Enzyme immunohistochemistry: review of technical aspects and diagnostic applications

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The era of immunohistochemistry was introduced by Coons et al[1] in 1941 when antibodies were successfully labeled with a fluorochromatic compound. Shortly thereafter, localization of tissue antigens was successfully accomplished with the use of fluorochromatic labels.[2] Initially a research tool, immunofluorescence became an essential diagnostic technique for the evaluation of many disease states, particulary autoimmune diseases mediated by immune complexes or autoantibody deposition.

It soon became clear that certain limitations such as special instrumentation requirements and lack of permanency were accorded immunofluorescent procedures. Consequently, immunohistochemical systems were developed that permitted the visual localization of a tissue antigen as a permanent preparation with the potential for visualization of adjacent tissue morphology. The successful conjugation of antibodies with enzymes and unlabeled antibody methods made immunomicroscopy practical. Both enzyme-labeled antibody and unlabeled antibody (antienzyme) methods allowed identification of tissue antigens by formation of permanent color products in histologic sections with excellent morphologic detail.[3,4]

This paper reviews the rationale underlying enzymatic immunomicroscopic procedures, tech-

niques currently available, characteristics of currently available chromogens, safety for personnel, quality control of immunohistochemical systems, and clinical diagnostic applications of the procedure.

Biochemistry of enzyme immunomicroscopy

Many different enzymes are potential antibody labels, including acid phosphatase, β-glucuronidase, 5′-nucleotidase, glucose oxidase, and horseradish peroxidase.[5–7] However, horseradish peroxidase has been the enzyme label used most frequently since it is readily available and relatively inexpensive; well-established conjugation methods have been developed for conjugation with antibody.[8]

The biochemical reaction that occurs at the histochemical level can be summarized in the equation below:

$$H_2O_2 \text{ (to be reduced)} + H_2R \text{ (Chromogen donor)} \xrightarrow{\text{Peroxidase}} R \text{ (Oxidized-chromogen donor)} + 2H_2O$$

The substrate, hydrogen peroxide, is important in the reaction only in that it accepts hydrogen from the chromogen. Ideally, the molecular change in the oxidized chromogen results in a reaction product insoluble in organic solvents which differs in color from its parent compound. The amount of substrate necessary to make the reaction proceed is very small, with usual useful working concentrations of hydrogen peroxide in most systems ranging from 0.0003% to 0.003%.

The proportion of conjugated antibody to enzyme is evaluated by the ratio of enzyme to antibody protein.[8] This is probably best expressed as a molar ratio implying the number of molecules of enzyme coupled to one molecule of antibody protein. At a ratio of three to four enzyme molecules per protein molecule there is loss of antibody binding, enzyme function, and penetration. For most enzyme-labeled antibody immunomicroscopy tests, a molar ratio of 1 enzyme molecule per protein molecule is adequate. This allows high function of both antibody and enzyme and good penetration (peroxidase + antibody = 40,000 + 160,000 = 200,000 molecular weight). For enzyme-linked immunosorb assay (ELISA) a molar ratio of between two- and three-enzyme molecules per molecule of antibody may be desirable. Currently, numerous commercial preparations of high-titer antibodies with optimum enzyme-protein conjugation ratios are available. However, lot-to-lot differences and variations between various companies' antisera exist and, therefore, the reactivity and specificity of every commercial reagent must be confirmed.

Tissue processing

The choice of type of tissue processing is largely dependent upon the individual microscopic system. Extracellular immune complexes and autoantibodies may be detected in paraffin-embedded tissue with the use of posttrypsinization techniques. Such preparations do provide superior morphology. However, frozen tissue is preferable for most studies since 10% to 25% of cases positive with cryostat frozen section immunofluorescence are negative even if dewaxed paraffin-embedded tissue is pretreated with trypsin.[9,10]

The effect of tissue fixation is especially important in the evaluation of lymphoproliferative disorders. It has been shown that fixation using any mordant solution markedly alters the immunoglobulin products associated with non-Hodgkin's and Hodgkin's lym-

phoma cells and reactive lymphocytes.[11,12] Although paraffin-embedded tissue sections may be counterstained to give excellent cellular detail in immunomicroscopic sections,[13] spurious immunostaining of non-Hodgkin's lymphomas not infrequently occurs.[11,14] Furthermore, immunostained cryostat frozen sections are amenable to counterstaining with hematoxylin and eosin or other counterstains permitting some definition of cellular morphology.[15]

For paraffin-embedded systems, reports vary widely as to the superiority of different fixatives.[16–22] It has been suggested that 2% formaldehyde is superior to 4% formaldehyde.[21] Some investigators have found cacodylate-buffered paraformaldehyde superior to Bouin's solution for cytoplasmic immunoglobulins and Bouin's best to preserve antigenicity of hormones.[22] In our experience, the best approach is to evaluate each immunomicroscopic system independently with regard to optimum fixative solutions.

When submitting tissue for paraffin embedding, an important consideration is the thickness of the original tissue specimen when placed in fixative. To ensure complete tissue penetration, 1- to 2-mm thick sections should be placed in abundant volumes of appropriate fixative.

The alleged problems of immunoglobulin diffusion and spurious staining said to occur with cryostat frozen section immunohistochemistry have not proved serious under close scrutiny.[11,13] Study of frozen section material yields reproducible results and observations consistent with well-established concepts of monoclonality in non-Hodgkin's lymphomas in most cases.[11,12,15,23] Furthermore, small amounts of alcohol used in paraffin embedding markedly alter the immunoglobulin phenotypes of proliferating lymphoid cells.[11,12] Both direct and unlabeled frozen section immunohistochemistry readily detect appropriate immunoglobulin phenotypes.[23–25]

Chromogens

Table 1 summarizes data currently available for chromogens used in immunoperoxidase methods. Each chromogen offers certain advantages but has some disadvantages, and many ques-

Table 1. Properties of chromogens

Immunohistochemical label	Solubility in organic solvents	Color	Carcinogenicity (laboratory animals)	Federal regulations of use
Fluorochromatic				
Fluoroscein isothiocyanate	Not applicable	Green	?	–
Tetramethyl rhodamine	Not applicable	Red	?	–
Enzymatic				
Benzidine dihydrochloride	–	Blue	+	+
DAB (diaminobenzidine)	–	Brown-black	?*	–
TMB (tetramethyl benzidine)	–	Blue	?†	–
HYR (Hanker-Yates reagent, p-phenylenediamine & pyrocatechol)	–	Black	?†	–
AEC (aminoethylcarbazole)	+	Red-brown	+	–

* One study has reported that diaminobenzide (3,3′,4,4′-Tetraminodiphenylether·4 HCl or 4,4′-Oxybis-o-phenediamine) did not act as a carcinogen in experimental animals.[31]

† Commercial sources of these reagents specify that this chromogen is noncarcinogenic but, to our knowledge, studies of carcinogenesis of these compounds sponsored by the federal government have not been done.

tions regarding the safety of these compounds (and fluorochrome markers) remain unanswered.[26] Benzidine dihydrochloride gives a stable blue color reaction product, which has been associated with greater sensitivity than other available chromogens.[27–29] Governmental regulations have made use of this compound impractical.[30]

The most widely used immunohistochemical chromogen has probably been 3,3-diaminobenzidine dihydrochloride monohydrate (DAB).[13] This reagent yields a brown to black color reaction product, which is not soluble in organic solvents and does not crystallize on the tissue sections. DAB is not currently regulated to our knowledge and in one study did not demonstrate carcinogenesis in experimental animals.[31] Tetramethylbenzidine (TMB) has been advocated as an alternative chromogen that has not been associated with carcinogenesis in laboratory animals, but crystallization on the tissue sections is a problem.[28,29,32]

The availability of multiple types of peroxidative chromogens yielding different color reaction products having different tinctorial properties allows the simultaneous visualization of more than one antigen in the same tissue section.[33–35] These results can be achieved without elaborate double incubation steps.[35] Similar double-labeling studies have also been done with the use of a combination of enzyme labels such as glucose oxidase and horseradish peroxidase.[36]

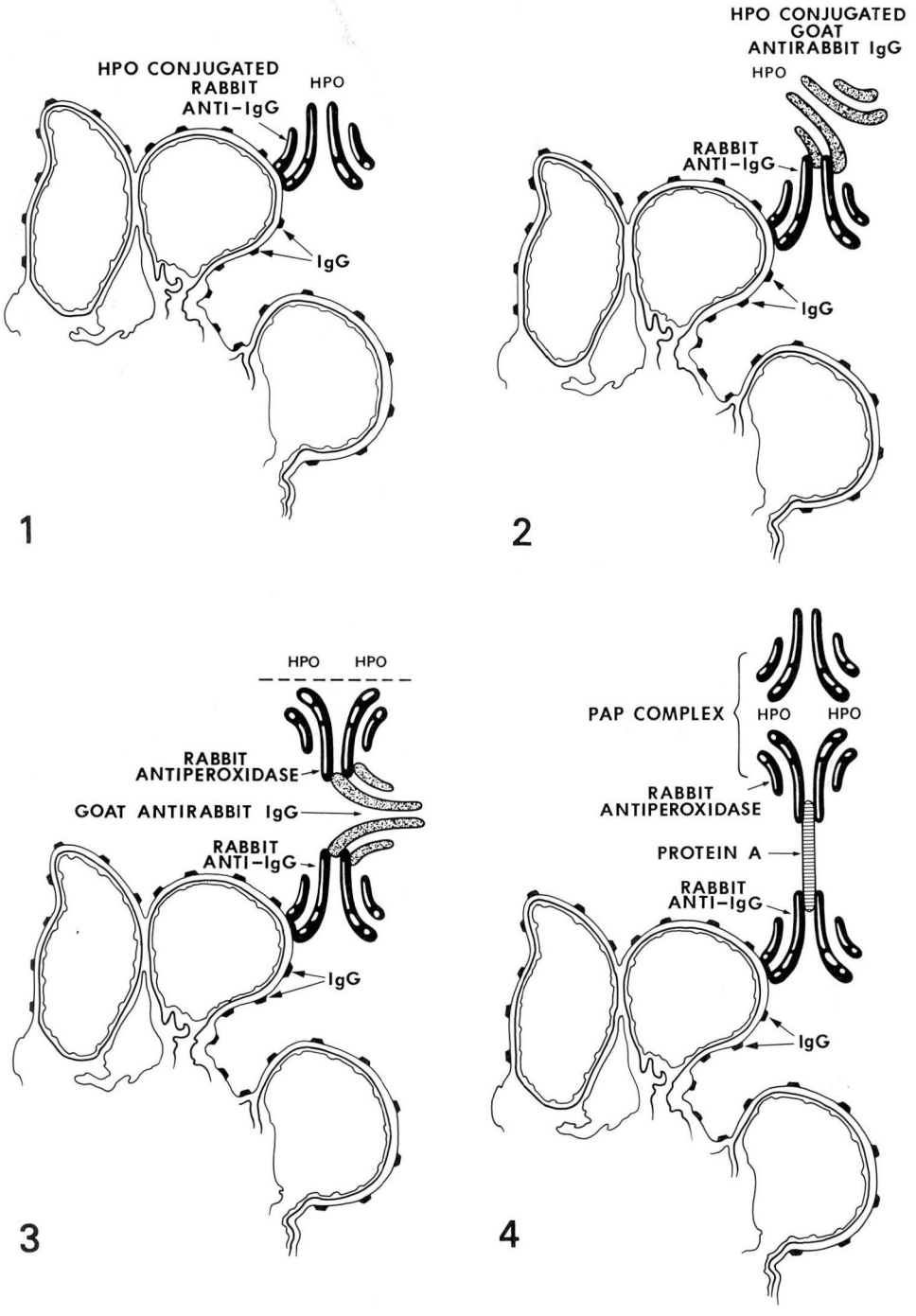
Aminoethylcarbazole (AEC) has also been advocated as a useful chromogen.[37,38] However, recent evidence suggests that carcinogenic potential in laboratory animals does exist, and this reagent may be regulated by the government in the future.[39]

Hanker et al[40] have developed a chromogenic reagent (p-phenylenediamine and pyrocatechol) that incorporates the better qualities of benzidine derivatives and that has no currently identified carcinogenic properties.[41] The biochemistry of this chromogen depends upon the peroxidation of aromatic alcohols in the presence of phenolic compounds.[40]

From the results of a recent study comparing nine methods for immunohistochemical chromogen systems it was concluded that TMB provided the greatest sensitivity and specificity.[32] However, these conclusions have been challenged, and Hanker-Yates reagent (HYR) has been suggested as a superior immunohistochemical chromogen.[42] Published reports have described variable methodology for HYR procedures, and the differential sensitivity of TMB and HYR may be attributable to minor technical variations. HYR reagent works well when a sequence of fresh substrate-chromogen solutions are used with addition of the substrate just prior to placing the sections into the chromogen solution.[41] At present one of the more useful reagents would appear to be HYR since it has no known carcinogenic potential to our knowledge,[42] and has been shown to work well in comparative immunomicroscopic systems.[41] All chromogens and fluorescent-labeled compounds should be handled as potentially hazardous reagents.

Enzyme immunomicroscopic procedures

Several enzyme immunohistochemical procedures are available and these are diagrammatically summarized in *Figures 1–5.* Once the enzyme has been localized at the antigen site by any of these procedures, the techniques for development of the substrate chromogen reaction product are the same regardless of the immunohistochemical technique chosen.



Figs. 1–4. Diagrams of four different immunoperoxidase methods to detect IgG in epimembranous immune complexes deposited in membranous glomerulonephritis. **Fig. 1**, direct technique; **Fig. 2**, indirect technique; **Fig. 3**, unlabeled peroxidase-antiperoxidase (PAP) technique; and **Fig. 4**, protein A modification of PAP technique.

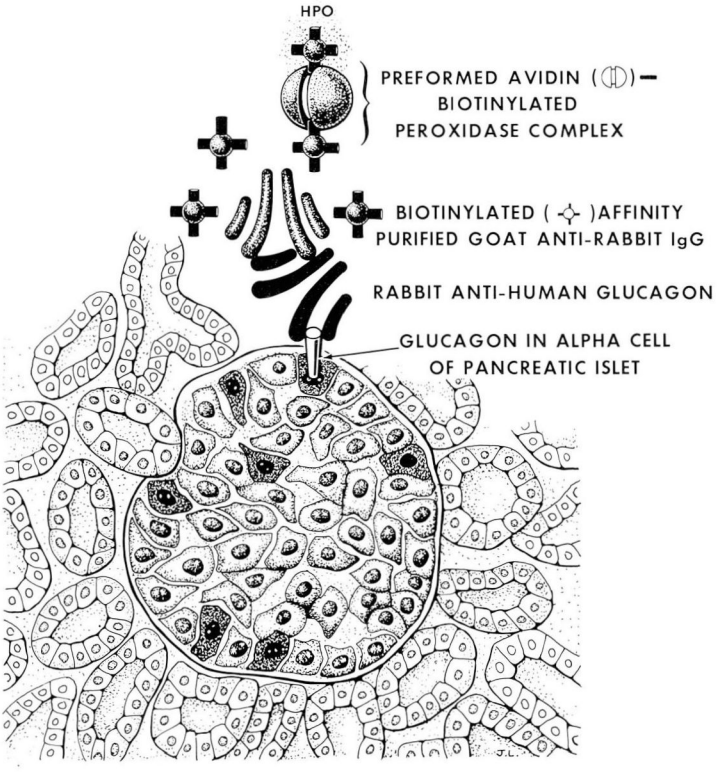


Fig. 5. Diagram of biotin-avidin "ABC" technique to detect glucagon within pancreatic alpha islet cells. Biotinylated affinity purified goat anti-rabbit IgG secondary antibody links the rabbit antiglucagon primary antibody to a preformed complex of avidin and biotinylated horseradish peroxidase.

Enzyme-labeled antibody methods

Direct method. The direct technique is the simplest immunomicroscopic procedure *(Fig. 1)*. The reagent consists of a specific antibody conjugated with enzyme. This enzyme-antibody conjugate is overlaid directly on the hydrated tissue section. Duration of incubation varies with the individual immunomicroscopic system. After washing of excess reagent from the tissue surface with an isotonic buffer system, the enzyme-substrate color reaction product is developed with one of the chromogens currently available.

The direct procedure that uses cryostat frozen sections is currently the technique of choice for studying renal tissue. Sensitivity of this procedure, although not as high as that for peroxidase-anti-peroxidase (PAP) procedure, is adequate for most clinical tissue studies. The direct technique also works well for detection of intracellular and surface membrane-associated immunoglobulins in lymphoproliferative disorders.[13]

Indirect method. The indirect immunoperoxidase (IMP) procedure does not differ from its immunofluorescent (IF) counterpart with respect to the basic technique *(Fig. 2)*. The primary unconjugated antibody binds specifically to its antigen in the tissue, and after washing off excess primary antibody from the surface of the tissue, the peroxidase-labeled secondary antibody is applied. Subsequently, the enzyme color reaction product is developed. Since the secondary antibody is labeled

with the enzyme, a color reaction product identifies the antigen focus in the tissue.

Unlabeled antibody methods

Triple antibody bridge method. For this method, primary antibody and antiperoxidase antibody are raised in the same animal, e.g., rabbit. A bridge antibody, e.g., anti-rabbit IgG, is applied in sequence after the primary antibody and before the addition of the antiperoxidase to the tissue surface. This secondary antibody "bridges" the primary and secondary antibody by virtue of its specificity for the immunoglobulin class in the primary and tertiary reagents. Finally, peroxidase is applied to the tissue section and the reaction product developed. Use of this particular reagent has been virtually eliminated by availability of the sensitive PAP unlabeled technique, which uses a preformed soluble PAP complex.

Unlabeled PAP method. The unlabeled PAP procedure is illustrated in *Figure 3.* This particular method differs from the triple antibody method only in that the tertiary reagent consists of a soluble complex of peroxidase and antiperoxidase. Excellent commercial sources of PAP are available. This particular procedure is generally more sensitive than the other available methods. However, the sensitivity of the labeled (indirect) antibody technique with the use of affinity-purified antibodies is about equal to that of the PAP method.[44] In some systems, the sensitivity of the PAP procedure approaches that of radioimmunoassay with useful working dilutions of the primary antibody approaching 1:100,000.[45] Rabbit PAP systems employ in sequence primary rabbit antibody against the tissue antigen in question, a bridge antibody consisting of goat or swine anti-rabbit IgG, and the soluble rabbit PAP complex. Similarly, the goat PAP system consists of a goat primary antibody, a bridge antibody, e.g., rabbit anti-goat IgG, and a soluble goat PAP complex.

Protein A modification of PAP method. Protein A from *Staphylococcus aureus* (SPA) has been shown to bind the Fc portion of IgG molecules of several species.[46] This particular reagent can be used as a conjugate with peroxidase as a "labeled secondary antibody" as a consequence of its Fc IgG binding.[47] Also, SPA can be substituted for bridge antibodies, e.g., goat anti-rabbit IgG or rabbit anti-goat IgG in the unlabeled PAP procedure *(Fig. 4).*[47–49] However, there are differences in relative avidity of SPA for the PAP complexes of different animal species; for example, rabbit and guinea pig PAP bind more completely than goat or rat PAP.[47–49]

Biotin/avidin lectin method

Recent evidence suggests that biotin/avidin enzyme immunohistochemistry compares favorably with established IMP techniques *(Fig. 5).*[50] The recently developed ABC lectin immunohistochemical system has been found to be 8 to 40 times more sensitive than the unlabeled PAP method, yields immunostained sections having negligible or no background staining, and is cost effective (about 5% of cost of average PAP procedure).[51] The ABC system uses in sequence unconjugated primary antibody, biotinylated affinity purified secondary antibody, and a preformed complex of avidin and biotinylated horseradish peroxidase as the tertiary reagent. The extraordinary sensitivity and specificity of this method are due to at least three factors: (1) avidin has high binding affinity for biotin; (2) the avidin-biotin binding reaction is essentially irreversible; and (3) unlike the second antibody of a PAP system (which must be present in excess since one of its two

potential antibody binding sites must be available to bind the PAP complex), biotinylated secondary bridge antibodies can be used in low concentrations since the biotin is already linked to the antibody.

Each of these methods has certain advantages and disadvantages. The indirect labeled and unlabeled antibody techniques, while offering increased sensitivity, require multiple procedural steps and involve additional reagents. Also, in some immunohistochemistry systems, increased sensitivity may be gained at the expense of specificity.

Background staining: the problem of endogenous peroxidases and pseudoperoxidases.

Both IF and IMP procedures are associated with certain predictable artifacts. Autofluorescence of certain materials in tissue must be recognized and interpreted for individual sections. Pseudoperoxidases such as hemoglobin and naturally occurring endogenous peroxidases in human tissue are similarly a source of misleading background staining in enzyme-labeled preparations. One of two approaches can be used to circumvent this problem. First, controls consisting of tissue not exposed to the specific primary antibody, but allowed to incubate with the substrate chromogen solution, will allow visualization of these endogenous peroxidases, and comparison can be made with specific immunostained sections. However, a more acceptable alternative is to destroy or consume the endogenous peroxidase either by preincubation of tissue sections with methanol H_2O_2 or by trypsin, protease, or pronase digestion.[9,10,52–56] Proteolytic enzyme pretreatment appears to enhance antigenicity by a mechanism that is not well established.

Nonspecific binding of antisera

Although methanolic H_2O_2 or enzyme pretreatment destroys or consumes endogenous peroxidases, the nonspecific absorption of heterologous serum to the tissue occasionally yields a problem in background staining. These problems can be eliminated by a combination of prolonged incubation with high dilutions of the primary antibody ($\geq$1:1000) and preincubation of rehydrated sections in nonimmune serum.[13]

Relative merits of immunomicroscopic methods

The disadvantages and advantages of fluorochromatic and enzyme-labeled techniques have been the subject of extensive debate. Since many of the initial IMP studies were done on paraffin-embedded tissues, for some time IF was thought to be a procedure most suited to frozen section material and IMP for fixed paraffin-embedded material. It is now known that either IF or IMP techniques are readily applied to fixed or frozen tissue sections. The initial lack of correlation observed between IF and IMP in studying lymphoproliferative disorders in frozen section material now appears to be a consequence of the methodology employed or antibody concentrations.[11,12,24] Also, the earlier problems encountered in differentiating granularity and linearity in renal biopsy specimens[57,58] were not observed in an evaluation of large numbers of kidney biopsies studied by comparative IF, IMP, and electron microscopy.[59–61]

Certain advantages are accorded the IMP procedure as compared with IF. IF preparations fade with repeated examination and storage, but immunohistochemical preparations yielding stable color reaction products do not fade.[13]

Also, it is not usually possible to visualize well simultaneously the immunostained antigen and adjacent tissue morphology in IF preparations. Conversely, IMP preparations are readily adaptable to a variety of counterstains enabling the observer to (1) more precisely locate the tissue antigen, and (2) evaluate such additional parameters as inflammatory response to the antigen. An IF microscope is necessary for examination of the IF preparations, and photographic documentation is necessary.

Previously, valid disadvantages were also accorded IMP procedures. These included the potential carcinogenic nature of the chromogens used with IMP, and the lack of reliable commercial reagents. Both of these objections are no longer valid, since diaminobenzidine (3,3′, 4,4′-Tetraaminodiphenylether · 4 HCl or 4,4′-oxybis-o-phenylenediamine) may not be a carcinogen[31] and at least one chromogen, HYR, is now available, which has no currently identified health hazard and yields excellent results. Several manufacturers currently distribute antibody-enzyme conjugates of excellent quality. Objections relating to the more complex nature of IMP procedures are no longer valid, since the direct technique using enzyme conjugates can be used for most studies that employ immunohistochemistry, i.e., renal diseases and lymphoproliferative disorders. The additional time required to develop the substrate chromogen reaction product is no longer than the additional time required for photography and cataloging of photographic slides for IF.

It is ideal to have the capability of doing both IF and IMP procedures. This allows the pathologist versatility in the selection of the appropriate procedure.

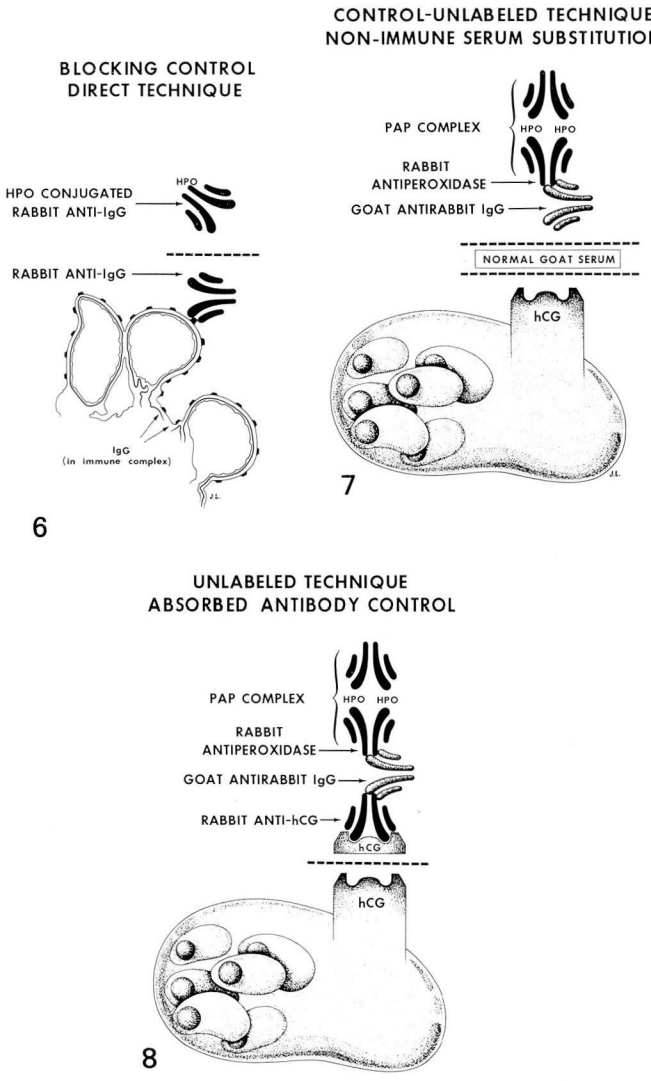
> It seems that the resistance to change from immunofluorescence to immunoperoxidase techniques for the routine examination of renal biopsy material cannot be explained in scientific terms but depends largely on emotional ties to a system which has been established for a considerable number of years.[60]

Quality assurance

Quality control of both IF and IMP reagents is an essential and often neglected part of fluorescent and enzyme immunohistochemistry. Commercially available antibody should not be assumed to be monospecific or of adequate immunoreactivity. The specificity and sensitivity of each reagent purchased should be evaluated when received. Each laboratory should have a protocol for evaluating all new antibodies entering the laboratory. The antisera should be dated when received, and evaluated by Ochterlony immunodiffusion, immunoelectrophoresis, competitive binding radioimmunoassay or immunohistochemistry with the use of preabsorption and postabsorption with antigen control tissues that have been well characterized with respect to the appropriate antigen. Many commercial antisera have package inserts that attest to the reliability of the reagents. However, there may be significant interlot variation and the reagent immunoreactivity may be altered by environmental factors during shipping. Individual techniques should be performed regularly to assure continued competence by technical personnel and adequate performance of reagents. A detailed record should be kept of all quality assurance tests and documentation of corrective actions taken.

Even when excellent standardization and characterization of antibody have been completed, rigorous in-run controls are necessary for valid interpreta-

tion of results. Several types of controls that may be used are summarized in *Figures 6–8*. The simplest control is omission of the primary antibody and substitution of nonimmune serum such as normal goat serum or normal swine serum for the primary antibody. This technique serves as a control for recog-



Figs. 6–8. Diagrams of three types of controls for immunohistochemistry. **Fig. 6**. Preincubation of tissue section with unconjugated antibody followed by addition of labeled antibody does not result in staining, since tissue antigen binding sites are occupied by the first reagent. **Fig. 7**. Substitution of nonimmune normal heterologous serum for the primary antibody is applicable to any immunohistochemical procedure, detects nonspecific binding of secondary or tertiary reagents, and profiles endogenous peroxidase staining. **Fig. 8**. In vitro preabsorption of primary antibody with antigen in question, followed by addition of filtered or centrifuged antiserum-antigen mixture to the section, is also applicable to any immunohistochemical procedure and is the best available negative control. This type of control detects lack of primary antibody specificity, as well as nonspecific binding of secondary or tertiary reagents and endogenous peroxidase activity.

nition of binding of secondary and tertiary antibodies to the tissue and for endogenous peroxidase in indirect and PAP methods, but cannot adequately assess monospecificity of the primary antibody. The direct IMP procedure can be controlled by preincubation with unlabeled antibody, preferably from the same antisera lots from which the conjugate was prepared. This type of control effectively blocks the labeled conjugate from reaching the antigen and although satisfactory for the direct procedure, cannot be applied to indirect or unlabeled modifications of the technique. The most reliable control for all IMP methods is an absorbed antibody control, in which the primary antibody is preincubated with exogenous antigen, thus binding all the available antibody-reacting sites. When the supernatant from the centrifuged mixture of bound antibody-antigen is applied to the tissue section, antibody is not available for the reaction and immunostaining does not occur.

Preabsorption may be necessary to remove nonspecific reactants or reactions with related antigens in the tissue. Not uncommonly, secondary antibodies, e.g., goat anti-rabbit IgG, or rabbit anti-goat IgG, will cross react with human immunoglobulins. In such instances, absorption with purified human gamma globulin followed by centrifugation of the antibody is necessary to assure specificity of the secondary antibody. For every procedure done on a day-to-day basis and for each tissue analyzed, an in-run control should be included for adequate verification of positive or negative results. Use of affinity purified antibodies may help insure specificity.

Finally, when a new commercial antibody is purchased or antibody is made available from other sources, checkerboard titrations with varying combinations of antibody dilutions can be used on control tissue sections to evaluate the optimal dilutions of each reagent.

Diagnostic applications

Immunohistochemical procedures have contributed greatly to the understanding of normal physiology and functional organization of many animal and human biologic systems. This paper will be restricted to reviewing the clinical diagnostic applications of IMP methods *(Figs. 9–16)*.

Renal immunohistochemistry

The value of immunomicroscopy in delineating deposition of immunoglobulin and complement components or autoantibody in glomeruli of patients with various autoimmune diseases is well established. Once a tool of academic interest, immunomicroscopy is now an essential diagnostic method that must be applied to every renal biopsy specimen. Patterns of IF have been shown to be highly reproducible and predictive histopathologically.[62]

Comparative studies of IMP and IF of glomerulonephritis were initially favorable.[8] Subsequently, studies of kidney biopsy specimens with the use of the direct technique and enzyme conjugates were associated with unacceptable background staining, and in some cases a distinction between linear and granular color reaction product was difficult.[57,58] Both IF and direct IMP procedures were compared to the unlabeled IMP technique, and the specificity and sensitivity of the unlabeled PAP procedure were demonstrated to be comparable to those of IF.[58] However, the length of the procedure and the expense of additional reagents make use of the PAP procedure for frozen renal tissue a poor choice.

In the past few years, improved tech-

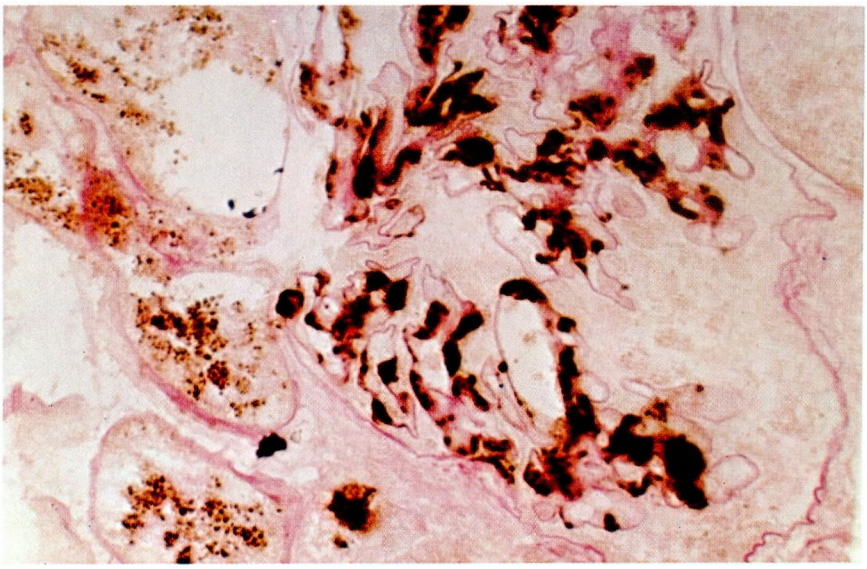


Fig. 9. Photomicrograph, IgA nephropathy, demonstrating confluent granular deposits of IgA in mesangial areas, (IMPAS × 160).

niques of enzyme conjugation have resulted in better commercial antibodies. Excellent reagents can be obtained from several commercial sources for identification of immunoglobulins and complement components. Three recent large series have described IMP results that compare favorably with IF performed on the same cases.[59–61] Immunostained frozen sections can be counterstained with periodic acid Schiff to locate more precisely sites of antigen deposition *(Fig. 9)*.[59]

Paraffin-embedded renal tissue, whether fixed in formalin or precipitative fixatives under the best of conditions, may not be adequate for demonstration of extracellular immune complexes or autoantibodies with standard immunohistochemistry. Since trypsin and pronase have been shown to enhance detection of tissue antigens, recent attempts have been made to use enzyme pretreatment for IF or IMP detection of extracellular immune complexes in paraffin-embedded tissue.[63–65] Similar results can be obtained with IMP with the use of enzyme-digested sections.[9,65,66] MacIver et al[9] have suggested that inconsistent detection of complement in previous studies may be a function of over trypsinization of tissue sections. These investigators demonstrated clear separation of granular and linear staining patterns, precise localization of deposits within glomeruli using posttrypsinization IMP, and a concordance rate with IF of 81%.[9] Optimum conditions for detection of complement were shown to be 0.05% trypsin for 40 minutes, a concentration also sufficient to detect immunoglobulin heavy chains and C1q.

For the present time, fresh tissue should still be used as the tissue of choice for renal immunomicroscopy. With the availability of excellent commercial antibodies and well-established techniques, the direct IMP procedure is probably the technique of choice. It is

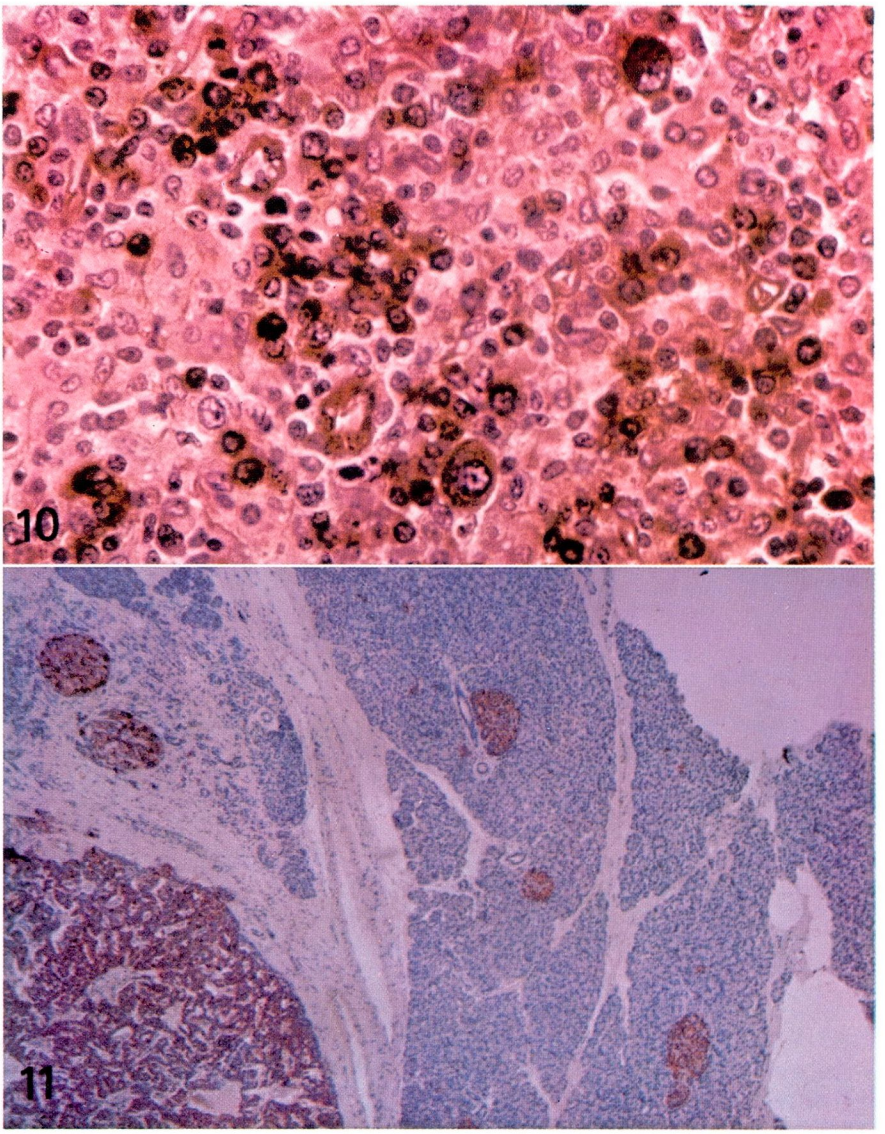


Fig. 10. Photomicrograph, B-cell immunoblastic sarcoma arising in plasmacytoid lymphocytic lymphoma, lymph node. The neoplastic cells (brown-black cytoplasm) are immunostained for kappa light chains. A serial section was negative for lambda light chains. Direct immunoperoxidase technique, counterstained with hematoxylin and eosin, (× 400).

Fig. 11. Photomicrograph, beta cell pancreatic apudoma. The neoplasm and adjacent normal islets contain immunoreactive insulin. Unlabeled PAP technique was used employing aminoethylcarbozole as the chromogen, (× 64).

not clear at this time whether trypsinized deparaffinized paraffin-embedded tissue will be acceptable as an immunomicroscopic preparation for most forms of glomerular disease, since in the hands of some investigators, enzyme pretreatment yields variable tissue digestion and inconsistent immuno-

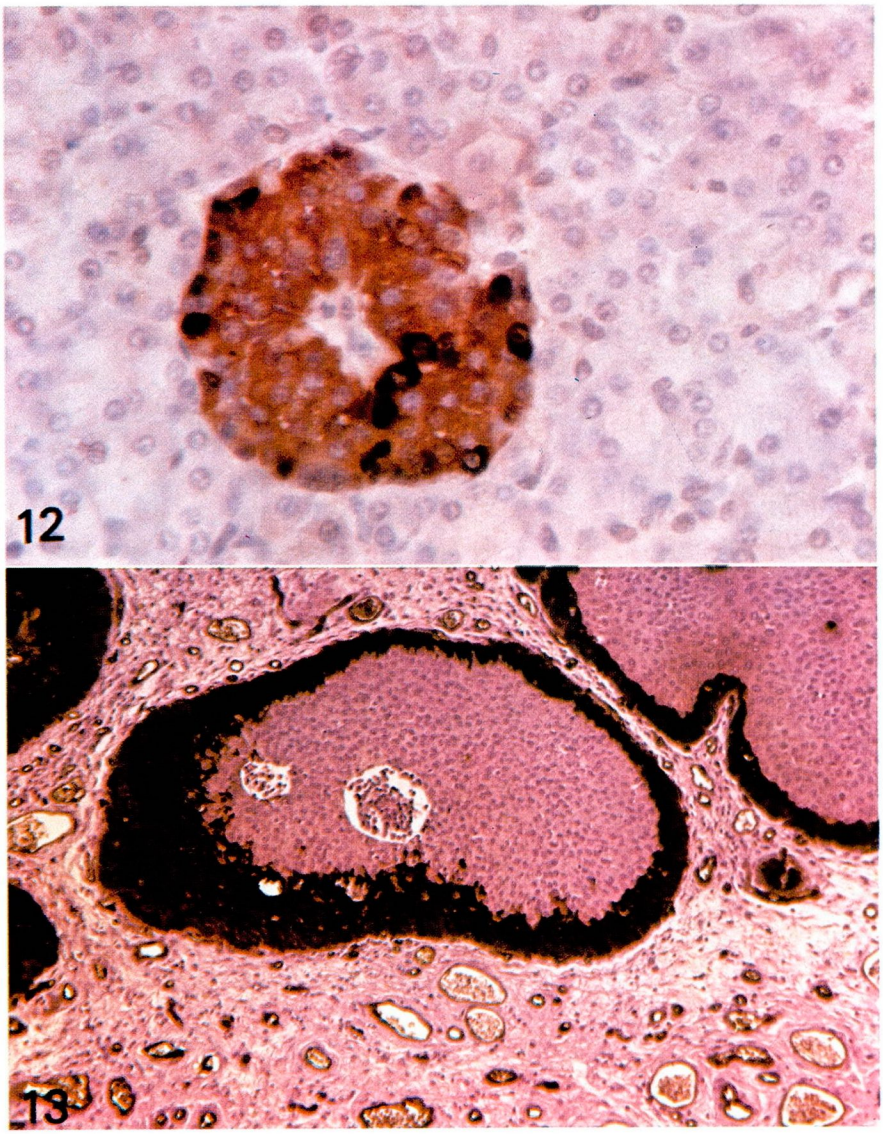


Fig. 12. Photomicrograph, double label study using the unlabeled PAP technique demonstrating glucacon (black) and insulin (red) within the same pancreatic islet, (× 160).

Fig. 13. Photomicrograph, bladder biopsy. Superficial transitional cell carcinoma at right extending over the residual nonneoplastic urothelium does not express blood group A antigen. Endothelial cells, erythrocytes, nonneoplastic urothelium express blood group A antigen (brown-black). Indirect immunoperoxidase technique, counterstained with hematoxylin and eosin, (× 160).

staining patterns due to poorly understood mechanisms that may include formation of antibody or protein moities of different antigenicity.[44]

Lymphoproliferative diseases

Immunohistochemistry has contributed greatly to our understanding of the

organization of the immune system and to architectural and functional alterations in its various components in a variety of disease states. The technology of immunohistochemistry has evolved parallel with increased knowledge about lymphoid neoplasia and has contributed significantly to the understanding of the nature of proliferating cells of malignant lymphoma. Immunohistochemistry of malignant lymphomas will eventually play a role similar to that of immunomicroscopy in evaluating renal disease.

Paradoxically, these techniques have contributed both to understanding and confusion regarding this group of entities. Thus, while documenting the presence of monoclonal cytoplasmic immunoglobulins in many non-Hodgkin's lymphomas,[14,67–72] these techniques as applied to paraffin-embedded tissues have yielded polyclonal staining of B-cell lymphomas in some reports.[14,68] Such observations are not in agreement with the clonal premise upon which most cancer immunology is based.[23,73–75]

Studies emphasizing the immunologic basis for classification of lympho-

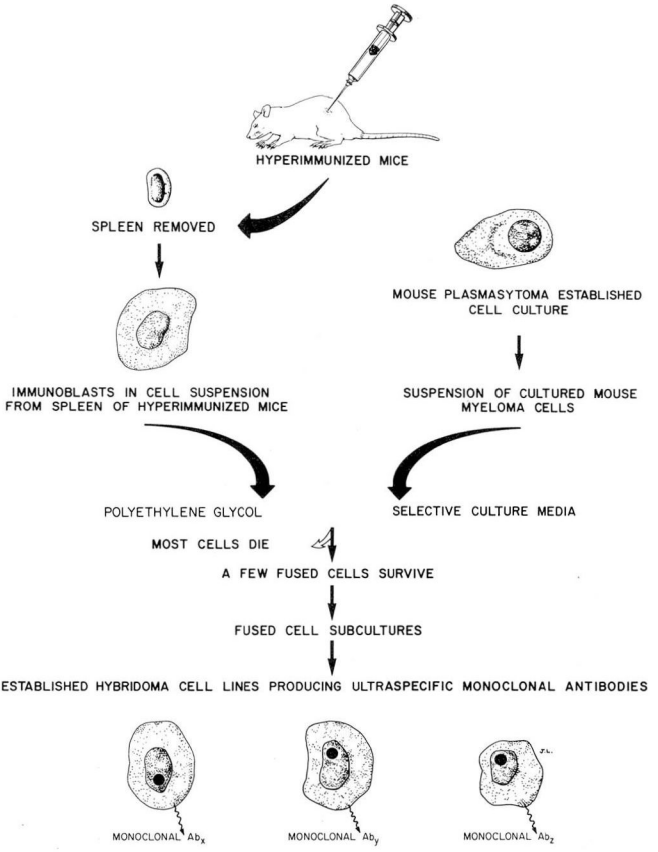


Fig. 14. Diagram of procedure used to obtain monoclonal hybridoma antibodies. Immunoblasts from hyperimmunized mice are fused with cultured mouse plasmacytoma cells in the presence of polyethylene glycol. Although most of the cells die, a few cells survive which contain the genetic content of both the stimulated immunoblasts and mouse myeloma cells. The fused cells are subcultured and cloned, reinjected into mouse peritoneal cavity, and ultraspecific monoclonal antibody harvested as ascitic fluid.

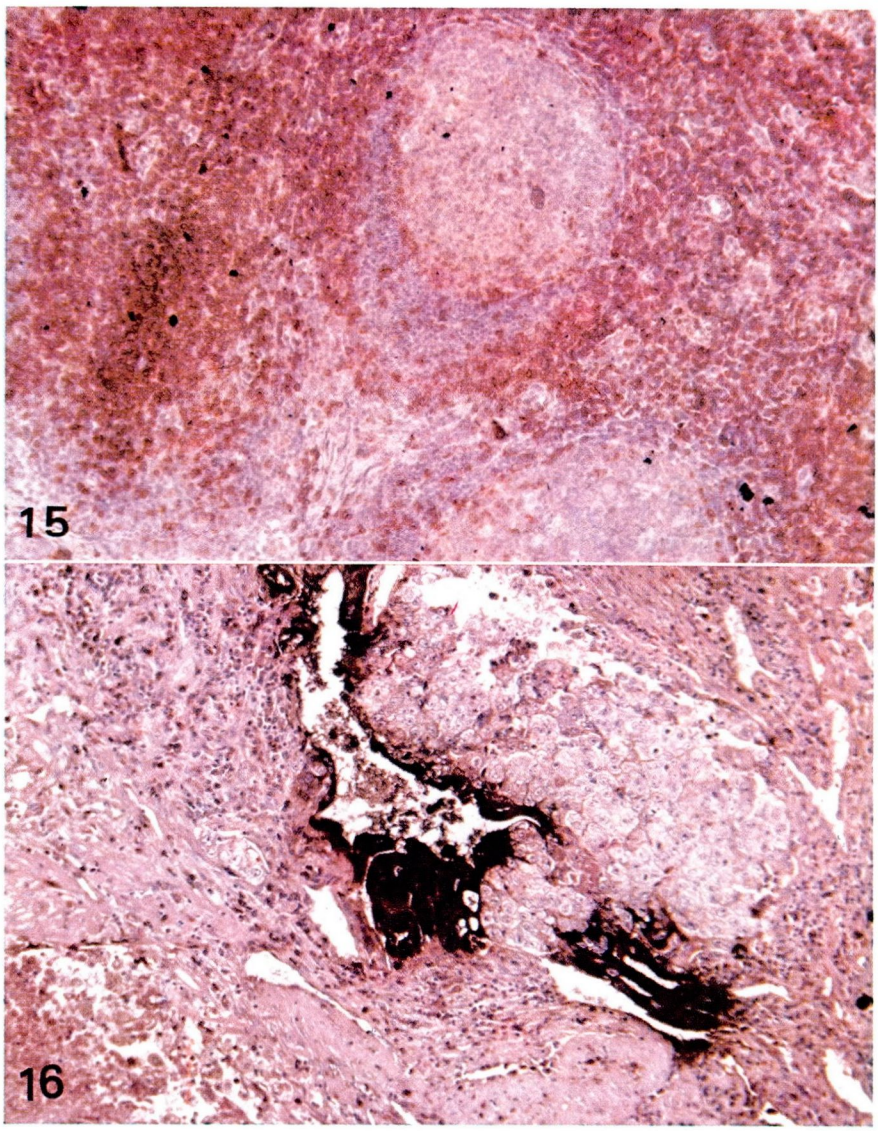


Fig. 15. Photomicrograph, reactive lymphoid hyperplasia, lingual tonsil. Red staining interfollicular T helper/inducer lymphocytes are identified using aminoethylcarbazole as the chromogen. Biotin-avidin "ABC" technique using mouse monoclonal hybridoma primary antibody specific for inducer/helper T lymphocytes, with methylene blue counterstain, (× 64).

Fig. 16. Photomicrograph, malignant mixed germinal neoplasm of testis. Neoplastic syncytia trophoblasts are immunostained for chorionic gonadotropin. Indirect immunoperoxidase technique using mouse monoclonal hybridoma primary antibodies specific for beta subunit of human chorionic gonadotropin, peroxidase conjugated affinity purifed goat anti-mouse IgG, and hematoxylin and eosin counterstained, (× 160).

mas attempt to draw parallels between the components of the immune system and the morphologic diversity of non-Hodgkin's lymphomas. Of the several different classifications available for subtyping of non-Hodgkin's lymphoma, only the Lukes-Collins classification,[69] which is currently available is directly dependent upon the identification of T- and B-cell marker expression by the neoplasm. However, immunologic data can be added to the Rappaport morphologic classification.[70]

Initial functional characterization of lymphomas was done principally by cell suspension (CS) studies.[75] With the use of CS techniques, classification as to T, B, or non-T/non-B origin can be effected in a relatively large number of cases.[73,74] In recent years, information regarding the reliability of CS studies has accumulated. In a number of B-cell lymphomas marking monoclonal with cryostat frozen section immunohistochemistry, polyclonality in CS has been observed.[76–78] This apparent discrepancy may be due to several factors, most likely selective loss of tumor cells or sampling error resulting in contamination of the suspension with nonneoplastic lymphocytes, particularly in nodular lymphomas in which a significant percentage of the lymphoid parenchyma may be spared by the neoplasm.

When the sensitive PAP immunohistochemical technique developed by Sternberger was initially applied to lymphoproliferative disorders, the use of immunologic markers to characterize non-Hodgkin's lymphomas was viewed as an academic curiosity rather than a clinically useful tool by many pathologists. Recent evidence suggests that when non-Hodgkin's lymphomas are approached from the standpoint of Lukes-Collins classification and interpreted in conjunction with surface marker analysis, data of distinct prognostic significance are obtained for subsets of lymphomas.[79] However, immunohistochemical study of paraffin tissue from patients with multiple myeloma has shown phenotypic expression of both kappa and lambda light chains in cells from patients with well-characterized circulating monoclonal immunoglobulins.[68] Thus, the initial enthusiasm for immunohistochemistry was tempered by these apparent anomalous staining patterns that violated basic concepts regarding monoclonality of B-cell neoplasia.[80]

A similar evolution of understanding of immunohistochemistry as applied to non-Hodgkin's lymphomas has been observed.[81–104] The spurious immunostaining patterns observed in a significant number of cases evaluated by paraffin-embedded immunohistochemistry raised serious questions about the validity of results obtained in this manner.[11,14,105] Initially, it was suggested that such results were a consequence of diclonal immunoglobulin production by the non-Hodgkin's lymphoma,[14] an explanation that is not in concurrence with the overwhelming body of evidence for monoclonality in human B-cell lymphomas.[23,106–108]

When results of frozen section and paraffin-embedded immunohistochemistry are compared, it becomes clear that the negative or spurious immunostaining patterns associated with paraffin-embedded tissue are probably a consequence of processing.[11,12,24,25] Currently, cryostat frozen section immunohistochemistry (CFSIH) provides the most sensitive and specific procedure for detection of monoclonal cell populations, since most non-Hodgkins lymphomas demonstrate monoclonal immunostaining with CFSIH.[11,15,23,25] However paraffin-embedded techniques provide su-

perior morphology in the minority of cases that mark in monoclonal fashion *(Fig. 10)*. Optimally, both frozen and paraffin-embedded tissues should be evaluated for each case. CFSIH is also helpful in delineating physiologic domains of lymphoid subpopulations, defining the nature of nonneoplastic lymphoid infiltrates, and in detecting malignant cellular populations in histologically reactive lymph nodes.[109-111]

Immunoglobulin negative non-Hodgkin's lymphomas consist of unusual lymphomas of T-lymphocytic and dedifferentiated B-lymphocytic origin, true null lymphomas of non-T non-B cytogenesis, and neoplastic proliferations of true tissue macrophages. More precise characterization of these unusual lymphoma subtypes can be accomplished by identifying immunohistochemistry profiles of lymphocyte differentiation antigens and elaborated substances *(Table 2)*. The growing availability of hybridoma monoclonal antibodies specific for lymphocyte subpopulations is increasing the accuracy with which determinations of cytogenesis are made.[112] Differentiated B-lymphocytic lymphomas are characterized by monoclonal surface membrane-associated immunoglobulin (CS or CFSIH), cytoplasmic immunoglobulins as detected by immunohistochemistry procedures on paraffin-embedded tissues in some cases, Ia and Ia-like antigens, and J piece expression.[11,113-118] Recognition of T-cell lymphomas has been previously based upon cytochemical expression of acid alpha-naphthyl acetate esterase or acid phosphatase activity in neoplastic cells.[119] However, monoclonal hybridoma antibodies monospecific for subsets of T lymphocytes are now available and are helpful in the recognition of these unusual lymphomas.[120-126] With the use of monoclonal antibodies and CFSIH, immunohistochemical phenotypes can be identified for most large cell lymphomas.[127-129]

Recognition of neoplastic proliferations of true tissue macrophages is perhaps the most difficult diagnostic challenge at present. Much of the current problem is a consequence of various morphologic criteria used and the arbitrary distinctions that have been established to distinguish between malignant histiocytosis and histiocytic lymphoma of true tissue macrophage origin. True tissue macrophages with maturation will express alpha-naphthyl acetate esterase activity in diffuse pattern, and are associated with the expression of muramidase and alpha-1-antitrypsin, Ia antigen, and polyclonal cytoplasmic immunoglobulins.[130-137] However, in poorly differentiated variants of malignant histiocytosis, the neoplastic cells may not express these proteins.[134,135] The most definitive evidence for tissue macrophage origin will be the identification of antigens peculiar to monocyte/macrophage differentiation on neoplastic cells with the use of monoclonal antibodies.

Terminal transferase, an enzyme present in lymphoblasts but absent in myeloblasts, may be helpful in subtyping the acute leukemas.[138,139] Both biochemical and IF techniques are currently used to identify the enzyme. Reliable immunohistochemistry methods for in situ demonstration of terminal transferase in tissue have not been developed to our knowledge. Results of initial studies of a cross-reacting antibody that preferentially immunostains myeloblasts in tissue suggest that this marker may also be helpful in subtyping leukemias.[140]

Since the monoclonal nature of B-cell non-Hodgkin's lymphomas has been well established, use of objective methods has been advocated to distinguish

Table 2. Immunohistology of lymphoproliferative disorders

	Ig	JP	Ia	OKT_3 Pan	OKT_8 Sup	OKT_4 Ind
Reactive hyperplasia						
Follicles						
Light zone	PC	+	+	OC	–	OC
Dark zone	PC	+	+	–	–	–
Sinus	PC, E	–	+	–	–	–
Paracortex	OC, DC	–	OC	+	+	+
Non-Hodgkin's lymphomas						
B cell lymphomas	MC	+	V	–	–	–
With Fc receptors	E	+	V	–	–	–
T cell lymphomas	–	–	V	+	+*	+*
With Fc receptors	E	–	V	+	+*	+*
Dedifferentiated B cell						
Lymphomas	–	–	+	–	–	–
With Fc receptors	E	–	+	–	–	–
Hodgkin's disease						
Plasma cells	PC	+	–	–	–	–
Reactive tissue macrophages	PC	–	+	–	–	–
Neoplastic cells	PC	–	NWE	NWE	NWE	NWE

Abbreviations: Ig = surface and/or cytoplasmic immunoglobulins (best assessed with cryostat frozen section immunohistochemistry). JP = J piece, OKI = monoclonal antibody specific for Ia antigen, OKT3. Pan = monoclonal antibody specific for all peripheral blood T lymphocytes, OKT8. Sup = monoclonal antibody specific for suppressor/cytotoxic T lymphocytes, OKT4. IND = monoclonal antibody specific for inducer/helper T lymphocytes, PC = polyclonal (both κ and λ light chains present), + = present, – = absent, OC = occasional cells, DC = dendritic cells, NWE = not well established, V = variable, MC = monoclonal (only one light chain present, either κ or λ), Fc = receptors for Fc portion of immunoglobulin), E = surface-associated immunoglobulin which can be eluted with acidic buffer.

* T cell malignant lymphoproliferations react with either T Supp. or T inducer/helper monoclonal antibody, depending on differentiation of the neoplasm.

reactive lymphoid hyperplasias from non-Hodgkin's lymphomas by characterizing surface immunoglobulin phenotypes.[141–144] Since CS may yield spurious results in non-Hodgkin's lymphomas, possibly due to contamination with nonneoplastic populations or selective loss of tumor cells, CSFIH should be used to help determine the biologic potential of the lymphoproliferative disorders.[141–144]

The origin of Reed-Sternberg cells in Hodgkin's disease has been the subject of extensive debate. For years the preferential involvement of the lymph node sinus and interfollicular zone and defects in cellular immunity were interpreted as evidence for T-cell or tissue macrophage origin. Immunohistochemistry studies by Taylor[14] and others[145–148] showed that Reed-Sternberg cells contain polyclonal IgG. These observations are consistent with a tissue macrophage origin, the cytoplasmic immunoglobulin probably representing engulfed exogenous polyclonal immunoglobulin. Tissue culture cell lines derived from Hodgkin's disease display cytochemical and immunologic features of macrophages, and when transplanted into experimental animals produce tumors with morphology resembling Hodgkin's disease.[149] Furthermore, CS from tissues involved by Hodgkin's disease contain Reed-Sternberg cells that have polyclonal cytoplasmic IgG, and

actively bind and internalize labeled exogenous immunoglobulins.[150] Although a tissue macrophage origin of Hodgkin's disease appears likely, definitive evidence could be obtained with immunohistochemistry staining with the use of lymphocyte-monocyte differentiation monoclonal antibodies. To our knowledge, such a study has not been done.

Endocrine systems

IMP procedures have contributed greatly to our understanding of the C-cell neoplasms and preneoplastic state of the thyroid gland. Wolfe et al[151] have delineated the distribution of C cells in the normal gland and in the thyroid gland of patients at risk for hereditary medullary carcinoma. When evaluated in this fashion, it has been shown that the middle and upper portions of the lateral thyroid lobes show marked increases and clustering of calcitonin-containing cells in this disease. The immunohistochemical demonstration of thyroglobulin within well-differentiated tumors of the thyroid gland of both papillary and follicular types has been shown to be helpful in confirmation of thyroidal origin, since tumors of nonthyroidal histogenesis examined did not show thyroglobulin synthesis.[152,153]

Immunohistochemistry studies have been helpful in evaluation of both normal and neoplastic pituitary tissue. It has been shown that adenomas of the adenohypophysis, although they may be tinctorially homogeneous, are immunohistochemically heterogeneous.[154] Immunohistochemistry studies are helpful in delineating the presence of neoplastic cells containing the hormone circulating in the patient. Thus, the documentation of prolactin in resection tissue from the anterior pituitary of a patient with hyperprolactinemia as assessed by radioimmunoassay is a helpful confirmatory study.[155,156] Study of the pituitary tissue of acromegalic patients has documented the presence of growth hormone within the neoplastic tissue, an observation corroborating radioimmunoassay results.[157] However, Fukaya et al[157] have also demonstrated occasional immunoreactive cells positive for prolactin and luteinizing hormone in an adenoma removed from a patient with acromegaly, raising questions about the significance of identifying other hormones within a particular tumor. Such immunoreactive cells may represent residual normal pituitary tissue. Conversely, these observations may suggest that pituitary adenomas are polyclonal neoplastic proliferations with secretion of one hormone dominating the clinical presentation. Recent cases of pituitary adenomas with ultrastructural and immunohistochemical evidence for heterogeneous cell populations have been described that were eosinophilic in tinctatorial differentiation but heterogeneous in their elaboration of growth hormone and prolactin.[158,159] It has been suggested that at least some of these described cases may involve technical problems and may not actually represent a stem-cell neoplasm.[160] Immunohistochemistry may prove to be especially helpful in the confirmation of hormonal homogeneity in small specimens of tissue removed as microadenomas from patients with Cushing's syndrome. Currently, this confirmation is based upon standard histochemical and clinical correlations.[161] Study of pituitary adenomas occurring in association with MEN I syndrome has confirmed that most of the adenomas are of either prolactin or growth hormone type.[162,163] In the rate occurrence of pituitary carcinoma metastatic to extracranial sites, immunohistochemistry techniques may offer confirmatory evidence for pituitary

origin of the neoplasm.[164] Of greater interest is the immunoreactivity demonstrable in chromophobe adenomas of the adenohypophysis. These tumors are usually characterized by multiple hormonally positive cells for most of the hormones endogenous to the anterior pituitary.[165] Immunohistochemistry techniques may also prove helpful in the characterization of "hypoplasias" or preadenomatous states.[166] Other applications of immunohistochemistry in the study of pituitary disease include the delineation of decreased hormonal synthesis or storage in gonadotrophs in patients with hemochromatosis.[167] Confirmation of elaboration of adenohypophyseal hormones by ovarian teratomas, oat cell carcinomas, and other extrapituitary tumors may also be done with the use of immunohistochemistry techniques.[168–171]

The use of immunohistochemistry in evaluation of the endocrine pancreas has yielded some interesting results *(Figs. 11, 12)*. In experimental animals, the distribution and relative frequency of different immunoreactive cell populations have been documented with IMP and IF.[172] Human pancreatic endocrine tumors are characterized by distinct ultrastructural features that allow subclassification of pancreatic apudomas.[173] Immunohistochemistry techniques have been used to identify the distribution of insulin-positive cells in hyperinsulinemic hypoglycemia of infancy,[174] to document the presence of hormones such as glandular kallikrein and cholecystokinin-pancreozymin within islet-cell populations[175,176] and to profile immunohistochemically the hormonal content of islet-cell tumors[177] *(Fig. 11)*.

Kurman et al[178–180] have used immunohistochemistry to localize elaborated steroid molecules in tumors of the ovary and the testis. With these techniques, both testosterone and estradiol were identified in Sertoli-Leydig cells and in primitive spindled cells in these tumors.[169–172] Estradiol was localized in granulosa cells and in luteinized theca cells, and nonluteinized stromal cells were negative for steroids.[178–181] Immunohistochemistry techniques may thus prove helpful in the subclassification of ovarian tumors based upon the predominant hormone elaborated, and also in the confirmation of gonadal stromal origin when such neoplasms occur in an extragonadal location.[180,181]

When carcinoid tumors are evaluated with immunohistochemistry techniques, positive immunoreactivity for multiple hormones is usually identified.[182] However, somatostatin immunoactivity ordinarily predominates and is usually associated with immunostaining for other hormones such as gastrin or calcitonin.[182] Most of the tumors studied in this fashion have been clinically silent with respect to hormonal elaboration.

The emergence of immunohistochemistry techniques specific for prostatic acid phosphatase have proved to be very helpful in the confirmation of prostatic origin of secondary metastasis.[183–187] Nadji et al[188] have also recently shown that an antibody can be raised against specific tumor antigens of prostatic carcinoma rather than the elaborated acid phosphatase product. The antibody thus derived is specific only for carcinoma of prostate histogenesis.

Oncodevelopmental antigens

Oncodevelopmental antigens are a group of substances that are produced early in fetal life, but which disappear with fetal maturation. These substances may reappear in the bloodstream in association with a malignant neoplasm apparently through derepression of the

genes responsible for production of these markers. It has been shown that the sensitive PAP technique is satisfactory to demonstrate the presence of human chorionic gonadotropin (HCG) in placental syncytial trophoblast and neoplastic trophoblastic elements.[189-191] Radioimmunoassay of beta subunit HCG is helpful in monitoring patients with gestational trophoblastic and gonadal germinal neoplasia.[192-196] Ideally, RIA baseline follow-up measurements are used in conjunction with immunohistochemical study of initially resected tumor tissue. This approach permits a precise morphologic and immunohistochemistry characterization of the tumor for definitive subtyping, and suggests which serum markers will be most useful for therapeutic monitoring.[197-200] The amount of choriocarcinomatous differentiation can be best assessed with immunohistochemistry for HCG and the degree of endodermal sinus and embryonal differentiation best assessed with α-fetoprotein immunohistochemistry. These techniques may also be helpful in confirming germinal nature of neoplasms occurring in an extragonadal location such as the intracranial vault.[201,202] However, these markers are by no means specific for germinal neoplasia since they may occur in a variety of nongonadal neoplasm.[203-211] Although perhaps of limited usefulness in confirming a germinal origin for a particular neoplasm, monitoring of markers may provide an indication of therapeutic success.[212]

Similar results have been observed for carcinoembryonic antigen (CEA). The immunohistochemistry method works well for the detection of CEA in tissue.[213] However, the elaboration of this oncodevelopmental antigen by a variety of neoplasms makes diagnostic usefulness limited. CEA expression has been suggested as a useful diagnostic tool in the assessment of lung tumors, since mesotheliomas have been shown with immunofluorescence not to express CEA, whereas bronchogenic carcinomas are associated with CEA elaboration.[214] Peeripheral serologic measurements of CEA may be useful in monitoring response to therapy in patients with breast, stomach, and colorectal cancer.[215-221] A distinction between benign colonic mucosa and dysplastic or frankly carcinomatous changes within the bowel mucosal tissue of patients with ulcerative colitis is theoretically possible with CEA immunostaining, but staining patterns are inconsistent.[222,223] It has also been shown that IMP techniques are able to identify certain CEA-positive cervical carcinomas before ovarian CEA concentration is elevated.[224] Van Nagell et al[225] have shown the usefulness of immunohistochemistry identification of CEA expression by ovarian cystadenocarcinomas and follow-up serologic measurements to assess therapeutic success. The presence of CEA seems to correlate best with mucinous rather than serous differentiation of ovarian neoplasms.[226-228]

An antibody reacting with gp52, a 52,000-dalton glycoprotein of the mouse mammary tumor virus has been shown to immunostain selectively breast carcinoma cells, not reacting with the normal breast tissue or other malignancies.[229] Autoantibodies having specificity for the same virus have been shown to occur in sera of breast cancer patients.[230]

Blood group antigens

The expression of ABO blood group antigens by different human tissues has been recognized for many years. Normal urothelium, for example, expresses ABO antigen in agreement with the phenotype of the patient's red blood cells. In

most urothelial neoplasia, the capacity to express the ABO blood group antigen is lost as the neoplasm becomes more aggressive.[231-234] In some superficial transitional cell carcinomas of the bladder, the natural history of the disease is not characterized by aggressive biologic potential, and thus it would be useful to have a means to identify this particular group of patients who require less aggressive therapeutic measures.[232] With the mixed red cell agglutination test, as assessed by passive red blood cell immunoadherence, it is possible to identify neoplasms that retain the ability to express blood group antigens on the tumor cells. The expressin of ABO antigen on neoplastic urothelium seems to indicate a less aggressive biologic potential, i.e., the tumor does not become invasive.[231-234] Passive red cell immunoadherence has also been used to identify secretion of fetal blood group antigens on polyps of the distal colon, and the loss of ABO isoantigen expression in histologically benign lesions and in mammary carcinoma.[235,236] ABO antigens in tissue can also be detected by indirect immunohistochemistry *(Fig. 13)*.

Infectious agents

Immunohistochemistry techniques are readily adaptable to the detection of hepatitis-B surface antigen in fixed and frozen tissue.[237-241] These techniques can be used to confirm the presence of surface and core hepatitis-B antigen in hepatic cirrhoses or hepatocellular carcinomas, although the relationship between the presence of the antigen and the pathogenesis of the disease remains problematic.[241-244] Recently, it has been shown that hepatitis surface antigen has an affinity for free and bound horseradish peroxidase, an observation that mandates strict use of controls in the procedure.[245] With IF, immunohistochemistry techniques have been used to identify the causative agent of non-A, non-B hepatitis in tissue that may also prove useful in IMP studies.[246]

Antisera specific for certain parasites, polyoma viruses, herpes virus, and varicella/zoster virus have been effective in the retrospective identification of infectious agents in tissue with the use of IF or IMP procedures.[247-253]

Other antigens

Other antigens detected in tissue with immunohistochemistry include the distribution of laminin,[254] ligandin,[255] and Factor VIII coagulation factor.[256] Gliofibrillary acidic protein can be used to characterize glial cell populations, and other intracranial mass lesions.[257-260] However, an inverse relationship between the degree of anaplasia and the intensity of immunostaining with anti-GFA antibody has been observed; thus, in poorly differentiated neoplasms, the technique may have limited usefulness.[258] Neoplastic Paneth cells have been identified with lysozyme antisera in an unusual variant of gastric carcinoma,[261] the presence of actin has been documented in meyloepithelial cells in the breast and other tissues of smooth muscle cell origin,[262,263] fetal red cells identified in placental intervillous thrombi,[264] myoglobin in normal and neoplastic human skeletal muscle,[265] keratin in a variety of normal human tissues,[266,267] and basement membrane antigen has been demonstrated in Wilm's tumor.[268]

Immunohistochemistry techniques have also been shown to be useful in serologic studies to detect antinuclear factor in serum,[269] cell surface antigens in CS,[270] thymus leukemia antigen expression on lymphoid cells,[271] and antilymphocyte antibodies.[272]

The future of enzyme immunohistochemistry

The future of diagnostic and investigational immunohistochemistry will be influenced by several things. The role of this specialized technique in the diagnostic surgical pathology laboratory will increase substantially as the techniques become more widely accepted and as more systems for application are developed. However, as the techniques become more widely used, the necessity for good controls and standardization of reagents and techniques become even more important.[45,273]

The federal government will undoubtedly play some role in the practical daily use of immunohistochemistry in clinical laboratories. Classification of benzidine as a carcinogen and subsequent strict regulations governing its use have had considerable bearing on the use of this reagent in cytochemistry. As outlined earlier in this paper, alternative chromogens are available. However, many chromogens currently in use in the United States, other than benzidine and fluorescent compounds have not been fully investigated with regard to their neoplastogenic potential. A framework for decision-making by the federal government regarding potential human carcinogens has been recently outlined.[274]

The immunohistochemistry detection of specific tumor antigens associated with one particular type of tumor offers great promise. Such antigens have been identified in association with ovarian carcinomas, melanoma, breast as evaluated by an antigen cross-reacting with mouse mammary tumor-related antigens, human cervical squamous cell carcinoma, and mesothelioma.[275-279]

The role of external photoscanning with radiolabeled antibody tracers will continue to grow. Immunohistochemistry may be helpful in the initial immunologic characterization of such tumors before noninvasive scanning procedures.[280,281]

Prolactin receptors have been profiled with the use of IMP methodology,[282,283] and the potential exists to develop peroxidase-labeled histochemical procedures for the detection of a variety of receptors including estrogen, progesterone, and testosterone.

Perhaps the most exciting development in immunohistochemistry relates to hybridoma technology. Since Köhler and Milstein's[284,285] reports of the successful fusion of specific antibody producing cells in culture with tumor cell lines, the applications of this particular biotechnology have increased greatly.[286] Hybridomas are produced by the fusion of a myeloma tumor cell line maintained in tissue culture with cell lines derived from splenic immunoblasts removed from hyperimmunized animals *(Fig. 14)*. Selective tissue cultures yield cloned hybridoma cell lines that elaborate monoclonal ultraspecific antibody. As long as the tissue cell lines can be maintained in culture, production of a standardized antibody is guaranteed. The fused cells may be inserted into mouse peritoneum where functioning hybridoma plasmacytomas grow, and ascitic fluid containing the monoclonal antibody can subsequently be harvested. Monclonal antibodies have been made with specificity for lymphocyte differentiation antigens *(Fig. 15)*, tumor specific antigens, α-fetoprotein, CEA, beta subunit of human chorionic gonadotropin *(Fig. 16)*, and many other substances,[276-279] and have contributed greatly to understanding of lymphocyte maturation and immunopathology of neoplasia.[287-305]

Summary

Immunohistochemical procedures have contributed greatly to our understanding of disease processes and have become a necessary tool in the evaluation of many disease states. Initial detection systems utilized IF markers. Enzyme immunohistochemical techniques developed during the past decade have circumvented many of the problems inherent in IF procedures.

This paper outlines the technical aspects and clinical diagnostic applications of enzyme-labeled immunohistochemistry. The availability of monoclonal antibodies and the adaptation of these reagents to immunohistochemistry systems will contribute greatly to further understanding of disease processes and will have continued utility in clinical diagnosis.

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