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Technical Aspects of Immunocytochemistry and its Application in Routine Histopathology

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Histological diagnosis is based on the macroscopic and microscopic appearance of the tissues, stained by hematoxylin and eosin, with the aid of various special stains. Usually this gives the pathologist sufficient information to make the diagnosis, but occasionally specific cells and their products need to be identified.

The introduction of fluorescent labelled antibodies[1] enabled these products to be demonstrated, but this technique, along with most histochemical methods, usually requires frozen material. This is not always available. The development of immunocytochemical techniques substituting an enzyme for the fluorescent label[2] was a great advantage, as these techniques could be carried out on routinely fixed paraffin embedded tissue. Peroxidase is the main enzyme label used since it is a small molecule which aids penetration into the tissues.

Immunofluorescence

Immunofluorescence is a widely accepted technique in the routine laboratory, especially in the study of renal and skin biopsies, and in the detection of autoantibodies. Although it is a sensitive method, it has a number of drawbacks. It requires fresh or specially fixed tissue, and it gives poor morphological detail. The preparations are not permanent and a special microscope is required.

Thus, the introduction of immunocytochemical techniques was a great advantage to pathologists, enabling the demonstration of many substances in routinely fixed, paraffin processed tissues.

Figure 1. Peroxidase Antibody Conjugate. Symbols: ■, ▼ = tissue antigens; ● = peroxidase

METHODS AVAILABLE

Direct Method

The primary antiserum against the antigen to be demonstrated is labelled with peroxidase or FITC. This is the shortest method, but is less sensitive than the following methods. (Fig. 1)

Indirect

The primary antiserum is unlabelled, and the second antiserum, directed against the species in which the primary antiserum is raised, is conjugated to peroxidase, FITC or alkaline phosphatase. This is a more sensitive method than the direct technique. It is also more versatile, because primary antisera can be raised against a variety of antigens, and either peroxidase or FITC conjugated antisera can be used as the second layer. (Fig. 1)

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No reader should utilize materials and/or undertake procedures discussed in HISTO-LOGIC articles unless the reader, by reason of education, training and experience, has a complete understanding of the chemical and physical properties of all materials to be utilized and the function of each material by which any procedure is accomplished.

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Figure 2. Enzyme Bridge Method. Symbols as in Figure 1.

Enzyme Bridge[3]

This is a three-step technique. The first and second layers are unlabelled. The third layer is an antiperoxidase antibody followed by free peroxidase. This is a time-consuming method. (Fig. 2)

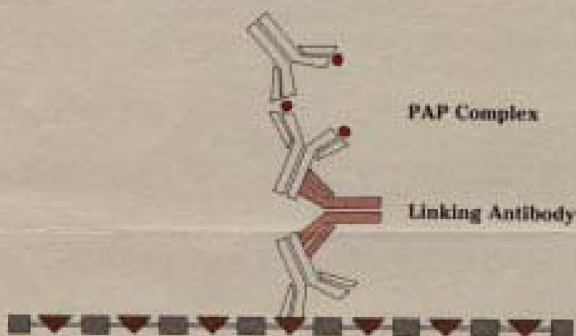

Figure 3. Peroxidase Anti-Peroxidase Method. Symbols as in Figure 1.

PAP[4]

In this method, the first and second layers are unlabelled, and the third layer is an immune complex of peroxidase-anti-peroxidase. The second layer is added in excess and acts as a bridge between the primary antiserum and the PAP complex. (Fig. 3)

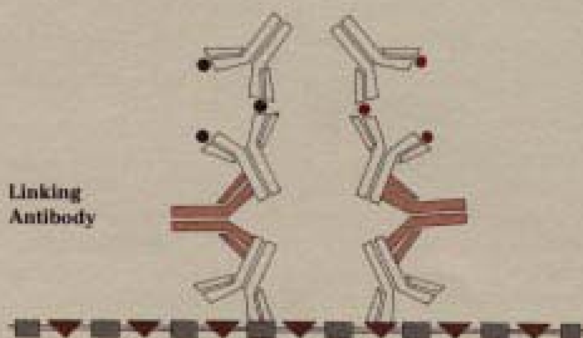

Figure 4. Simultaneous Double-Labelling. Symbols as in Figure 1 and ● = Alkaline Phosphatase.

Simultaneous Double-Labelling

In this method, two substances can be demonstrated in the same tissue section by using antibodies raised in different species. One is labelled with peroxidase and demonstrated with 3:3 diaminobenzidine (DAB); the other is labelled with alkaline phosphatase and localized by naphthol capture, this showing each substance as a different color. (Fig. 4)

Figure 5. Labelled Antigen. Symbols as in Figure 1 and ◆ = Labelled Free Antigen.

Labelled Antigen[5]

The primary antiserum is applied to the section followed by the antigen itself, which is labelled. It is only suitable where enough antigen is available for labelling. This technique is shorter and more specific than the PAP method, with no cross-reaction or collagen staining. (Fig. 5)

MODES OF APPLICATION

Immunohistochemistry can be carried out on cytological smears, frozen and paraffin sections, imprints and tissue culture. Frozen sections are used mainly with fluorescent labelled antisera, but the results are not permanent, and peroxidase techniques on frozen section or imprints often give a heavy background stain. This is reported to be reduced by treating the slides with 20% acetic acid solution prior to incubation in the primary antiserum.[6] In smears and frozen sections, both cytoplasmic and surface immunoglobulins are demonstrated.

In tissue section, cytoplasmic, nuclear and interstitial antigens can be demonstrated. The morphological detail seen in paraffin sections is one of the advantages of the method.

It is also possible to carry out immunoperoxidase techniques on resin embedded material. Aradite/epon mixtures[7] require that the resin is removed with matured saturated alcoholic sodium hydroxide prior to staining. The antisera are used at higher concentrations and incubation times are extended.

When applied to electron microscopy, the tissues are either stained as blocks, on grids or on thick resin sections. In each case the tissue is incubated in the primary antisera overnight at 4°C, and subsequent antisera are applied for extended periods. After staining with DAB, the tissue is treated with osmium to give electron dense granules. Antisera may be conjugated to ferritin or colloidal gold, with the latter conjugate using the different sizes of gold particles in double-labelling techniques.

TECHNICAL ASPECTS

Fixation

It is thought that fixation and processing may denature or alter the antigenicity of the tissue components, so that specificity and sensitivity are impaired. Prompt fixation is essential for consistent results, since poor fixation causes loss of antigenicity or diffusion of the antigens into the surrounding tissue. Due to the high sensitivity of the PAP technique, frozen sections are not necessary. A wide range of fixatives may be used to demonstrate various antigens. Many of the antigenic determinants may be lost during fixation, but enough remain to enable them to be visualized using the PAP technique. There is not one fixative that can be considered ideal for the demonstration of all antigens. Some may still require fresh frozen sections. However, in general, many antigens can be demonstrated after fixation in formal saline, formal mercury, Zenker's or Bouins'. (Some antigens can be localized after osmium fixation, and the periodate-lysine-paraformaldehyde fixative gives good preservation of antigens and can be used for both light and electron microscopy.)

Certain fixatives can be used only if enzyme digestion is carried out on the section prior to immunostaining, e.g., isotonic formaldehyde solutions. Other fixatives give good results without the use of enzymes, e.g., formal mercury, Bouins' and Carnoy's. Very good results are obtained if 2 - 10% acetic acid is added to formal saline. A combination of acetic acid, formal saline and formal mercury gives excellent results with bone marrow specimens.[8]

Because many antigens can be demonstrated after formal saline fixation, it is possible to carry out retrospective studies.

Processing

Following fixation, the tissue blocks are paraffin processed. In some cases the tissues may be washed in buffer to reduce background staining. Bone marrow trephines may be decalcified in EDTA for about two days and then paraffin processed. Processing has little effect on the PAP method.

Cutting

The thickness and quality of the sections is important. The average thickness for immunohistochemistry is 4mu. Adhesives are not always necessary, but slides coated with chrome-gelatine or egg albumin solutions may be used, if required.

The sections are drained and dried briefly on a hot plate, and then dried in an incubator overnight at 60°C. Lower temperatures can be used, depending on the type of tissue.

Enzyme Digestion

Proteolytic enzymes have been introduced into the PAP method with the aim of obtaining more consistent results and reducing background staining. However there are some problems. The major problem is the variance of the results due to the fixation of the tissues. This applies to different antigens as well as to the same antigens in a section, as a result of uneven masking of the determinants during fixation. Batches of enzymes vary in their reactivity and decay with time. Therefore, tissue sections require varying lengths of time in the enzyme to get optimum demonstration of an antigen.

The enzymes that have been investigated are trypsin,[9] protease type VII[10] and pepsin.[11]

Protease has been used as a 0.05% solution pH 7.4 at 37°C, and requires a short time to unmask the antigenic sites, but type VII digests tissue rapidly and type I takes too long. Type VII demonstrates immunoglobulins well and is consistent in its results, but it is expensive.

Trypsin is used as a 0.1% solution in 0.1% calcium chloride pH 7.6 at 37°C. Batches of this enzyme vary and digestion times range from 15 minutes to 45 minutes. It is, however, less expensive than protease.

Pepsin is used as a 0.4% solution in 0.1 M HCl for two hours at 37°C. This is a very long digestion time, although it may be preferable for some antigens.

Immunoperoxidase staining is usually improved after treatment with a proteolytic enzyme, although some antigens may be destroyed. The length of digestion will vary with the length of fixation of the tissue. Careful control of the enzyme reactivity is required, but, in general, antisera may be used at higher dilutions which will therefore reduce background staining.

Endogenous Peroxidase Blocks

Peroxidase is found under normal conditions in red blood cells and granulocytes. At first, this endogenous peroxidase was stained red using alpha-napthyl pyronin, distinguishing it from the brown antigen/antibody complex. It is now usually inhibited before staining. There are a number of methods available: 0.5% H_2O_2 in methanol 30 minutes, 3% H_2O_2 in water, 3-10 minutes periodic acid, followed by sodium borohydride, 1% acid alcohol and methanol/picric acid. Probably the most commonly used blocking method is hydrogen peroxide/methanol. Some tissue antigens may be sensitive to blocking agents, in which case it is best to leave the sections blocked.

Antisera and Controls

To obtain the best possible results, the antisera must

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Observations on the Demonstration of Amyloid When Using Different Methods

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Following are some observations we have made concerning the use of different methods to demonstrate amyloid deposits in tissue sections, using either paraffin or plastic resin embedding.

Demonstration of amyloid in sections is sometimes difficult since it is subject to the influence of many factors, including the type of amyloid, its concentration, and the nature of the lesion or tissue. In addition, the embedding technique plays an important role in the demonstration of amyloid deposits. The staining technique used in this laboratory is a modification of the original technique by Bennhold[1]. Our modified technique is performed as follows:

Procedure for Staining:

1. Cut paraffin sections at 6 microns.
2. Deparaffinize slides in xylene, hydrate through graded alcohol to water.
3. Wash slides in double distilled water for 2 minutes.
4. Stain slides in hematoxylin for 5 minutes (compound solution of equal amounts of Mayer's hemalum & Harris' hematoxylin solutions).
5. Wash slides in running tap water for 30 seconds.
6. Differentiate slides in 0.25%-hydrochloric acid-alcohol solution for 30 seconds.
7. Wash slides in running tap water for 10 minutes.
8. Stain slides in Congo red solution for 20 minutes (0.5 gram Congo red, CI 22120, in 100 ml of 50% ethyl alcohol).
9. Wash slides in running tap water for 8 minutes.
10. Dehydrate slide in alcohol 70%, 80%, 95% and absolute alcohol, for 2 minutes each.
11. Clear in xylene, 2 changes.
12. Mount cover slip with resinous media.

Results:

With polarized light microscopic examination: Young amyloid deposits give grass green fluorescence against dark backgrounds. The older deposits give bright green to white fluorescence.

With darkfield fluorescence microscopy: For this type of examination, we recommend the use of oil-immersion lenses and relatively light filters (lens type: Fluoresz 170/0.17 by Leitz [Wetzlar] Lamp HBO 50).* Beam the light in the green area using a BP 546/12 filter dichroic mirror FT 580 and barrier filter LP 590 (Filter packet Zeiss 487715).** The amyloid deposits, when present, give a bright red fluorescence against a dark background.

Principally, the same technique can be used for the demonstration of amyloid deposits in tissues embedded in plastic resins. We recommend either E.F.L. 67, or Sorval JB 4 resins, as routinely used in our laboratory. In our experience, sections cut at 5 microns are the most desirable for this type of examination.

This was determined during our initial experimentation in adapting the Congo red method to plastic embedded sections. We found that the positive amyloid kidney control tissue, which gave good results after paraffin embedding and cutting at 6 microns, were negative when embedded in plastic and cut at 2 microns. These sections were negative with the use of both polarized light and fluorescence techniques.

The experiment was repeated many times with different biopsies, with the same results. The only exception was the type of amyloid present in medullary carcinoma of the thyroid. In this type of lesion, clear-cut positive results were obtained in a 2 micron plastic section with polarized light examination of preparations conjugated with Congo red. This was observed as green granular structures. Examination of sections of the same blocks with the fluorescence microscope, stained with thioflavine and/or conjugated with Congo red, gave negative results. Apparently the structure of amyloid deposits in the medullary carcinoma of the thyroid is of a different type of nature than that of amyloid deposits in other lesions.

Technique for Conjugation of Amyloid Deposits in Paraffin

The conjugation of amyloid deposits in paraffin sections with thioflavine as performed in our laboratory can be carried out as described below. This procedure includes modifications of the original technique described by Burns.[2] It has not been adapted to plastic resin sections.

Procedure

1. Cut paraffin embedded sections at 7 microns.
2. Deparaffinize slides in xylene, 2 changes.
3. Hydrate through graded alcohols to water.
4. Stain slides in hematoxylin for 5 minutes.
5. Wash slides in running tap water, 30 seconds.
6. Differentiate slides in 0.25% hydrochloric acid-alcohol solution for 30 seconds.
7. Wash slides in running tap water for 10 minutes.

8. Conjugate with thioflavine solution for 5 minutes. This solution can be prepared as follows: Mix 0.4 ML HCl with 100 ML double-distilled water. Then dissolve in this solution 1 gram thioflavine T (C.I. 49005). The stock solution keeps well for a few months at room temperature.
9. Wash slides in running tap water for 30 seconds.
10. Dehydrate slides quickly in alcohol 50%, 70%, 95% and absolute alcohol.
11. Clear slides in xylene, 2 changes.
12. Mount coverslip with resinous media.

For examination with the fluorescence microscope using HBO 50 lamps, beam the light in the blue area of the spectrum: BP 450-490, using a dichroic mirror FT 510 and a barrier filter LP 520 (available in filter packet Zeiss 487709).**

Results:
Grass green fluorescence at sites of amyloid deposits against dark backgrounds.

Differentiating Primary and Secondary Amyloidosis Procedure:
Differentiation between primary and secondary amyloidosis can be achieved by pretreatment of the sections with potassium permanganate solution ($KMnO_4$) before conjugation with Congo red. The details of the procedure are as follows:

1. Cut paraffin embedded sections at 7 microns.
2. Deparaffinize slides in xylene, hydrate through graded alcohol to distilled water.
3. Treat slides with the following potassium permanganate solution for 3 minutes:
   0.5% aqueous potassium permanganate 35.0 ml
   5.0% aqueous sulfuric acid (prepare fresh) 5.0 ml
4. Wash slides in double-distilled water for 30 seconds.
5. Decolorize slides in an aqueous solution of 3% oxalic acid for 1 to 2 minutes.
6. Wash slides in running tap water for 5 minutes.
7. Stain slides in hematoxylin for 5 minutes.
8. Wash slides in running water for 30 seconds.
9. Differentiate slides in 0.25% HCL-alcohol solution for 30 seconds, followed by running tap water for 10 minutes.
10. Conjugate with Congo red solution for 20 minutes.
11. Wash slides in running tap water for 10 minutes.
12. Dehydrate through alcohol: 70%, 80%, 95% and absolute alcohol.
13. Clear slides in xylene, 2 changes.
14. Mount coverslip with resinous media.

Results:
Specimens from patients with primary amyloidosis show amyloid deposits (positive fluorescence) after treatment with $KMnO_4$. In case of secondary amyloidosis, the results with both the fluorescence technique and the dark field polarized light become negative. For control, use sections from the same blocks conjugated with Congo red but without pretreatment with $KMnO_4$. These should be positive in both conditions (primary or secondary amyloidosis.).

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1. Bennhold; *Pathological Techniques* (1961) New York: F. B. Mallory, Hafner Pub. & Co.
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*Leitz **Zeiss

Tissue Adhesive for Immunoperoxidase

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Immunoperoxidase has become a widely used staining procedure in histology laboratories, and the use of trypsin,[1] a digestive enzyme, is of vital importance in many reactions in order to expose antigenic sites. In our laboratory, we use 0.1% trypsin* diluted in 0.1% $CaCl_2$ for 30 minutes at room temperature. A common problem we have encountered following trypsinization, however, is that tissue sections will often slip off the slides. Presented below are two simple procedures we have discovered to keep paraffin sections adhered to glass slides.

The first procedure employs Elmer's glue diluted to 3 percent. A single drop of this is smeared on a clean slide and works quickly to keep the section in place. It should be noted, however, that a fine film which is created by the glue will sometimes give a cloudy background on stained slides.

More recently we have used an alternative procedure which we consider to be even better. Poly-L-Lysine[2]* is diluted 75 mg per 1 ml distilled water, and then flooded on wiped microscopic slides and left to stand for at least one hour. Slides are then placed in a rack, rinsed well with tap water, and dried thoroughly. This procedure renders slides with a clean background and no cloudiness.

In comparing the two procedures, the one advantage of using Elmer's glue is its quickness as an adhesive. Poly-L-Lysine, however, is preferred because it results in cleaner slide background. This, in turn, assures better interpretation of immunoperoxidase staining reaction.

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2. Mazia, D., Schatter, G. and Sale, W. (1975) *J. Cell Biology* 66, 198.

*Sigma Chemical Company

The Phenol Crystal Violet Stain for Differential Staining of Gram Positive and Negative Bacteria

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A procedure for demonstrating Gram Negative and Positive bacteria that has proven to work well for the author is described below. Before examining this procedure, a review of the general concepts relating to the Gram stain may be helpful.

The Gram stain emerged in 1884, when Dr. Christian Gram applied iodine as a *counterstain* to bacteria which had been treated with gentian violet. His observations that certain bacteria remained colored after *alcoholic dehydration* became one of the most important discoveries in microbiology. Although this staining mechanism has been disputed over the years, the following concepts have been generally accepted in recent times:

1. The primary dye (i.e., gentian or crystal violet, etc.) has no specific affinity for any component of the bacterial cell.

2. Iodine's sole purpose in staining is to precipitate the primary dye. Suggestions that iodine is responsible for the oxidation of cell structures, or the binding of the primary stain to oxidized cell structures, have been disproven.

3. The use of accelerators (e.g., phenol or aniline) is of significant value, particularly in tissue pathology where staining is not confined to cultured cells. While accelerators do not play a direct chemical role in the staining mechanism, it would appear that they aid the Gram positive cell in absorbing more of the primary dye.

4. As indicated through substantial evidence, iodine in an alcoholic solution diffuses much slower through the cell wall of a Gram Positive organism than it does through the Gram Negative. The precipitated dye, which is insoluble in the presence of iodine, will therefore remain in the Gram Positive cell until the iodine has been completely extracted by the decolorizer. This is the fundamental principle of the Gram stain: It is a physical reaction based on the difference between the permeability of Gram Positive and Negative cells to alcoholic solutions of iodine.

5. Magnesium ribonucleate is found only in Gram Positive cells and may be responsible for the difference in the permeability of these cells to solutions of alcoholic iodine.

I have found the following procedure to be quite useful and reproducible for demonstrative Gram Negative and Positive bacteria:

Fixation:
10% buffered neutral formalin.

Microtomy:
Cut sections at 5 to 6 microns.

Solutions:

1% Crystal Violet Solution (Stock)

100% alcohol	100.0 ml
Crystal violet	10.0 gm
Distilled water	900.0 ml

10% Phenol Solution (Stock)

Phenol crystals	25.0 gm
100% alcohol	250.0 ml

Crystal Violet-Phenol Solution (Working)

1% crystal violet solution (stock)	50.0 ml
10% phenol solution (stock)	0.5 ml

This working solution keeps well for 3 days.

0.5% Fast Green Solution (Stock)

Fast green	2.5 gm
100% alcohol	500.0 ml

1% Safranin Solution (Stock)

Safranin	5.0 gm
100% alcohol	500.0 ml

Fast green-safranin Solution (Working)

0.5% fast green solution (stock)	2.0 ml
1% safranin (stock)	20.0 ml
Distilled water	40.0 ml

This working solution keeps well for 4 hours.

1% Acetic Acid Solution

Acetic acid	1.0 ml
100% alcohol	100.0 ml

Staining Procedure:

1. Hydrate sections to distilled water in the usual manner.
2. Stain slides for 2 minutes in crystal violet-phenol working solution.
3. Rinse slides in distilled water until clear.
4. Place slides in Gram's iodine for 2 minutes.
5. Rinse slides in distilled water until clear.
6. Decolorize slides in 100% acetone until no more visible dye is released. (This step is not critical, but takes only 10-15 seconds.)
7. Rinse slides briefly in water.
8. Stain slides for 2 minutes in fast green-safranin working solution.

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3. The use of accelerators (e.g., phenol or aniline) is of significant value, particularly in tissue pathology where staining is not confined to cultured cells. While accelerators do not play a direct chemical role in the staining mechanism, it would appear that they aid the Gram positive cell in absorbing more of the primary dye.

4. As indicated through substantial evidence, iodine in an alcoholic solution diffuses much slower through the cell wall of a Gram Positive organism than it does through the Gram Negative. The precipitated dye, which is insoluble in the presence of iodine, will therefore remain in the Gram Positive cell until the iodine has been completely extracted by the decolorizer. This is the fundamental principle of the Gram stain: It is a physical reaction based on the difference between the permeability of Gram Positive and Negative cells to alcoholic solutions of iodine.

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1% crystal violet solution (stock)	50.0 ml
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This working solution keeps well for 3 days.

0.5% Fast Green Solution (Stock)

Fast green	2.5 gm
100% alcohol	500.0 ml

1% Safranin Solution (Stock)

Safranin	5.0 gm
100% alcohol	500.0 ml

Fast green-safranin Solution (Working)

0.5% fast green solution (stock)	2.0 ml
1% safranin (stock)	20.0 ml
Distilled water	40.0 ml

This working solution keeps well for 4 hours.

1% Acetic Acid Solution

Acetic acid	1.0 ml
100% alcohol	100.0 ml

Staining Procedure:

1. Hydrate sections to distilled water in the usual manner.
2. Stain slides for 2 minutes in crystal violet-phenol working solution.
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5. Rinse slides in distilled water until clear.
6. Decolorize slides in 100% acetone until no more visible dye is released. (This step is not critical, but takes only 10-15 seconds.)
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be used at the correct dilution. This is found by carrying out a range of dilutions on the same tissue, with all other steps being constant. To get a clear picture, a compromise is made between the staining of the positive cells and the background. Each time a new batch of antisera is started, it must be titrated to find its optimum dilution. Too strong of a solution will result in a false negative — a prozone effect. Most antisera are incubated on the sections for 30 minutes at room temperature. However, some antisera may require longer periods of incubation, in which case they are left at 4°C for anywhere up to 24 hours. It is possible that incubating the sections at 37°C will shorten the incubation time.

Controls

Any immunological staining will be only as good as the antibodies used. Many antisera contain impurities causing cross-reactions, and the PAP technique may detect these impurities. Monoclonal antibodies would solve this problem, since they are well characterized and recognize just one antigenic determinant. However, to obtain a meaningful result with most other antisera, controls must be carried out as follows:

1. Replacement of the primary antiserum with a normal serum of the same species.
2. Replacement of the primary antiserum with another primary antiserum directed against a different antigen.
3. Absorption of the primary antiserum with purified antigen.

The last control is the most useful specificity control if the purified antigen is available. The antigen must be pure enough to prevent it from absorbing both wanted and unwanted specificities, giving a false impression of monospecificity.

Monoclonal Antibodies

The methodology for producing monoclonal antibodies was developed by Kohler and Milstein[35] from the basic concept of hybrid cell growths pioneered by Harris in 1965.[36] Polyclonal antiserum contains antibodies directed against a number of antigenic determinants. In theory, a single antibody-producing cell grown in culture would produce a single specific antibody. In practice, however, normal antibody-secreting cells do not grow in culture. In the creation of monoclonal antibodies malignant tumor cells which can be cultured are fused to normal lymphocytes, forming hybrid cells, which produce one type of antibody. Individual hybrid cells are then cloned, each clone producing identical antibodies to a single antigenic determinant, i.e., monoclonal. Therefore, whereas polyclonal antisera will recognize different parts of each antigen and each batch will vary, a monoclonal antibody will always recognize the same antigenic determinant and the batches will be identical.

The advantages of monoclonal antibodies are mainly in specificity and the reduction of background staining. One disadvantage lies in the fact that the antigenic determinant is a relatively small amino acid sequence, and thus it is possible to find the same sequence elsewhere in the body and therefore get cross-reaction that does not appear with polyclonal antibodies where minor structural similarities are masked by hundreds of other antibodies to the other antigen sites. This is usually obvious because the sites of a cross-reaction are quite different from the specific staining. Some antibodies can be used only on frozen sections due to their high specificity, but some also work on paraffin embedded tissue. With monoclonal antibodies it has been possible to distinguish subsets of B and T lymphocytes and macrophages, and to detect subtle differences of viruses, e.g., influenza.

Demonstration of Enzyme

Diaminobenzidine (DAB) is probably the best peroxidase substrate for both light and electron microscopy, although there are various alternatives. Alpha-naphthyl pyronin gives a red color which can be dehydrated and cleared, but it is not electron dense. 3-amino 9 ethyl carbazol also gives a red color but cannot be dehydrated and is not electron dense, although it is quite widely used. Hanker-Yates is another substrate which can be substituted for DAB giving a similar color (brown).

If alkaline phosphatase is used as the labelling enzyme, different staining methods are required. Mason and Sammons[37] used naphthol AS phosphate at pH 9.0 with fast blue BBN, or fast red TR. Fast blue BBN gave the better contrast when used with DAB in a double-labelled technique. None of these reagents produce reaction products which can be dehydrated. However, a simultaneous capture method of Stutte using hexanitrogenated New Fuchsin and naphthol AS-TR phosphoric acid produces a red color and can be dehydrated.[12]

Counterstaining

The nuclei are usually lightly stained with hematoxylin after DAB, and if differentiation is required this does not affect the reaction product. Carrazzis' or Mayer's hematoxylin does not usually require differentiation. Other stains may be used as long as they do not mask the peroxidase or alkaline phosphatase reaction product.

Background Staining

The demonstration of antigens by immunocytochemical methods depends on the contrast between the staining intensity of the antigen and the surrounding tissues.

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be used at the correct dilution. This is found by carrying out a range of dilutions on the same tissue, with all other steps being constant. To get a clear picture, a compromise is made between the staining of the positive cells and the background. Each time a new batch of antisera is started, it must be titrated to find its optimum dilution. Too strong of a solution will result in a false negative — a prozone effect. Most antisera are incubated on the sections for 30 minutes at room temperature. However, some antisera may require longer periods of incubation, in which case they are left at 4°C for anywhere up to 24 hours. It is possible that incubating the sections at 37°C will shorten the incubation time.

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2. Replacement of the primary antiserum with another primary antiserum directed against a different antigen.
3. Absorption of the primary antiserum with purified antigen.

The last control is the most useful specificity control if the purified antigen is available. The antigen must be pure enough to prevent it from absorbing both wanted and unwanted specificities, giving a false impression of monospecificity.

Monoclonal Antibodies

The methodology for producing monoclonal antibodies was developed by Kohler and Milstein[35] from the basic concept of hybrid cell growths pioneered by Harris in 1965.[36] Polyclonal antiserum contains antibodies directed against a number of antigenic determinants. In theory, a single antibody-producing cell grown in culture would produce a single specific antibody. In practice, however, normal antibody-secreting cells do not grow in culture. In the creation of monoclonal antibodies malignant tumor cells which can be cultured are fused to normal lymphocytes, forming hybrid cells, which produce one type of antibody. Individual hybrid cells are then cloned, each clone producing identical antibodies to a single antigenic determinant, i.e., monoclonal. Therefore, whereas polyclonal antisera will recognize different parts of each antigen and each batch will vary, a monoclonal antibody will always recognize the same antigenic determinant and the batches will be identical.

The advantages of monoclonal antibodies are mainly in specificity and the reduction of background staining. One disadvantage lies in the fact that the antigenic determinant is a relatively small amino acid sequence, and thus it is possible to find the same sequence elsewhere in the body and therefore get cross-reaction that does not appear with polyclonal antibodies where minor structural similarities are masked by hundreds of other antibodies to the other antigen sites. This is usually obvious because the sites of a cross-reaction are quite different from the specific staining. Some antibodies can be used only on frozen sections due to their high specificity, but some also work on paraffin embedded tissue. With monoclonal antibodies it has been possible to distinguish subsets of B and T lymphocytes and macrophages, and to detect subtle differences of viruses, e.g., influenza.

Demonstration of Enzyme

Diaminobenzidine (DAB) is probably the best peroxidase substrate for both light and electron microscopy, although there are various alternatives. Alpha-naphthyl pyronin gives a red color which can be dehydrated and cleared, but it is not electron dense. 3-amino 9 ethyl carbazol also gives a red color but cannot be dehydrated and is not electron dense, although it is quite widely used. Hanker-Yates is another substrate which can be substituted for DAB giving a similar color (brown).

If alkaline phosphatase is used as the labelling enzyme, different staining methods are required. Mason and Sammons[37] used naphthol AS phosphate at pH 9.0 with fast blue BBN, or fast red TR. Fast blue BBN gave the better contrast when used with DAB in a double-labelled technique. None of these reagents produce reaction products which can be dehydrated. However, a simultaneous capture method of Stutte using hexanitrogenated New Fuchsin and naphthol AS-TR phosphoric acid produces a red color and can be dehydrated.[12]

Counterstaining

The nuclei are usually lightly stained with hematoxylin after DAB, and if differentiation is required this does not affect the reaction product. Carrazzis' or Mayer's hematoxylin does not usually require differentiation. Other stains may be used as long as they do not mask the peroxidase or alkaline phosphatase reaction product.

Background Staining

The demonstration of antigens by immunocytochemical methods depends on the contrast between the staining intensity of the antigen and the surrounding tissues.

Specific background staining may be present when the antigen is in both the cells and the tissue fluid, or when the fixation has been delayed and the antigen has diffused into the surrounding tissues.

Background staining of the collagen is quite common and may be due either to the Fc portion of the immunoglobulins being attracted to the basic groups in the collagen fibres, or to IgG binding to collagen during formalin fixation and processing. This can be minimized by using Fab fractions of immunoglobulin antisera and by application of an immunoglobulin that does not react or interfere with the specific antiserum, i.e., non-immune serum. Optimally diluted antisera, or incubation for 24 to 48 hours with very dilute antisera, will also keep background staining to a minimum. The tissue itself can be washed prior to fixation, and in some cases enzyme digestion of the sections may reduce background staining.

Applications

The immunocytochemical methods allow the examination of specific cell products or extracellular materials in tissue sections. Some of these products can be measured by other methods, such as radioimmunoassay, but those cannot show the morphology or distribution of the product.

One very useful application of the technique is in the field of lymphomas, to distinguish B-cell lymphomas (which tend to be monoclonal), from reactive proliferations or carcinomas (which are often polyclonal). Monoclonal antibodies are used to determine the cell types in a lymphoma which may aid in the treatment of the patient. Much work has been done on lymphomas of the gastro-intestinal tract where alpha-1-antitrypsin and muramidase are useful histiocytic markers.

The demonstration of hormones gives information concerning the histiogenesis of neoplasms of the endocrine system. Ectopic hormone production can be studied, and this can be confirmed by radioimmunoassay of tissue extracts, but this requires large amounts of fresh tissue. Immunocytochemical techniques are advantageous in that they detect these substances in fixed tissue.

Enzymes can be located in tissue by their antigenicity rather than their reaction with standard histochemical techniques, providing they survive fixation and processing. Muramidase is seen in many epithelial cells, myeloid cells and histiocytes. Prostatic acid phosphatase is seen in metastatic adenocarcinoma arising from the prostate gland.

Neuron specific enolase (NSE) has been suggested as a specific marker for neural elements and APUD cells, and it has been used to demonstrate carcinoid cells in the appendix,[13] and in melanomas and neuroblastomas to distinguish them from lymphomas.

Oncofetal antigens are present in adults in raised levels associated with certain tumors, alpha-feto-protein in hepatocellular carcinoma and yolk sac tumors of the testis and ovary, and carcinoembryonic antigen (CEA) in many carcinomas, e.g., colon, lung, breast, etc.

Tissue specific markers are found in one tissue only and are therefore useful in determining the histogenesis of a neoplasm. Myoglobin in muscle will be present in rhabdomyosarcomas. Factor VIII in endothelial cells and megakaryocytes helps with vascular tumors. Actin, although widespread in intracellular distribution, is used to show blood vessel invasion of a neoplasm.

Epithelial membrane antigen is seen in epithelial cells and is present in increased amounts in carcinomas, depending on the degree of differentiation. It can be used to distinguish between lymphomas and epithelial tumors and in the identification of micrometastases. Antibodies to intermediate filament proteins are also helpful in diagnosing carcinomas, lymphomas and sarcomas.

Alpha-1-antitrypsin is produced in the liver and is seen in large amounts in alpha-1-antitrypsin deficient patients, along with hepatoma and yolk sac carcinomas. It can also be used as a histiocytic marker.

Immunocytochemical techniques can be applied to the demonstration of micro-organisms, although usually they are used to stain viral antigens. HBsAg (surface) and HBcAg (core) antigen can be studied along with herpes simplex, measles, Epstein-Barr virus and cytomegalovirus in immuno-suppressed patients.

Conclusion

The demonstration of tissue-specific components in paraffin sections by immunocytochemical techniques is therefore a great advantage to pathologists as an aid to diagnosis. The introduction of monoclonal antibodies has enabled the identification of lymphoma types and helps in the correct therapy for the patient.

Many commercially available antisera have the potential to be useful in the diagnosis of cancer and micrometastases and possibly, in the future, precancerous lesions.

This text has been written using information gained from practical experience, with additional information from the references which follow.

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Continued from Page 216

9. Transfer slides directly to absolute alcohol for 10-15 seconds.
10. Differentiate fast green-safranin in 1% acetic acid solution until no more dye is visibly released (about 10-15 seconds).
11. Rinse slides briefly in 100% alcohol.
12. Clear in xylene and mount coverslip with resinous media.

Results:

Gram positive organisms — blue
Gram negative organisms — red
Cytoplasm - collagen — green
Nuclei — red

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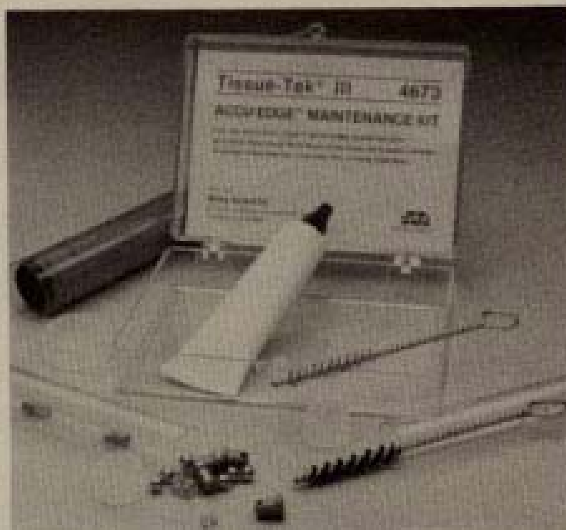

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