CHAPTER

ONE

PRINCIPLES OF IMMUNOHISTOCHEMISTRY

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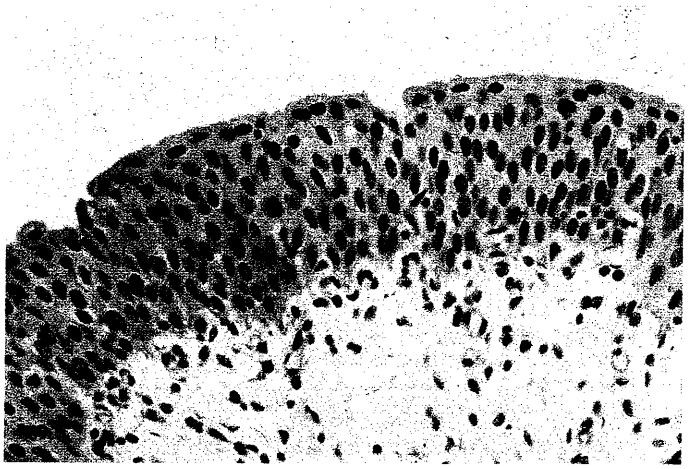

Immunohistochemistry involves the use of antibodies to locate antigens in tissue. Because molecules are restricted in distribution to specific cell types, the ability to identify molecules through antigenic sequences has become a powerful technique in diagnostic surgical pathology. In current diagnostic practice, antigens are most often localized to sections of tissue or to the surfaces of cells for visualization at the light microscopic level. Although there is no current role in diagnostic pathology for localization of antigens at the ultrastructural level, or for simultaneous immunostaining for multiple antigens, the potential of these special techniques is sufficiently great to warrant their inclusion in this chapter.

GOALS

The goal of immunohistochemistry is a more precise characterization of cells, infectious agents, and macromolecules than nonimmunologically based histochemical stains can usually provide. The following types of tissue characterization can be performed.

Differentiative features of morphologically poorly or undifferentiated cells, either neoplastic or hyperplastic, are determined. The precision of determining histogenesis varies with the cell and tissue type. With some differential diagnoses, the identification of a single antigen suffices, ie, localization of keratin is generally diagnostic of epithelial differentiation. In other cases, the tissue should be analyzed for multiple antigens because of the variability of expression of sets of antigens, ie, the distinction between adenocarcinoma and mesothelioma (Fig. 1.1).

A corollary is the hypothesis that batteries of antigens are specific for both cell type and tissue of origin. For example, the patterns of different keratins appear to be characteristic of epithelial cells of different tissues (Moll, *1982*; Cooper, *1985*). Specifically, lung adenocarcinoma lacks a 44 kd keratin that is immunohistochemically detectable in squamous-cell carcinoma (Banks-Schlegel, *1984*). However, current evidence suggests that characterization of carcinomas by their pattern of either immunoreactive or extractable keratins is not tissue specific. Ovarian surface carcinomas of similar histology have different patterns of immunoreactive keratins (Nagle, *1983*), and esophageal carcinomas differ in the types of extractable keratins (Grace, *1985*) (Fig. 1.2). Batteries of unrelated antigens may also differentiate tumors of different histogenesis, ie, of mesothelioma vs. adenocarcinoma (Battifora, *1985*).

Hyperplastic and neoplastic processes, typically of endocrine tissues, can be functionally characterized. For example, the cells comprising islet-cell tumors and pituitary adenomas can be identified by their hormonal content.

Quantification of elements in disease processes is performed. The relative percentages of cells expressing certain antigens correlate with tumor behavior, independent of staging and other histologic information. For example, an increased number of Langerhans cells in carcinomas may be associated with a better prognosis (Lauriola, *1984*), and an increased percentage of prostate carcinoma cells containing the *ras* sarcoma virus gene product p21 correlates with a higher Gleason's grade of differentiation (Hand, *1984*).

The percentage of tumor cells containing certain antigens may predict other disease. For example, medullary carcinoma of the thyroid characterized by large numbers of calcitonin-immunoreactive cells is a feature of multiple endocrine neoplasia syndrome I (MEN I); in contrast, sporadic medullary carcinomas contain only small numbers of calcitonin-positive tumor cells (Lippman, *1982*).

The apparent concentration of antigen in tumor cells may predict the aggressiveness of a tumor. Melanomas with increased S-100 immunoreactivity seem to have a worse prognosis (Rode, *1984*). The concentration of certain antigens predicts growth qualities of tumors. For example, the quantity of estrogen receptor predicts responsiveness to hormone therapy (McCarty, *1985*).

The ratios of cell types enable us to obtain more precise diagnosis of disease. For example, the relative number of plasma cell subtypes, identified by immunoglobulin heavy-chain subtype, helps to distinguish Crohn's colitis from ulcerative colitis (Bosman, *1979*).

The identification of infectious agents is performed. The diagnosis of medical and metabolic diseases by the distribution of molecules, eg, of immunoglobulins in renal and skin disease (see Chapter 10) and of α_1-antitrypsin in cirrhosis of the liver (see Chapter 7), is determined.

The precise cell and organelle localization of molecules is done most accurately with immunohistochemistry. For example, by immunohistochemistry the estrogen receptor has

PERCENTAGE OF TUMORS CONTAINING REACTIVE CELLS FOR VARIOUS ANTIGENS

	Antigen		
Tumor	**EMA**	**CEA**	**Leu-MI**
Mesothelioma	<10%	<10%	0
Adenocarcinoma	>90%	>90%	>50%

Figure 1.1. *The percentage of tumors containing a significant number of immunoreactive cells for each respective antigen—EMA (milk-fat globule protein, or epithelial membrane antigen), CEA (carcinoembryonic antigen), and Leu-M1. (From Otis,* 1987.*)*

been localized to the nucleus (King, *1984*). Biochemical assays of tissue extracts do not provide information about distribution of antigens. For example, the heterogeneity of distribution of proteins in the liver has been determined by immunohistochemistry (Gaasbeek Janzen, *1985*). Immunohistochemical information provides new criteria for diagnosing and evaluating disease.

This chapter will discuss the principles of all aspects of immunohistochemistry. Details of the methodology will be covered in Chapter 2.

ANTIGEN

Definition

An antigen is any molecule that has generated an antibody response (*anti*body *gen*erator). The part of the antigen that reacts with a given antibody is the specific antigenic determinant or epitope. A molecule typically has multiple different potential epitopes, although an antibody binds only one specific epitope.

Most antigens are proteins, although any type of chemical can be antigenic. For example, the antigen identified in neural tissues by the Leu-7 antibody is a carbohydrate (see Chapter 11), and some antigens recognized by lupus antibodies are phosphodiesters (Lafer, *1981*).

Antigenicity

Antigenicity is the presence of antibody binding activity. Because antigenicity is dependent on the physicochemical nature of the three-dimensional structure of the antigen, it is influenced by the chemical and physical forces of tissue processing. "Optimal" antigenic preservation implies retention of antigenicity that is identifiable with available antibodies. The possibility of altered antigenicity depends on the particular antigen and the effect of the various procedures of tissue handling—autolysis, fixation, and embedding.

Autolysis

Ischemia and cell death, which occur after tissue is devitalized, initiate the release of lysosomal proteolytic enzymes. By metabolizing cell components, these enzymes irreversibly alter their molecular structure and, hence, alter their antigenicity. To minimize autolysis of devitalized tissue, the endogenous proteolytic enzymes must be rapidly inactivated, either by fixation or by maintaining the tissue at a sufficiently low temperature.

Fixation

The purpose of fixation is threefold: inactivation of autolytic mechanisms, with retention of tissue architecture; immobilization of molecules to prevent their artifactual relocalization to other cell compartments or to prevent diffusion from the tissue; and increase in tissue rigidity for possible sectioning without embedding.

The precision with which fixatives localize molecules varies with the antigen, the fixative, and the conditions of fixation. For example, inaccurate localization of secretory component (SC) to plasma cells may have resulted from prolonged (24 hr) incubations in immunostaining reagents of sections in which SC was not completely immobilized (Brandtzaeg, *1981*). In general, smaller antigens are more subject to such relocation. The degree of loss of antigens from tissue varies with the fixative. Protein losses of up to 6%, 8%, and 10% have been reported with 10% formalin, Carnoy's solution, and ethanol, respectively (Mays, *1984*). The condition of fixation may further minimize protein loss. Using phase partition (where the tissue is in the organic phase and the aldehyde fixative is in the aqueous phase), virtually no loss of protein occurs (Mays, *1984*).

The manner of fixation also affects the precision of localization. Conditions that accelerate fixation—heat, agitation, and the more rapid delivery of fixative to tissues, such as by perfusion—optimize localization of antigens. The patchwork pattern of immunostaining of immersion-fixed liver for several liver proteins suggests that different liver-cell populations synthesize different proteins. In contrast, the uniform staining of perfusion-fixed liver for these proteins suggests that all hepatocytes synthesize the proteins (Feldmann, *1985*).

A consequence of the chemical reactions of fixation is altered antigenicity. The degree to which antigenicity is altered depends on both fixative and antigen. Formaldehyde greatly alters the antigenicity of keratin intermediate filaments (Figs.

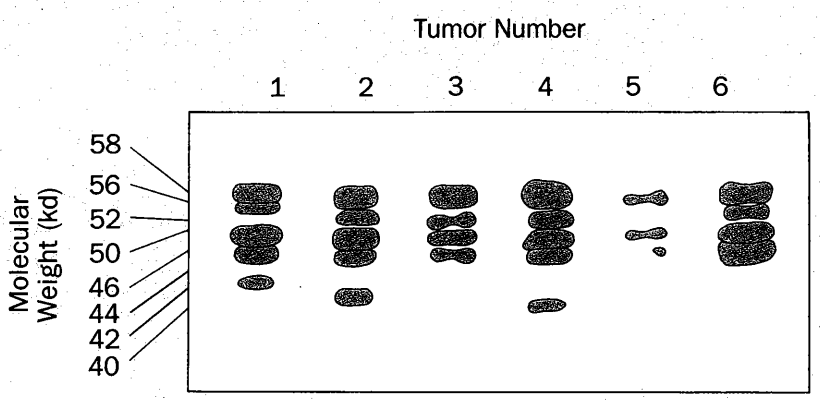

Figure 1.2. *Carcinomas of similar histology may produce different keratins, exemplified in this schematic of immunoblots from six esophageal squamous carcinomas. Although the production of keratins of molecular weights 56, 50, and 46 kd was common to the six tumors, only some of these synthesized additional keratins of molecular weights 58, 52, 44, 42, and 40 kd. Antikeratin antibodies specific to any of the latter three keratins would not have immunoreacted with all of the tumors. (From Grace,* 1985*).*

1.3—1.5); in contrast, neither formaldehyde nor ethanol changes the antigenicity of peptide hormones to readily available antibodies. Furthermore, the length of fixation correlates with the degree of alteration of antigenicity (Battifora, *1986*) (Fig. 1.6). A beneficial effect of alteration of protein structure is inactivation of endogenous enzyme activity, a potential source of false positivity.

The conditions of fixation also affect antigenicity. Fixation in an isotonic solution at neutral pH optimizes retention of native antigenicity.

The more common protocols and agents for fixation (Fig. 1.7) are as follows.

Freezing. Freezing optimizes retention of native antigenicity and, when done rapidly, precisely localizes antigen. The main disadvantages of immunostaining sections of frozen, nonfixed tissue are as follows:

1. There may be diffusion of antigens, especially of small molecules, to different cell compartments or even out of the tissue.
2. The inactivation of endogenous enzymes is reversible.
3. Structure may be significantly distorted by ice-crystal formation; ultrarapid freezing with dehydration can correct this problem (Livesey, *1987*).
4. There is labor intensiveness, especially with ultrathin cryosectioning (Tokuyasu, *1980*)
5. Frozen sections more easily detach from slides.

Alcohols. Alcohols and acetone fix antigens and enzymes by denaturation. Antigenicity is altered minimally. But the disadvantages are similar to freezing: translocation of antigens may occur, and endogenous materials are not inactivated. As with frozen tissue, ultrastructural preservation is poor.

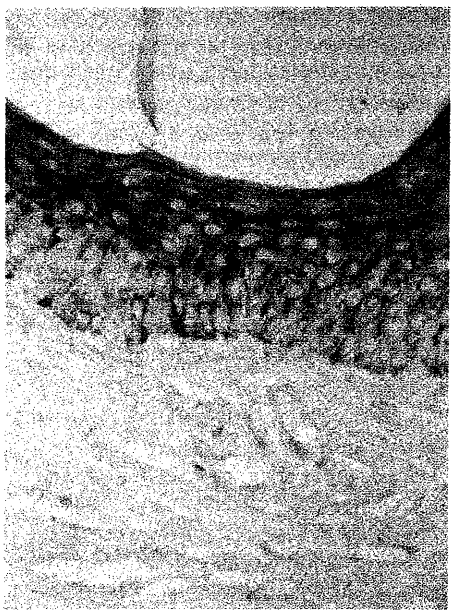

Figure 1.3. *Section of formaldehyde-fixed, paraffin-embedded skin immunostained with antikeratin antibodies. Note the variable immunoreactivity of keratinocytes, particularly in the basal cell layer.* (DAB-PO)

Aldehydes. The most common aldehydes used for fixation are formaldehyde (Fox, *1985*) and glutaraldehyde (Bullock, *1984*). Both fix by primarily covalently cross-linking proteins between α- and ε-amino groups.

Ultrastructural preservation is better with glutaraldehyde because the two aldehyde groups provide extensive cross-linking. Although tissue penetration is slow, (eg, 0.35 mm/hr in liver) (Hopwood, *1967*), cross-linking is rapid, providing complete fixation of tissue in close contact with the fixative.

In contrast, formaldehyde cross-links more slowly and therefore fixes more slowly; however, it penetrates tissues more rapidly. Furthermore, because it contains only single aldehyde groups, there is less alteration of antigenicity. Combinations of these two aldehydes can be used to maximize both ultrastructural preservation and retention of native antigenicity.

Formaldehyde can be combined with other fixatives. With picric acid, it improves fixation of basic proteins and histones. Tissue can also be fixed primarily with a combination of periodic acid (which oxidizes carbohydrate groups to aldehydes) and polymeric complexes of lysine and formaldehyde (Hixson, *1981*).

Osmium. Osmium tetroxide, which is a tetrapolar compound, cross-links lipids and proteins through chelation of multiple sites (Bullock, *1984*). Alteration of antigenicity from the native form is greatest with osmium, owning both to extensive cross-linking and to protein cleavage, although osmium can subsequently be removed by metaperiodate treatment (Hearn, *1985*).

Mercuric Compounds. Mercuric salts provide good light microscopic fixation (but bad ultrastructural preservation) with typically better retention of antigenicity than do pure al-

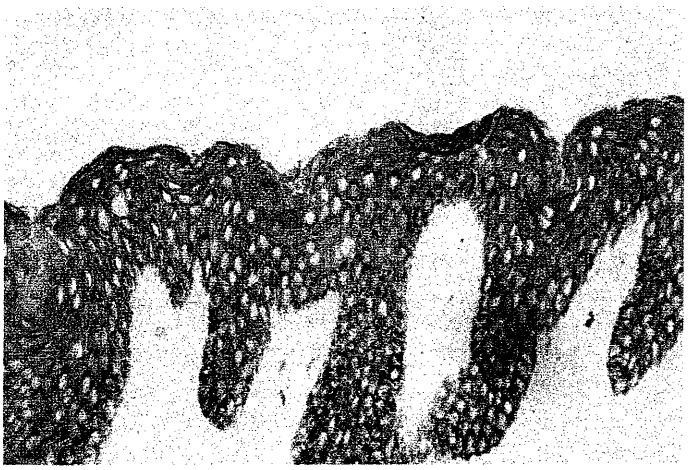

Figure 1.4. *In contrast, all keratinocytes of ethanol-fixed, paraffin-embedded skin immunoreact with the same antibodies. This indicates that formaldehyde fixation, rather than the process of paraffin embedding, has degraded immunoreactivity.* (DAB-PO)

dehyde fixatives. Because mercuric chloride penetrates tissue only slowly and may shrink tissue markedly, it is often combined with other fixatives:

1. B5 is a formalin–mercuric chloride mixture, neutralized with sodium acetate.
2. Zenker's is a solution of mercuric chloride, potassium dichromate, and acetic acid. Black mercuric precipitates, which can potentially confuse interpretation of immunostains, may be removed.

The factors that influence choice of fixative include: efficiency; preservation of light-microscopic morphology; preservation of native antigenicity; preservation of ultrastructural morphology; and retention of proteins.

In a diagnostic setting, efficiency and preservation of light microscopic morphology are dominant considerations. Accordingly, formaldehyde is the common fixative. However, when the goal is to maximize the precise localization of antigen, the particular fixative used and the method of fixation should be carefully considered. Because the preservation of immunoglobulins (either free, cell-bound, or as immune complexes) and of hematopoietic cell surface antigens is labile and is also dependent on fixation conditions (Warnke, *1979*; Jacobsen, *1980*), we are able to optimize retention of antigenicity by freezing tissues that might be analyzed for these antigens.

Embedding

Both the conditions of embedding and the type of embedding medium potentially alter antigenicity. Heat, which is necessary for infiltration of tissues by paraffin and which is a product of polymerization of methacrylates and epoxy resins, can po-

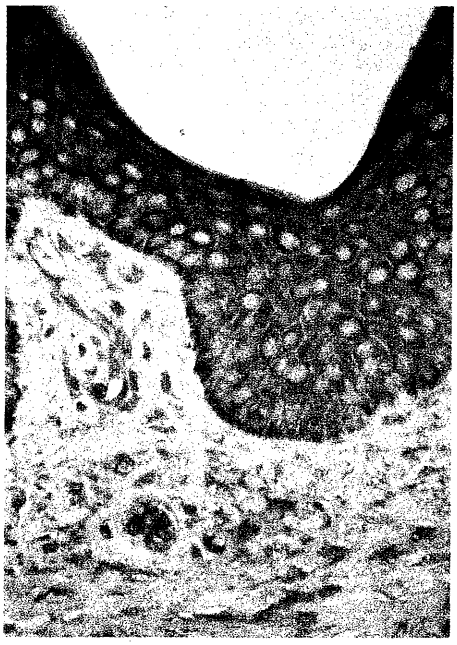

Figure 1.5. *A section of formaldehyde-fixed, paraffin-embedded skin incubated with Pronase-restored uniform immunoreactivity to keratinocytes. This section is a parallel section to that in Figure 1.3. "Digestion" of tissue sections with such an enzyme can restore intermediate filament reactivity.* (DAB-PO)

DURATION OF TRYPSIN DIGESTION FOR OPTIMAL IMMUNOSTAINING

	Duration of Formaldehyde Fixation		
	1 Day	1 Week	6 Weeks
Squamous mucosa	60	180	180
Pancreas, ducts	30	30	120
Kidney, tubules	10	30	30

Figure 1.6. *Longer fixation times necessitate longer digestion of the tissue section in a protease (0.1% trypsin, in this case) to recover immunoreactivity to a monoclonal antikeratin antibody. The trypsin digestion time, which varies with the type of tissue, is given in minutes. (Modified from Battifora,* 1986.*)*

EFFECTS OF VARIOUS FIXATIVES

	Histology	Ultrastructure	Immunoreactivity	Recoverable?
Unfixed	0 – +	0	+ +	
Ethanol	+ +	0	+ +	
Acetone	+ +	0	+ +	
Formaldehyde	+ +	+	+ / + +	Yes
Glutaraldehyde	+	+ +	0	Sometimes
Bouin's	+ +	?	+ / + +	Yes
B-5	+ +	0	+ / + +	Yes
Osmium tetroxide	+ +	+ +	0	Sometimes

Figure 1.7. *The effect of a given fixative on each parameter is indicated on a scale from 0 (worst) to + + (best). "Recoverable?" indicates the degree to which antigenicity can be recovered by such maneuvers as digesting the tissue section with an enzyme. This is a chart of generalizations. In practice, the effect of each fixative on the immunoreactivity of a given antigen should be assessed.*