution of diagnostic markers is significantly changing the clinical practices of the surgical pathologists and this has demonstrated the improvement of disease diagnoses by the use immunochemistry.

Conflict of Interest

The authors declare that there is no conflict of interest.

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