



IMMUNOHISTOCHEMISTRY – A REVIEW ARTICLE

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ABSTRACT

Over the last two decades, immunohistochemistry has had an immense impact on the practice of diagnostic pathology. The identification of specific or highly selective cellular epitopes in routinely processed paraffin wax embedded tissues with an antibody and appropriate labeling system is now a routine procedure in most cellular pathology laboratories in the developed world. Immunocytochemistry is helpful in vast number of cases where the morphology and clinical data alone do not allow firm diagnosis of the type of disease present in the tissue section. Occasionally a pathologist is faced with a diagnosis that could have a profound effect on the patient, in such cases immunohistochemistry is used to confirm the diagnosis so as to ensure that the aggressiveness of the treatment given is not given in error. Since immunohistochemistry involves specific antigen-antibody reaction, it has apparent advantage over traditionally used special and enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique and widely used in many medical research laboratories as well as clinical diagnostics.

KEY WORDS

Antigen, Antibody, Monoclonal antibodies, Polyclonal antibodies.

1. INTRODUCTION

IMMUNOCYTOCHEMISTRY

Immunocytochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method. In some parts of the world the term 'immunohistochemistry' is employed when using tissue sections and 'immunocytochemistry' for cytological preparations.

Antigens

An antigen has been defined as any substance which when introduced into the body, stimulates the production of the antibody with which it reacts

specifically and in an observable manner. An antigenic protein, carbohydrate or lipid molecule bears one or more antibody-binding sites. These are highly specific topographical regions composed of a small number of amino acids or monosaccharide units and are known as antigenic determinant groups or epitopes.

Antibodies

Following introduction of the antigen into the body, antibodies appear in the serum and tissue fluids and react with the antigen specifically and in some observable manner. Antibodies belong to the class of serum proteins known as immunoglobulins. They are formed in the humoral immune system by plasma cells. the end cell of B lymphocyte transformation after

recognition of a foreign antigen. There are five types of antibody found namely IgA, IgD, IgE, IgG and IgM.

Antibody-Antigen Binding

The amino acid side-chains of the variable domain of an antibody form a cavity, which is geometrically and chemically complementary to a single type of antigen epitope. The analogy of a lock (antibody) and key (antigen) has been used, and the precise fit is required which explains the high degree of antibody-antigen specificity seen. The associated antibody and antigen are held together by a combination of hydrogen bonds, electrostatic forces and van der Waals' forces.

2. PRODUCTION OF PRIMARY REAGENTS

Polyclonal Antibodies

It was De Mey & Moeremans who designed the system of production of the Polyclonal antibodies. These antibodies are produced by immunizing an animal with a purified specific molecule (immunogen) bearing the antigen of interest. The animal will mount a humoral response to the immunogen and the antibodies so produced can be harvested by bleeding the animal to obtain immunoglobulin-rich serum. The animal will produce numerous clones of plasma cells (polyclonal). Each clone will produce an antibody with a slightly different specificity to the variety of epitopes present on the immunogen. Some of these antibodies may cross-react with other molecules and will need to be removed by absorption with the appropriate antigen or can be eliminated by dilution process¹.

Monoclonal Antibodies

The development of monoclonal antibodies has revolutionized immunocytochemistry by increasing enormously the range, quality and quantity of specific antisera. The method combines the ability of a plasma

cell transformed B lymphocyte to produce a specific antibody with the in vitro immortality of a neoplastic myeloma cell line; a hybrid with both properties can be produced. With the technique of cloning, this cell can be grown and multiplied in cell culture or ascitic fluid to unlimited numbers. By careful screening, hybrids producing the antibodies of interest without cross-reactivity to other molecules can be chosen for cloning.

3. THE TECHNIQUES EMPLOYED ARE

1. Traditional Direct Technique:

This is the oldest and the traditional technique developed. In this technique the primary antibody is conjugated directly to the label. The most popular direct conjugates are those, which are labeled with a fluorochrome. Horseradish peroxidase and alkaline phosphatase directly labeled antibodies are occasionally used

Advantages

The advantage of the directly labeled antibodies is that they are simple to use as they only require one application of reagent, followed by the appropriate chromogen substrate solution.

Disadvantage

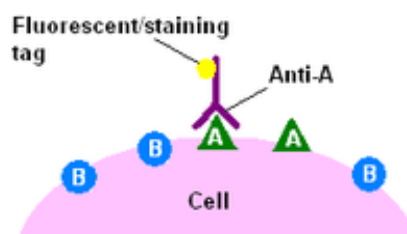
The main disadvantage is low sensitivity of the reaction.

2. New Direct Technique (Enhanced Polymer One-step Staining method)

In this technique a large number of primary antibody molecules and peroxidase enzymes are attached to a dextran polymer "backbone".

Advantages

- It is rapid, especially for frozen section immunocytochemistry.
- Sensitive enough to demonstrate small amounts of antigen.



Diagrammatic representation of direct immunohistochemical method

2. Indirect Techniques:

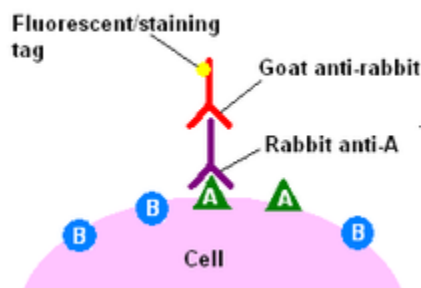
In this technique the antigen of interest is detected by the following manner. The unconjugated primary

antibody is applied, followed by a labeled antibody directed against first antibody

Horseradish peroxidase labeling with chromogen substrate is used.

Advantages:

- It is a more sensitive technique than the equivalent traditional direct methods
- It is also rapid and relatively inexpensive



Diagrammatic representation of indirect immunohistochemical technique

5. TISSUE PROCESSING IN IMMUNOHISTOCHEMISTRY

Fixation: Tissue preparation is the cornerstone of immunohistochemistry. To ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation is essential. However, inappropriate or prolonged fixation may significantly diminish the antibody binding capability. There is no one universal fixative that is ideal for the demonstration of all antigens. However, in general, many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded tissue sections. The discovery and development of antigen retrieval techniques further enhanced the use of formalin as routine fixative for immunohistochemistry in many research laboratories.

Sectioning:

Paraffin Wax Sections:

Since its introduction, paraffin wax has remained the most widely used embedding medium for diagnostic histopathology in routine histological laboratories. Accordingly, the largest proportion of material for immunohistochemistry is formalin-fixed, paraffin-embedded. Paraffin sections produce satisfactory results for the demonstration of majority of tissue antigens with the use of antigen retrieval techniques. Certain cell antigens do not survive routine fixation and paraffin embedding. So, the use of frozen sections still remains essential for the demonstration of many antigens. However, the disadvantage of frozen sections includes poor morphology, poor resolution at higher magnifications, special storage needed, limited retrospective studies and cutting difficulty over paraffin sections.

6. PROTOCOL FOR PARAFFIN-EMBEDDED SECTIONS:

A. Conventional deparaffinization and dehydration sequence:

1. Incubate sections in Xylene: 2 to 3 changes, 5 min. each.
2. 100% absolute ethanol: 2 changes, 3 min. each
3. 95% ethanol: 2 changes, 3 min. each
4. 80% ethanol: 3 min.
5. 50% ethanol: 3 min.
6. Rinse with distilled water, PBS, or Tris buffer: 2 changes, 3 min. each.

Note: Once sections have been rehydrated, do not allow them to dry.

B. Place slides in prewarmed (37°C) 0.1% trypsin in PBS for 5–60 min. or 0.4% pepsin in 0.01N HCl for 30 min. to one hour. Follow by rinsing with distilled water

C. If peroxidase conjugate is used, endogenous peroxidase should be blocked at this stage. Peroxidase activity results in the decomposition of hydrogen peroxide (H_2O_2). It is a common property of all hemoproteins such as hemoglobin, myoglobin, cytochrome and catalases. Suppression of endogenous peroxidase activity in formalin-fixed tissue entails the incubation of sections in 3% H_2O_2 for 8–10 min. Methanolic H_2O_2 treatment (1 part 3% H_2O_2 plus 4 parts absolute methanol) for 20 min. can also be used, but it is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from their carrier glass.

D. Wash twice with PBS.

E. Proceed with immunostaining procedure (see Antibody Staining section).

Whole Mount Preparation:

Small blocks of tissue (less than 5 mm thick) can be processed as whole mounts. The advantage of whole mount preparations is that the results provide three-dimensional information about the location of antigens without the need for reconstruction from sections.

However, the major limitation of using whole mounts is antibody penetration may not be complete in the tissue, resulting in uneven staining or false negative staining. So, Triton X-100 or saponin treatment is used routinely for whole mount immunohistochemistry to enhance penetration of the antibody.

7.UNMASKING OF ANTIGEN SITES

In the routine histology techniques requires tissues to be fixed in a preserving fluid such as 10 per cent formal saline to prevent putrefaction and autolysis. Subsequently most material is then routinely processed to paraffin wax to facilitate section cutting. This series of events masks some of the antigens, usually by formalin cross-linking or even destroys some antigenic epitopes.

In order to prevent destruction of some of the labile antigenic sites several modifications have been employed.

Tissues to be studies can be subjected to frozen sections, lightly fixed in acetone or a specialized processing schedule may be required.

8.ANTIGENS RETRIEVAL TECHNIQUES:

Various methods have been introduced to reveal the masked antigen to make them available for the reaction with the antibodies. They are of the following types;

- Proteolytic enzyme digestion method
- Microwave antigen retrieval method
- Microwave and trypsin antigen retrieval method
- Pressure cooker antigen retrieval method

A. Proteolytic Enzyme Digestion

The most popular enzymes employed today are trypsin and protease. The theory behind the unmasking properties of these proteolytic enzymes is not fully understood, but it is generally accepted that the digestion somehow breaks down formalin cross-linking and hence the antigenic sites are uncovered. Pure trypsin has a very limited digestion effect on formalin-fixed paraffin section but the impurities in crude trypsin such as chymotrypsin are the active ingredients. So, the commercial companies constantly monitor and

standardize the chymotrypsin levels in the batches of trypsin they supply. Alternatively, users can choose to employ pure chymotrypsin.

Disadvantages

- Digestion time has to be tailored to formalin fixation time.
- Some antigens such as cytokeratins are retrieved over a broad digestion time range whilst others, such as heavy and light chain immunoglobulins are only retrieved to a reliable diagnostic level over a narrow digestion time range. So, the retrieval time is very critical and poses difficulty in maintaining time.
- Under-digestion results in very little staining.
- Over-digestion can produce very severe tissue damage with many cells losing their cytoplasmic structures.
- Many antigens are not retrieved or can even be destroyed by proteolytic digestion.
- The specificity of some primary antibodies can be altered.
- Some laboratory workers are allergic to trypsin powder.

B. Microwave Antigen Retrieval

It is a relatively new and revolutionary technique. The microwave oven heating retrieves many antigens thought previously to be either lost or destroyed by routine histological processing techniques.

Mechanism: It involves the boiling of dewaxed formalin-fixed paraffin sections in various solutions. E.g. 0.01 M-citrate buffer (pH 6.0).

Initially boiling took place in a Coplin jar. The limited volume of fluid allowed for rapid and uniform heating, but it was preferred to use larger volumes of fluid so that more slides can be heat pre-treated together. This technique requires sections to be placed on slides with a strong adhesive to reduce section damage or loss.

Adhesives such as Vectabond (Vector Laboratories) or aminopropyltriethoxysilane (APES) are recommended.

C. Pressure Cooker Antigen Retrieval

This system has replaced microwaves to pressure cooker.

Advantages:

It overcomes the problems with microwave method like constant attention required to ensure that the sections do not dry. It is less time consuming. In microwave technique larger numbers of slides using bigger containers does suffer from inconsistencies. Occasionally, the optimized microwave oven heating

time are unable to consistently recover antigens on certain slides. This is thought to be due to the microwave oven having 'hot spots' and 'cold spots'. The pressure cooking method does not suffer from such inconsistencies.

D. Steamer Antigen Retrieval Technique

This method was introduced by Pasha et al in 1995. It is a quick method and is popular in some parts of the world.

Advantage:

Less damaging to tissues than the other heating methods.

Disadvantages:

Appears to be less efficient than either microwave oven heating or pressure-cooking. Times in excess of 40 minutes are sometimes required.

E. Combination of Microwave Antigen Retrieval and Proteolytic Digestion

Combination of microwave antigen retrieval in the coplin jar followed by 30 seconds trypsin digestion enables more reliable identification of some critical antigens. Trypsin can be replaced by chymotrypsin in this method.

Advantages:

Light chains in plasma cells and amyloid are more easily demonstrated. Labeling of other antigens. e.g. high mol. Weight cytokeratin are much improved.

Disadvantages:

As microwave oven pre-treatment causes sections to be highly sensitive to proteolytic digestion and so tissues can easily be damaged by excessive trypsinization. The staining distribution with some antibodies can be severely altered.

9.BLOCKING THE BACKGROUND STAINING:

Background staining may be specific or non-specific. Inadequate or delayed fixation may give rise to false positive results due to the passive uptake of serum protein and diffusion of the antigen. Such false positives are common in the center of large tissue blocks or throughout tissues in which fixation was delayed. Antibodies, specially polyclonal antibodies, are sometimes contaminated with other antibodies due to impure antigen used to immunize the host animal. The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining

is usually uniform and can be reduced by blocking those sites with normal serum.

10.CONTROLS:

Special controls must be run in order to test the protocols and for the specificity of the antibody being used.

Positive Control is to test for a protocol or procedure used. It will be ideal to use the tissue of known positive as a control. If the positive control tissue showed negative staining, the protocol and procedure need to be checked until a good positive staining is obtained.

Negative Control is to test for the specificity of the antibody involved. First, no staining must be shown in the omission of the primary antibody or the replacement of the specific primary antibody by a normal serum (must be the same species as primary antibody). This control is easy to achieve and can be used routinely in immunohistochemical staining.

Second, the staining must be inhibited by adsorption of the primary antibody with the purified antigen prior to its use, but not by adsorption with other related or unrelated antigens. This type of negative control is ideal and necessary in the characterization and evaluation of new antibodies, but it is sometimes difficult to obtain the purified antigen, therefore it is rarely used routinely in immunohistochemical staining.

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