CHAPTER

ONE

SPECIFICITY
ONLY

PRINCIPLES OF IMMUNOHISTOCHEMISTRY

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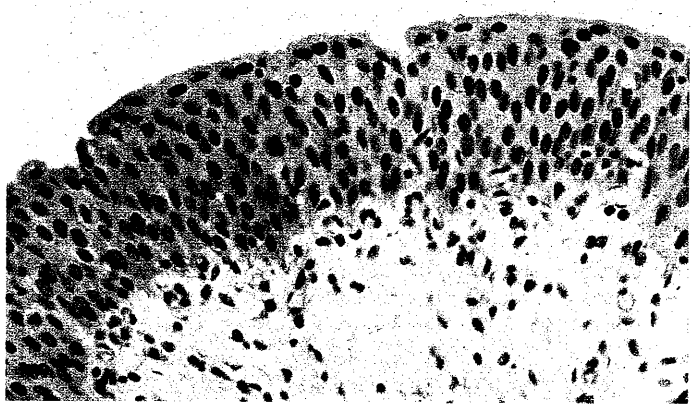

to varying levels of illumination should be known. In contrast to the eye (and to photographic emulsions), which responds logarithmically to illumination, the response of digital cameras is closer to linearity (Inoue, *1986*).

Adequacy of the detection system for identifying a sought antigen is checked with a positive control. This is a section previously shown to contain the antigen, under identical conditions of immunohistochemistry. Selecting an adequate positive control can be difficult for antibodies to a new antigen; a negative immunostain of a tissue reported to contain the antigen cannot be fully interpreted and the distinction cannot be made between inactive antibody and inactive or absent antigen. In such a case, there is little choice but to immunostain other tissues reported to contain the antigen (at a range of antibody dilutions) or to return the antibody to the supplier.

Enhancement Methods

Diaminobenzidine reactivity can be enhanced by counterstaining with heavy metals such as osmium (Graham, *1966*), colloidal gold followed by silver, and nickel or cobalt (Figs. 1.29 and 1.30). Repeat bridges, ie, with subsequent peroxidase–antiperoxidase, also enhance detectability (Vacca, *1975*) (Fig. 1.31).

SPECIFICITY

Nonspecific immunoreactivity represents deposition of reaction product at a site other than the location of the desired antigen. Such false positivity can arise either from antibody binding or from nonantibody-specific binding.

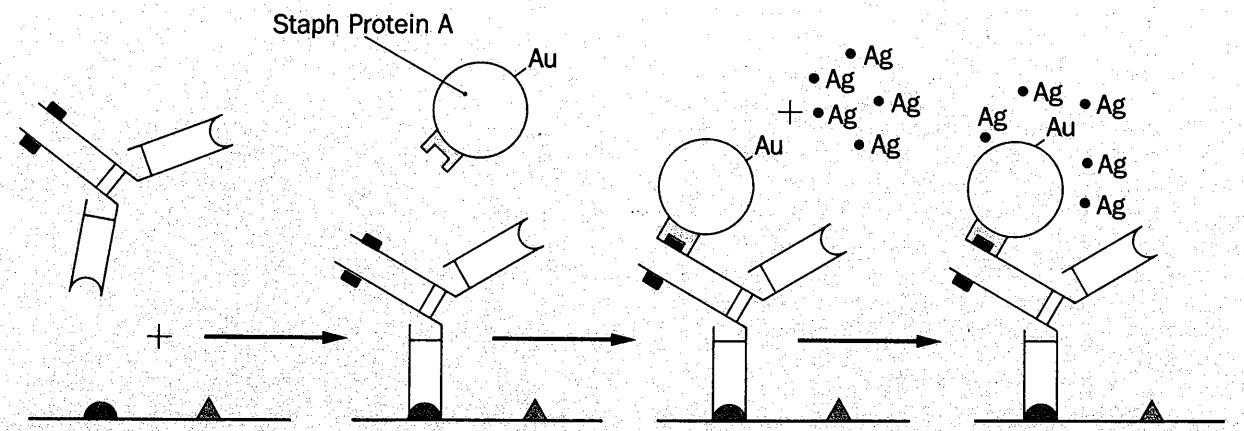

Figure 1.29. *Silver-enhanced, gold-labeled Staph Protein A immunohistochemistry. The primary antibody must contain a Staph Protein A-binding site, to which the Protein A–gold complex binds. Subsequent development of silver (Ag) deposits black reduced silver grains at the site of sought antigen A.*

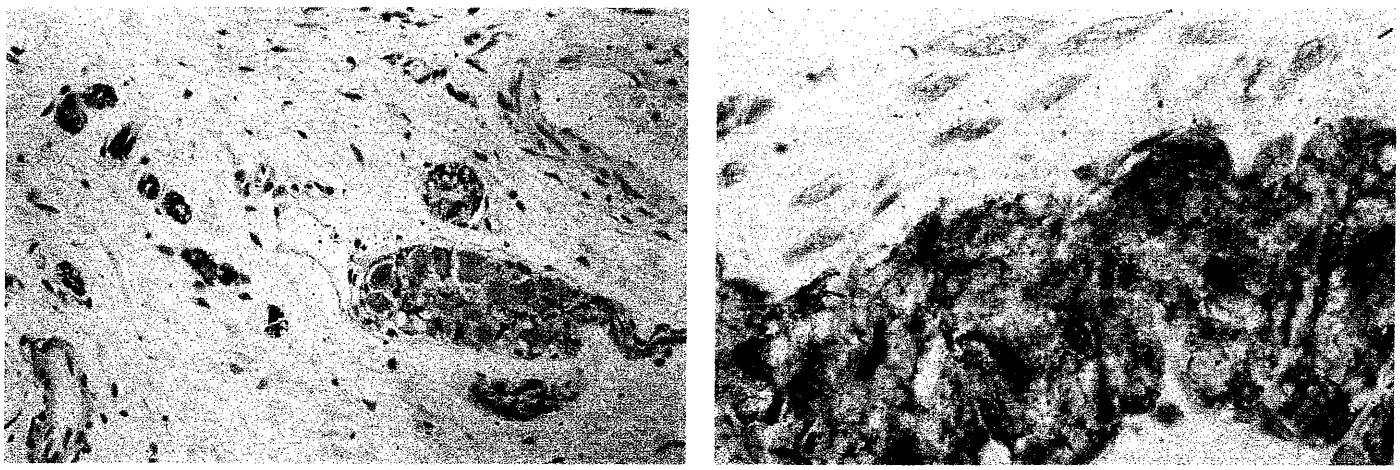

Figure 1.30. (Left) *Section of colon reacted with anti-S-100 protein, followed by Staph Protein A complex and silver. Granules of reduced silver stain nerve sheath cells in Auerbach's plexus black.* (Right) *Amplification of PAP staining. At higher magnification, the granular nature of the silver precipitate is seen. There is virtually no nonspecific binding.*

Immunologic Nonspecificity

Here, immunologic nonspecific reaction refers to the successful immunohistochemical localization of an antigen which provides a false result. Sources of such false positivity include the following.

Sequence Homology

Many molecules share partial amino acid identity, ie, gastrin/cholecystokinin, the intermediate filaments, α_1-AT/α_1-ACT, and S-100/calmodulin. Antibodies to the homologous sequence may localize both molecules. Unless the investigator is aware of both the partial potential antigenic identity of these two molecules and the specificity of the antibody to the common antigen determinant, she/he may falsely conclude that one molecule has been specifically localized. For example, localization of antineurofilament activity to nuclei could represent cross-reactivity with nuclear lamin, which is partially homologous with the intermediate cytoplasmic filaments (see Chapter 4) (Figs. 1.32 and 1.33).

Similar Antigenicity

There need not be amino acid identity for there to be antigenic similarity. For example, Leu-7 monoclonal antibody binds any proteins containing a certain carbohydrate group of restricted configuration, and some lupus antibodies bind to a phosphodiester epitope regardless of whether the epitope is on DNA or cardiolipin (Lafer, *1981*).

Contaminating Antibodies

The primary antibody preparation may contain various antibodies in addition to the one expected. If directed towards endogenous molecules, these are termed "autoantibodies." They may be present congenitally. Six percent of hybridomas from plasma cells of newborn mice produce autoantibodies, most frequently directed to such cytoskeletal proteins as tubulin and actin (Dighiero, *1985*). Virtually all adult humans contain antibodies to the 200,000 kd neurofilament protein; these immunohistochemically localize to neurons at 1:50 dilutions (Stefansson, *1985*). Apparently normal people have a 4% to 8% incidence of antivimentin and/or antikeratin antibodies.

Rabbits are known to have endogenous antikeratin antibody activity. That a polyclonal antiserum may be localizing an antigen other than the desired antigen was probably the explanation for reported immunostaining of epidermal cells and of proven squamous-cell carcinomas using a polyclonal anti-factor VIII-related antibody (Wilson, *1984*) (Fig. 1.34).

False positivity owing to contaminating or autoantibodies can be corrected by purifying antibody preparations. When pure antigen is available, affinity chromatography can remove spe-

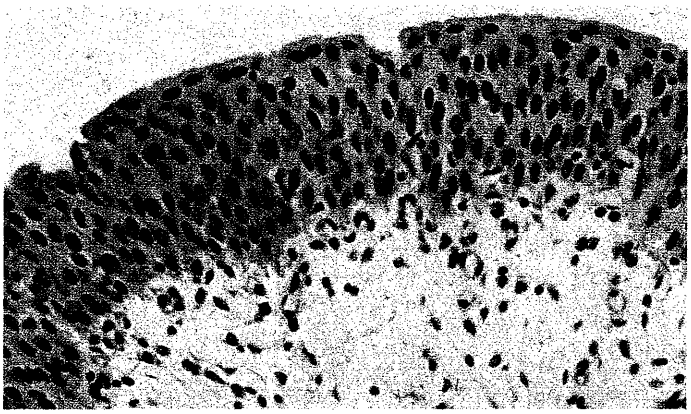

Figure 1.32. *Nuclei of urothelium localizing antineurofilament antibody. One explanation is that the nuclear lamin proteins of these urothelial nuclei share a specific epitope with neurofilament protein.* (DAB-PO; H-counterstained.)

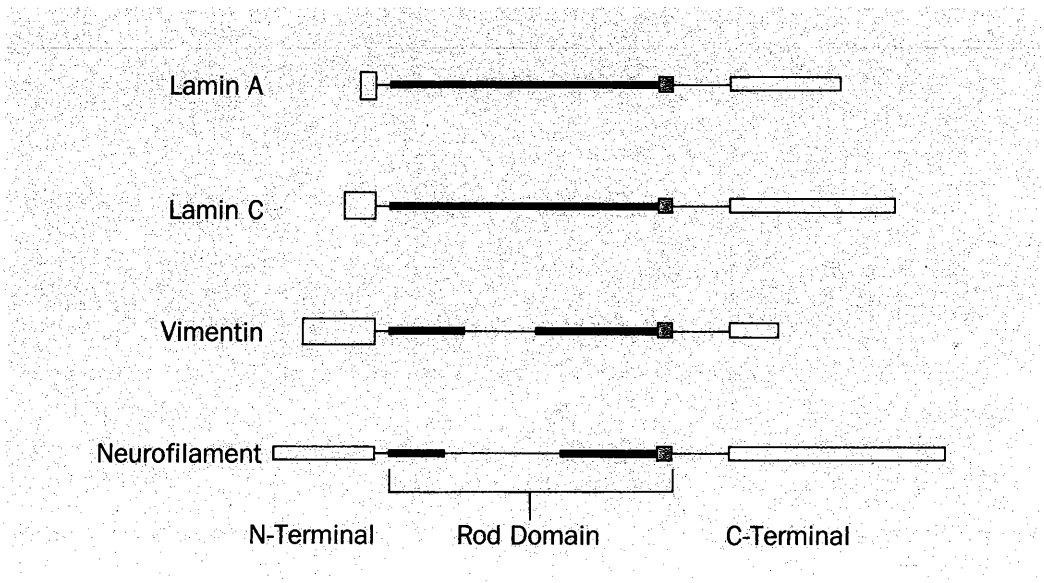

Figure 1.33. *Schematic of areas of extensive amino acid homology between the nuclear-membrane-associated proteins lamin A and lamin C. The cytoskeletal filaments vimentin and neurofilament protein are indicated in red. These four proteins differ at the amino and carboxy terminals. (McKeon, 1986).*

cific antibodies from a solution of multiple antibodies. When the specific antigen is unknown, the specific antibody cannot be isolated, but it can be concentrated using methods that isolate immunoglobulins, such as ammonium precipitation of immunoglobulins or separation with a Protein A column.

False positivity due to epitope identity cannot be corrected because successful antibody–antigen binding in these instances represents immunologic activity. Solutions of polyclonal antibodies containing multiple antigen-specific antibodies can be purified of antibodies that cross-react by affinity chromatography using the cross-reacting molecule as the immunoreactant. Only antibodies directed to unique epitopes will remain.

Nonimmunologic Nonspecificity

Many sources of non-antibody-binding false positivity exist. Fc portions of whole immunoglobulins may bind receptors of Fc phagocytes and mast cells (True, *1981*). Fc receptors are labile and readily inactivated by fixation. Complement-binding IgG molecules may localize to complement already present in tissue. (Buffa, *1979*). Complement is also labile. Certain antibodies and gut endocrine cells have an electrostatically mediated affinity that can mimic antibody–antigen reactivity (Grube, *1980*). Changes in the solution pH and salt concentration of reagents abolish this activity. Free aldehyde groups from incompletely reacted fixatives may nonspecifically bind antibodies to tissue (Farr, *1981*).

Neurohormonal peptides such as ACTH and vasoactive intestinal polypeptide have binding affinity for several reagents, including peroxidase-labeled immunoglobulin, Protein A, and streptavidin. Preincubation with excess ACTH(1-24) or use of poly-L-lysine in diluents will be corrective (Scopsi, *1986*b).

We have not suffered these sources of false positivity, which can be controlled for, in part, by using an irrelevant antibody as a positive control, and minimized with high dilutions of antibodies.

Endogenous Label Activity

Peroxidase. Hemoproteins with an iron porphyrin prosthetic group have peroxidase activity, which is highly variable and affected by factors that can be readily controlled in immunoperoxidase staining. The peroxidase activity of peroxidases in epithelial cells (mammary gland and secretory endometrium), megakaryocytes, and mast cells, catalase in liver, cytochrome C, myoglobin, and hemoglobin is suppressible by routine fixation and dehydration of tissues (Fahimi, *1979*; Escribano, *1987*).

The peroxidase activity in red cells, neutrophils, eosinophils, basophils, and histiocytes can be suppressed by multiple techniques that destroy or inactivate enzymatic activity (True, *1981*). (Fig. 1.35).

TUMORS IMMUNOSTAINED WITH ANTI-FACTOR VIII-RELATED ANTIGEN ANTIBODY

	Intensity of Immunostain		
Type of Tumor	**Strong**	**Weak**	**Absent**
Angiosarcoma	0	2	1
Squamous-cell carcinoma	3	3	0
Renal-cell carcinoma	2	0	1

Figure 1.34. *Number of cases of respective tumors immunostained with an anti-Factor VIII-related antigen antibody that also had anti-keratin activity. (From Wilson,* 1984.)

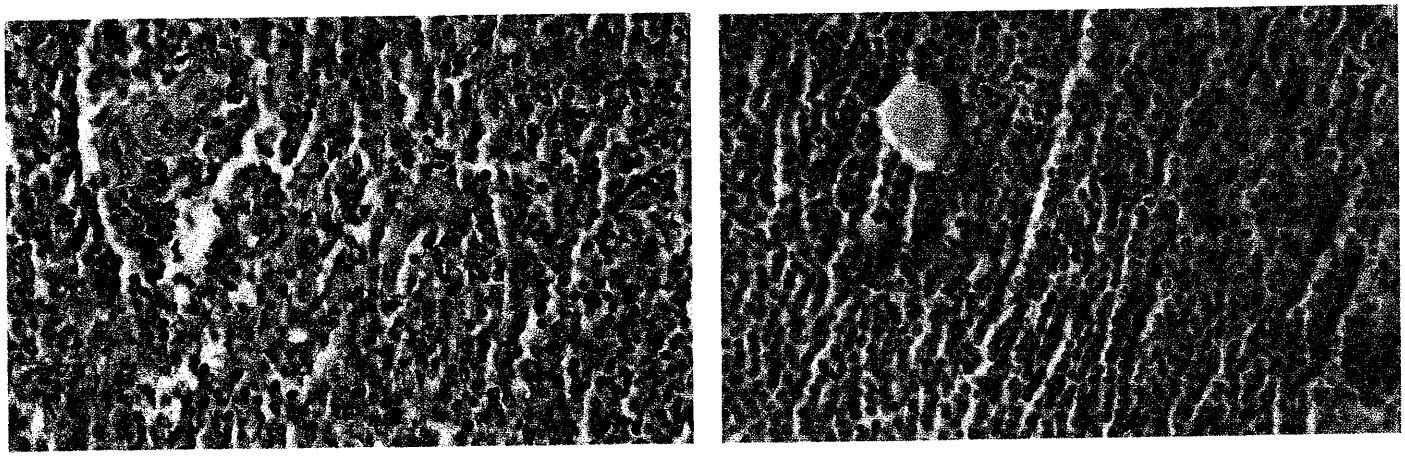

Figure 1.35. (Left) *Section of spleen incubated with antikeratin antibody. Reaction product is seen associated with red cells and neutrophils, because endogenous peroxidase activity was not suppressed.* (DAB-PO; H-counterstained.) (Right) *After endogenous peroxidase activity in an adjacent section of spleen is suppressed, red cells and neutrophils exhibit virtually no staining.* (DAB-PO; H-counterstained.)