PREFACE

Practical Immunopathology of the Skin begins with a discussion of the science behind immunopathology and an explanation of the immunoperoxidase technique and its most frequently used modifications. Issues of tissue preparation, antigen retrieval techniques, and pitfalls that occur in the laboratory will be addressed.

Following the introductory sections dealing with technique and laboratory issues, a working library of antibody probes is introduced. The antibodies are arbitrarily divided into categories based upon the types of cells they help to characterize. However, as is the case with any categorization system, there is some overlap, and antibodies might fit easily into more than one category. In these situations, I opted for the location that fits best into my strategy scheme when deciding upon an antibody profile. For instance, anti-cytokeratin 20 is an anti-cytokeratin antibody, but is used most commonly to identify Merkel cell carcinomas, neuroendocrine tumors. Thus, I have chosen to discuss this antibody in the chapter addressing markers of neuroendocrine cells. For each antibody discussed, I offer a small introductory paragraph that provides a general overview of the known information about the targeted antigen and its role in cellular function. I then progress to a discussion of the diagnostic utility of the probe, attempting to highlight the uses and potentially confounding features of each. When available, sensitivities and specificities for these markers in identifying various neoplasms are cited. More specific technical aspects of each antibody, including any personal experiences we have encountered with the antibodies in our laboratory, are mentioned. The discussion of each potential probe is summarized with a terse statement of its potential uses in a diagnostic dermatopathology laboratory.

The final section of *Practical Immunopathology of the Skin* is a series of vignettes taken from my practice. I have selected a range of real cases designed to exemplify a strategy for the employment of immunopathology. For each scenario, clinical history is presented along with the photomicrographs from the original, routinely stained microscopic sections. These sections are sometimes less than ideal and they have been chosen for this reason in order to fully demonstrate the benefits of immunopathology. A differential diagnosis is constructed based upon the available information and a strategy for solving the diagnostic dilemma is presented in tabular form. Results of the staining procedures are presented and there is a concluding statement explaining some specifics of the case.

It is my hope to keep the book on the level of a practical, "user's manual" rather than that of an in-depth, scientific treatise on the subject. I believe that the theoretical aspects of immunopathology are well covered by other authors and I will make frequent reference to these works throughout the book.

Bruce R. Smoller, *MD*

2 Immunoperoxidase
The Technique

Tissue Preparation

Fixation

Tissue specimens must be fixed properly in order to attain adequate histologic sections. There are currently many types of fixative available for this purpose. The various types of fixative have different methods of stabilizing the tissues, thus resulting in different effects on the immunopathology process. Some of the commercially available fixatives are alcohol-based, while others contain heavy metals as the main operative component. However, virtually all diagnostic dermatopathology laboratories use a formaldehyde-based fixative for routine tissue fixation. These fixatives are readily available, relatively inexpensive, and provide good fixation for most tissues and clinical situations. As other fixatives such as mercury-based, picric acid, and alcohol-based fixatives are not widely used in dermatopathology, their inherent problems pertaining to immunopathology will not be discussed in this volume. For more specific information addressing these specific issues, the readers are referred to more generalized textbooks of immunopathology *(1)*. It is important to be aware of the different problems if other fixatives are used. In many places, mercuric acid fixatives are used for biopsies of suspected cutaneous lymphoma, especially when hematopathology laboratories are involved in the initial work-up.

The most widely used fixative in dermatopathology laboratories consists of 4% formalin solution, made by diluting 40% formaldehyde into a 10% solution with neutralized, buffered saline. Fortunately, formalin fixation allows for excellent immunoreactivity with a large number of diagnostically useful antibodies. Formalin works by crosslinking proteins within the cells of the tissue by forming hydroxymethyl groups *(2)*. This serves to "mask" some of the cellular antigens, but these effects can be reversed during the immunoperoxidase procedures.

Formalin penetrates tissue specimens relatively slowly. Ideal fixation is essential for optimal immunostaining results. Fixation time is therefore a very important variable to consider in tissue preparation. A 3-mm punch biopsy of skin takes approx 8 h to fully fix, and this is the ideal fixation time for subsequent immunostaining procedures. Overfixation can also result in diminished tissue immunogenicity *(1)*. For this reason, it is usually not advisable to fix tissue sections for greater than 24 h. As with many chemical reagents, newly mixed formalin solution would ideally be created each day. As the buffered formalin solution ages, immunostaining results may be adversely affected. However, in most laboratories, the formalin supply is kept current by rapid turnover of specimen bottles, rather than the daily creation of fresh formalin solution. For this reason, the age of the fixative used is rarely a factor in the immunopathology laboratory. It is important to maintain a near neutral pH for the solution, as acidic pHs can diminish immunostaining. (Extensive decalcification with acetic acid may decrease tissue antigenicity, though this procedure is seldom used in dermatopathology.)

Many immunomarkers do not recognize antigens in tissue following formalin fixation. However, few of these are in use in routine diagnostic dermatopathology. Previously, altered antigen recognition occurred with many of the antibodies directed against lymphocyte surface antigens. However, antibodies have been developed that can recognize these same (or related) antigens in formalin-fixed tissue sections. Other antibodies such as keratin-specific anticytokeratin antibodies are now being developed that can recognize specific cytokeratins in fixed tissue sections.

A little used, but valuable, technique is microwave tissue fixation. Subjecting fresh tissue immersed in a dilute aldehyde solution to microwave irradiation allows rapid fixation with good preservation of morphology and preservation of antigens. In many cases, antibodies can recognize these antigens, without the pretreatment procedures such as enzymatic digestion (*see* the following section) sometimes required with formalin-fixed tissue *(3)*.

Paraffin Embedding

Virtually all diagnostic dermatopathology laboratories generate microscope slides from tissue sections embedded in paraffin. This part of the processing involves placing the fixed tissue sections into

paraffin that has been heated to a liquid state and then cooling the properly embedded tissue specimen back to a solid state. While varying the temperatures and types of paraffin used in this process affect immunolabeling results, in most laboratories, the embedding process is standardized. If these conditions remain constant, subsequent immunolabeling techniques can be established within the laboratory to maximize staining results. Thus, this step in the tissue processing rarely contributes significantly to the quality of immunostaining results.

Immunostaining can be performed adequately and reproducibly on archived tissue sections. There is a suggestion that at least some antibodies lose some sensitivity when used on blocks that have been stored for long periods. However, antigen retrieval techniques (described in the Enzymatic Epitope Retrieval section) can often enhance the sensitivities. In most cases, this is not a significant problem, though it should be kept in mind when attempting to evaluate staining results on older cases.

Slide Preparation

Immunolabeling techniques require tissue sections to be placed on special types of glass slides. Because of the repeated incubation and washing steps required in the various processes (*see* below), paraffin-embedded tissue sections often become dislodged and wash off of ordinary glass microscope slides. There are several types of specially prepared microscope slides designed to overcome this problem. Slides coated with poly-L-lysine, or positively charged slides can be purchased that are much more effective at retaining tissue sections throughout the immunostaining procedure. Coating ordinary slides with a slight film of common glue will also solve the problem, but this step requires more effort of the laboratory personnel. In addition, the quality control will not be as uniform with locally produced "sticky" slides.

Drying the slides is an additional step required to assure that tissue sections remain attached to the slides. After cutting the sections to be immunolabeled, the slides are placed in a conventional oven (at $<60°C$) for at least an hour. Most laboratories heat the slides for longer than this. There is marked variation from laboratory to laboratory at this point in the specimen preparation, but some type of slide drying is considered essential in most laboratories.

Techniques for Antigen Visualization

There are many different laboratory techniques that have been developed that are designed to enable the visualization of cellular antigens. All of the techniques are variations on the theme of an indirect immunopathologic method. Over the past 25 yr, investigators have continued to modify this basic process in order to maximize sensitivity and specificity while keeping background staining to a minimum.

The basic laboratory strategy is depicted in Diagram 1. A commercially prepared "nonlabeled" primary antibody from a nonhuman source directed against a specific antibody found in human tissue (i.e., rabbit anti-human S100 protein) is put onto a glass slide containing tissue to be examined. The slide is then washed in a buffering solution. Antibody that is bound to the specifically sought antigen will remain on the tissue sections and all unbound antibodies will be washed away. A second antibody directed against immunoglobulins from the first animal, created in different species from the first animal (i.e., goat anti-rabbit IgG) is placed on the tissue sections with a large excess of antibody. Following an incubation period, the slide is again washed with a buffer solution, rinsing away the unbound antibodies. These secondary antibodies are labeled with a reagent such as peroxidase to allow visualization of the developing antibody complex. This method enables a wide range of unlabeled primary antibodies to be used in conjunction with one (or several) secondary antibodies that not are specific for any primary human antigens, but simply react with immunoglobulin from a nonhuman source. These "labeled" antibodies are ultimately enzymatically linked to the colorimetric end product that is seen by the interpreter. The modifications in the immunopathology methods are largely concerned with the bridging (secondary) antibodies and the end-point precipitates. There are also several points during which "blocking" steps can be added to decrease background staining, especially important with the secondary antibodies.

Diagram 1A–C. *(opposite page)* Sample protocol for immunoperoxidase staining method. **(A)** Cells within tissue sections are incubated with a mouse anti-human (i.e., S100 protein) antibody (M). **(B)** Excess, unbound mouse anti-human antibodies are rinsed off with buffer, leaving only antibody specifically bound to the human cellular antigen. **(C)** The tissue sections are then incubated with rabbit anti-mouse immunoglobulin antibodies (R).

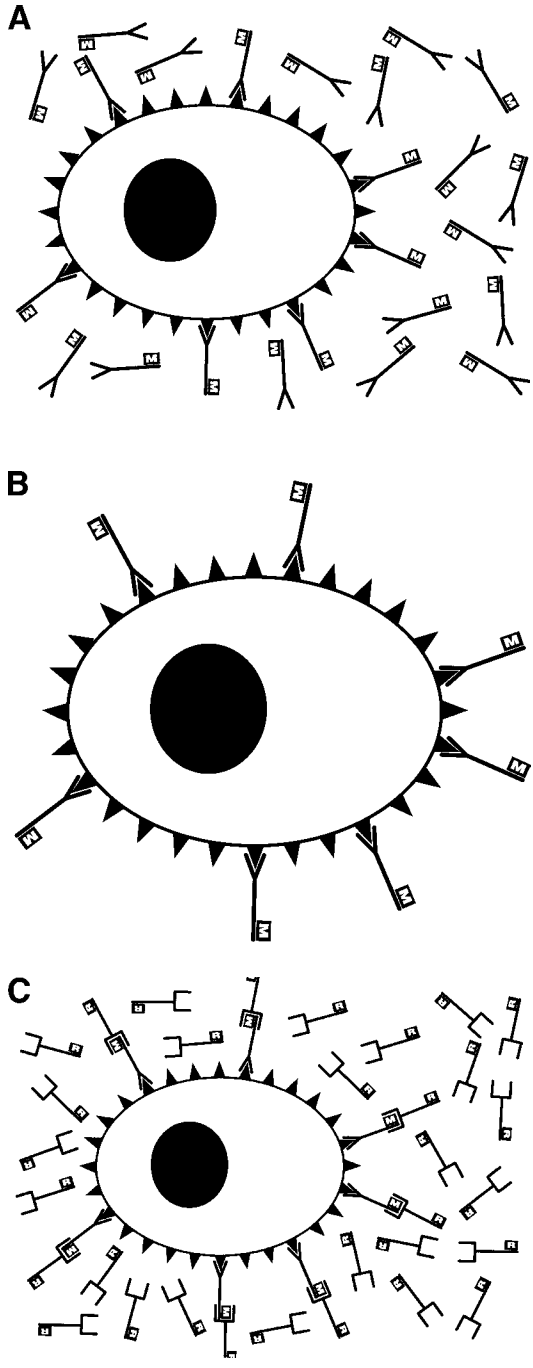

Diagram 1A–C.

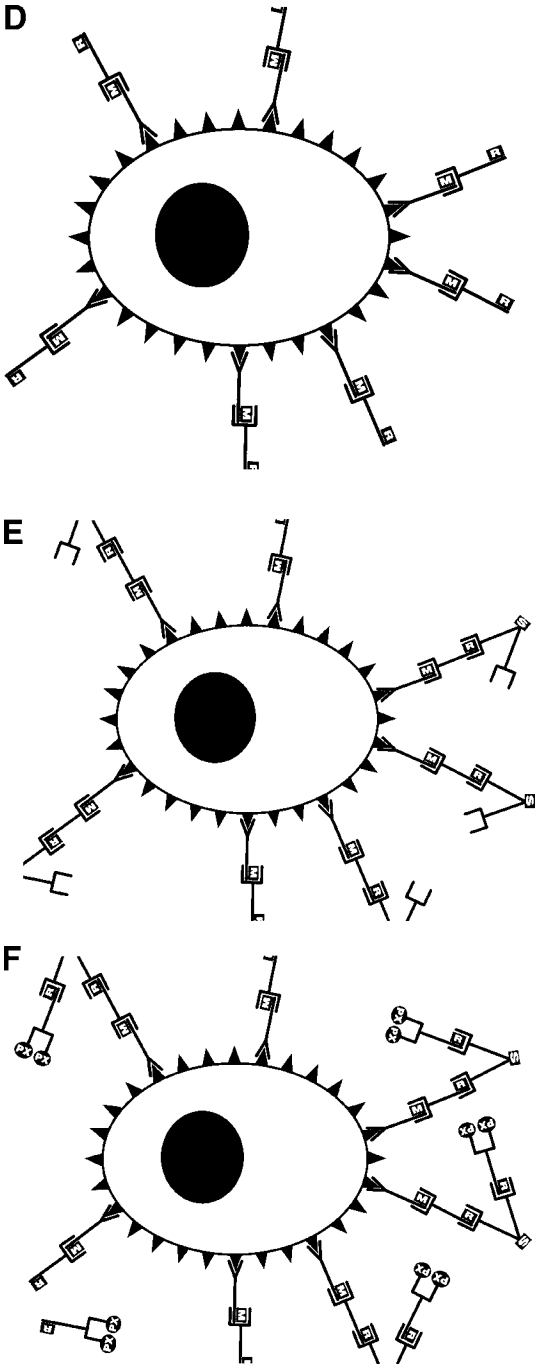

Diagram 1D–F.

Diagram 1D–F. *(opposite page)* Excess, unbound rabbit antibodies are rinsed off, leaving only rabbit antibody that specifically bound to the mouse immunoglobulins. **(E)** Tissue sections are incubated with swine anti-rabbit antibodies (S), the so-called bridge antibodies. (Excess, unbound swine antibodies are rinsed, not shown). **(F)** A preformed peroxidase anti-peroxidase complex (PX), formed on rabbit antibodies are incubated with the tissue sections and bind to unbound receptors on the swine antibodies. (Excess, unbound conjugated rabbit antibodies are rinsed, not shown.)

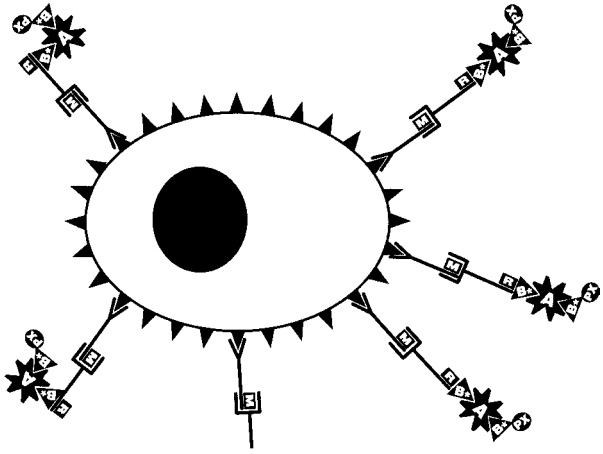

Diagram 2. Avidin-biotin-peroxidase method. The diagram shows the final stage in the avidin-biotin-peroxidase modification of the immunopathology technique. Specific mouse anti-human (i.e. desmin) antibodies are bound to a cell. A rabbit-anti-mouse IgG is complexed with biotin and binds to the bound mouse immunoglobulin. The tissue sections are incubated with avidin and with biotin conjugated to peroxidase. (Intermediate steps are not shown, but are similar in principle to those demonstrated in Diagram 1.)

The most commonly employed immunopathology technique at this time is the biotin-streptavidin immunoperoxidase complex (B-SA) method (Diagram 2). The standard, unlabeled primary antibody is applied to tissue sections and unbound antibody washed off the sections. In this modification of the immunostaining technique, the secondary antibody directed against the species from which the primary antibody is derived is labeled on one end with the vitamin biotin. Following an incubation with the tissue section, excess, unbound antibody is washed away. Streptavidin, a 60-kD analog of avidin with multiple high-affinity binding sites for biotin, is added to the tissue sections, followed by biotinylated peroxidase. This com-

plex is incubated with the tissue sections containing primary antibody and biotinylated secondary antibody. This incubation takes place in conditions of great streptavidin excess, thus enabling the formation of large peroxidase complexes. The final colorimetric precipitate proceeds in a manner identical to the original peroxidase anti-peroxidase method. The avidin-biotin-immunoperoxidase method is thought by some to be more sensitive than earlier methods *(4)*.

Colorimetric End-Products

Diaminobenzidine (DAB)

The most commonly used colorimetric precipitate used in immunostaining laboratories is diaminobenzidine. This reagent yields a strong brown precipitate that is easily visualized with any type of counterstain (or without) on tissue sections, serving as an oxidant for the horseradish peroxidase- H_2O_2 complex (Fig. 1). It has the advantage of being relatively sensitive and permanent. The precipitate does not fade over time. One disadvantage is that the compound is a carcinogen, but this is not a major concern for most laboratories, as it is believed that the risks from exposure to DAB are relatively small. There are a variety of modifications that can be used to enhance the intensity of the DAB end product. Increasing the incubation time of the tissue section with the DAB and hydrogen peroxidase to form the precipitate will increase the staining intensity, but often will increase background staining in parallel. Incorporation of heavy metals into the DAB mixture will increase the signal:noise ratio effectively. Copper sulfate is the most commonly used heavy metal, but osmium tetroxide, cobalt chloride, and nickel have also been used to enhance the DAB intensity *(5)*.

Aminoethylcarbazol (AEC)

Another frequently used chromagen is aminoethylcarbazol (AEC). AEC produces a bright red color that is easy to visualize and to photograph. It also has the advantage of not being carcinogenic. However, the major drawback to AEC is that the color tends to fade over time. The intensity of the staining decreases over months, and the chromagen tends to bleed into the surrounding tissue. Thus, the slides cannot be regarded as permanent records. In our laboratory, AEC has repeatedly proven to yield less sensitive results than DAB.

One great advantage to AEC over DAB is when immunostaining is required for cells that contain abundant melanin and/or hemosid-

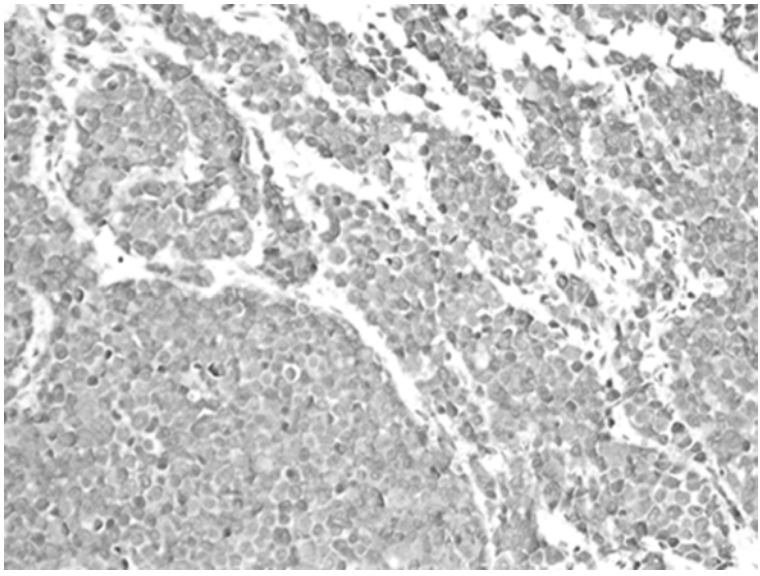

Fig. 1. Diaminobenzidine (DAB) yields a dark brown precipitate that contrasts with the blue counterstain. (*See* color plate 1 appearing in the insert following p. 22.)

erin (Fig. 2). It can be quite difficult to distinguish immunostaining from these endogenous pigments when DAB is used as the chromagen, as all appear brown on tissue sections. However, the AEC provides a nice contrast between a red, positive immunostaining reaction and endogenous brown pigment. In some cases, we have found the use of AEC as a chromagen helpful in detecting true staining in heavily pigmented cells. (Another technique is to bleach the tissue sections with H_2O_2 prior to immunolabeling. The S100 protein will survive this pre-treatment, though antigens recognized by MART-1 and HMB-45 do not survive very well.)

Alkaline Phosphatase

Some laboratories prefer an alkaline phosphatase-based colorimetric system. This system yields a brilliant red end product. It is not as widely used as the systems described earlier.

Antibodies

A primary antibody that identifies a given antigen is applied to tissue sections. This primary antibody is developed in an animal other than a human being and is directed against an antigen found on human

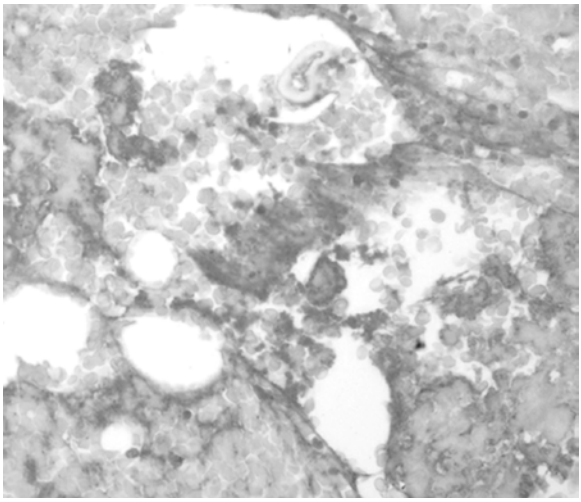

Fig. 2. Aminoethylcarbazol produces a bright red end product that contrasts with the blue counterstain. Further, this reagent allows distinction from endogenous melanin that may be present in the tissue sections. (*See* color plate 2 appearing in the insert following p. 22.)

cells. These antibodies can be either monoclonal or polyclonal and are produced by a variety of methods. Monoclonal antibodies are more specific than are polyclonal antibodies, but are more likely to be adversely affected by fixation problems *(6)*. Polyclonal antibodies tend to have higher binding affinities for specific antigens than do their monoclonal counterpart *(7)*. Most commonly, hybridoma technology using mice is the method used for generating monoclonal antibodies. In diagnostic dermatopathology, only commercially available antibodies are used. Thus, an in-depth discussion on the methods for developing new antibodies is outside the scope of this volume. It is important to realize, however, that every antibody has its own specificity and sensitivity. Each manufacturer's antibodies have unique staining properties. It is imperative to learn the behavior pattern of each antibody before employing it in routine clinical work, both in the printed materials supplied by the manufacturers as well as performance of the antibodies in the laboratory using them. Extensive testing should be performed on each new antibody in order to develop maximum performance in each individual laboratory. It is not sufficient simply to use the manufacturer's suggested protocols, as performance varies widely from location to location.

A secondary antibody directed against the primary antibody is required for all the antigen detection methods described. These antibodies are most commonly anti-immunoglobulin antibodies directed against immunoglobulins that comprise the primary antibody. These secondary antibodies must be somewhat species-specific and cannot react with human tissue. Several companies now produce secondary antibody cocktails that recognize immunoglobulins from several species, and can be used with a large number of primary antibodies.

Commercially available antibodies have a recommended shelf life. It is essential that outdated antibodies be discarded and not used for diagnostic purposes. In addition to being a laboratory regulation enforced by several accreditation boards, results obtained with outdated antibodies may not be reproducible, leading to erroneous results and compromising patient care.

Controls

As with every laboratory test, it is imperative that appropriate controls be performed with each immunostaining procedure. Several types of controls are essential in order to permit the accurate interpretation of immunostaining results. Controls take the form of internal controls and external controls. For immunostaining procedures, probably the most important controls are internal. For each antibody tested, it is imperative that cell types that are expected to label with an antibody demonstrate the appropriate positive staining reaction on the tissue being examined (a positive internal control). It is equally important that cell types that are not expected to react with a given antibody are appropriately negative on the tissue section being examined (negative internal control). These controls provide the information that the tissue section has been fixed in a manner that preserves the antigen in question. It also provides the information that the staining procedure worked. Further, it allows the interpreter to see the specific antibody working as expected.

In addition to the internal controls, most laboratories have several different types of external controls that are run with each immunolabeling procedure. In order to assure that the immunostaining procedure worked appropriately, tissue sections taken from a specimen known to be reactive with a given antibody are run in parallel with the sections being tested diagnostically. This tissue serves as a positive external control. In many laboratories, a stan-

dardized "sausage-like" tissue section comprised of a wide range of cell types serves as the positive external control. This type of tissue specimen can be prepared locally or purchased commercially. If the external control specimen stains appropriately but there is no staining whatsoever on the experimental tissue (including cells that should label with the antibody), it is likely that there is a problem with antigen recognition on the tissue to be examined. This result might suggest over- or underfixation, or several other problems. Additional antigen retrieval steps (*see* the Enzymatic Epitope Retrieval section) might be indicated. In addition, a negative external control specimen may be run. In most cases, this is a tissue section that undergoes all of the immunostaining procedural steps except that instead of the primary antibody being tested, an irrelevant antibody (or normal immune serum) is used. Any staining in this section can thus be regarded as nonspecific staining. Should this be significant, extreme caution should be used in interpreting the specimen being examined.

It has been suggested that the use of a ubiquitous antibody such as vimentin is helpful in assuring the antigenicity of each tissue section examined *(8)*. Vimentin labels cells that are present in virtually every specimen examined. Lack of any staining with vimentin on a slide suggests the likelihood of decreased antigen preservation and should preclude interpretation of immunostaining results.

While there are many other types of controls that can be undertaken, in most situations these are the only controls run during the immunostaining procedures. All control tissues should be examined prior to examining the test specimen so that the immunostaining results can be interpreted fairly.

Automation

The last decade has witnessed the development of automated slide stainers capable of performing immunopathologic procedures. The great advantage to these machines is their reproducibility. Preprogramming a set of incubation times and antibody dilutions essentially eliminates run-to-run variations. There are many commercially available immunostainers currently available. Each of these has its own strengths and weaknesses, and allows the laboratory personnel varying levels of control and abilities to modify individual programs.

For the usual dermatopathology laboratory that performs at least a moderate volume of immunostains, automated immunostainers have become a cost-effective part of the laboratory resources. An automated slide stainer has built-in computer technology that enables the operator to program protocols for a large number of antibodies. This is done at the time that any new antibody is brought into the laboratory and added to diagnostic work-ups. Each of the antibodies is initially analyzed in the laboratory, and the protocol for optimizing each one is established and programmed into the immunostainer prior to performing the tests on any cases. Optimization generally requires parameters such as dilutions, incubation times, and pretreatment conditions. While individual cases may require some manual "tweaking" of this program, the protocol for each antibody ideally is established to allow for best results in the vast majority of cases. The immunotechnologist prepares the tissue sections (including any required pretreatments described elsewhere) and places the entire day's slides to be immunostained onto the machine. (Most machines can hold up to 40–50 slides for a single run.) The immunostainer is preprogrammed for the day's run, taking great care to correctly correlate the slide number with the required antibodies. Once the prepared slides have been placed onto the slide racks and the computer appropriately programmed, the immunostaining procedures automatically occur. The stainer simultaneously performs the individualized protocols for each of the antibodies requested, varying the incubation times, dilutions and other parameters as preprogrammed. However, the protocol for each primary antibody remains essentially identical from run to run. The immunostainer thus provides standardization and is a labor saving device.

Additional assets to automated slide stainers include the ability to perform routine histochemical stains, direct immunofluorescence procedures, and in some cases, many of the steps required for *in situ* hybridization and polymerase chain reactions (PCR).

Modifications in Antigen Retrieval

Enzymatic Epitope Retrieval

Formalin fixation, paraffin embedding, and other tissue processing techniques alter tissue in many ways. Cellular proteins are crosslinked and masked. In some cases, this prevents primary anti-

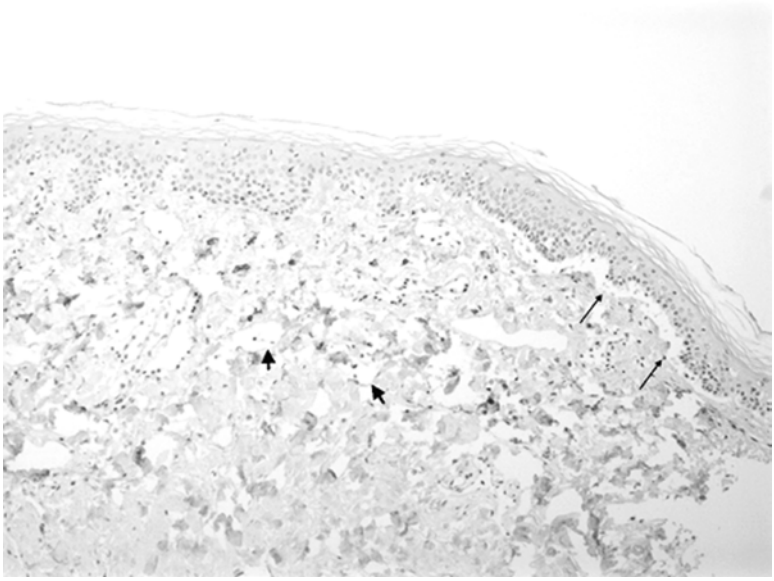

Fig. 3. Enzymatic pretreatment may result in loss of morphologic detail.

bodies from recognizing the specific cellular antigens. Overfixation in formalin can be partially reversed with the use of enzymatic digestion. Protease digestion unmasks some crosslinked immunoreactive sites and may be necessary to maximize performance of some antibodies used on routinely processed tissue sections. Protease incubation is also reported to decrease nonspecific background staining *(9)*. Incubation of tissue sections with trypsin, pepsin, pronase, ficin, DNAse, or protease is often used for this purpose *(10)*. Different incubation protocols are necessary for each of the reagents, and for each antibody to be tested. Further, it is generally recommended that the longer a tissue specimen be fixed in formalin, the longer it needs to be enzymatically pretreated. In actual practice, laboratories seldom pay heed to this rule, as there is a general assumption that all skin biopsies are fixed for approximately the same amount of time in a given laboratory setting. It is only when individual immunostaining results appear that this step is varied.

The major drawback to enzymatic tissue digestion is the loss of morphologic detail (Fig. 3). Overtreatment results in extensive tissue artifacts and may preclude diagnosis. It also may result in

increased non-specific staining by fragmenting antigens into smaller antigenic fragments found on many cells other than the initially intended cells *(11)*.

Enzymatic epitope retrieval was the first type of antigen-enhancing technique described. Since the development of heat-induced epitope retrieval techniques, enzymatic pretreatments are used much less commonly.

Heat-Induced Epitope Retrieval

Heat-induced epitope retrieval (HIER) enhances staining with many antibodies and is required for some to recognize antigens on fixed tissue sections *(12)*. The tissue is incubated in a buffered, preheated solution. Water baths can be heated with pressure-cookers, microwave ovens, steamers or other types of devices. Actual protocols vary widely in terms of temperature, duration of heating and pH of the buffered solutions. However, standard protocols can be developed for routine laboratory usage that will enable generalization across most antibodies. Alkaline pHs of 8 or 9 enhance antigen retrieval, but run the risk of causing tissue damage. The literature is replete with articles explaining the relative advantages and disadvantages of shorter and longer incubation times, higher and lower incubation temperatures, methods of heating, and compositions of buffered media. In most diagnostic laboratories, however, one or several uniform methods can be established that will attain relatively good staining results with a wide range of antibodies, if not the absolute best for any given probe. For some antibodies, HIER has proven to be superior to enzymatic pretreatment. However, this is not the case with all antibodies. Gown and co-workers have published their experiences with a large number of antibodies *(13)*.

Frozen Section Immunopathology

In recent years, there has been a move to incorporate immunolabeling into Mohs' surgery procedures in attempt to better evaluate tumor margins. Immunolabeling to identify residual pagetoid cells, be they malignant keratinocytes, melanoma cells, or tumor cells from Paget's disease is becoming more prevalent. Certainly, immunoperoxidase technology works exceedingly well on fresh, frozen tissue. However, the immunotechnician must work with an

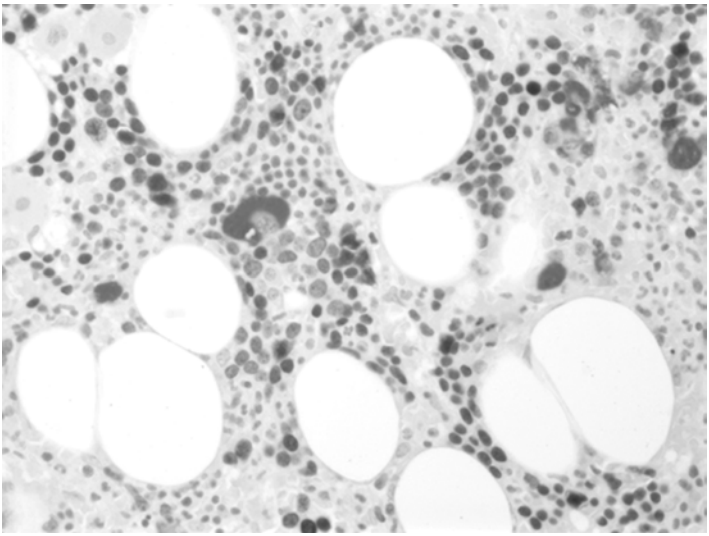

Fig. 4. Double labeling with kappa and lambda antibodies demonstrates separate populations of plasma cells in this specimen. (*See* color plate 3 appearing in the insert following p. 22.)

entirely different set of antibody dilutions and incubation times, as none of the protocols established for routinely fixed tissue will provide optimal results for frozen section analysis. Robinson has advocated the use of anti-S100 protein and HMB-45 immunostaining in order to achieve better margin control in examining narrow excisions for lentigo maligna *(14)*. Immunostains have also been used to unmask tumor cells hidden by a dense inflammatory reaction when performing frozen section examination *(15)*. A review of the use of frozen section immunoperoxidase techniques in Moh's micrographic surgery details many of the current uses for this procedure *(16)*.

Double Labeling

It is technically possible to perform double labeling with two antibodies on a single tissue section. If the primary antibodies to be used are from the same species, the immunostaining procedures are performed sequentially, varying the final colorimetric precipitate. If primary antibodies from different species are used, the immuonstaining procedures can be performed simultaneously (Fig. 4; *17*).

Table 1

Excess immunostaining	*Potential solutions*
Tissue dried at some point in processing	Redo making certain to keep wet at all times
Excessive enzymatic digestion	Decrease incubation time with enzymes
Excessive HIER	Decrease time of HIER incubation
Edge effect	Look at tissue away from edges
Too concentrated antibodies (primary or sencondary)	Decrease concentrations or incubation times
Too long incubations	Decrease incubation times at several steps in protocol
Too concentrated DAB	Decrease concentration of DAB or incubation time at this step
Tissue necrosis	Choose another area of slide without necrosis to interpret
Nonimmunologic binding to collagen	Find more cellular area with less collagen in tumor mass
Endogenous peroxidase not completely extinguished	Increase length of time for blocking
Overfixation	Decrease incubations or antibody concentrations
Insufficient immunostaining	*Potential solutions*
Tissue sections too old-deteriorated antigen preservation	Increase incubation with enzymes or HIER
Overfixation	Increase incubation with enzymes or HIER
Outdated antibodies (primary or secondary)	Try vimentin antibody to check tissue; restain with new antibodies if tissue is not the problem
Too dilute antibody concentrations (Primary or secondary)	Vary concentrations of primary and secondary antibodies (separately)
Need for antigen retrieval technique	May be required even when package insert claims otherwise
Too dilute DAB concentration	Increase concentration of DAB or incubation time

Pitfalls in the Interpretation of Immunopathology

As is the case with every laboratory test, there are many potential pitfalls in the interpretation of immunopathology results (*see* Table 1). Poor fixation can result in false negative readings if appropriate attention is not paid to internal and external controls. The presence of necro-

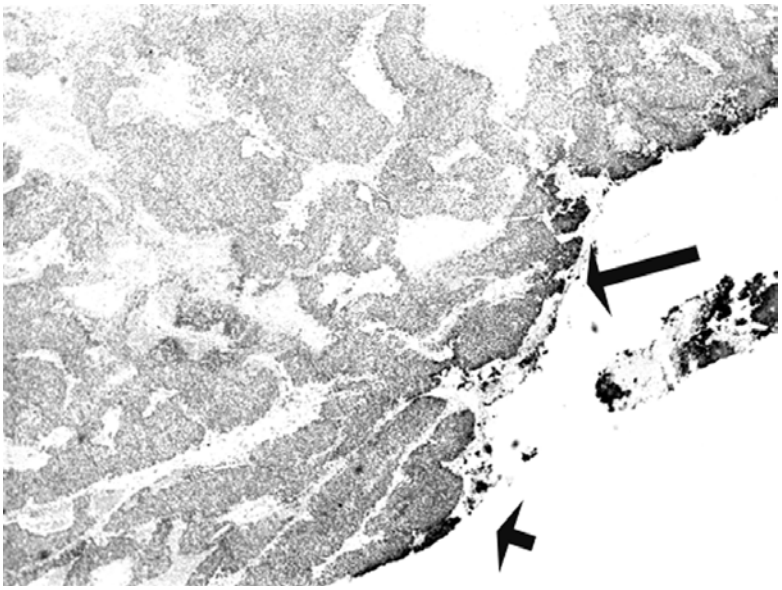

Fig. 5. The presence of necrosis contributes to a high background staining owing to nonspecific adsorption.

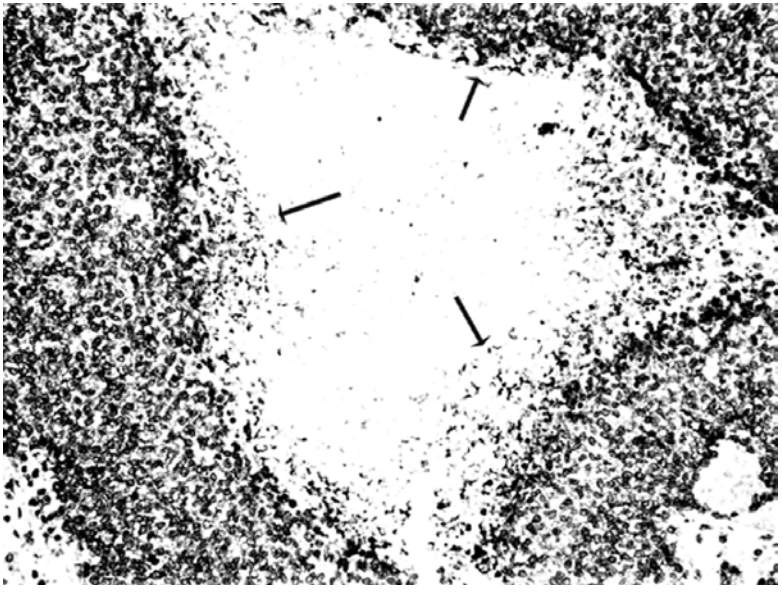

Fig. 6. An "edge effect" is often present on tissue sections. The edges of the tissue appear to be immunoreactive. This staining is not real and should be interpreted with caution.

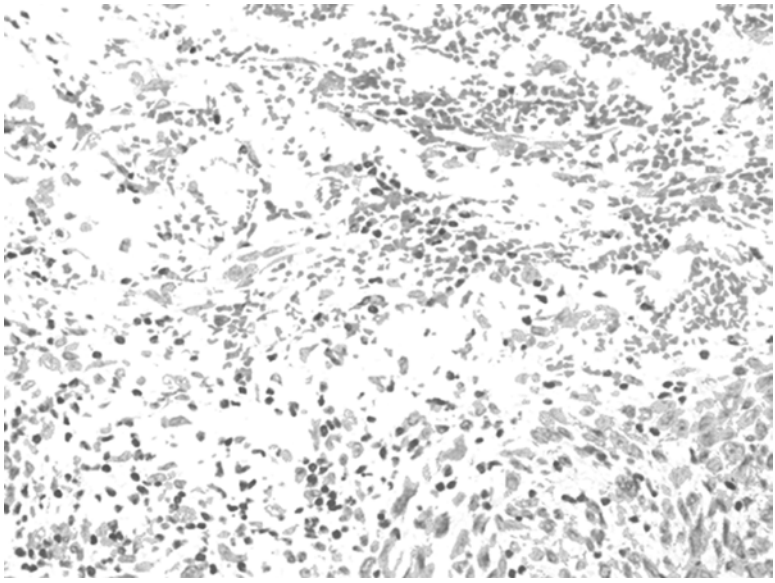

Fig. 7. Endogenous peroxidase activity is present in erythrocytes and granulocytes and may interfere with interpretation. (*See* color plate 4 appearing in the insert following p. 22.)

sis within tissue sections can result in nonspecific staining, resulting in false positive interpretations (Fig. 5). There is often an "edge-effect" such that the edges of tissue sections appear to have positive staining when none is present (Fig. 6). Similarly, drying of the tissue at any point along the staining procedure can result in excessively high backgrounds and false positive readings. Failure to extinguish endogenous peroxidase activity and erythrocytes and granulocytes can also result in increased nonspecific staining (Fig. 7). It is crucial to be aware of each of these situations prior to making a final interpretation of the immunostains.

References

1. Taylor, C. R. (1994) *Immunomicroscopy*, 2nd ed., Philadelphia: W. B. Saunders.
2. Fox, C. H., Johnson, F. B., Whiting, J., and Roller, P. P. (1985) Formaldehyde fixation. *J. Histochem. Cytochem.* **33,** 845–853.
3. Login, G. R., Schnitt, S. J., and Dvorak, A. M. (1987) Rapid microwave fixation of human tissues for light microscopic immunoperoxidase identification of diagnostically useful antigens. *Lab. Invest.* **57,** 585–591.
4. Hsu, S.-M., Raine, L., and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. *J. Histochem. Cytochem.* **29,** 577–580.

5. Brigatti, D., Budgeon, L. R., and Unger, E. R. (1988) Immunocytochemistry is automated: Development of a robotic workstation based upon the capillary action principle. *J. Histotechnol.* **11,** 165–170.
6. Yelton, D. E. and Scharff, M. D. (1981) Monoclonal antibodies: A powerful new tool in biology and medicine. *Ann. Rev. Biochem.* **50,** 657–680.
7. Davidoff, M. S. (1986) Immunocytochemistry: Possibilities for detection of different tissue antigens and establishiment of the functional role of cells. *Acta Histochem. Suppl. Band.* **23,** 175–193.
8. Battifora, H. (1991) Assessment of antigen damage in immunohistochemistry. The vimentin internal control. *Am. J. Clin. Pathol.* **96,** 669–671.
9. Reading, M. (1977) A digestion technique for the reducation of background staining in the immunoperoxidase method. *J. Clin. Pathol.* **30,** 88–90.
10. Brozman, M. (1978) Immunohistochemical analyses of formaldehyde and trypsin- or pepsin-treated material. *Acta Histochem.* **63,** 251–260.
11. Heyderman, E. (1979) Immunoperoxidase technique in histopathology: Applications, methods and controls. *J. Clin. Pathol.* **32,** 971–978.
12. Shi, S. R., Key, M. E., and Kalra, K. L. (1991) Antigen retrieval in formalin-fixed paraffin-embedded tissues: An enhancement method for immunohistochemical staining based upon microwave heating of tissue sections. *J. Histochem. Cytochem.* **39,** 741–748.
13. Gown, A. M., de Wever, N., and Battifora, H. (1993) Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl. Immunohistochem.* **1,** 256–266.
14. Robinson, J. K. (1994) Margin control for lentigo maligna. *J. Am. Acad. Dermatol.* **31,** 79–85.
15. Jimenez, F. J., Grichnik, J. M., Buchanan, M. D., and Clark, R. E. (1995) Immunohistochemical techniques in Mohs micrographic surgery: their potential use in the detection of neoplastic cells masked by inflammation. *J. Am. Acad. Dermatol.* **32,** 89–94.
16. Mondragon, R. M. and Barrett, T. L. (2000) Current concepts: the use of immunoperoxidase techniques in mohs micrographic surgery. *J. Am. Acad. Dermatol.* **43,** 66–71.
17. Nakane, P. K. (1968) Simultaneous localization of multiple tissue antigens using the peroxidase labeled antibody method: A study on pituitary glands of the rat. *J. Histochem. Cytochem.* **16,** 557–560.