Immunohistochemistry

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IMMUNOHISTOCHEMISTRY AS A LABORATORY TEST

Although they do not publicize it, pathologists have long recognized their fallibility.[1] As a result, more objective means of validating morphologic judgments have been sought. Stains using histochemical methods are of value in accentuating morphologic features but do not provide objective evidence of the lineage or biologic potential of a cell. The objective of immunohistochemistry is to use antibodies to identify antigens, increasing the specificity of the stain for the tissue with which it reacts. In doing so, immunohistology has transformed surgical pathology from a highly subjective discipline into a much more objective science, while still taking advantage of the light microscope and standard morphologic practices.

Immunohistochemistry, as the name implies, is the combination of histology and immunology. The resulting technique is a powerful tool that not only enables pathologists to detect whether particular antigens are present within a given cell but also allows the identification of the micro-anatomic (cellular) location of the antigen. These abilities permit the lineage of cell populations to be identified, an important consideration when confronted with a poorly differentiated neoplasm of undetermined origin. The technique is also useful in defining distinct populations of cells within the same lineage and defining functional differences. In addition, this technique preserves the histologic architecture and enables the pathologist to confirm that the positive cells are the cells in question. This confirmation is not possible with molecular methods, such as reverse transcriptase polymerase chain reaction or standard flow cytometry methods.

Immunohistochemistry is used by a variety of disciplines to study a wide range of questions. This chapter discusses the application of this technology in surgical pathology, in which immunohistochemistry has had a profound and fundamental impact on the practice of pathology.[1,2]

Technical Considerations

Immunohistochemistry has the potential to transform surgical pathology from a subjective art to an objective science, based on the recognition of cells by microscopic methods. Although this potential has resulted in its almost universal use, immunohistochemistry has not produced uniformly high standards of practice.[3,4] Therefore, certain technical considerations must be borne in mind to ensure the accuracy of results. It is self-evident that the quality of an immunohistochemical stain depends on the integrity of an antibody-antigen interaction and on the extent to which the relevant antigen has been preserved during tissue fixation and processing.[1] There is a high degree of variability in the way tissues are initially prepared. These variations include differences in the fixative used; the amount, age, and pH of the fixative; how long the tissue sat unfixed before being placed in fixative; the thickness of the tissue when first placed in fixative; and the time the tissue is left in fixative. All these variables, which may not alter the results of routine hematoxylin-eosin (H&E) staining to a significant degree, can lead to widely discrepant results when it comes to immunohistochemistry. These variables cannot always be predicted or remedied, but they can be mitigated to a great extent by the advent of successful and relatively simple antigen retrieval methods (discussed later).[5,6]

In addition to the variables in tissue fixation, as in all other laboratory tests, the reagents and techniques used must be optimized and thoroughly validated to ensure consistent, reliable, and clinically meaningful results. When developing an immunohistochemical protocol, it is impor-

tant for each laboratory performing a test to validate every reagent used. This validation includes a determination of the specificity and the optimal working dilution of each primary antibody, secondary antibody, linking antibody, labeling reagent, and substrate. Repeat validation is required for each new lot of reagents because of variations in origin, composition, concentration, and specificity that can occur among different lots even when supplied by the same company.[1] Also, reputable manufacturers should be used. The higher standards and more rigorous quality control of products from the better manufacturers have been counterbalanced by the concurrent proliferation of smaller manufacturers that are able to produce or otherwise acquire and market large numbers of different antibodies through monoclonal antibody technology, molecular engineering, and the like. In addition to the need for high-quality antibodies and reagents, proper incubation times and ideal temperatures for each antibody must be determined. The optimal buffering agent must be determined, as well as the need for any predigestion techniques or antigen enhancement procedures.

Premanufactured Kits

Premanufactured, all-inclusive kits have been marketed in an attempt to simplify the performance of immunohistochemistry; for example, these kits obviate the need for each individual clinical laboratory to validate each reagent because preoptimized working dilutions and recommended working protocols are provided. Nevertheless, there are some pitfalls that should be kept in mind when working with premanufactured kits. The protocols and reagents have been formulated to work on the prototype tissue used by the manufacturer and may not be as effective on the actual tissues tested because of laboratories' different fixation and processing protocols, all of which may have adverse effects on the results. Adjustments in the recommended protocol are necessary to optimize the kits in each individual laboratory, which effectively means that the kits must be customized, and any changes made in the manufacturer's protocol require that the *entire* staining procedure be revalidated by the performing laboratory.[1] This procedure may be complicated by the fact that many of the working dilutions of the reagents supplied are already at the critical level of sensitivity.

Automated Staining

Another method that can potentially enhance consistency and reproducibility is automation. A variety of automated immunostaining systems are now commercially available. The theoretical advantages of these systems over manual staining techniques include improved reproducibility, facilitation of interlaboratory comparisons, reduced reagent expenses, and increased technician productivity. Automation does not mitigate the need to thoroughly validate each step of the staining procedure or the need to evaluate every reagent used to ensure high-quality, consistent results. The same quality control issues that apply to manual staining apply to automated systems. As with manual staining, it is important that a complete reevaluation be performed if there is any departure from the validated protocol.[1] Auto-

mation does not guarantee an optimal result. Finally, automation cannot replace the pathologist, who must choose the appropriate antibodies and then interpret the final result.

Automated Image Analysis

There is a growing need to be able to quantify immunohistochemical staining results, which is probably best accomplished by automated image analysis. Current systems, including the Automated Cellular Imaging System II (ACIS II Clarient, Aliso Viejo, Calif), have the ability to assess marker positivity in terms of both percentage positive and intensity of staining. The ability to quantify markers more precisely is especially important in the identification of targets of therapy. One example of this is Her-2/*neu*. When Her-2/*neu* protein expression by immunohistochemistry was compared with gene amplification by fluorescence in situ hybridization using routine manual methods and the assistance of a digital microscope, both accuracy and reliability were improved when the digital microscope was used.[7,7a] Precise quantification of hormone receptors may also be important because there is evidence that in patients with high levels of hormone receptors, the addition of cytotoxic chemotherapy has a deleterious effect on outcome.[8,9] Another exciting development in the field of automated cellular imaging is spectral imaging. This technology allows multiple markers to be assessed on the same slide—even on a single cell. Computer software can isolate a single chromogen from other chromogens present based on its emission spectrum. Automated cellular imaging provides greater objectivity and reproducibility and thus minimizes interobserver discrepancies.

Positive and Negative Controls

Immunohistochemical tests performed and interpreted in the absence of the appropriate controls are valueless and even dangerous. Minimal controls should include a tissue known to express the particular antigen of interest, processed in a manner analogous to that of the unknown tissue (the positive control), and a second section of the test specimen in which the primary antibody is replaced either by diluent or, better, by an irrelevant antibody of the same isotype, from the same species, and at the same concentration (the negative control). In the positive control, only cells expected to express the antigen should show positivity; all other cells and structural elements should be negative. In the negative control, there should be no specific staining. The "sausage" technique, in which samples of multiple tissues are gathered into a single tissue block, is a useful control method.[10]

Controls are performed for a variety of purposes; in addition to indicating whether a reaction occurred (or not), they are essential for judging the nature of the reaction. A vast array of immunohistochemical tests are judged not by a *positive* or *negative* result but by the intensity and localization of the result (a good example is Her-2/*neu* and hormone receptor analysis in breast cancer). Immunohistochemistry results should never be interpreted in the absence of the known positive results because the assessment of *quality* and *quantity* of the reaction is essential.

Results and Reporting

Interpretation of the results of immunohistochemical stains is the province of the surgical pathologist and is best accomplished by pathologists who have the appropriate level of experience not only in the morphologic aspects of diagnosis but also with regard to immunohistochemical findings. As in any other area of pathology, experience matters: A pathologist with little experience with immunohistochemistry, who runs a few different tests each week or month, will obtain very different results from a pathologist who performs and interprets immunohistochemical tests on a daily basis. As the impact of immunohistochemistry on surgical pathology increases, these differences will become more profound.

As described previously, many factors influence the results. All these factors must be considered by the pathologist in interpreting the findings. Negative, weak, or uninterpretable results should lead to a repetition of the test after the use of antigen retrieval.[11,12] One measure of antigen preservation is to test for expression of the intermediate filament vimentin, a fixation-sensitive protein that is typically expressed by vascular or connective components; this technique often serves as an internal indicator of the conservation or loss of antigenicity.[11] Test results may also be affected by technical artifacts and by the nature of the tissue under study. For instance, if tumor cells are crushed, false-positive or nonspecific staining may be encountered. Nonviable areas of tissue from a necrotic tumor may also be a source of false-positive results, attributable in part to leakage of serum proteins (e.g., immunoglobulins). The subcellular distribution of immunoreactivity is critical to the interpretation of immunohistochemical results. For example, Her-2/*neu* shows membranous staining, whereas antibodies to estrogen and progesterone receptors produce nuclear staining. When unexpected staining patterns are observed with an antibody, the results should be discounted.[1] To interpret the results effectively, the pathologist must have extensive knowledge of the staining patterns of the primary antibodies under consideration, including a detailed knowledge of tissue specificity and subcellular localization of the antigen, and an awareness of technical variables. Each laboratory performing immunohistochemical staining should have established written criteria for determining and reporting positive and negative findings for each immunohistochemical stain, with particular reference to stains that are expected to produce cell surface membrane, cytoplasmic nuclear, or extracellular staining. Although it seems obvious, it often is overlooked that staining should be recorded as *positive* only if it occurs in the expected cellular or tissue location.

Validation and Proficiency Testing

The Food and Drug Administration's increased attention to the reagents used in immunohistochemistry has undoubtedly contributed to an improvement in their quality.[13] It is highly recommended that all laboratories performing diagnostic immunohistochemistry participate in the College of American Pathologists' certification program, which includes a checklist of the essential elements required for a successful immunostaining program.[7a] With regard to staff qualifications, the National Society of Histotechnologists has focused its efforts on continuing education and certification programs for technologists performing immunohistochemical staining.

Federal law requires a high degree of testing and validation. In the United States, laboratories performing immunohistochemistry are required under the Clinical Laboratory Improvement Amendments of 1988 to validate the performance of their test reagents for accuracy, specificity, sensitivity, and precision.[14] First, the testing procedure is optimized (as described earlier), and performance expectations are established. During the validation process of each analyte, multiple slides with known pathology (generally 20 representative cases) are evaluated with the optimized procedure to assess the accuracy of diagnostic staining, sensitivity of signal, and reproducibility. Validations that meet specifications must be signed by qualified individuals, and the documents are maintained in the laboratory. Quality control and proficiency testing must be performed to monitor performance.

Limitations

Although immunohistochemistry is an extremely valuable technique in experienced hands, its limitations must be recognized for it to be used to its maximum potential.

Experience

Although immunohistochemistry is more objective than routine morphologic examination, the experience of the pathologist assessing the slides is critical. A firm understanding of the principles of immunohistochemical staining is necessary because the reporting pathologist must be equipped to deal with the unexpected and conflicting results that inevitably occur. To evaluate the immunohistochemical slides properly, the pathologist must have a firm understanding of the limitations of antibodies in terms of their technical aspects as well as their inherent specificity, sensitivity, and expected subcellular location.

Availability of Antibodies

The advent and refinement of the hybridoma technique for the production of monoclonal antibodies have produced a large number of available antibodies. Often a newly developed antibody is hailed as exquisitely specific. In time, however, most are found to be considerably less specific than initially hoped, generally because the antigen the antibody detects has a wider distribution than expected. This fact does not negate the usefulness of the antibody in question, but it may mean that panels of antibodies must be used in conjunction with standard morphologic features and clinical history.

Loss of Antigenicity in Stored Cut Paraffin Sections

Many studies have shown that a loss of antigenicity can occur on cut paraffin sections that have been stored for varying lengths of time.[15-17] Among the antibodies studied, those most adversely affected by storage include p53,[15,17]

MIB1,[16,17] factor VIII–related antigen,[15] estrogen receptor,[15] bcl-2,[15] p27kip1,[16] CD-44s,[16] and androgen receptor.[16] In many cases, the use of carefully selected and tested antigen retrieval techniques can compensate for this loss.[17]

Antigen Retrieval

Formalin is the most widely used fixative in surgical pathology. Cross-linking of proteins is the essential feature of formalin fixation. This cross-linking interferes with the antigen's ability to react with the primary antibody. In 1991 the antigen retrieval technique was developed.[12,18] This technique is a heat-induced modification of the protein conformation that allows the antigen to be accessible again for chemical reactions, in this case, antibody binding. Hydrolysis of cross-linking resulting from formalin fixation probably plays a major role in this modification process.[19-22] The application of antigen retrieval to sections derived from formalin-fixed, paraffin-embedded blocks produces consistent results of acceptable quality,[6] although a few antigens remain undetectable even after antigen retrieval has been performed. Antigen retrieval methods have revolutionized immunohistochemistry and have become a standard part of diagnostic immunohistochemistry in surgical pathology. These methods result in higher sensitivity and more consistent antibody reactivity. Antigen retrieval technology has led to a proliferation of protocols that may produce different results in different laboratories. The successful application of these methods allows the detection of some antigens that were previously undetectable in paraffin sections, rendering much of the early literature (prior to 1993) obsolete. This fact continues to escape the notice of some practicing pathologists, leading to errors of interpretation (Fig. 5-1).

It should be recognized that two major factors influence the effectiveness of antigen retrieval: the conditions under which heating takes place, and the pH value of the buffer solution used during the heating process.[5,11,23] The most critical factor is the combination of the temperature and the duration of heating, which have a reverse correlation. Based on these two factors (heating conditions and buffer pH), a test battery approach has been developed to establish optimal antigen retrieval protocols for immunostaining on archival paraffin-embedded tissue sections.[1,6,23] A typical test battery consists of nine serial sections of a specimen known to express the antigen under study. The sections are evaluated with buffer at three different pH values (e.g., pH 1 to 2, 7 to 8, and 10 to 11) and three heating conditions (e.g., 90°C, 100°C, and 120°C) for various lengths of time (or some other comparable heating versus time schedule). The best result is selected as the optimal retrieval condition for that antigen. In the event that a satisfactory result is not obtained, other variations may be explored, including different buffer solutions and more or less vigorous heating methods.[19,23] Protocols for antigen retrieval differ in their effectiveness for retrieving certain antigens, and a single universally effective retrieval method does not exist. Many laboratories use more than one method for different antibody and antigen combinations. Overall, citrate buffer at pH 6.0 has the broadest applicability for the widest range of antigens, although several studies have demonstrated that the use of higher pH retrieval solutions yields satisfactory results.[21,24] Retrieval solutions with lower pH values, TRIS (tromethamine) buffer at pH 8.0, and EDTA (ethylenediaminetetraacetic acid)-NaOH solution (pH 8.0), are effective in certain special situations.[25-27] The selection of heating method (water bath, steamer, microwave, pressure cooker, or autoclave) is influenced by custom and availability.

CURRENT APPLICATIONS OF IMMUNOHISTOCHEMISTRY

Diagnostic Tool for Tumors of Unknown Origin

Immunohistochemistry has become an integral and essential part of surgical pathology. It is applied to define tumor
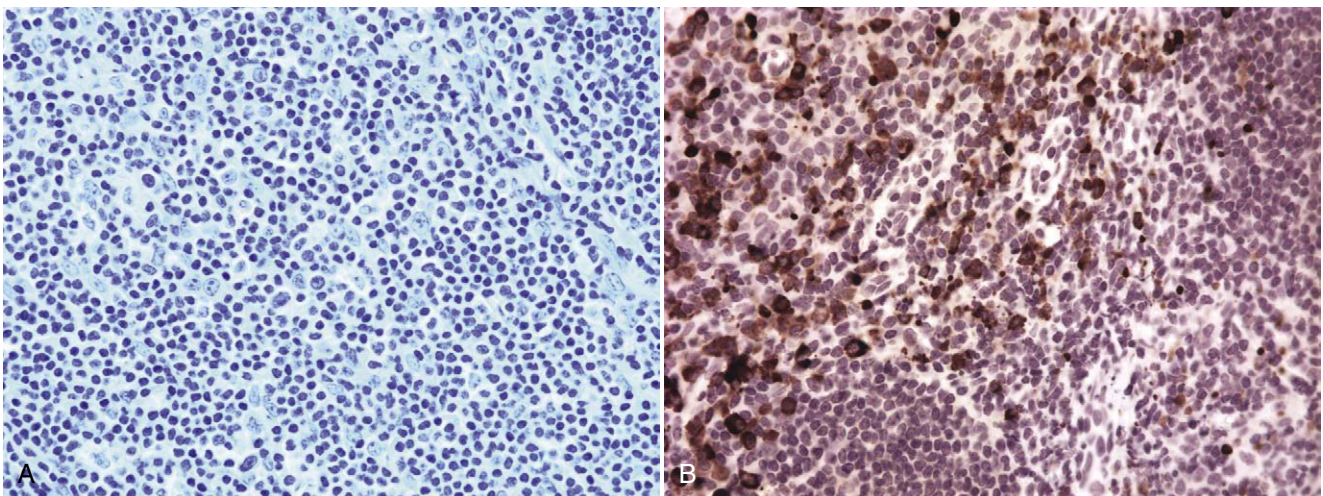

Figure 5–1 ■ Section of lymphoid tissue stained with antibody against lambda light chain without antigen retrieval, showing no positive cells (**A**), and with antigen retrieval, showing scattered positive cells (**B**).

origin, establish prognosis, and determine treatment response. In this textbook, the role of immunohistochemistry in defining the origin, prognosis, and treatment response of tumors is discussed in the chapters devoted to the specific organ systems; therefore, a full discussion is not provided here. Because the evaluation of tumors of unknown origin does not fall under any particular organ system, it is discussed in this chapter.

Tumors are classified most often by their tissue of origin (e.g., breast, colon, prostate) or histogenetically (e.g., tissue of epithelial, mesenchymal, or neural origin). A tumor cannot be staged accurately, and proper therapy cannot be administered, without such classification. Although accepted and fairly reproducible criteria exist for the morphologic diagnosis of most tumors, there is inherent subjectivity in any morphologic evaluation. It is well recognized that morphologic features often overlap among different entities and that one disease can present with myriad histologic pictures. Most tumors can be classified correctly by routine histologic techniques when the clinical situation is clear (e.g., a breast mass); however, an important subset of tumors defies morphologic interpretation. The magnitude of this problem is substantial. The diagnosis of "metastatic cancer of unknown primary site" is the eighth most common cancer diagnosis and may represent up to 15% of cancers at large hospitals.[28] Much more common is the diagnosis of "tumor of uncertain origin." This occurs when (1) the tumor is first identified in a metastatic site, and the primary site is not apparent; (2) the tumor is so poorly differentiated that no specific morphologic features can be identified; (3) the morphologic appearance of the tumor is compatible with more than one distinct tissue (e.g., epithelial versus lymphoid origin); and (4) the histogenesis of a tumor is clear (e.g., adenocarcinoma), but the primary site is in question. This distinction has important consequences to the patient.

Test Selection

Immunohistochemical tests should be performed with a defined objective in mind. The results of a single immunohistochemical procedure can be misleading not only because of variables in the staining procedure but also because of unanticipated patterns of reactivity of certain antibodies.[1] Although myriad antibodies are available, the choice in a particular case should be judicious and designed to address the diagnostic possibilities. The use of too few antibodies rarely provides sufficient information to support a specific diagnosis and can produce misleading information. Antibodies should be selected on the basis of their ability to affirm or exclude considerations in the differential diagnosis. This so-called problem-oriented approach is based on the selection of appropriate panels of antibodies. When selecting antibodies, factors that should be considered include the clinical history, morphologic features of the tumor, and results of other tests that may have been performed, including serologic and radiographic tests. Pathologists can find guidance in the literature and in a few specialized textbooks that address the use of immunohistochemistry,[2,29] but this is a rapidly evolving field. The limited antibody panels of a few years ago are inadequate to deal

with tumors of unknown or uncertain primary sites today. With this in mind, the panels presented here must be considered elementary guides.

Panel Approach: Basic Principles

When evaluating tumors of uncertain origin by immunohistochemistry, certain basic guidelines of interpretation must be kept in mind. A positive staining reaction is generally more helpful than a negative one because a lack of immunoreactivity may represent a technical problem with the tissue or the way it was fixed, as discussed earlier. The more poorly differentiated a tumor is, the less likely it is to express tissue differentiation antigens. There is often staining variation within a tumor; by extension, variations in staining patterns may be seen between the primary tumor and the metastatic focus. Most important, the final diagnosis should never depend on immunohistochemistry alone; it must be made using all the clinical, serologic, radiographic, morphologic, and epidemiologic data available. Other techniques, such as the assessment of specific DNA alterations, are becoming increasingly important adjuncts to the pathologic evaluation. Although immunohistochemical evaluation is essential, it is only one of many tools that must be used in the evaluation of pathologic processes.

The application of a primary panel of antibodies to characterize tumor histogenesis (epithelial, mesenchymal, neural, or hematopoietic) is often the first step. When this panel has been established, additional antibodies can be used to identify the tumor type more specifically. Included in the first tier would be antibodies against pan-keratin, vimentin, S-100 protein, neuron-specific enolase (NSE) and CD45 (common leukocyte antigen) to differentiate epithelial, mesenchymal, melanomatous, neural, and lymphoid malignancies (Table 5-1; Fig. 5-2).

Intermediate Filaments

The expression of intermediate filament proteins, which function as the supporting cytoskeleton in normal and neoplastic cells, is extremely useful in the initial assessment of tumors of unknown primary origin.[1] There are five major classes of intermediate filaments, based on protein composition and cellular distribution: cytokeratin, vimentin, desmin, neurofilament, and glial fibrillary acidic protein (GFAP).[30] Most neoplasms show the predominant expression of one or more of these intermediate filaments. Carcinomas usually express cytokeratin; sarcomas, melanomas,

TABLE 5–1

Screening Immunophenotypes of Undifferentiated Neoplasms

AE1/AE3	Vimentin	CD45	S-100	Tumor Type
+	–/+	–	R	Carcinoma
R	+	–	–/+	Sarcoma
–	–	+	–	Lymphoma
–	+	–	+	Melanoma

+, always positive; –, negative; –/+, mostly negative; R, rare positive cells.
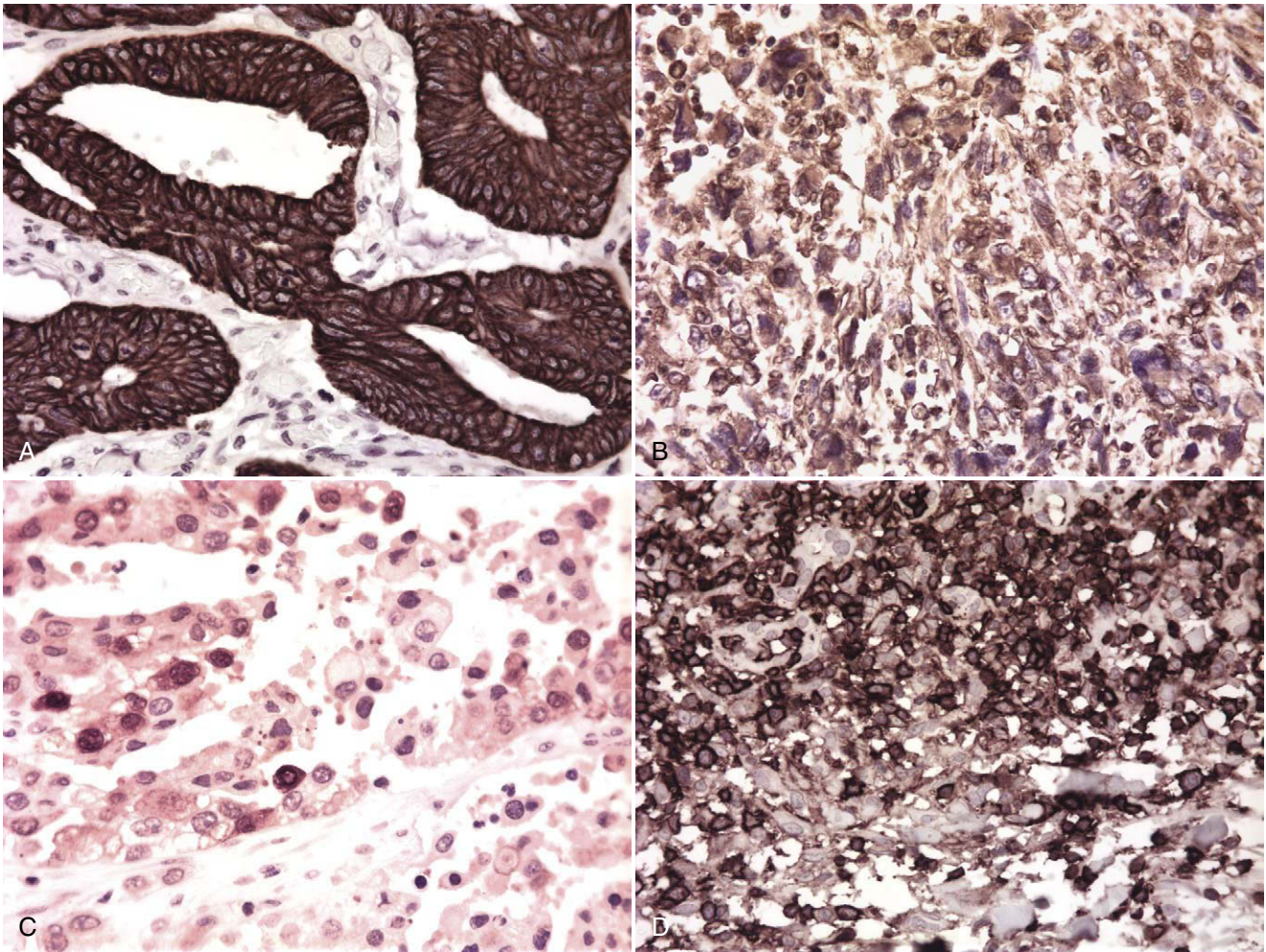
Figure 5–2 ■ **A,** Colon carcinoma showing keratin positivity, which is typically seen in carcinomas. **B,** Malignant fibrous histiocytoma showing vimentin positivity, which is typically seen in sarcomas. Note the fine reticular pattern of the intermediate filaments in the giant cells. **C,** S-100–positive melanoma. Note the nuclear reactivity, characteristic of melanoma. **D,** CD45 (common leukocyte antigen)–positive lymphoma.

and lymphomas are generally vimentin positive; myogenic tumors are characteristically positive for desmin or muscle actins and vimentin; and glial tumors are predominantly positive for GFAP.[1] Some tumors characteristically coexpress more than one intermediate filament (e.g., renal and thyroid carcinomas contain keratin and often vimentin), whereas others show aberrant or no intermediate filament expression. Immunohistochemical markers for intermediate filaments on tumors of uncertain origin are one of the most useful and productive ways to begin classifying the lesion. Anaplastic neoplasms can be characterized as keratin positive (carcinomas, mesotheliomas), vimentin positive (sarcomas, lymphomas, melanomas), or neurofilament and GFAP positive (neuroendocrine, neural, and astrocytic tumors).[1]

Keratin-Positive Tumors

Cytokeratins are present in almost all epithelial cells and are highly sensitive markers for carcinomas. In a generic sense, malignant cells expressing keratin positivity indicate an epithelial origin. Antibodies against keratin are also

extremely useful as markers for occult metastases (micrometastases) in the peripheral blood, bone marrow, and lymph nodes (discussed later).

There are more than 20 different subtypes of cytokeratin found in human epithelial cells. These subtypes are distinguishable by their molecular weight and isoelectric pH.[31] Monoclonal antibodies specific for many of these subtypes have been developed. Carcinomas of different types tend to express characteristic keratin profiles.[32] There is a general correlation between the complexity of the epithelium from which the tumor is derived and the complexity of the keratin subunits expressed. Low-molecular-weight or nonsquamous keratins appear early in development and predominate in tumors derived from simple, nonstratified epithelium (e.g., ductal carcinoma of the breast, gastrointestinal adenocarcinoma). High-molecular-weight or squamous keratins appear in more complex stratified epithelium and predominate in tumors derived from stratified epithelium (e.g., squamous cell carcinoma). Some tumors, such as those derived from pseudostratified columnar epithelium, contain a mixture of high- and low-molecular-weight keratins, with a predominance of the latter. In some instances, especially in extremely poorly differentiated

tumors, as few as 5% of tumor cells may express keratin reactivity.[1]

When a tumor of uncertain primary site has been defined as epithelial by either immunohistochemistry or morphology, it is important to attempt to define its specific origin. This presents a problem when, for example, a patient with a prior history of breast carcinoma presents with a lung mass that, on biopsy, is adenocarcinoma. Determining whether the lung mass represents a primary pulmonary tumor or metastasis from the breast has enormous consequences in terms of patient outcome and choice of specific treatment. Although the immunohistochemical evaluation of primary epithelial tumors is problematic, advances have been made. Monoclonal antibodies against keratin subtypes may help determine the origin of certain poorly differentiated neoplasms. Hepatocellular carcinoma (positive for AE3 and CAM 5.2 but negative for AE1) can be distinguished from bile duct carcinoma and adenocarcinoma metastatic to the liver (positive for AE1).[33] In particular, the differential expression of cytokeratins 7 and 20 (CK7, CK20) is extremely useful in the characterization of epithelial neoplasms (Fig. 5-3).[32,34-36] These patterns are not absolute, but they can be useful guides in establishing origin.

Cytokeratin 5/6 (CK5/6) has received considerable attention recently. CK5/6 has been found to be positive in the majority of squamous cell carcinomas, basal cell carcinomas, thymomas, salivary gland tumors, and biphasic malignant mesotheliomas and in a subset of endometrial adenocarcinomas, transitional cell carcinomas, and pancreatic adenocarcinomas.[37] CK5/6 is rarely positive in adenocarcinoma of the lung and has therefore been used to distinguish malignant mesothelioma from pulmonary adenocarcinoma.[37,38] In addition, p63 is frequently seen in squamous cell carcinoma and transitional cell carcinoma, whereas mesothelioma is uniformly negative for p63. Therefore, positive immunostaining for both p63 and CK5/6 is highly predictive of a primary tumor of squamous epithelial origin.[39,40] Because p63 is also known to immunoreact on the basal cell nuclei in benign prostate glands, this marker can be used to distinguish prostate cancer from benign mimics.[41]

There is an increasing array of *tissue-specific* markers, such as prostate-specific antigen (PSA) and thyroglobulin, as well as *tissue-associated* markers, such as GCDFP-15 and mammaglobin for breast epithelium,[42,43] OC-125 for ovary,[44] uroplakins for urothelium,[45,46] and synaptophysin for neuroendocrine lesions (Fig. 5-4).[47] Table 5-2 summarizes many of these tissue-associated antibodies. It is important to keep in mind patterns of antigenic coexpression, which can lead to erroneous assessments. Also, as mentioned earlier, the more poorly differentiated the neoplasm, the less likely it is to express tissue-specific or -associated antigens. Nevertheless, the specific origin of an epithelial neoplasm of uncertain primary site can be elucidated in an increasing proportion of cases through careful evaluation of morphology, clinical data, and antigen expression.

Keratin-Negative Tumors

LYMPHOMAS

Lymphomas often present morphologically as undifferentiated malignant neoplasms. A large cell lymphoma may be difficult to differentiate from carcinoma or melanoma. Anaplastic large cell lymphomas occasionally react with antibodies against keratin or epithelial membrane antigen. Similarly, small cell lymphomas often resemble other tumors, such as small cell undifferentiated carcinoma. Immunohistochemistry can be invaluable in classifying a tumor as lymphoid in origin. Immunohistochemistry is also used widely to aid in the subclassification of non-Hodgkin's lymphoma (discussed in detail in Chapter 41). Another important application of immunohistochemistry is the phenotyping of a lesion to determine the immunoglobulin light-chain expression. One can distinguish between malignant and benign lymphoid proliferation by demonstration of light-chain (or heavy-chain) restriction.

CD45 is an excellent screening marker to determine whether a tumor is lymphoid in origin. Staining is characteristically membranous. Some neoplasms of lymphoid origin may not express CD45, and rare nonlymphoid neoplasms may show cytoplasmic staining for CD45. More specific lymphoid markers such as CD3, which marks T cells, and CD20, which marks B cells, may further delineate a lesion. Immunohistochemistry can also be used in cases of Hodgkin's disease, in which markers useful in identifying Reed-Sternberg cells include CD15, CD30, and BLA-36.[48,49] Figure 5-5 illustrates an algorithmic approach to the immunohistochemical diagnosis of malignant lymphomas.

MELANOMAS

Melanomas are typically (but not always) negative for cytokeratin[50] and positive for vimentin. S-100 protein is a sensitive marker for melanoma and occurs, in almost all cases, in a nuclear pattern (see Fig. 5-2C). S-100 is not specific for melanoma, however, and is seen in a variety of lesions, including Langerhans histiocytes, many sarcomas, and certain carcinomas.[51,52] Positive immunoreactivity for HMB-45, Melan A, or tyrosinase, which are much more specific markers for melanoma and melanocytes, can confirm the diagnosis.[53] This topic is discussed in detail in Chapter 49.

SARCOMAS AND SOFT TISSUE TUMORS

A diagnosis of sarcoma is worth considering when a spindle cell neoplasm expresses vimentin but not keratin, CD45, or HMB-45. Immunohistochemical analysis can delineate a specific type of sarcoma such as myogenic and fibrohistiocytic tumors, tumors with neural differentiation, and vascular sarcomas. The differentiation of soft tissue tumors by immunohistochemistry is discussed in Chapter 46. The major subtypes are described briefly here.

Myogenic sarcomas react with antibodies to muscle-specific actin and desmin. Antibodies against myoD-1 smooth muscle actin show preferential reactivity with leiomyosarcomas.[54] Alveolar soft part sarcomas typically coexpress vimentin and desmin.[55] Tumors derived from skeletal muscle often contain myoglobin; positive immunoreactivity for muscle-specific actin, desmin, and myoglobin indicates that the tumor in question may be a rhabdomyosarcoma.

Fibrohistiocytic tumors, including malignant fibrous histiocytoma, are the most common form of soft tissue sarcoma in adults. Malignant fibrous histiocytoma reacts with vimentin and lacks significant reactivity for keratin. These lesions
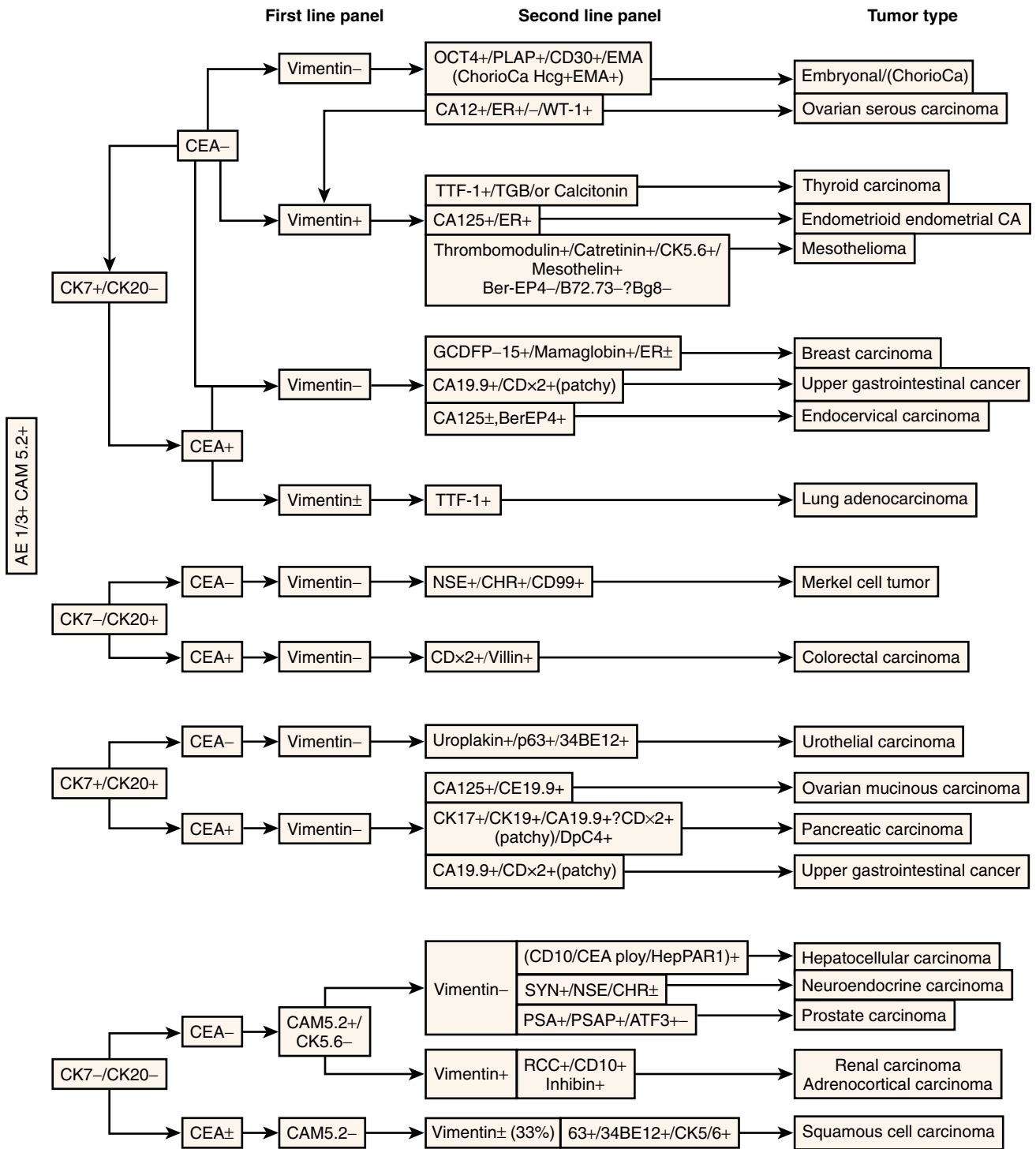
First line panel **Second line panel** **Tumor type**

AE 1/3+ CAM 5.2+

CK7+/CK20−
- CEA−
  - Vimentin−
    - OCT4+/PLAP+/CD30+/EMA (ChorioCa Hcg+EMA+) → Embryonal/(ChorioCa)
    - CA12+/ER+/−/WT-1+ → Ovarian serous carcinoma
  - Vimentin+
    - TTF-1+/TGB/or Calcitonin → Thyroid carcinoma
    - CA125+/ER+ → Endometrioid endometrial CA
    - Thrombomodulin+/Catretinin+/CK5.6+/Mesothelin+ Ber-EP4−/B72.73−?Bg8− → Mesothelioma
- CEA+
  - Vimentin−
    - GCDFP−15+/Mamaglobin+/ER± → Breast carcinoma
    - CA19.9+/CD×2+(patchy) → Upper gastrointestinal cancer
    - CA125±,BerEP4+ → Endocervical carcinoma
  - Vimentin±
    - TTF-1+ → Lung adenocarcinoma

CK7−/CK20+
- CEA− → Vimentin− → NSE+/CHR+/CD99+ → Merkel cell tumor
- CEA+ → Vimentin− → CD×2+/Villin+ → Colorectal carcinoma

CK7+/CK20+
- CEA− → Vimentin− → Uroplakin+/p63+/34BE12+ → Urothelial carcinoma
- CEA+ → Vimentin−
  - CA125+/CE19.9+ → Ovarian mucinous carcinoma
  - CK17+/CK19+/CA19.9+?CD×2+(patchy)/DpC4+ → Pancreatic carcinoma
  - CA19.9+/CD×2+(patchy) → Upper gastrointestinal cancer

CK7−/CK20−
- CEA− → CAM5.2+/CK5.6−
  - Vimentin−
    - (CD10/CEA ploy/HepPAR1)+ → Hepatocellular carcinoma
    - SYN+/NSE/CHR± → Neuroendocrine carcinoma
    - PSA+/PSAP+/ATF3+− → Prostate carcinoma
  - Vimentin+
    - RCC+/CD10+ Inhibin+ → Renal carcinoma Adrenocortical carcinoma
- CEA± → CAM5.2− → Vimentin± (33%) | 63+/34BE12+/CK5/6+ → Squamous cell carcinoma

Figure 5–3 ■ Algorithm for carcinoma of unknown primary site.

may also coexpress α_1-antitrypsin, α_1-antichymotrypsin, HAM-56, and CD68. α_1-Antichymotrypsin is not specific for malignant fibrous histiocytoma and can be seen in other sarcomas, some carcinomas, and melanomas.[56-58]

Neurogenic tumors, including malignant peripheral nerve sheath tumors and schwannomas, are positive for antibody against S-100 protein, myelin basic protein, and Leu-7.[59,60] Although most benign neurogenic tumors (schwannomas and neurofibromas) contain S-100, less than half of malignant peripheral nerve sheath tumors contain detectable S-100 protein.[61]

Normal and neoplastic vessels can be identified with endothelial markers such as factor VIII–related antigen and *Ulex europaeus* lectin. Antibodies to factor VIII–related antigen react only with endothelial cells and megakaryocytes and are more specific than *Ulex europaeus*. Other
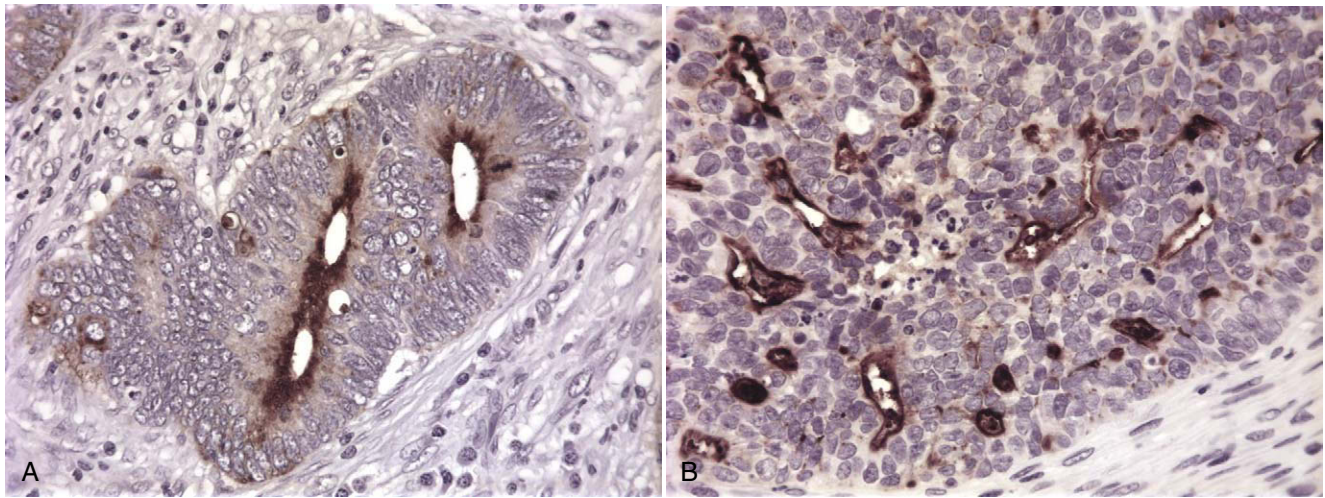
Figure 5-4 ■ **A,** Carcinoma of the colon showing CEA positivity. **B,** Ovarian carcinoma showing OC-125 positivity.

markers of endothelial cells include CD34 and CD31; these are more sensitive but less specific than factor VIII.

Other tumors to be considered in the differential diagnosis of anaplastic spindle cell tumors include liposarcomas, chondrosarcomas, osteogenic sarcomas, fibrosarcomas, and synovial sarcomas. Although certain variants of liposarcoma may be diagnosed by histologic criteria alone, the diagnosis of pleomorphic liposarcoma is aided by immunohistochemistry. Most liposarcomas react with vimentin and S-100 but are nonreactive for HMB-45, in contrast to most

First Choice Antibody Panel	Second Choice Antibody Panel				Additional Antibodies	Consistent with Tumor Type
CD3−; CD20−/+	**CD10**	**CD34**	**CD79a**		Precursor B-cell Neoplasms	
	−	+	−/+		CD22−/+; TdT+	Pro-B–ALL (B-1)
	+	+	+		CD22+/−; TdT+	Common ALL (B-II, early pre-B)
	+	−/+	+		CD22+/−; TdT−/+	Pre-B–ALL (B–III, late pre-B)
CD3− / CD20+	**CD5**	**CD10**	**CD23**	**CD43**	Mature B-cell Neoplasms	
	+	−	−	+	Cyclin D1+	Mantle cell lymphoma
	+	−	+	+	Cyclin D1−; CD38+/−	CLL/SLL
	−	+	+/−	−/+	BCL2+/−; BCL6+	Follicular cell lymphoma
	−	+	−	+/−	BCL2−; Ki67+; BCL6+	Burkitt's lymphoma
	−	−/+	−	−/+	CD25+; DBA44+; TRAP+;	Hairy cell leukemia
	−	−	−/+	−/+	Correlation with morphology is critical	MALT lymphoma
	−	−	−	−		B-cell prolymphocytic leukemia
						Lymphoplasmacytic lymphoma
					CD45+; BCL6+/−; EMA+/−; Bob1 or OCT2+	NLPHD
CD3− / CD20−	**CD30**	**CD38**	**CD45**			
	+/−	+	+		CD138+; K/L (Clonal)	Plasma cell neoplasm
	+	−	−		ALK+; BCL6+/−; EMA+/−; CD4+/−	Anaplastic large cell lymphoma
	+	−	R/− (focal)		CD15+; Fascin+; EBV+/−; Bob1/OCT2+	Classic Hodgkin's lymphoma
	−/+	−	+/−		S100+; CD1a+; CD68−/+	Langerhans cell histiocytosis
					CD68+; CD4+; CD43+	True histiocytic neoplasm
					CD21+; CD23+; CD35+; S100−/+; CD4−	Follicular dendritic cell neoplasm
					S100+; CD4+/−; CD68−/+	Dendritic cell neoplasm other than follicular
	−	−	+/−		MPO+; Lysozyme; CD34−/+: C-kit+; TdT −/+	Acute myeloid leukemia

Figure 5-5 ■ Diagnostic algorithm for hematolymphoid neoplasms.

TABLE 5–2

Antibodies Useful in Determining the Origin of Undifferentiated Tumors and Tumors of Uncertain Primary Site

Panel	Antibodies	Panel	Antibodies
Undifferentiated tumors	Pan-keratin CD 45 (CLA) S-100 Vimentin	Liver panel	Alpha fetoprotein α_1-Antitrypsin α_1-Antichymotrypsin Nonsquamous keratin Hepatitis B surface antigen Hepatitis B core antigen
Carcinoma panel	Pan-keratin CK5/6 AE1 AE3 CAM5.2 MAK6 Squamous keratin (HMW) Nonsquamous keratin (LMW)	Mesothelioma panel	Squamous keratin Nonsquamous keratin CEA-negative EP4 (epithelial antigen)-negative CD15 (Leu M1)-negative Epithelial membrane antigen B72.3 Secretory component Vimentin OC-125
Sarcoma panel	Pan-keratin Vimentin S-100 Desmin CD45 (CLA) Actin (muscle/HHF-35-MSA) Actin (smooth muscle specific—SMA) Myoglobin (skeletal muscle) LN-5 (histiocytes) Lysozyme (histiocytes) LN-6 (nonlymphoid vimentin) Factor VIII antigen (endothelial cells) CD34 (vascular antigen) CD31 (vascular antigen) Ulex (vascular antigen) O13 (Ewing's sarcoma/PNET)	Melanoma panel Central nervous system/neural panel	S-100 HMB-45 Melan A Vimentin Pan-keratin Glial fibrillary acidic protein (GFAP) Neurofilament S-100 NSE Vimentin Pan-keratin Synaptophysin
Breast panel	BRST-2 (GCDFP) Mammaglobin Cu-18 (breast-related antigen) Lactalbumin	Neuroendocrine panel	NSE Chromogranin Serotonin Neuron-endocrine Synaptophysin Nonsquamous keratin CD57 (Leu 7, HMK 1) Vasointestinal peptide
Prognosis (breast carcinoma)	Estrogen receptor (monoclonal) Progesterone receptor (monoclonal) Her-2/*neu* (c-*erb* B-2) p52 (luminal epithelial antigen) p53 Factor VIII antigen	Pituitary hormone panel	Adrenocorticotropic hormone (ACTH) Follicle-stimulating hormone (FSH) Growth hormone Luteinizing hormone Prolactin Thyroid-stimulating hormone (TSH) α-Subunit PIT-1
Lung panel	CK7 CK20 TTF-1	Pancreatic panel	Amylase Insulin
Prostate panel	Prostate-specific antigen (PSA) Prostatic acid phosphatase (PAP) Androgen receptor 34BE12 (SK)		Glucagon Gastrin Somatostatin CDX-2
Gastrointestinal panel	CEA COTA CDX-2 CK7 CK20	Urothelial panel	p63 Uroplakin
Kidney/bladder panel	Renal antigen CEA p53		
Ovary panel	COTA OC-125 (CA-125) Estrogen receptor Progesterone receptor		

CEA, carcinoembryonic antigen; CLA, common leukocyte antigen; COTA, colonic ovarian tumor antigen; HMW, high molecular weight; LMW, low molecular weight; NSE, neuron-specific enolase; PNET, primitive neuroectodermal tumor.

melanomas. The same pattern is seen for chondrosarcomas.[62] Osteosarcomas react with vimentin. Antibodies to the so-called osteonectin or osteosarcoma antigens also may assist in the diagnosis.[62] Fibrosarcomas are rare neoplasms that express only vimentin. Figure 5-6 provides the immu-

nostaining patterns characteristic of anaplastic spindle cell tumors.

Cytogenetic profiling of sarcomas of unknown origin is becoming common. Tumors with known abnormalities include endometrial sarcoma, myeloid sarcoma, synovial
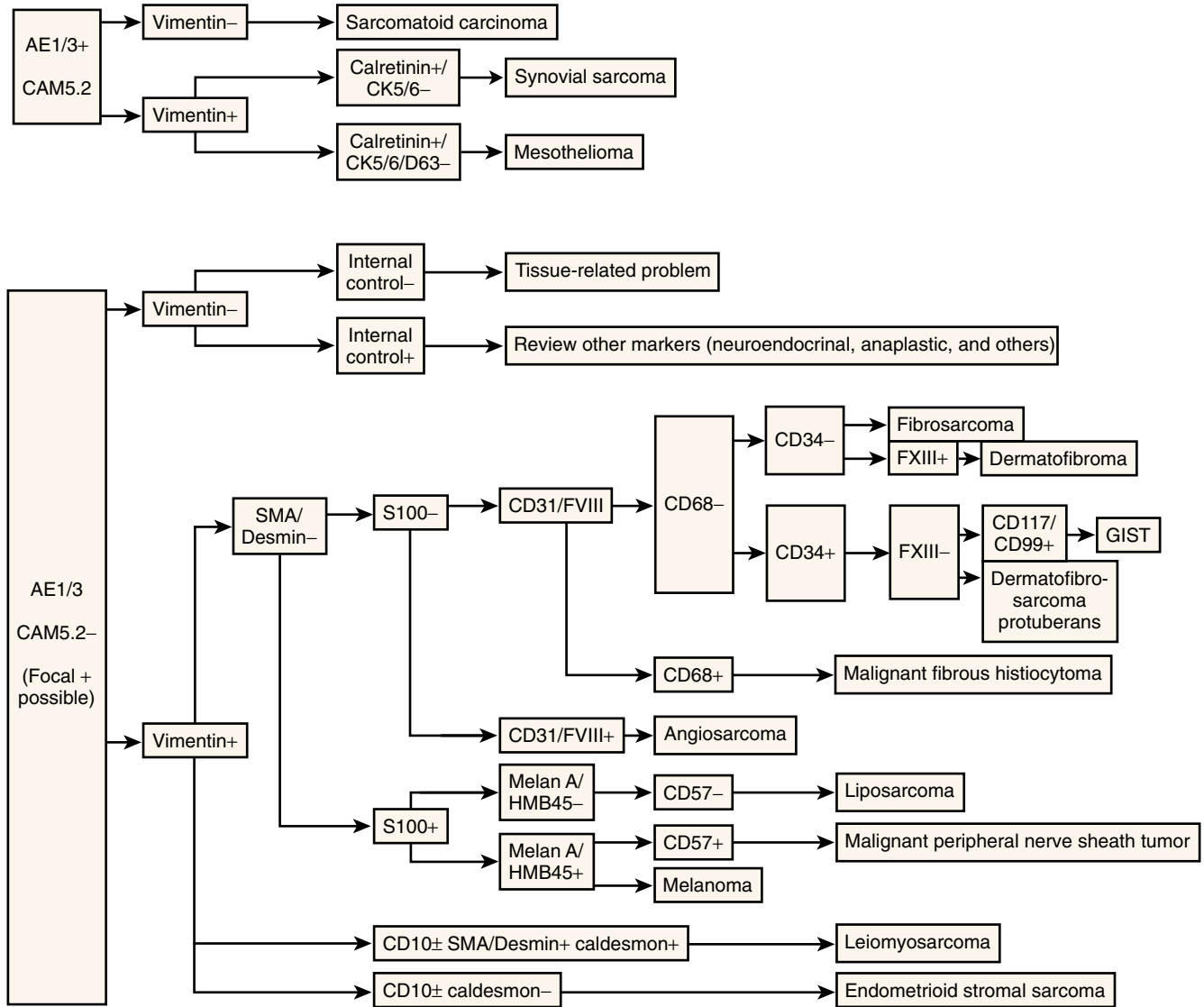
Figure 5-6 ■ Diagnostic algorithm for spindle cell tumors.

sarcoma, Ewing's sarcoma, and visceral clear cell sarcoma, among others.[63-70] This topic is discussed in more detail in Chapter 46.

NEURAL AND NEUROENDOCRINE TUMORS

Neural and neuroendocrine tumors may be classified as neural tumors, neuroepithelial tumors, or neural neoplasms of mesenchymal origin. These three categories are based on the different predominant intermediate filaments found in the cytoplasm of these lesions. Neural tumors usually express neurofilament, NSE, chromogranin, and synaptophysin.[71-73] Examples include neuroblastomas, paragangliomas, and pheochromocytomas. Neuroepithelial tumors coexpress keratin and neuroendocrine markers. These tumors include carcinoids, Merkel cell carcinomas, and small cell carcinomas. Neural neoplasms of mesenchymal origin, which consist of primitive neuroectodermal tumors, Ewing's sarcomas, and medulloblastomas, are positive for vimentin and may express NSE and Leu-7.[74] A good marker for small cell tumors such as Ewing's sarcoma, primitive neuroectodermal tumor, and peripheral neuroepithelial tumor is O13, which identifies the CD99 (p30/32, mic2, HBA71) antigen.[75]

NSE, although a sensitive marker for neuroendocrine tumors, lacks specificity and can be seen in a wide variety of tumor types. Chromogranin and synaptophysin are more specific than NSE but lack sensitivity. Chromogranin tends to be positive in better-differentiated neuroendocrine tumors but is less often positive in the more poorly differentiated tumors, such as small cell carcinoma.[72,76-78] Table 5-3 provides immunostaining patterns of endocrine tumors, and Figure 5-7 illustrates an algorithm used to distinguish small round cell tumors.

GFAP-POSITIVE TUMORS

GFAP is expressed by glial cells and is seen in astrocytomas, ependymomas, medulloblastomas, some oligodendrogliomas, and choroid plexus tumors.[79,80] It has also been reported in a few extracerebral tumors, including pleomorphic adenomas of the salivary gland,[81] neurofibromas, and schwannomas.[82] Intracerebral tumors in which GFAP is not expected to be positive include meningiomas, lymphomas, and

TABLE 5–3
Dominant Immunophenotypes of Endocrine Neoplasms

CAM 5.2	CEA	Chromogranin	Serotonin	Synaptophysin	TTF-1	Vimentin	Additional Markers	Tumor Type
+	–	+/–	–/+	+	–	–	Calcitonin –/+; Somatostatin –/+ Gastrin –/+	Carcinoid
+	–/+	–/+	–	+	–/+	–/+		Neuroendocrine
+	–	–/+	–	+	–	–	Insulin +	Insulinoma
+	–	+	–	+	–	–	Glucagon +	Glucagonoma
+	–	–/+	–	+	–	–	Somatostatin +	Somatostatinoma
+	–	+	–	+	–	–	Gastrin +	Gastrinoma
+	+	+	–	+	+	+	Calcitonin +	Medullary, thyroid
+	–	+	–	+	–	–	PTH +	Parathyroid
+	–	+/–	–	+	–	–	CK20 +	Merkel cell
–/+	–	+/–	–	+	–	+	S-100 +/–	Paraganglioma, pheochromocytoma
–/+	–	–	–	+	–	+	Melan A +; inhibin +	Adrenal cortical

CEA, carcinoembryonic antigen; PTH, parathyroid hormone.

metastatic carcinomas. GFAP is discussed in more detail in Chapter 51.

Molecular and Genetic Markers of Tumor Origin

Cancer results from defects in gene structure, expression, or both. Alterations in chromosome and DNA structure—including cytogenetic changes, point mutations, deletions, amplifications, translocations, and DNA methylation—are being identified at an increasing rate. Characterization of these defects is becoming an important component of tumor evaluation, particularly in terms of prognosis and response to treatment. In addition, genetic defects highly characteristic of specific tumor types are being identified. The identification of DNA alterations is also becoming an increasingly important component in the evaluation of tumors of uncertain primary site. Molecular and genetic evaluation is particularly useful in the evaluation of hematopoietic and soft tissue tumors.[83,84] Recently, a highly sophisticated evaluation of hundreds of genes using so-called microarray chip technology was used to identify gene expression differences in several tumor types[84-86] and will no doubt become an important component of tumor evaluation in the future.
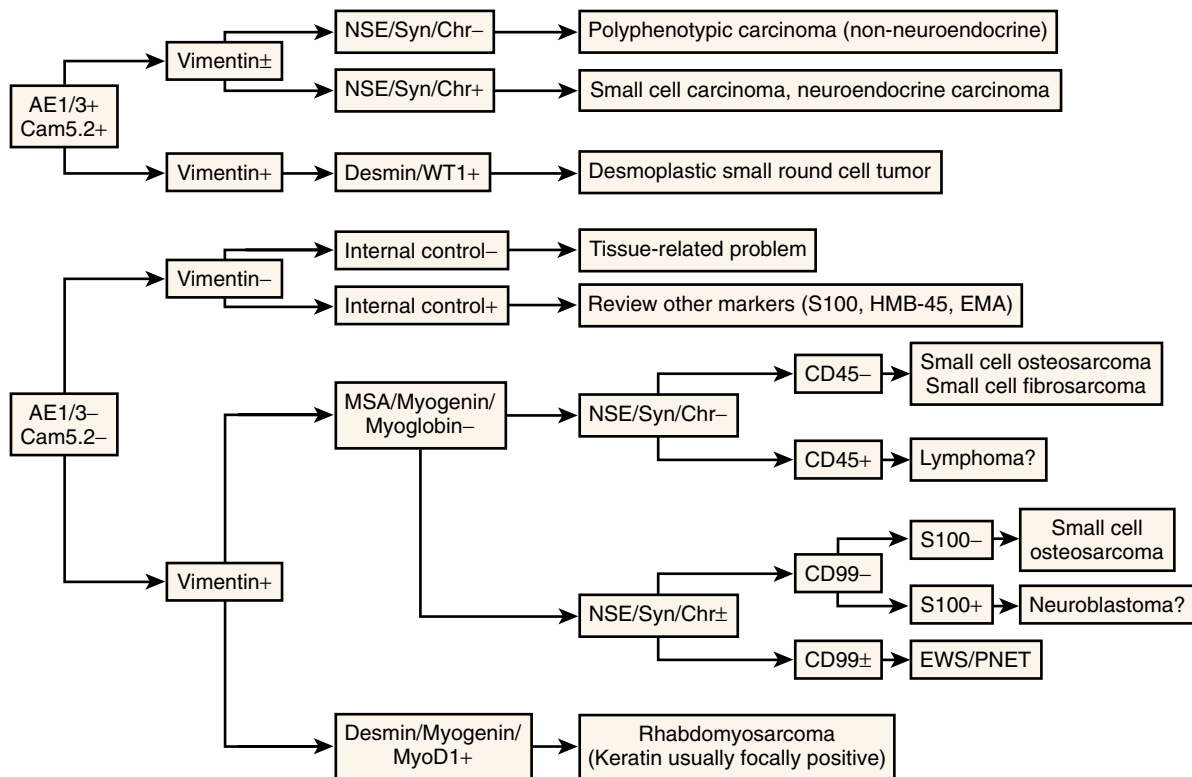

Figure 5-7 ■ Diagnostic algorithm for small round cell tumors.

Transcription factors are proteins involved in the regulation of gene expression that bind to the promoter elements upstream of genes and either facilitate or inhibit transcription. They may be tissue specific, or they may be present in more than one tissue type. Even the so-called tissue-specific transcription factors are, however, usually not restricted to a single tumor type. Examples include thyroid transcription factor-1 (TTF-1), which is found in the thyroid and lung, and the pituitary transcription factor PIT-1, which is found in the placenta as well as the pituitary gland. Nevertheless, they can be useful in determining the primary site of tumors of unknown origin (see Table 5-2).

TTF-1

TTF-1 belongs to a family of homeodomain transcription factors and plays a role in regulating genes expressed within the thyroid, lung, and diencephalons. TTF-1 is considered a reliable marker for distinguishing primary tumors of the lung, including adenocarcinoma (75%), non–small cell carcinoma (63%), neuroendocrine and small cell carcinoma (>90%), and squamous cell carcinoma (10%),[87-90] and thyroid from other tumor types. However, primary adenocarcinoma of the colon is positive in some cases.[36,91,92] The pattern of reactivity is nuclear. Hepatocytes and hepatocellular carcinoma reportedly show cytoplasmic positivity.[93]

CDX-2

CDX-2 is a homeobox gene that encodes a transcription factor involved in the development of intestinal epithelium. It is expressed in normal colonic epithelium and in most colorectal adenocarcinomas and is a useful marker to identify colorectal metastases.[94-96] CDX-2 is also useful in extramammary Paget's disease; the endodermal subtype is positive for CDX-2, whereas the cutaneous subtype is negative.[97]

ATF3

Activating transcription factor 3 (ATF3) is a member of the basic leucine zipper/cyclic adenosine monophosphate–responsive element binding protein family of transcription factors. Studies indicate that ATF3 is an androgen-regulated gene that stimulates cell proliferation. ATF3 protein detected by immunohistochemistry is present in prostate tumors; it has increased expression in high-Gleason-score disease and in tumors refractory to therapy.[98]

Prognostic Markers in Cancer

One of the outstanding achievements of modern medicine is the ability to predict the behavior of tumors based on specific clinical and pathologic criteria. Tumor stage and grade provide only general estimates of outcome for a particular patient, however. Current clinical and pathologic staging parameters cannot identify those patients who are destined to experience relapse or those whose disease will be cured by local therapy alone. These considerations have obvious consequences for the patient and enormous economic implications.

Efforts are under way to identify enzymes, oncogenes, or tumor suppressor genes whose presence or absence may predict more accurately the biologic behavior of tumors.

Such studies represent a fundamental shift in the means by which tumor behavior is defined—a change from an outcome-based empirical analysis (i.e., prediction of what a tumor will do based on what it has done) to one focused on tumor biology (i.e., predictions of behavior based on specific genetic alterations). The immunohistochemical analysis of tumors is also undergoing a profound shift in emphasis. Although initial studies focused on defining tumor histogenesis, the goal of much current research is to reveal the biologic potential of tumors, providing a more scientific basis for patient management.

The use of advanced technologies to define tumor prognosis is described in detail throughout this book. Presented here are some general principles that pertain to the prognostic evaluation of all tumors.

Occult Metastases

The most important factor affecting the outcome of patients with invasive cancers is whether the tumor has spread either regionally (to regional lymph nodes) or systemically. A proportion of patients with no evidence of systemic dissemination as evaluated by routine methods (careful pathologic, clinical, biochemical, and radiologic evaluation) develop recurrent disease. In addition, the success of adjuvant therapy is assumed to stem from its ability to eradicate occult metastases before they become clinically evident.[99] Immunohistochemistry is commonly used to identify occult metastatic cancer cells in the bone marrow, peripheral blood, and lymph nodes of patients with cancer. Although many of the initial studies focused on breast cancer,[100-102] tumors from other organs such as the stomach,[103,104] colon,[105,106] prostate,[107,108] lung,[109,110] nervous system,[111] and skin[112] have been investigated. Immunohistochemical methods are based on the ability of monoclonal antibodies to distinguish between cells of different histogenesis (e.g., epithelial cancer cells versus the hematopoietic and stromal cells of the bone marrow and lymph nodes). The results indicate that it is possible to identify occult metastatic cancer cells in lymph nodes and bone marrow before their detection by other methods and that the presence of these cells may be an important risk factor for disease recurrence (Fig. 5-8).

The most widely used monoclonal antibodies to detect occult metastatic carcinoma cells are directed toward epithelium-specific antigens. These antibodies do not react with normal hematopoietic or stromal cells present in the bone marrow or lymph nodes. None of the antibodies used in any study is specific for cancer; all react with normal and malignant epithelial cells. They are useful because they can identify an extrinsic population of epithelial cells in bone marrow or lymph nodes, where there are normally no epithelial elements. The reported sensitivity of immunohistochemistry ranges from the detection of 1 epithelial cell in 10,000 to 2 to 5 epithelial cells in 1 million hematopoietic cells.[100,113]

A potentially more sensitive approach for the detection of occult metastasis is the reverse transcriptase polymerase chain reaction (RT-PCR) technique, which has been applied to several malignancies using a variety of marker transcripts as targets. Since the first study by Smith and colleagues in 1991,[114] many authors have reported molecular diagnoses
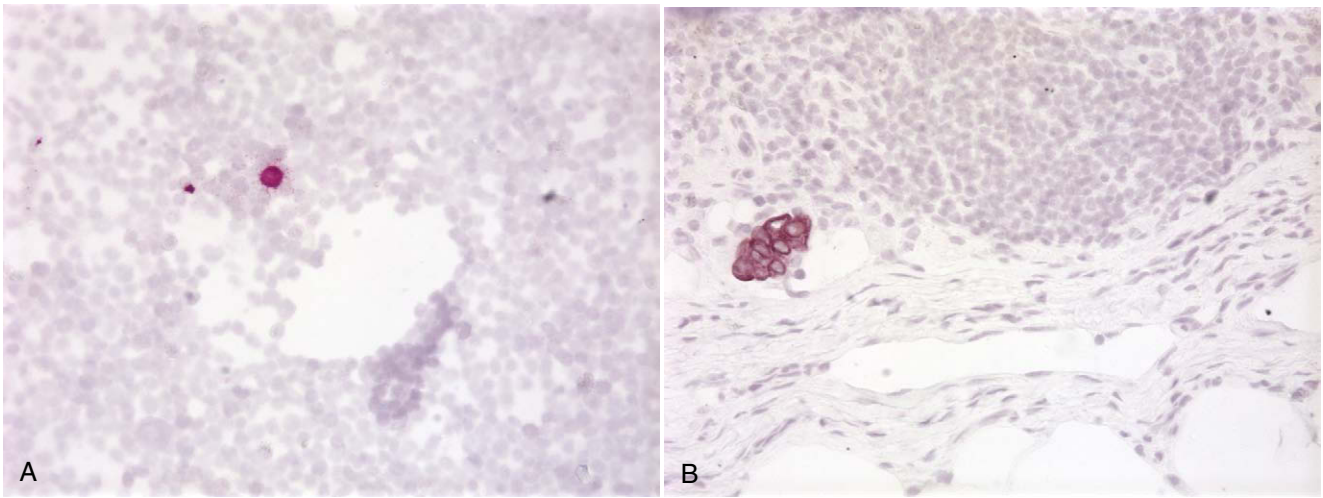
Figure 5–8 ■ **A,** Bone marrow aspirate with a single keratin-positive cell from a patient with lung cancer. **B,** Section of lymph node shows a small focus of early metastatic breast cancer.

in the lymph nodes, blood, and bone marrow in cancer patients.[42,107,115-119] Application of RT-PCR in regional and sentinel lymph nodes has been described for a number of cancers, including melanoma, colorectal cancer, and cancers of the prostate, breast, and lung.[118,120-124] Many of these compare immunocytochemical-based detection with RT-PCR for sensitivity and conclude that RT-PCR may achieve enhanced detection, provided the target markers are sufficiently specific. Various formats of RT-PCR assays[125-127] have also been used to detect disseminated tumor cells in the bone marrow of patients with cancers of the breast, colon, and lung, among others. With the exception of some organ-specific markers such as maspin or mammaglobin for breast cancer[116,128] or uroplakins for urothelial tumors,[129] most of the molecular targets used in these RT-PCR assays lack the requisite specificity owing to illegitimate expression in nontarget hematopoietic cells.[130-132] Unlike immunohisto-chemistry, morphologic confirmation of the cells in question to verify tumor origin is not possible with RT-PCR. RT-PCR has also been used to enhance the sensitivity of the detection of tumor cells in the peripheral blood in a variety of cancers, including prostate, breast, gastrointestinal tract, colorectal, and head and neck cancers and melanoma.[117,133-135] Concerns about nonspecificity owing to illegitimate transcription of target genes in the nontarget hematopoietic cells also apply to the blood, which has hampered the use of these assays in routine clinical diagnosis.

Bone Marrow and Peripheral Blood

In breast cancer, the bone marrow is the single most common site of metastasis, and 80% of patients with recurrent tumors develop bone marrow metastases at some point during the evolution of their disease.[136] Immunohistochemistry can show the presence of occult metastases in the bone marrow in approximately 10% to 45% of patients with low-stage disease.[102,127,137-143] Several studies have addressed the clinical significance of these early metastatic cells in the bone marrow, including a pooled analysis of more than 4700 patients.[143] They have found that the presence of such cells is an independent prognostic indicator of disease-free survival and overall survival.[144-146] Occult metastases in the

bone marrow are also prognostically important in other malignancies, including primary non–small cell lung cancer,[109,110,147,148] esophageal and gastric cancers,[103] colorectal cancer,[149] and neuroblastoma.[111] The finding of positive cells in the bone marrow of patients with colorectal cancer—a tumor that rarely shows overt metastasis to the bone—indicates that this may be a general indicator of tumor dissemination. In addition, the prognostic significance of occult metastatic cells in the blood is under investigation.

Peripheral blood has the advantage of being easier to access than bone marrow. However, detection rates are considerably lower than with bone marrow, a fact that has hampered studies to date.

In the detection of tumor cells in the bone marrow and blood, epithelial cell adhesion molecule in conjunction with immunomagnetic enrichment has been used to detect circulating tumor cells in breast cancer patients. Circulating tumor cells in the blood have been used to monitor response to therapy in patients with metastatic cancer.[150,151,151a] The presence of epithelial adhesion molecule–positive cells before and after the initiation of therapy was found to be an independent prognostic factor. Other markers used in patients with breast cancer include mammaglobin, epidermal growth factor receptor, and carcinoembryonic antigen.[116,152,153] Although none of these markers is entirely specific for the detection of metastatic breast cancer, and although the sensitivity of peripheral blood is less than that of bone marrow, there is growing evidence that the detection of occult metastatic cells in the peripheral blood has a negative impact on prognosis.[154] A recent study found that the presence of five or more tumor cells in the peripheral blood from patients with breast cancer examined upon the initiation of therapy was important in predicting outcome.[151]

Peripheral blood from patients with colorectal,[106] stomach,[104] prostate,[155] and skin [155,156] cancer have also been studied. Markers that have been studied in colorectal cancer include cytokeratins,[106] carcinoembryonic antigen,[157,158] apolipoprotein,[159] and CD44v6.[160] PSA messenger RNA (mRNA) is the most commonly used marker in patients with prostate cancer.[155,161] Tyrosinase mRNA is the marker

of choice for detecting circulating tumor cells in patients with melanoma.[161,162]

Lymph Nodes

Studies undertaken to detect occult lymph node metastases by routine histologic methods have generally been performed by cutting serial sections from all paraffin blocks containing lymph nodes, followed by routine staining and microscopic review.[163] Several studies simply reviewed the original histologic slides. Newer studies involve cytokeratin immunohistochemistry on one or more lymph node sections. PSA immunohistochemistry has also been used to confirm the prostatic origin of cytokeratin-positive cells in the lymph nodes of patients with prostate cancer.[108] All these studies have shown that deposits of tumor can be detected using these methods. In previously determined node-negative cases of breast cancer, 7% to 33% convert to node-positive status after review. Neville and colleagues[164] found the mean conversion rate to be approximately 13%. Although virtually all studies have shown that lymph node metastases can be overlooked, there has been surprising disagreement about the prognostic importance of these occult tumor deposits.[165-167] However, it is now widely accepted that the detection of occult lymph node metastases is an important predictor of outcome in patients with histologically node-negative cancer.[168-170] In a key study (Ludwig Trial V), occult breast cancer metastases were detected by immunohistochemistry in 20% of patients and were associated with significantly poor disease-free and overall survival in postmenopausal patients but not in premenopausal patients.[168] Additional studies in patients with breast cancer found occult lymph node metastases to be predictive of a poorer outcome.[169,170] Studies in patients with lung,[171,172] prostate,[107,108] and colorectal[173,174] cancer suggest that occult metastases in the lymph nodes in these patients may also predict a worse prognosis.

The finding that occult bone marrow and lymph node metastases are prognostically important has motivated several major clinical trials, notably by the American College of Surgeons Oncology Group, in breast cancer (Z0010) and lung cancer (Z0040). The advent of the use of sentinel lymph node biopsy in tumor surgery (for breast cancer and melanoma) has caused physicians to examine these lymph nodes by more sensitive techniques, owing to the limited material available for histologic review.[175] It is likely that the detection of occult metastases will soon be the general standard of care; this is true at many institutions that treat large numbers of patients with cancer.

Oncogenes, Growth Factors, and Receptors

HER-2/NEU

Her-2/*neu* (or c-*erb* B-2) is a proto-oncogene. The gene encodes for a protein (185 kD) that shows homology with epidermal growth factor and displays tyrosine activity. Amplification of the gene coding for Her-2/*neu* has been described in breast, ovarian, prostate, gastric, salivary gland, lung, colon, and squamous cell carcinoma.[176-186] When overexpressed, the protein accumulates at the cell membrane and is seen as a crisp membrane stain; a cytoplasmic staining pattern is not associated with protein or gene overexpression.[176,183]

Although Her-2/*neu* overexpression and amplification have been described in several tumor systems, it has been studied most extensively in the breast. Her-2/*neu* overexpression occurs in 10% to 34% of primary breast carcinomas[187] and is restricted to cancer cells. There is an inverse association between Her-2/*neu* amplification and the expression of estrogen and progesterone receptors. Her-2/*neu* overexpression is also associated with high-grade tumors[176,188] and is considered an adverse prognostic indictor in patients with breast cancer.[187] The presence of Her-2/*neu* overexpression is associated with resistance to tamoxifen therapy[189-191] and to CMF (cyclophosphamide, methotrexate, 5-fluorouracil) adjuvant chemotherapy but is associated with an increased response to regimens that use high-dose doxorubicin.[192-195] Recent studies have linked amplification of the Her-2/*neu* and topoisomerase IIα genes to the effects of anthracyclines. Preliminary data suggest that coamplification of these two genes may identify a subgroup of high-risk breast cancer patients who might benefit from individually tailored and dose-escalated adjuvant anthracyclines.[196,197] Her-2/*neu* can also be assessed through amplification of the gene by fluorescence in situ hybridization.

EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor (EGFR) belongs to a family of growth factor receptors involved in normal growth. The gene is located on chromosome 7p12. It is the receptor for epidermal growth factor and is a member of the receptor tyrosine kinase family. It is closely related to Her-2/*neu*, Her-3, and Her-4. EGFR is known to be involved in carcinogenic processes such as cell proliferation, apoptosis, angiogenesis, cell motility, and metastasis. The expression of EGFR has been examined in a wide variety of tissues, and in many cases, increased expression of EGFR is predictive of tumor progression (e.g., cancer of the breast, esophagus, adrenals, lung, bladder, thyroid, and gastrointestinal tract and glioblastoma multiforme).[198-205] In addition to immunohistochemical methods, fluorescence in situ hybridization has been used successfully to identify EGFR mutation or deletions on formalin-fixed, paraffin-embedded tissue.[206]

EGFR is also showing promise as a therapeutic target. Studies are under way in lung and colorectal cancer to determine the usefulness of targeting EGFR for anticancer therapy.[207,208]

Tumor Suppressor Genes and Gene Products

The primary characteristics of tumor suppressor genes are that they encode normal cellular products involved in growth control, and both alleles must be inactivated for loss of function (i.e., loss of tumor suppression) to occur. The most well known are retinoblastoma (Rb) protein, p53, p27, p21, and p16. The two best characterized are the *Rb* and *p53* genes. Both are thought to be involved in growth control through the regulation of transcription.

RETINOBLASTOMA GENE

The *Rb* gene is located on chromosome 13q14 and is dysfunctional in a number of types of cancer. Its normal func-

tion is to prevent the replication of damaged DNA; it does so by preventing cell replication by binding and inhibiting the transcription factor E2F.[209,210] The retinoblastoma protein (pRb) is activated when it is dephosphorylated and inactivated when it is phosphorylated. Alterations in this gene have been described in many human tumors, including retinoblastoma, osteosarcoma, other sarcomas, leukemias, lymphomas, and certain carcinomas, including breast, lung, prostate, bladder, kidney, and testicular carcinoma.[29,211,212] Gene alterations are associated with advanced tumor grade and stage in a variety of tumors.[211,213] Alterations in the *Rb* gene correlate with loss of expression of pRb as determined by immunohistochemistry.[214] Assessment of *Rb* gene loss by immunohistochemistry is based on the loss of detectable nuclear staining for pRb. There is growing evidence that gene alterations may identify tumors that have a higher risk of developing metastases.[215] Loss of heterozygosity, mutations, or deletions of the *Rb* gene usually result in the loss of pRb expression, which has been regarded as an indicator of loss of pRb function in human tumors. In addition to loss of pRb expression, aberrantly high pRb expression indicates a loss of pRb function in bladder tumors compared with moderate pRb expression.[210,215] It has been shown that tumors with pRb overexpression demonstrate pRb hyperphosphorylation, mediated in part by the loss of p16 expression or overexpression of cyclin D1.[210]

P53

The *p53* gene is located on chromosome 17p13.1. The p53 protein is expressed by all normal cells, but the half-life of the normal protein is so short (6 to 30 minutes) that it does not accumulate in levels high enough to be detected by standard immunohistochemical techniques. Mutant p53 protein, by contrast, has an extended half-life, accumulates, and is readily detectable in the cell nucleus; mutation is indicated by positive staining. Alterations of the *p53* gene are extremely common in human cancer and have been described in bladder, colon, lung, breast, and other carcinomas; astrocytomas; leukemias; sarcomas; and mesotheliomas.[1,29,213,216,217] Because of the importance of *p53* alterations in human cancer and the ease of detecting *p53* mutations by molecular or immunohistochemical methods, *p53* alterations have been the focus of intense examination. As with *Rb* alterations, *p53* alterations are associated with tumors of high histologic grade and a high proliferative index. There is growing evidence that, at least for some types of tumors, *p53* alterations identify patients with shorter disease-free intervals and poorer overall survival.[217,218]

CYCLIN-DEPENDENT KINASE INHIBITORS

The cyclin-dependent kinase inhibitors are a family of cell cycle regulators. Their primary function seems to be the formation of stable complexes with cyclin-dependent kinase proteins and the subsequent inhibition of the cell cycle. These complexes inactivate the catalytically operative units. Among the most well known and clinically relevant are p21, p27, and p16.

p21

A member of the WAF/CIP/KIP family of cyclin-dependent kinase inhibitors, p21 is probably the best characterized. It acts as a regulator of epithelial carcinogenesis and differentiation and is thought to play an important role in tumor suppression by regulating cell cycle progression, DNA replication, and DNA repair.[29,219] The protein expression of p21 has been studied in a variety of tumor types, including breast,[220] gastric,[221] ovary,[222] colorectal,[223] and bladder[213,224] carcinomas. The alteration of protein expression assessed by immunohistochemical methods has been associated with higher tumor grade and worse prognosis in patients with bladder cancer.[123,213]

p27

The p27 inhibitor is involved in the regulation of the cell cycle at the G_1-S transition, ultimately through the inhibition of pRb phosphorylation.[225] Mutations in the human *p27* gene appear to be rare.[226] Loss of p27 expression is associated with colon, breast, prostate, and gastric cancer progression.[227-231]

p16

Also known as p16^{INK4} and CDKN2A, p16 is a tumor suppressor protein encoded on the INK4a/ARF locus of chromosome 9p21, which is one of the most frequent sites of genetic loss in human cancer.[232] Numerous studies have found abnormal p16 protein in a variety of tumor types, including melanomas; gliomas; esophageal, pancreatic, lung, and bladder carcinomas; and certain types of lymphomas.[232-240] In addition, p16 is known to regulate *Rb*, and immunohistochemical expression of pRb and p16 is inversely correlated in a variety of tumors.[241,242]

COMBINED EFFECTS OF P53, P21, AND PRB

It is known that, individually, p53, p21, and pRb are independent predictors of time to recurrence and overall survival in patients with bladder cancer.[215,217,224] Efforts have therefore been made to examine these determinants in combination.[123,213] In one study, patients were analyzed according to whether none, one, two, or all three markers were positive. The 5-year survival rates were 70%, 58%, 33%, and 8%, respectively. These data suggest that alterations in p53, p21, and pRb act in cooperative or synergistic ways to promote bladder cancer progression.

TUMOR ONCOGENE CYCLIN D1

Cyclin D1 plays a key role in the regulation of the G_1-S transition phase of the cell cycle. It has been linked to a number of different cancers, including colorectal, esophageal, gastric, lung, head and neck, and pancreatic cancer.[243]

Predicting Response to Therapy

Although a major purpose of the molecular assessment of cancer is to better understand the risk for disease progression, advanced technologies are also being used to understand the specific patterns of response and resistance to therapeutic regimens. The traditional means of determining appropriate systemic treatment generally involved histogenic classification. It has long been recognized, however, that response to hormonal therapy can be predicted specifically by molecular determinants (e.g., the expression of estrogen and progesterone receptors in breast and other cancers of reproductive organs).[244]

Tumors arising from the breast, prostate, endometrium, and ovary are known to be regulated by steroid sex hormones (estrogens, androgens). It was discovered that removing the source of hormones that control tumor growth (by oophorectomy, orchiectomy, or chemical methods) sometimes resulted in dramatic tumor remission.[244] Growth regulation was found to be associated with the amount of specific hormone receptors: Tumors that expressed high levels of these receptors tended to respond well to hormone ablation, whereas those with few or no receptors tended not to respond to this type of therapy. Accurate methods for determining the presence or absence of hormone receptors are essential for determining the best method of treatment.

The availability of monoclonal antibodies to estrogen, progesterone, and androgen receptors has made immunohistochemical detection of hormone receptor status the current method of choice. These immunohistochemical methods can be performed on formalin-fixed, paraffin-embedded tissue and on cytology specimens. Immunohistochemical antireceptor assays allow one to predict breast cancer's response to hormonal treatment.[245,246] Tumors that do not express estrogen or progesterone receptors have a low probability of responding to hormonal manipulation, whereas estrogen receptor– and progesterone receptor–positive tumors have a high probability of responding to such treatment. Many practitioners believe that the only relevant result for hormone receptors in breast cancer is "positive" or "negative." However, some investigators have shown that the level of hormone receptor is important as well.[8,9] Although a proportion of patients with low levels of hormone receptor will respond to hormone therapy, most benefit from the addition of systemic cytotoxic chemotherapy. In contrast, in patients with high levels of hormone receptor, the addition of cytotoxic chemotherapy has a deleterious effect on outcome.[8,9]

Recently, attention has focused on expression of the estrogen receptor subtypes α and β and on various isoforms of the β subtype. It has been found that estrogen receptor α–negative tumors express significant levels of estrogen receptor $\beta 1$ and $\beta 5$ and that their expression levels are no different from levels in estrogen receptor α–positive tumors.[246] Therefore, these two estrogen receptor isoforms may be potential molecular targets for designing chemopreventive drugs to treat estrogen receptor α–negative breast cancers.

P-glycoprotein is a transmembrane protein of 170 kD that has been associated with intrinsic and acquired resistance to certain chemotherapeutic agents, particularly anthracyclines and vinca alkaloids. P-glycoprotein also may play a role in tumor progression and has been associated with blood vessel invasion and lymph node metastases.[2] Some tumors inherently express P-glycoprotein, whereas other tumors acquire expression only after exposure to certain chemotherapeutic agents.[2] Overexpression is associated with failure of chemotherapy.[2,247]

Other predictors of response to specific forms of chemotherapy are being explored. The prevailing view has been that p53 alterations should result in a chemoresistant phenotype. This view is based on a body of evidence showing that wild-type p53 is required for entrance into the apoptotic pathway at the G_1- to S-phase transition.[248,249] Because chemotherapy works through the induction of apoptosis, p53 alterations may result in resistance to such agents. We are conducting a clinical trial concerning the role of p53 in predicting progression and response in patients with bladder cancer.[250] It is also possible that p53 may promote chemoresistance by other mechanisms, such as through induction of the multidrug resistance (MDR-1) gene.[249,251] In tumors in which p53 alterations confer increased (selective) chemosensitivity, combining agents that have different actions (e.g., DNA damage versus inhibition of the G_2M checkpoint) may have synergistic effects on tumor cell killing, a finding that has important implications in the design of new combination chemotherapy regimens.[252]

The expression of thymidylate synthase in colorectal tumors predicts resistance to the most common type of systemic chemotherapy used in that disease, 5-fluorouracil.[253,254] As mentioned earlier, Her-2/neu overexpression in breast cancer predicts resistance to hormone therapy in estrogen receptor–positive tumors[189,190,255] and resistance to some types of chemotherapy, but increased sensitivity to doxorubicin-based regimens.[193,194] Her-2/neu and EGFR are specific targets of antibody-directed therapy. In the case of Her 2/neu, it seems that only those tumors that overexpress the target are likely to respond to such therapies.[256-258] In the case of colorectal cancer, therapy directed against EGFR appears to work only in tumors with wild-type KRAS; tumors with mutant KRAS do not respond to EGFR therapy.[258a] Similar findings are seen in lung cancer.[258b]

The ability to predict the specific response of individual tumors to chemotherapeutic agents can have a profound effect on treatment decisions for patients with cancer. It is not difficult to envision the day when drug selection is based on the resistance patterns of individual tumors to specific agents. Treatment decisions will become less organ based and will better reflect the biology of the tumors.

Infections

Traditionally, the stains available to surgical pathologists to identify infectious organisms in tissue sections consisted of Gram stain, variations of the acid-fast stain, periodic acid–Schiff, and silver stains. There is now a wide range of immu-
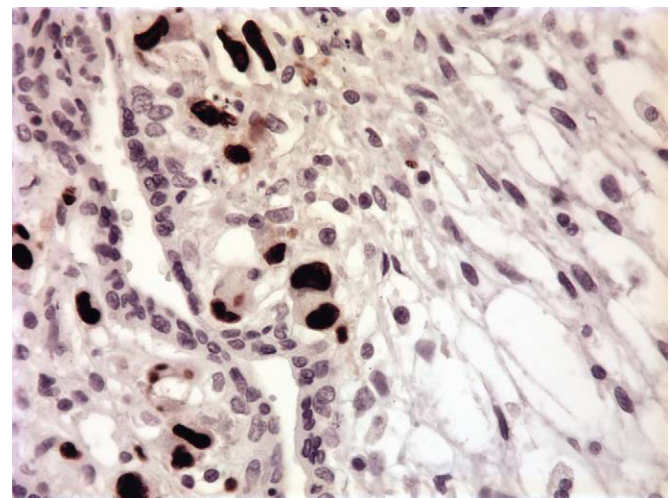

Figure 5–9 ■ Placenta showing infection with cytomegalovirus by immunohistochemistry.

TABLE 5–4
Infectious Agents for Which Antibodies Are Available for Use on Paraffin Sections

Adenovirus	Influenza
Aspergillus	*Klebsiella*
Baboon endogenous virus	*Legionella*
Blastomyces	*Leishmania*
Borrelia burgdorferi	Lymphocytic choriomeningitis
Buffalo pox virus	virus
Campylobacter coli	Measles antigen
Campylobacter jejuni	Moloney virus
Campylobacter spp.	Mouse mammary tumor virus
Candida	antigen
Chylamydia	Mycobacteria
Coccidioides	*Mycoplasma*
Coronavirus	*Parainfluenza*
Cryptococccus neoformans	*Pneumoncystis carinii*
Cryptosporidium	Polio
Cytomegalovirus	Polyomavirus
Distemper virus	*Pseudomonas aeruginosa*
Entamoeba histolytica	Rabies virus
Epstein-Barr virus	Respiratory syncytial virus
Escherichia coli	Rotavirus
Fasciola hepatica	Rubella
Friend's virus	*Salmonella*
Giardia	*Shigella*
Helicobacter pylori	Shope's fibroma virus
Hepatitis A virus	*Staphylococcus*
Hepatitis B core antigen	*Streptococcus*
Hepatitis B surface antigen	SV40 virus
Hepatitis C virus	*Toxoplasma gondii*
Herpes simplex virus 1 and 2	*Treponema pallidum*
Histoplasma capsulatum	*Trichophyton*
Human immunodeficiency virus	Varicella-zoster virus
(HIV-1)	*Yersinia*
Human papillomavirus	

nohistochemical or in situ hybridization techniques available for the detection of specific types of organisms within fixed paraffin sections. Although culturing techniques remain the most important method for diagnosing most infections, immunohistochemical methods are as effective as, or even superior to, culture and routine H&E methods for the detection of certain infectious organisms such as cytomegalovirus (Fig. 5-9), mycobacteria, *Toxoplasma*, *Pneumocystis carinii*, *Histoplasma capsulatum*, *Helicobacter pylori,* and human papillomavirus.[259-269] Table 5-4 summarizes some of the infectious agents that can be identified by immunohistochemistry.

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