REVIEW

IMMUNOCYTOCHEMISTRY IN DERMATOPATHOLOGY: METHODOLOGY

Sujata Raj, E Calonje, B S Bhogal, D Ramnarain, M M Black

Immunohistochemical staining methods are so widely used today that they can be truly referred to as just another special stain. These techniques are used to recognize, classify and understand the pathogenesis of a variety of skin processes, ranging from the identification of poorly differentiated tumours to the classification of autoimmune diseases. It is the purpose of this review article to provide an understanding of these immunoenzyme techniques.

Key Words: Immunoperoxidase, Immunoenzyme, Direct method, Indirect method, PAP method, ABC method

Introduction

Greater insights into many dermatological diseases have been made possible by the use of light and electron microscopy in dermatopathological examination. The development of immunohistologic techniques like immunofluorescence, immunoenzyme methods and immunoelectron microscopy in the past few decades has contributed even further to the identification and clarification of different diseases.

The success of the immunofluorescence technique encouraged the development of an alternative method of antibody labelling that partially would avoid some of its disadvantages viz, the need of a specialised microscope and lack of permanence but yet allow visualisation of the antigen-antibody complex.[1,2] A variety of labels, mostly enzymes, can be chemically tagged to antibodies in lieu of fluorescein isothiocyanate, hence the name immunoenzyme technique.

Immunocytochemistry is the identification of a tissue constituent in situ by means of a specific antigen-antibody reaction, tagged by a microscopically visible label. Provided that a suitable antibody can be produced and the antigen in the tissue preserved, there is no limit to the substances that may be localised by this procedure.

Basic Mechanism

The enzyme (peroxidase) in the presence of it's substrate (hydrogen peroxide) changes the colour of a suitable chromogen/label that is attached to the specific antibody used (Fig. 1).

Labels

Horseradish peroxidase is most frequently used as it is readily available, inexpensive, easily conjugated with an antibody and unlikely to cross react with human antigens.[3] These techniques are therefore commonly referred to as the immunoperoxidase methods. Other enzymes which have been used include alkaline phosphatase and glucose oxidase. With peroxidase, the most frequently used

From St John's Institute of Dermatology (UMDS), St Thomas' Hospital, London.

Address correspondence to : Dr M M Black Department of Immunofluorescence, St John's Institute of Dermatology, St Thomas' Hospital, Lambeth Palace Road, London SE1 7EH.

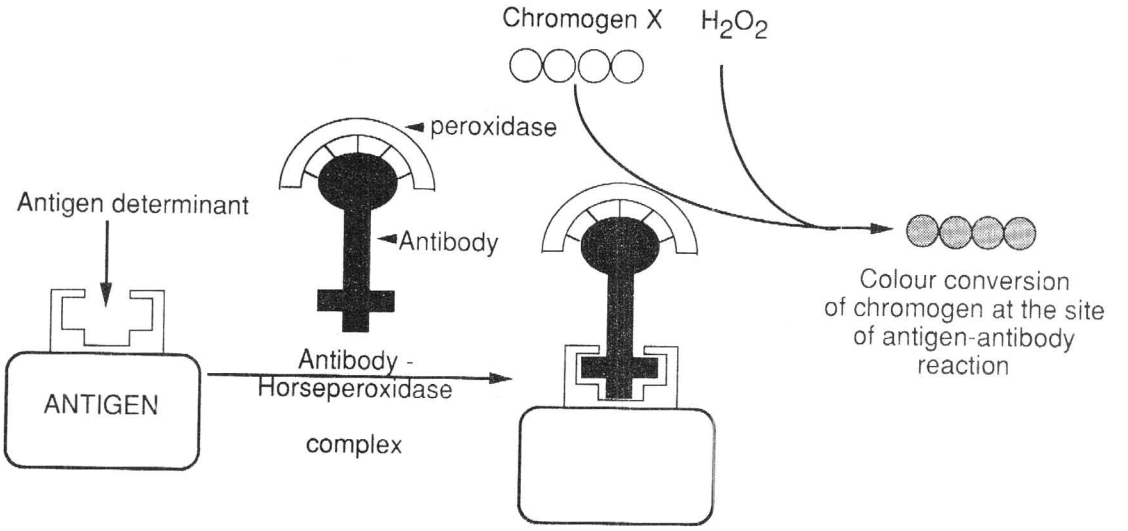

Fig. 1. Schematic diagram of IP technique.

chromogen is diaminobenzidine (DAB), a substrate which yields an insoluble brown polymer at the site of antibody binding when acted upon by peroxidase. It does not fade and contrasts well with haematoxylin stain. Other substrates which can be used are 3-amino 9-ethylcarbazole (red), 4 chloro-1-naphthol (blue), 2,2' oxy diethanol 4 chloronaphthol (black). In the skin the latter ones are useful as their reaction products do not resemble melanin (unlike DAB), however their products are more soluble in inorganic solvents and less reliable for daily use.[4,5] Colloidal gold/silver have also been used.

Tissue Handling/Storage/ Processing

Proper handing of the specimen plays a very important part. Delay in fixation of the tissue after sampling can result in drying of the surface of the tissue block leading to false positive reactions at the periphery. A similar artefact is seen at the margins when specimens are removed by thermal devices like electrocautery. The immunocytochemical techniques can be applied to both frozen (cryostat) and paraffin sections. Some antibodies react only on frozen sections and this must be taken into account and a portion of the biopsy should then be frozen. Cryostat sections are preferred for demonstrating surface markers or other cell membrane antigens. There are several methods for freezing the tissues-snap freezing of small tissue blocks (5x5x3 mm) in cold hexane/ isopentane/liquid nitrogen. The tissue is placed on a cork wafer and then a drop of embedding medium OCT i.e. Optimum Cutting Temperature) is added, followed by immersion in a cold hexane bath maintained at -60°C. This results in rapid freezing (in 20 seconds or less) of the tissue.[6] Sections are cut 3-5 microns thick and collected on slides precoated with poly-l-lysine. The slides are air dried and then fixed in cold absolute methanol or cold acetone.

However specimens are usually submitted in an aqueous solution of formaldehyde. This unfortunately results in the loss of antigens in tissue section and therefore, in routine cases, the application of this technique must be limited to those antigens

which are stable to formaldehyde fixation and paraffin embedding procedure. Usually the extracellular antigens are completely lost during fixation but several intracellular antigens are still detectable, provided the pH of formalin is maintained. It is therefore recommended to use buffered formalin.[7]

Formaldehyde fixes the tissue by reacting with basic amino acids forming cross linking methylene bridges, resulting in low permeability of the cells to macromolecules. This makes it difficult for the antibodies to penetrate and reach the now hidden antigens in the cytoplasm. Treatment of the tissue with an enzyme such as trypsin or protease at 37°C (proteolytic digestion) can expose these cryptic antigenic sites.[8] However, great care is required in the use of these proteolytic enzymes as over treatment will damage the tissue and affect the adherence of sections to the glass slide. Over trypsinisation may even destroy the antigen resulting in false negative results or expose cross reacting antigens thereby giving false positive results. Some antigens resist fixation with formalin and therefore can be stained without prior enzymatic digestion. The antigen retrieval technique may be used instead of enzymatic digestion to rescue antigens from formalin fixed tissue. This involves heating the tissue sections in a microwave oven at over 100°C in the presence of a citrate buffer solution.[9]

Several other factors that may adversely affect the results are[10] (a) inadequate fixation leading to false positive or false negative results, (b) poor penetration of the fixative due to the large size of the block/thick fibrous capsule/insufficient time allowed/slow penetrating fixative. Here the central portion of the section may be falsely positive or negative whilst the periphery is allright. (c) Overfixation resulting in loss of cellular antigens. The optimal fixation time being 12-18 hours in formalin for a section of size 10x10x3 mm. If the tissue is likely to be in the fixative for over 24 hours then it should be kept at 4°C in the fridge. Infiltration and embedding with paraffin should be done at temperatures between 56-60°C, as temperatures over 60°C result in reduced intensity of staining at the edges.

Tissue Sectioning

Sections cut are 3-5 microns thick. Thick, folded, torn (from microtome) sections are undesirable as they lead to false positive/false negative results. The temperature of the water bath should not exceed 60°C. Glass slides coated with an adhesive i.e. poly-L-lysine/chrome-alum/silane are always used to prevent loss of tissue sections during processing. Although egg albumin is routinely used for other histochemical staining procedures (H&E), it is not used in immunohistochemistry as the avidin content in the egg may interfere with the Avidin-Biotin-enzyme Complex (ABC) technique. Drying of slides in the dry oven should always be done below or at 60°C to avoid tissue denaturation.

Prestaining

Endogenous peroxidase activity is a result of the peroxidase present in the tissuei.e. in all haemoproteins such as haemoglobin (RBC), myoglobin (muscle), cytochrome (granulocytes, especially high in eosinophils, monocytes). This peroxidase reacts with the hydrogen peroxide substrate and gives a nonspecific background staining. It can be remedied by incubation of tissue sections in 3-6% hydrogen peroxide prior to the addition of the antibody enzyme complex.[11] Levamisole is used to block endogenous alkaline phosphatase activity.

Staining Reagents

It is imperative to purchase these from a reliable source in the concentrated form and store in the freezer at 4°C. The quality i.e purity and specificity of the reagents used, their dilution, diluents and incubation times should be properly controlled.

Controls

Positive controls (tissues which are known to contain the target protein) are processed identically to the unknown. This is done to ensure quality control. The internal control is ideal because the variability of tissue preparation, processing and staining are eliminated e.g. cyto-keratin in a squamous cell carcinoma will be seen to stain normal tissue elements such as the sebaceous glands, hair and eccrine apparatus in addition to the keratinocytes.

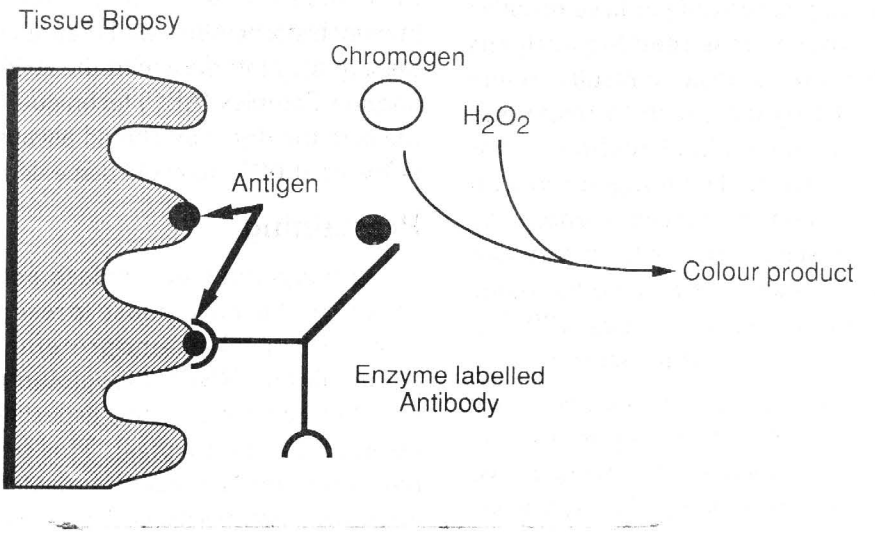

Fig. 2. Direct method.

Staining Methods

Numerous variations in the immunoperoxidase technique exist and they are as follows:

(1) Direct Method

In this technique an enzyme labelled primary antibody is used to detect the antigen in the tissue (Fig. 2).[12]

Advantages: This method is quick and there is less nonspecific reactivity.

Disadvantages: Less sensitive than the multistep methods as little signal amplification is achieved due to the use of a single antibody.

(2) Indirect Method

This is a multi-step technique. An unconjugated primary antibody binds to the antigen. An enzyme labelled secondary antibody directed against the primary antibody (now the antigen) is then applied, followed by another enzyme labelled tertiary antibody (Fig. 3).

Advantages: This is more sensitive than the direct method. The addition of another antibody serves to further amplify the signal. If multiple layers of antibodies are added then there is additional enzyme at the site of the tissue antigen thereby producing greater colour intensity.

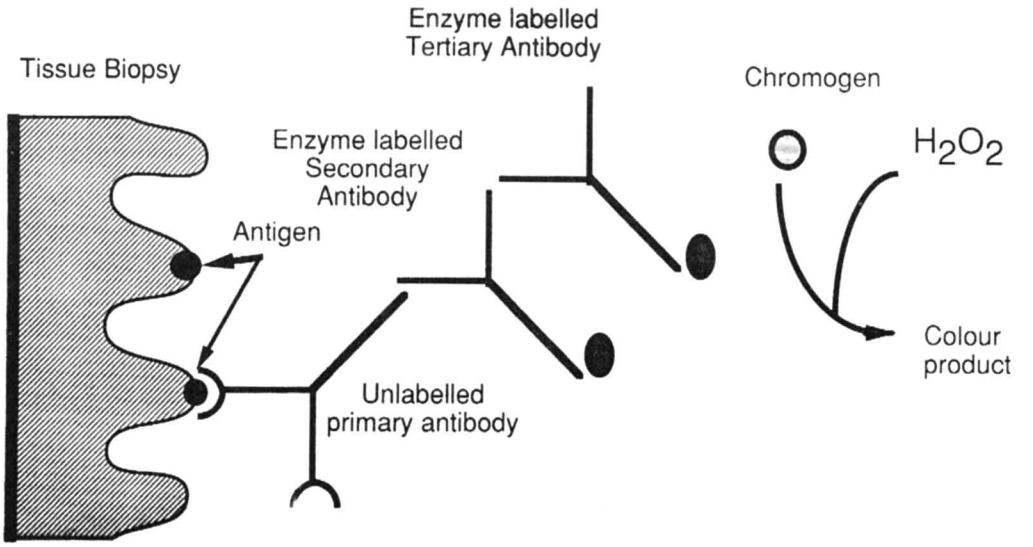

Fig. 3. Indirect method.

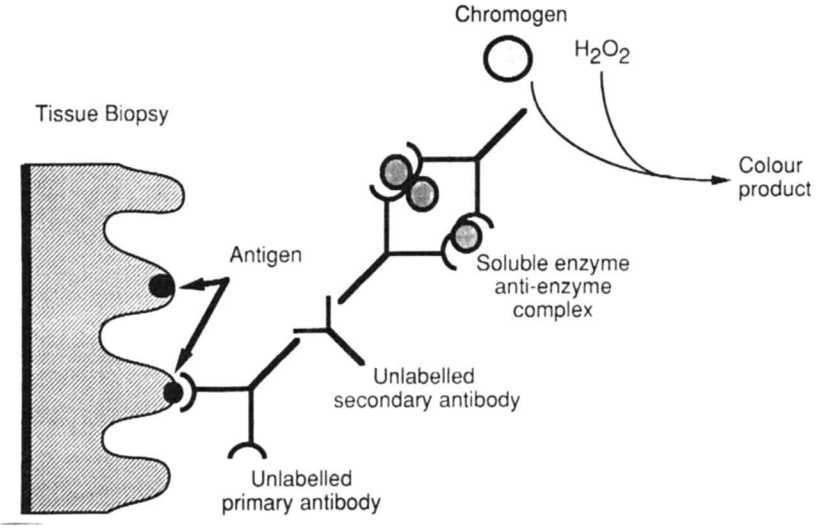

Fig. 4. Soluble enzyme immune-complex method.

(3) Soluble Enzyme Immune Complex/ Unlabelled Antibody Method

The unconjugated primary antibody is followed by the secondary antibody. Finally the soluble enzyme-antienzyme complex is added (Fig. 4). The soluble enzyme immune complex techniques are named after the particular enzyme immune complex they use, for example:

PAP method-Peroxidase-antiperoxidase[13,14]

APAAP-Alkaline phosphatase-anti alkaline phosphatase.

Advantages: Efficiency and specificity are improved as it is unlikely that nonspecifically bound secondary antibody will bind to the tertiary reagents with high affinity. Sensitivity is increased by having increased number of enzyme molecules. In addition, this technique

gives excellent results on formalin fixed, paraffin embedded specimens.

(4) Avidin Biotin Method

This utilizes the high affinity of avidin for biotin.[15] Each molecule of avidin has 4 binding sites for biotin. In this method a biotinylated antibody acts as the link antibody. The primary antibody is followed by the biotinylated secondary antibody. Finally the preformed avidin-biotin-enzyme complex (ABC) is added (Fig. 5).

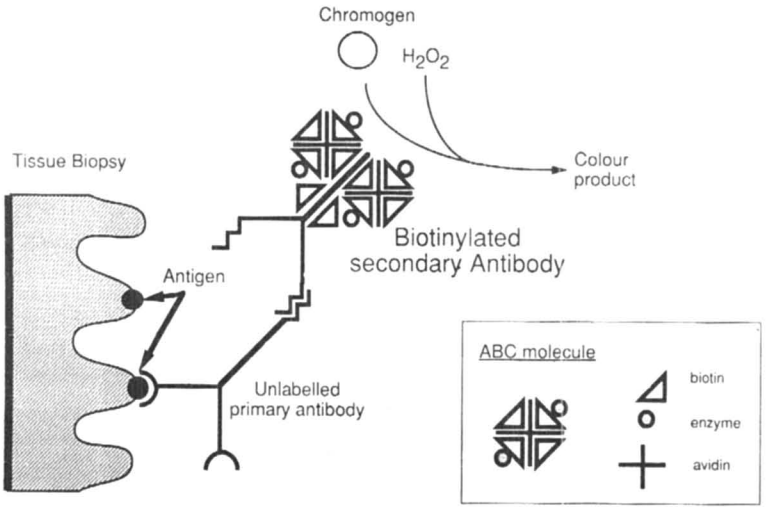

Fig. 5. ABC method.

Advantages : The strong affinity of avidin for biotin makes these methods more sensitive than the direct and the indirect methods. Excellent results can be achieved on fixed, paraffin embedded specimens. Of all the immunocytochemical techniques, the PAP and ABC have gained the widest acceptance.

Interpretation

This may be the most difficult aspect, it requires both skill and experience because of the many pitfalls of these techniques. Even with appropriate controls, nonspecific staining in the dermal connective tissue, epidermis and on the membranes is a common nuisance. Pre-incubation with high concentration of some protein, like normal serum/bovine albumin and use of the highest possible dilution of the primary antibody helps to reduce the background staining.

When DAB is used as the chromogen, a positive reaction is characterized by brown granules at the site of the antigen-antibody reaction. Majority of the immunohistochemical cell markers are intracytoplasmic or on the cell surface membrane except for some viral antigens which are entirely intranuclear and S 100 and Neuron Specific Enolase (NSE) which are both intranuclear and intracytoplasmic.[10]

In individual cells the brown granules may occupy all of the cytoplasm, perinuclear area alone or simply one of the poles of a cell. Diffuse pale brown or a single tone yellow staining of neoplastic cells is in all likelihood nonspecific. The only exceptions to this rule are the pattern of staining for NSE and alpha-1-anti chymotrypsin. These are diffuse and homogenous.[10]

Pigments like melanin and haemosiderin

which on H & E appear brown are often misinterpreted as a positive reaction. It is therefore better to use a chromogen like 3-amino 9-ethylcarbazole which gives a contrasting red colour.

Necrotic tissue nonspecifically absorbs antibodies and exhibits undesirable background staining. Therefore tissue blocks should be chosen from areas with little haemorrhage and necrosis. Another problem of interpretation occurs with passive absorption or active phagocytosis of antigen by histiocytes which then become immunoreactive. A similar phenomenon occurs in the superficial and intermediate cells of the epidermis which exhibit a nonspecific reaction regardless of the antibody used. The free edges of the section as well as the surface membrane (vesicle, pustule) often give a false positive reaction.

Carcinogenicity

Although there is conflicting evidence in literature, 3,3'-diaminobenzidine has not been clearly established as a carcinogen.[16] It is now available ready weighed, which overcomes the particular problem of aerosol formation and inhalation. Large quantities of the substrate can also be prepared in advance using gloves in the fume cupboard and the solution stored frozen without loss of activity.[8]

Conclusion

These techniques require skill in tissue preparation, staining procedures and interpretation but are an invaluable diagnostic tool when faced with a histological diagnostic dilemma.

Acknowledgements

Sujata Raj was a research fellow in the Department of Dermatopathology at the St John's Institute of Dermatology, London, and a recipient of a research grant from the Rameshwardasji Birla Smarak Kosh Foundation.

References

1. Patterson JAK. Immunofluorescence technique in dermatopathology. J Cutan Pathol 1983; 10:439-47.
2. Nakane PK, Pierce GB Jr. Enzyme labelled antibodies: preparation and application for the localization of antigens. J Histochem Cytochem 1966; 14:929-31.
3. Penneys NS. Immunoperoxidase methods and advances in skin pathology. J Am Acad Dermatol 1984; 11:284-90.
4. Straus W. Peroxidase procedures: technical problems encountered during their application. J Histochem Cytochem 1979; 27; 1349-51.
5. Hanker JS, Yates PE, Metz CB, Rustioni A.A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochemical Journal 1977;9:789-92.
6. Rickert RR, Maliniak RM. Intralaboratory quality assurance of immunohistochemical procedures. Recommended practices for daily application (review).Arch Pathol Lab Med 1989; 113:673-69.
7. Holden CA, MacDonald DM. Immunoperoxidase techniques in dermatopathology (review). Clin Exp Dermatol 1983;8:443-57.
8. Hautzer NW, Wittkuhn JF, McCaughey WTE. Trypsin digestion in IPO staining.J Histochem Cytochem 1980;28:52-3.
9. Shi SE. Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1981; 39:741.
10. Nadji M. Immunoperoxidase technique: I. Facts and artefacts (review). Am J Dermatopathol 1986; 8:32-6.
11. Streefkerk JG. Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. J Histochem Cytochem 1972; 20:829-31.
12. Graham RC, Karnovsky MJ. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966; 14:291-302.
13. Sternberger LA, Joseph SA. The unlabelled antibody method. J Histochem Cytochem 1979; 27:1424-9.

14. Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG. The unlabelled antibody enzyme method of immunochemistry. Preparation and properties of soluble antigen antibody complex (horseradishperoxidase-antihorseradish peroxidase) and its use in identification of spirochaetes. J Histochem Cytochem 1970; 18:315-33.
15. Hsu SM, Raine L, Fanger H. A comparative study of the PAP method and avidin biotin complex method for studying polypeptide hormones with antibodies. Am J Clin Pathol 1981; 75:734-8.
16. Weisberger EK, Russfield AB, Homburger F, et al. Testing of 21 environmentals aromatic amines or derivatives for long term toxicity or carcinogenicity. J Environmental Pathol Toxicol Oncol 1978; 2:325-56.