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Advanced diagnostic methods in oral and maxillofacial pathology. Part II: Immunohistochemical and immunofluorescent methods

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The practice of pathology is currently undergoing significant change, in large part due to advances in the analysis of DNA, RNA, and proteins in tissues. These advances have permitted improved biologic insights into many developmental, inflammatory, metabolic, infectious, and neoplastic diseases. Moreover, molecular analysis has also led to improvements in the accuracy of disease diagnosis and classification. It is likely that, in the future, these methods will increasingly enter into the day-to-day diagnosis and management of patients. The pathologist will continue to play a fundamental role in diagnosis and will likely be in a pivotal position to guide the implementation and interpretation of these tests as they move from the research laboratory into diagnostic pathology. The purpose of this 2-part series is to provide an overview of the principles and applications of current molecular biologic and immunologic tests. In Part I, the biologic fundamentals of DNA, RNA, and proteins and methods that are currently available or likely to become available to the pathologist in the next several years for their isolation and analysis in tissue biopsies were discussed. In Part II, advances in immunohistochemistry and immunofluorescence methods and their application to modern diagnostic pathology are reviewed. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;93:56-74)**

The analysis of DNA, RNA, and proteins, obtained from diagnostic specimens, is currently revolutionizing the practice of surgical pathology and heralds a new era of diagnostic and prognostic tests that will greatly influence our day-to-day clinical decision making. The diagnosis of cancer and many other diseases is fundamentally based on the microscopic study of cells and tissues and remains the standard by which all other diagnostic tests are measured. Nevertheless, the era when the pathologist relies entirely on the examination of tissue sections stained by histochemical methods is gradually being replaced by a time when advanced immunologic and molecular techniques augment disease diagnosis and classification. Part I of this series provided an overview of many of the most important molecular tools emerging in diagnostic pathology. Part II will review the principles and application of the 2 most important protein-based analyses in diagnostic pathology: immunohistochemistry and direct immunofluorescence.

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IMMUNOHISTOCHEMICAL METHODS IN DIAGNOSTIC PATHOLOGY

Fundamentals of immunohistochemistry

The application of immunologic research methods to histopathology has resulted in marked improvement in the microscopic diagnosis of neoplasms. Although histologic analysis of hematoxylin and eosin (H & E)–stained tissue sections remains at the core of the practice of head and neck surgical pathology, immunohistochemistry has become a powerful tool in the armamentarium of the pathologist. It affords a significant advantage in the diagnosis of difficult and equivocal tumors, where it augments traditional tissue histomorphologic, histochemical, and electron microscopic study. Immunohistochemistry has also provided insight into tumor histopathogenesis and has contributed to more accurate determination of patient prognosis.

Predictable tumor expression of many of the same antigens (*a macromolecular protein or polysaccharide that can bind to an antibody molecule*) as their cells of origin or normal tissue counterparts validates the principle of tumor classification by immunohistochemistry. Distinguishing between undifferentiated neoplasms of different origins is achieved through the detection of tumor antigens by using known antibodies. Some caution is necessary because antigens may be shared by several tumor types and on occasion may react with several distinct antigens and antibodies. It is also important to note that this technique does not in itself differentiate between benign and malignant neoplasms and, as yet, no single antibody can consistently identify a specific type of malignant lesion.

Many significant advantages are associated with the use of immunohistochemistry in surgical pathology. First, the technique can be performed on routinely prepared tissue sections, permitting the pathologist to work in a familiar microscopic environment while linking morphology with immunologic phenotype. Second, it is a very sensitive system that can detect antigens expressed at relatively low levels, and, if carefully selected, antibody-antigen binding can be very specific. Third, the technical aspects are the most favorable: Equipment costs are low and only a small amount of laboratory space is needed. Further, the technique is relatively straightforward and easily learned.

Of utmost importance is careful interpretation of immunohistochemically stained slides. This is typically subjective in nature, although a semiquantitative scale (eg, 1+ to 4+) is sometimes used. Staining intensity, percent, and morphologic features of positive cells must be considered when an evaluation of staining reactions is performed. Moreover, the distribution of positive staining relative to cell structure (nucleus, nuclear envelope, cytoplasm, or plasmalemma) must be considered because nonspecific staining frequently may show incorrect antigen localization. Positive controls are necessary for stain interpretation. Internal positive controls are particularly helpful; when they are not present, external positive controls are needed to confirm staining specificity. In any event, immunohistochemical studies must be interpreted only in the context of the impression made after review of H & E sections.

Principles and technical advances

The selection of antibodies for immunohistochemical testing is made on the basis of their tumor specificity and the likelihood that they will react with the tumor under evaluation. After tissue sections are incubated with the prospective antibodies, positive reactions (tumor antigen–antibody binding) are identified through the application of one of several detection systems. Those that have the greatest sensitivity use a secondary antibody, reactive against the primary antibody, which is conjugated or linked to an enzyme marker. This system tends to be very sensitive because it allows for the attachment of a relatively large number of enzyme molecules, such as peroxidase, at the antigen site. The color of the reaction is determined by the selection of a precipitating chromogen, usually diaminobenzidine (brown) or aminoethylcarbazole (red), with which the enzyme reacts.[1]

For a laboratory to produce uniform immunohistochemical results, standardization of immunohistochemical technique is critical. Inconsistencies can often be directly related to improper tissue fixation and processing, inadequate unmasking of antigenic epitopes (ie, the part of an antigen that combines with the antigen-binding site of an antibody molecule), and/or low sensitivity of the detection system.

Tissue fixation. Although antigens are best preserved in frozen tissue, good—if not excellent—results can be achieved with formalin-fixed tissue through the application of newer antigen retrieval methods and recently developed antibody preparations. Many of these newer antibodies are also effective in the recognition of leukocyte *c*luster of *d*ifferentiation (*CD*) antigens, giving this technique considerable utility in the classification of lymphoid tumors. Formalin-fixed tissues provide the pathologist with a readily available test material, applicability of familiar and cost-effective laboratory methods, and results characterized by well-preserved microscopic detail.

Immunohistochemical studies are most often performed on specimens fixed in neutral-buffered formalin because this is the most extensively used fixative. However, the effects of fixation, including protein-protein and protein-nucleic acid cross-linking and calcium ion bonding, mask or damage epitopes through alteration of the protein 3-dimensional structure. These changes can often be overcome by one of several antigen-retrieval methods. Other problems include delayed or extensive fixation times, inadequate tissue dehydration, and/or excessive paraffin-embedding temperatures (> 56°C). These preventable factors can contribute significantly to poor staining, resulting in weak or false-negative results.[2,3]

Assurance of fixative neutral pH is important, because acidic solutions reduce antigenicity. Delay in the placement of excised tissue into fixative may reduce antigen expression. Ideally, tissue specimens should be small (ie, 0.5 cm^3) and placed in fixative immediately. To ensure complete but not excessive fixation, tissue should be immersed in formalin for approximately 24 hours, but not more than 48 hours. Tissue received from outside

sources, as is the case for many outpatient biopsy services, may suffer significant antigenic compromise, necessitating careful selection of antibodies, an effective unmasking procedure matched to the antibody, and selection of a sensitive detection system.

Antigen retrieval. Antigen retrieval is the process by which antigenic epitopes, made unavailable because of fixation-associated protein cross-linking, are rendered accessible to antibodies for binding. Antigen retrieval (epitope unmasking) in formalin-fixed tissue can be achieved either through enzyme digestion or heating of sections. The method of choice depends on the antigen and antibody under study and is usually determined by trial and error testing to observe which method gives the best staining result.[4] Automated immunostainers have reduced some of the vagaries of immunohistochemistry. Unfortunately, technique protocols for available antibodies necessarily vary from one laboratory to another, making interlaboratory standardization and reproducibility difficult to attain.

Enzymatic digestion of tissue sections for epitope unmasking is typically accomplished through incubation in a solution of protease, trypsin, or pepsin.[5] All are effective, but they may result in increased background staining. In general, enzyme digestion provides less intense staining results than heating. There are several methods to heat sections, and all require a chelating buffer, such as citrate or ethylenediaminetetraacetic acid, to keep tissue moist and stabilize antigens. Sections may be heated in a microwave oven, pressure cooker, or waterbath. It has been our experience that a 95°C to 99°C waterbath technique (Dako Corp, Carpinteria, Calif) is the most gentle and provides the greatest consistency of results.

Antigen amplification. Several systems are available for detecting antigen-antibody reactions. Those that have the greatest sensitivity require the attachment or conjugation of an enzyme marker to a secondary antibody and a tertiary complex. Traditionally, alkaline phosphatase–antialkaline phosphatase and avidin-biotin-peroxidase systems, in which there are 3 linkage layers (primary antibody–secondary antibody–marker enzyme complex), have been widely used, with excellent results. A recent innovation that takes advantage of dextran technology reduces the number of linkage layers to 2 (EnVision; Dako Corp). A large number of enzyme molecules (peroxidase or alkaline phosphatase), as well as numerous secondary antibody molecules, are conjugated to a dextran backbone and used as the second link to the primary antibody. This method, now routinely used in our laboratory, is more sensitive than traditional systems, eliminates nonspecific biotin staining because biotin is not part of the system, and reduces laboratory time.

The object of layering or linkage of primary and secondary antibodies is to achieve attachment of as many enzyme molecules at the antigenic site as possible, thus increasing the intensity of the color reaction in the tissue section. Peroxidase is the most widely used enzyme marker because of its convenience and clarity of reaction. Alkaline phosphatase is sometimes used, especially when it is desirable to stain for 2 antigens in the same section. The color of the antigen-antibody reaction is determined by the selection of chromogen or substrate with which the enzyme reacts. Diaminobenzidine, which is water-insoluble, produces a brown color, and water-soluble aminoethylcarbazole produces a red color. These are the 2 most common chromogens utilized in this technique.

Antibodies. Cytoplasmic, nuclear, and cell membrane proteins represent the targets for antibodies used in immunohistochemical tumor classification. Whether polyclonal or monoclonal antibodies are used depends on the availability and effectiveness of individual antibody preparations. The high specificity, intense staining, and low background obtained with monoclonal preparations is usually desirable but may occasionally be a detriment because of reactivity with only a single antigenic epitope. If the epitope to which the monoclonal antibody reacts is damaged or altered in the neoplastic cells, a false-negative result may occur. Polyclonal antibodies, by contrast, react with more than one epitope, potentially increasing the odds of a positive reaction. However, occasional cross-reaction with unrelated antigens and increased background staining may render interpretations of test results problematic.

Immunohistochemical applications: Diagnostically challenging oral malignant neoplasms

Immunohistochemistry has been shown to be an effective adjunct to H & E diagnosis in a majority of equivocal tumor cases, through the establishment of a definitive diagnosis or through confirmation of H & E section impression.[6] Immunohistochemistry is typically applied to cases when the definitive diagnosis cannot be established on the sole basis of findings in H & E sections. Diagnostically difficult tumors generally fall into one of the morphologic subsets listed in Table I.

Most head and neck neoplasms listed in Table I can be identified by their pattern of reactivity with several antibodies, some of which are more specific than others. It is logical and cost-effective to begin an immunohistochemical study with a small panel of antibodies that serves to distinguish between major groups of neoplasms (eg, epithelial, connective tissue, melanocytic). On the basis of these results, a more focused sequence of immunostaining with specific

Table I. Subsets of diagnostically difficult head and neck tumors

Subsets	*Tumors*
Small round-cell tumors	Squamous cell carcinoma, adenocarcinoma, rhabdomyosarcoma, melanoma, Langerhans cell disease, neuroendocrine carcinomas, olfactory neuroblastoma, Ewing's tumor/PNETs, Merkel cell tumor, lymphoid tumors
Large round-cell tumors	Squamous cell carcinoma, adenocarcinoma, rhabdomyosarcoma, melanoma, lymphoid tumors, paraganglioma
Spindle cell tumors	Spindle cell carcinoma, rhabdomyosarcoma, leiomyosarcoma, neurosarcoma, fibroblastic and myofibroblastic tumors, Kaposi's sarcoma
Metastatic jaw tumors	Lung, gastrointestinal, breast, kidney, prostate
Minor salivary gland tumors	Polymorphous low-grade adenocarcinoma, adenoid cystic carcinoma, mixed tumor, monomorphic adenoma

PNETs, Peripheral neuroendocrine tumors.

antibody preparations can be performed for precise tumor classification. For example, an initial panel for round-cell tumors might include anti-S-100 protein, antikeratin cocktail, antileukocyte common antigen (CD45) cocktail, desmin, and possibly chromogranin to separate melanocytic, epithelial, lymphoid, skeletal muscle, and neuroendocrine neoplasms. An initial panel for spindle cell tumors might include anti-S-100 protein, antimuscle actin, and anti-CD34 to assist in the separation of neural/melanocytic, smooth muscle, and endothelial neoplasms. Oral neoplasms and the antibodies most commonly used to confirm their diagnosis by immunohistochemistry are listed in Table II.

Molecular marker descriptions

Epithelial marker, keratins. Immunohistochemical staining for cytokeratins, known generically as *keratins*, is frequently done to help confirm the epithelial lineage of a poorly differentiated carcinoma, adenocarcinoma, or spindle cell carcinoma (Fig 1). Thus, keratin antibodies, used as an antikeratin cocktail (mixture of antibodies to low and high molecular-weight keratins) are typically used as part of a general screening panel for undifferentiated tumors.[7]

The cytokeratins represent a group of structurally related intermediate filament proteins. They are subdivided into 19 subsets depending on their molecular weights (M_r), which vary from 40,000 to 68,000. Cytokeratins are identified either by molecular weight or by numerical designation, 1 through 19.[8] Generally, keratin subtypes expressed by epithelial neoplasms are similar but not identical to those expressed by their presumed cells of origin. However, as dedifferentiation occurs, there may be a general shift to the production of lower molecular-weight subtypes, or more of one subtype may be produced at the expense of another.[9] Abnormal expression of subtypes has been described in epithelial cells of dysplastic and in situ carcinoma lesions of the oral mucosa, suggesting the possibility of markers of premalignancy.[10] Metastatic epithelial cells generally express keratin profiles that are similar to those of the primary tumor. This antigen fidelity may be exploited to gain insight into the primary site of lesions metastatic to the jaws (see next paragraph).

Table II. Tumor-associated antigens that are useful in immunohistochemical diagnosis

Neoplasm	*Antigens*
Carcinomas	Keratins
Adenocarcinomas	Keratins
Salivary gland tumors	S-100 protein, actins, calponin
Rhabdomyosarcoma	Desmin, myoglobin, actin, myogenin, muscle-specific actin
Leiomyosarcoma	Smooth muscle actin
Neurosarcoma	Neurofilaments, S-100
Angiosarcoma and Kaposi's sarcoma	CD31, CD34, factor VIII–related antigen
Melanoma	HMB45, S-100 protein, MART-1 (Melan-A)
Langerhans cell disease	CD1a
Lymphomas	CD45, CD45RB isoform
B-cell lymphomas	CD20, CD791, CD45RA isoform
T-cell lymphomas	CD3, CD43, CD45RO isoform
Anaplastic large cell (Ki-1)	CD30 (Ber-H2 clone), ALK-1
Hodgkin's disease (RS cells)	CD15, CD30
Plasma cell myeloma	κ/λ light chains
Leukemic infiltrates	TdT, myeloperoxidase
Paraganglioma and neuroendocrine carcinoma	Synaptophysin, chromogranin, neurofilaments
Olfactory neuroblastoma	Synaptophysin, chromogranin, neurofilament
Merkel cell tumor	Synaptophysin, chromogranin
Ewing's sarcoma and PNETs	CD99
Solitary fibrous tumor	CD34, CD99, Bcl-2

TdT, Terminal deoxynucleotidyl transferase; *RS*, Reed-Sternberg.

Because squamous cell carcinomas produce a wide range of keratin subtypes, it is necessary to use a broadly reactive antikeratin cocktail in the identification of carcinomas. Antibodies to low molecular weight keratins are invaluable in the identification of undifferentiated carcinomas and adenocarcinomas. It is important to note that antibodies to keratins, as well as to other tissue antigens, appear to have limited potential in separating benign from malignant lesions, because expression of these antigens may be independent of tumor differentiation and clinical behavior.

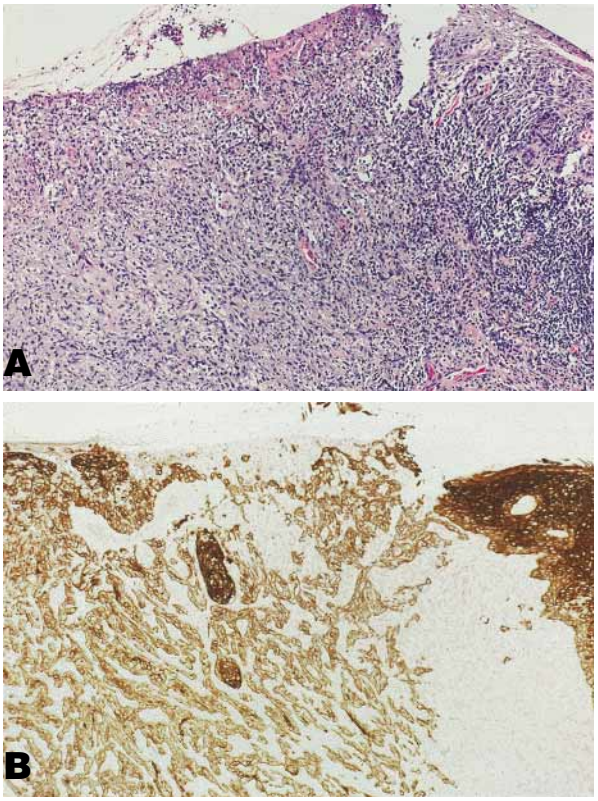

Fig 1. **A,** Spindle cell carcinoma from the palate of a 79-year-old woman (×250). **B,** Positive immunohistochemical staining with antikeratin "cocktail." Note the normal epithelium to the right and invasive neoplasm center and left (×250).

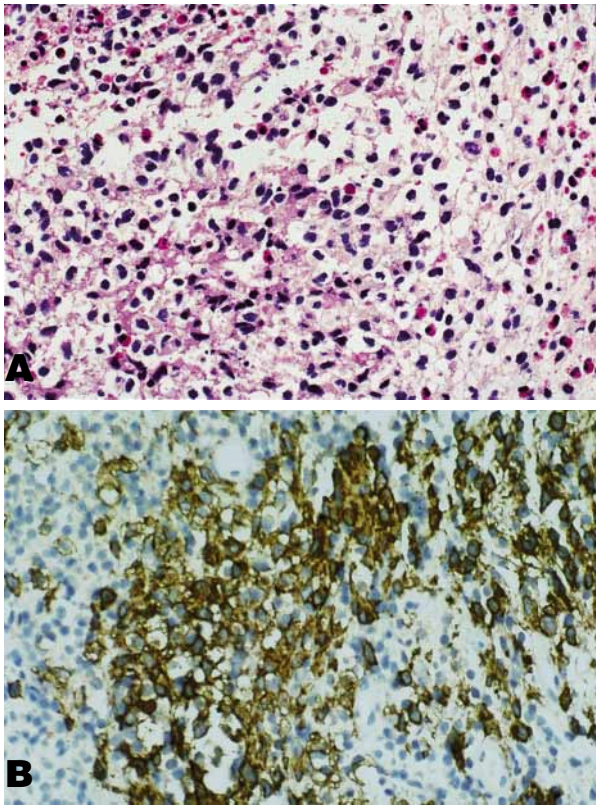

Fig 2. **A,** Langerhans cell disease discovered at the apex of the mandibular left second premolar tooth (×400). **B,** Immunohistochemical stain for CD1a antigen identifies Langerhans cells in the tissue (×400).

In general, keratin typing of salivary gland tumors by immunohistochemistry has been of little value in tumor diagnosis because all tumors—benign and malignant—contain keratin filaments. Once antibodies to the 19 specific keratin subtypes become available, studies may reveal diagnostically useful information. Currently, antibodies to glial fibrillary acidic protein (GFAP), actin proteins, and S-100 protein are of some help in salivary gland tumor diagnosis, although H & E microscopic pattern is still the most important diagnostic criterion.

Crossreactive keratin expression may also be evident in moderate to well-differentiated neuroectodermal carcinomas, but not in paragangliomas. Also, synovial sarcomas may stain positive for keratins.

General mesenchymal marker, vimentin. Staining for cytoplasmic filaments, including vimentin, desmin, GFAP, and neurofilaments, can aid in the precise identification of connective tissue tumors, including sarcomas. Vimentin, an M_r 57,000 intermediate filament, is found in most mesenchymal cells, lymphoid cells, and neural crest cells, including melanocytes, Langerhans cells, and nevus cells, as well as in their neoplastic counterparts. However, coexpression of vimentin with other intermediate filaments is not uncommon in head and neck tumors, such as spindle cell carcinoma.[11] Because of this coexpression and the wide variety of neoplasms that demonstrate antibody reactivity to vimentin, these intermediate filaments tend to be of only limited value in the diagnosis of tumors by immunohistochemistry.

Muscle markers: Desmin, actins, myoglobin, myogenin. Muscle differentiation in a neoplasm can be established by demonstrating the expression of desmin, actin, or myoglobin proteins. Desmin is an intermediate filament of approximately M_r 53,000 found in muscle cells and myofibroblasts. Desmin and myoglobin immunoreactivity are helpful in the diagnosis of tumors of muscle origin, especially rhabdomyosarcoma. Antibody to myogenin, one of several myogenic differentiation proteins, may be of value in the identification of rhabdomyosarcoma.[12] Actin is a small cytoplasmic filament, approximately 5 nm in diameter, that has contractile properties. Six actin isotypes differen-

tiate smooth muscle, striated muscle, and nonmuscle cells. Anti–muscle-specific actin (clone HHF35) generally provides good sensitivity and intensity for detection of leiomyosarcoma.[13] Anti–muscle-specific actin is also effective in staining myoepithelium of the salivary gland.[14] However, because most salivary gland neoplasms contain myoepithelial cells, this antibody is of limited value in the classification of these lesions. Nonetheless, newly developed antibodies against smooth muscle proteins (α–smooth muscle actin, smooth-muscle myosin heavy chains, and calponin) have been shown to be effective in separating positively staining adenoid cystic carcinoma from negatively staining polymorphous low-grade adenocarcinoma.[15,16]

Neural markers: S-100, GFAP, neurofilaments, and CD57. S-100 protein, originally isolated from nerve cells of the brain, derives its name from its 100% solubility in ammonium sulfate. Once thought to be unique to the central nervous system, it has subsequently been identified in numerous other cells outside the CNS, including Schwann cells, chondrocytes, Langerhans cells, and some nevus cells.

The antibody to S-100 protein stains a wide array of unrelated neoplasms, including neural tumors, paraganglioma, some salivary gland tumors, granular cell tumor, Langerhans cell disease, chondrosarcoma, some muscle tumors, and approximately 95% of melanomas. It should be noted that CD1a is a specific marker for normal Langerhans cells and the pathologic cells in Langerhans cell disease (Fig 2). A monoclonal antibody reactive to CD1a is now available and is effective for immunohistochemical analysis of formalin-fixed tissue, replacing the less-specific anti-S-100 protein for the confirmation of Langerhans cell disease.

Despite the diversity of reactions, polyclonal antibodies to S-100 protein can still provide useful information in the diagnosis of head and neck neoplasms. The results, however, are valid only when taken in the context of staining within a panel of antibodies and in relationship to findings in H & E sections.

Neural differentiation of a tumor can be confirmed with antibodies to either GFAP or neurofilaments. GFAP is an intermediate filament of M_r 51,000 that is typically found in glial cells and their neoplastic counterparts.[17] Occasionally, GFAP immunoreactivity may be demonstrated in cells of the peripheral nervous system, particularly in Schwann cells. Correspondingly, tumors such as neurofibroma and neurosarcoma may also contain these filaments. Myoepithelial cells of salivary glands and salivary gland neoplasms—in particular, mixed tumor—also express GFAP.[17] By contrast, neurofilaments are found in neurons. These intermediate filaments may be encountered in neoplasms of the central nervous system, as well as of the peripheral nervous system. Neuroblastoma (also identified by antibody NB84), olfactory neuroblastoma, ganglioneuroma, paraganglioma, and Merkel cell tumor also express neurofilament antigens.

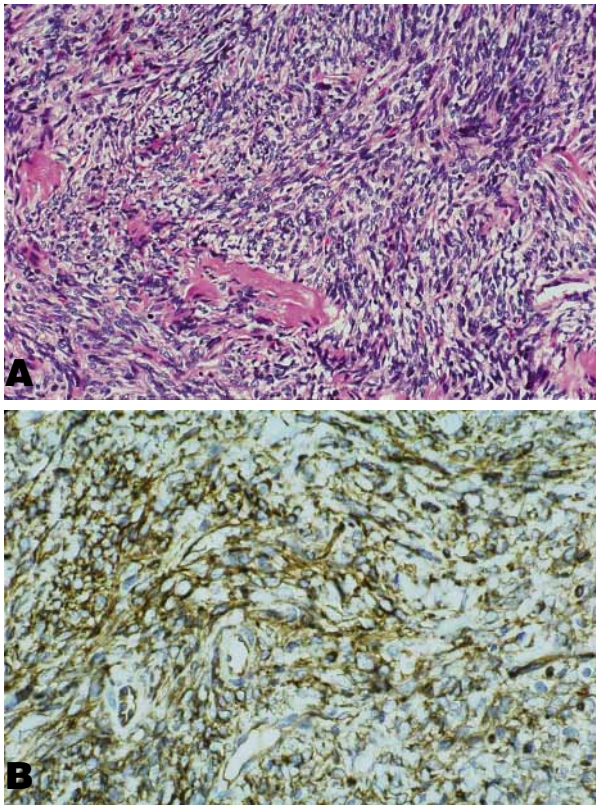

Fig 3. **A,** Solitary fibrous tumor from the buccal mucosa of a 46-year-old woman (×400). **B,** Positive immunohistochemical stain for CD34 helps confirm diagnosis (×400).

CD57 is expressed by natural killer cells but also somewhat inconsistently in some neural tumors, such as neurofibroma and granular cell tumor. Anti-CD57 cross-reacts with myelin-associated glycoprotein. This antibody may have some utility in confirming neural origin of some benign and malignant tumors.

Endothelial markers: CD31, CD34, and factor VIII–related antigen. Vascular differentiation of a tumor can be established with the antibodies to CD31 (PECAM), CD34, and factor VIII–related antigen. In diagnostic problems in which Kaposi's sarcoma or other neoplasms of vascular origin are being considered, any of these 3 antibodies may be used, although anti-CD34 seems to be the most consistent marker of endothelial cells in Kaposi's sarcoma.[18] As our own work has shown, anti-CD34 is also useful in confirming solitary fibrous tumors (Fig 3).[19]

Melanocytic markers: HMB45, MART-1 (Melan-A), and S-100 protein. Melanoma, especially when

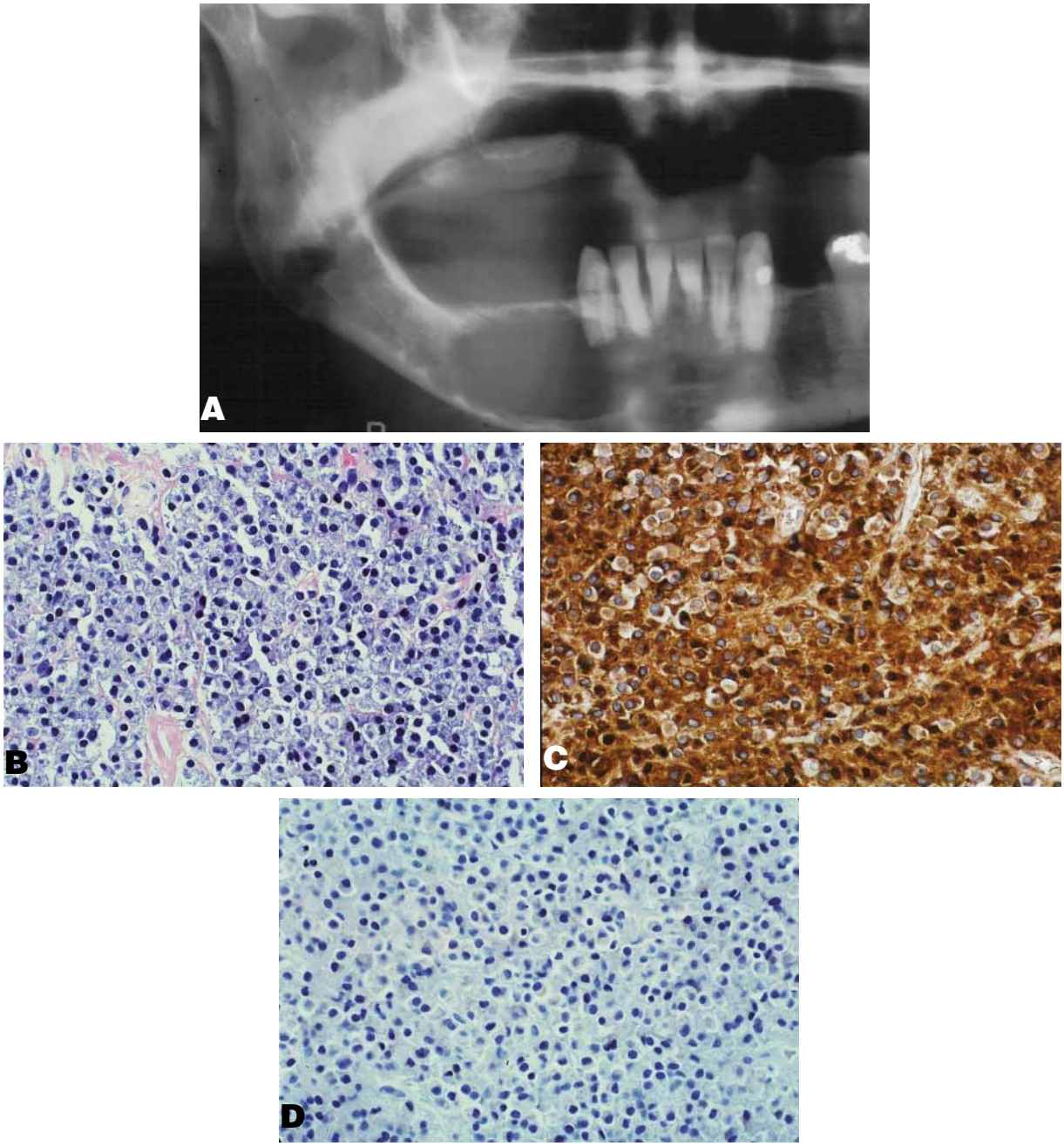

Fig 4. **A,** Multiple jaw radiolucencies in a 61-year-old woman representing previously undiagnosed multiple myeloma (courtesy of Dr Steven Rowan, Merced, Calif). **B,** Histologic section showing atypical plasmacytoid cells (×400). **C,** Positive immunohistochemical stain for κ light chains (×400). **D,** Negative staining for λ light chains (×400).

amelanotic, can histologically mimic other malignancies, and is often included in the histopathologic differential diagnosis of poorly differentiated neoplasms. Three reliable antibodies that react with proteins expressed by melanoma are HMB45, MART-1, and anti-S-100 protein. These reactions do not involve antigens directly linked to melanin formation, making such immunohistochemical analysis effective in distinguishing pigment-poor melanomas from other tumors with similar microscopic appearance. Staining with these antibodies may also be useful in locating occult tumor cells in tissue sections, aiding in the evaluation of depth of invasion and detection of metastasis.

HMB45 reacts with an intracellular antigen in a variable number of cells in approximately 90% of melanomas. Although highly specific for melanoma, some nevi may be reactive. Normal melanocytes are typically nonreactive. Recently, some nonmelanoma tumors (ie, lymphoma, adenocarcinoma, angiomyolipoma) have also been shown to react to HMB45.[20,21]

A recently developed antibody to a transmembrane protein on melanoma cells recognized by T cells (anti-MART-1 or Melan-A) has been shown to be useful in the diagnosis of melanoma.[22] Because this antigen (protein) is preserved in formalin-fixed tissue, it can be used when S-100 and HMB-45 stains are equivocal, or in lieu of HMB-45.

Lymphoid markers: κ and λ, CD3, CD15, CD20, CD30, CD45, CD68, CD79a, ALK-1, and TdT. The diagnosis of B-cell differentiated lymphomas, especially plasmacytoid forms, can be confirmed through the identification of a monoclonal κ or λ population. Although this is most easily done on frozen sections, it is often possible, immunohistochemically, to stain cytoplasmic light chains in paraffin-embedded tissues (Fig 4). Significant alteration in the normal 30%/70% κ-λ ratio typical of non-neoplastic B-cell populations would be strong evidence of a monoclonal population of cells. Predominance of one form of light chain is termed *light chain restriction*.

Leukocyte CD markers are typically used to subclassify lymphomas. Until recently, lymphocyte subtypes could be identified only in frozen tissue. Several new antibodies directed against various lymphocyte antigens have been developed for use in routinely processed tissue. Although these new antibodies cannot help us distinguish benign from malignant proliferations, they can be used to help us distinguish between B- and T-cell lymphomas. Because these new agents are not completely lineage-specific, some cross-reactions may occur, making the use of antibody panels necessary to avoid misinterpretation of false-positive reactions.[23-26]

The antibodies most widely used to determine T- and B-cell differentiation are CD3 and CD20 (clone L26), respectively (Table II). The most commonly used CD3 antibody is a polyclonal preparation reactive with an epitope located on the cytoplasmic domain of the CD3 protein. CD43, also a T-cell marker with some cross-reactivity with B-cells, is sometimes used to confirm lymphomas of T-cell lineage. CD20 is a highly reliable B-cell marker, and L26 is generally regarded as an effective antibody for identification of normal B-cells, staining approximately 95% of these cells. L26 is highly useful in identification of B-cell lymphoma and some lymphomas that are nonreactive to anti-LCA (CD45). L26 is believed to be reactive to an intracellular membrane–associated epitope of the CD20 antigen. In unusual B-cell lymphoma cases where there is no detectable expression of CD20, CD79a may be detected and can be used for confirmation of B-cell lineage. CD79a is a dimeric protein that is part of the B-cell receptor complex. It is expressed early in B-cell development and persists to the plasma cell stage.[27]

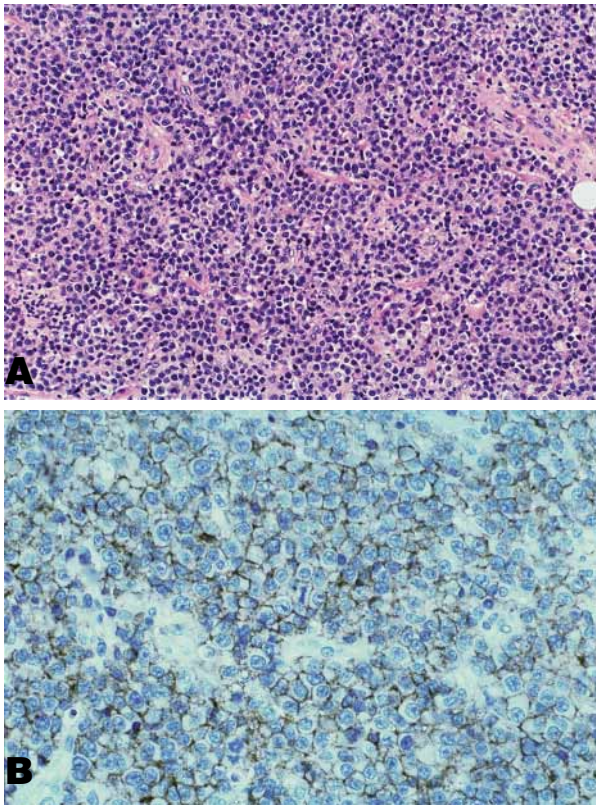

Fig 5. **A,** Lymphoma from the tuberosity and palate of a 73-year-old man (×250). **B,** Positive membrane staining of tumor cells for CD45 (×400). Stains for both CD3 and CD20 were negative.

Leukocytes typically can be stained with antibodies to CD45 (Fig 5). CD45 and its 3 isotypes—RA (clone 4KB5), RB, and RO (clone UCHL1)—show reactivity to antibodies in formalin-fixed tissue. The CD45RA isoform is found on B cells, CD45RB on both B and T cells and CD45RO on T cells only. Most T-cell lymphomas can be identified with antibody to CD45RO; fewer than 1% of B-cell lymphomas are reactive. Anti-CD45 cocktail is particularly useful in the evaluation of undifferentiated "round cell" tumors, because it stains lymphoma but not carcinoma, sarcoma, or melanoma. In contrast to anti-κ and anti-λ antibodies, anti-CD45 findings do not enable us to distinguish between benign and malignant proliferations.[25]

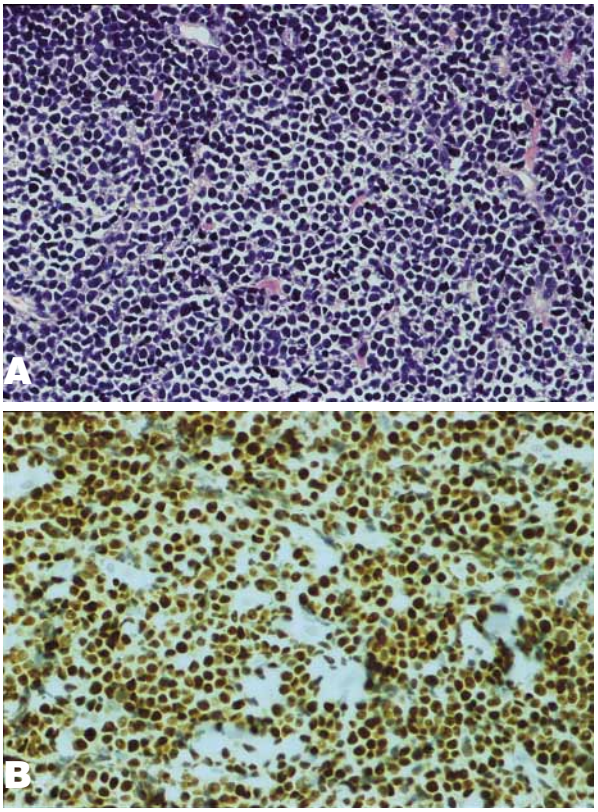

Fig 6. **A,** Leukemic infiltrate in the gingiva of a 16-year-old girl (×250). **B,** Positive immunohistochemical stain for terminal deoxynucleotidyl transferase (TdT), to confirm a leukemic infiltrate (×400).

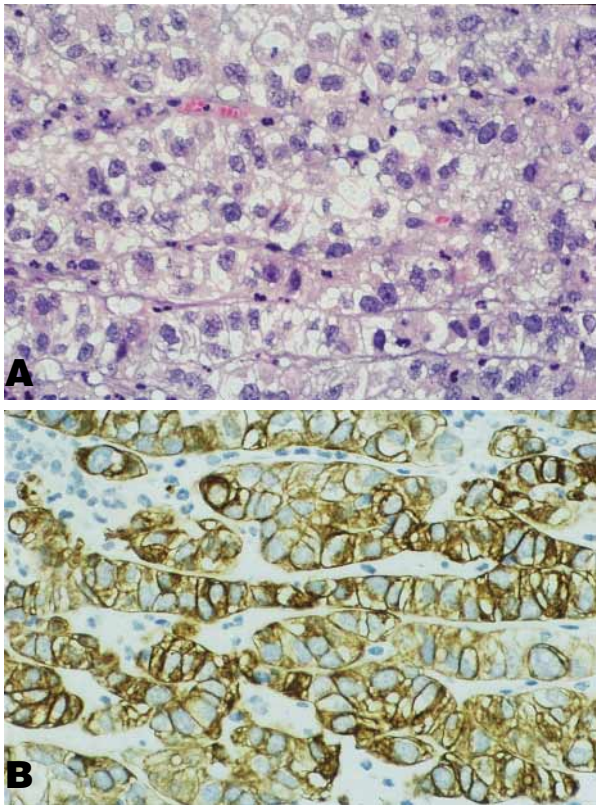

Fig 7. **A,** Metastatic tumor to the gingiva in a 72-year-old man with no history of previous malignancy (×400). **B,** Positive immunohistochemical stain for CK7 (×400) and a negative stain result for CK20 were observed, suggesting the lung as a possible primary site; this was subsequently confirmed clinically.

Antigens CD15 and CD30 can be expressed by Reed-Sternberg cells in Hodgkin's disease. More important to the pathologist is the membrane expression of CD30 by tumor cells of anaplastic large cell lymphomas, also known as *Ki-1 lymphomas*, named after the antibody clone that identifies them. These tumors also express cytoplasmic aberrant tyrosine kinase related to chromosomal translocations, usually t(2;5). Antibody (ALK-1) to this enzyme is also useful in the identification of a majority of anaplastic large-cell lymphomas.[28]

Terminal deoxynucleotidyl transferase (TdT) is an enzyme that may be expressed by acute leukemia cells, especially those of acute myeloid and acute lymphoblastic leukemias. Immunohistochemical staining for this enzyme can be helpful in the identification of leukemic infiltrates in oral tissues, and it may aid in the separation of leukemias from lymphomas (Fig 6). However, some caution must be exercised in the interpretation of TdT-stained slides, because not all acute leukemias will express TdT. A correlation between TdT expression and prognosis has not been shown.[29,30] Granulocyte differentiation can be confirmed with antibody to myeloperoxidase or with histochemical stain for chloroacetate esterase (Leder stain).

Traditional methods for identification of macrophages in paraffin-embedded tissue have included the use of polyclonal antibodies reactive to intracellular enzymes such as lysozyme, antitrypsin, and antichymotrypsin. Newer monoclonal antibodies that are reactive to macrophage membrane antigens are proving to be desirable alternatives to the traditional polyclonal antibodies to these intracellular enzymes. These antibodies are helpful in the separation of macrophages from neoplastic cells. CD68, a glycoprotein found in lysosomes and to a lesser extent on surface membranes, is highly expressed by macrophages and neutrophils. KP1 seems to be the most reliable antibody for detection of the antigen. Other macrophage-associated antibodies (ie, MAC 387, HAM-57, PG-M1) are less commonly used but are available for confirmation, if necessary.

Neuroendocrine markers, synaptophysin and chromogranin. Catecholamine production is a common characteristic of neuroendocrine cells. These neurotransmitter

substances are found in cytoplasmic neurosecretory granules (dense core granules) and provide morphologic and chemical evidence of cell origin. Synaptophysin and chromogranin are neurosecretory-associated proteins that have been used for development of monoclonal antibodies specific for paraganglioma, neuroendocrine carcinomas, Merkel cell tumor, medullary carcinoma of the thyroid, and olfactory neuroblastoma.[31]

Ewing's tumor and peripheral neuroendocrine tumor marker, CD99 (MIC2 gene product). Ewing's tumor and peripheral neuroendocrine tumors (PNETs) are closely related—if not identical—neoplasms. They share similar chromosomal translocations, predominantly t(11;22) translocations, that result in a novel fusion gene encoding for a chimeric oncoprotein that appears to act as a transcription factor. These tumors also express cell surface glycoprotein p30/32 (CD99) encoded by the MIC2 gene. O13, the monoclonal antibody that binds this glycoprotein, is helpful in the identification of this rare group of tumors when analyzing formalin-fixed tissues.[32] Interpretation of round-cell tumors must be made with the knowledge that some lymphomas and rhabdomyosarcomas may also stain positive with this antibody, although LCA and muscle markers can be used for separating these cases. It should be noted that solitary fibrous tumors also stain positive for CD99. Definitive diagnosis of Ewing's tumor/PNETs can be made with either cytogenetic, fluorescence *in situ* hybridization, or reverse transcriptase polymerase chain reaction analyses to identify the characteristic chromosomal/molecular defects in these tumors.[33]

Metastatic tumor markers, CK7, CK20, and villin. Application of immunohistochemistry can, on occasion, be helpful in determining the organ from which epithelial neoplasms metastatic to the jaws originated. By using stains for cytokeratins 7, 20, and villin (the actin-binding protein in microvilli), primary sites of origin can be reasonably well predicted (Fig 7). The scheme in Table III has been substantiated in several studies.[34,35] In addition, monoclonal antibody to prostate-specific antigen may be effective in the identification of metastatic adenocarcinoma of the prostate in formalin-fixed sections.

Minor salivary gland tumor markers, S-100 protein and actins. Immunohistochemistry is presently of minimal value in the microscopic diagnosis of minor salivary gland tumors, due, in part, to the varying participation of cells with both epithelial and myoepithelial differentiation in all the tumors. Antigenic markers lack specificity in these tumors; however, there are some quantitative differences that occasionally may be diagnostically helpful. S-100 protein is typically expressed to a greater degree in polymorphous low-grade adenocarcinoma than in adenoid cystic carcinomas. On the other hand, muscle-specific actins are expressed to a greater degree in adenoid cystic carcinoma than in polymorphous low-grade adenocarcinoma, reflecting greater myoepithelial differentiation in adenoid cystic carcinomas. Newly developed antibodies reactive to smooth muscle proteins, such as calponin, may prove of considerable value in terms of distinguishing these tumors. Actins are also expressed in mixed tumor (pleomorphic adenoma) but minimally so in monomorphic adenoma.

Table III. Immunohistochemical staining profile of metastatic epithelial malignancies

Tumor	*Antigenic profile*
Lung (adeno)	CK7+/CK20–/villin+
Lung (SCC)	CK7–/CK20–
Colon	CK7–/CK20+/villin+
Breast	CK7+/CK20–/villin–
Kidney	CK7–/CK20–/villin–
Prostate	CK7–/CK20–/PSA+

PSA, Prostate-specific antigen.

Tumor markers of patient course and outcome

It has been determined that specific genes are altered in oral cancers. Their encoded proteins, many of which can be detected with standard immunohistochemical methods, may serve as markers of lesions with high risk of progression to malignant disease—or they may be predictive of patient response to treatment and survival. Among the most promising candidate genes and proteins known to be dysregulated in oral cancer are those associated with cell proliferation. Overexpression of the cell cycle–associated oncoproteins cyclin D1 and MDM2, as well as underexpression of the tumor suppressor proteins p53, p16, and p27, may be important tumor markers.[36-40] Although these genes have the potential to be predictive of patient course and outcome, they have yet to be evaluated.

Regulators of apoptosis (ie, programmed cell death; a normal physiologic process whereby individual cells die, which maintains total cell numbers within a physiologically appropriate range) have also been a focus of cancer investigations, and their detection may have practical value. In some oral cancers the antiapoptotic proteins Bcl-X and Bcl-2 are overexpressed.[41,42] Moreover, expression of the proapoptotic protein Bax has been positively correlated with increased sensitivity to chemotherapeutic agents in head and neck carcinomas.[43] The prognostic value of these proteins is undetermined in oral cancers.

Several other oncoproteins that function in regulation of cell growth and in transport of signals from the cell

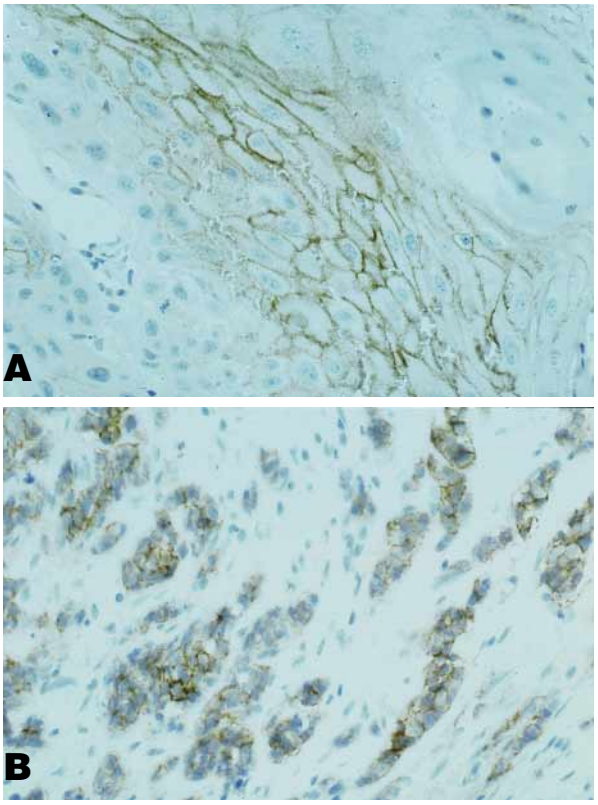

Fig 8. **A,** Positive membrane staining for HER2/neu (*c-erb*B-2) in squamous cell carcinoma of the pharynx (×400). **B,** Positive HER2/neu staining in metastatic cancer of the breast, an organ in which there is a proven positive correlation between staining and poor clinical outcome (×400).

membrane to the nucleus are also frequently altered in many oral cancers. These include growth factors such as fibroblast growth factor and growth factor receptors such as EGFR and HER-2/neu (c-*erbB-2*). EGFR and HER-2/neu are oncogenes that encode transmembrane receptors that are believed to increase cell cycle, cell motility, and angiogenesis (ie, the formation of new blood vessels). Overexpression is seen in many types of malignancy, including head and neck carcinomas.[44-48] Squamous cell carcinomas that overexpress EGFR are associated with a poorer outcome than those that do not overexpress this receptor.[49,50] HER-2/neu is known to be overexpressed in approximately 25% of breast cancers and is an important prognostic marker associated with reduced overall survival. Whether HER-2/neu is a prognostic marker for head and neck carcinoma is yet to be determined. A recently developed polyclonal antibody and laboratory protocol (DAKO Hercep Test; Dako Corp) for formalin-fixed sections may provide some answers. In preliminary unpublished studies in our laboratory, 20% of oral and oropharyngeal squamous cell carcinomas overexpress HER-2/neu (Fig 8). Potentially, the signaling pathways of EGFR and HER-2/neu could be therapeutic targets in head and neck cancer patients. Monoclonal antibodies to these proteins may be able to inhibit growth of tumors overexpressing these receptor proteins.

Other proteins involved in signal transduction (ie, the process whereby interactions between a receptor on the cell surface and its ligand are transmitted to the nucleus of the cell), such as ras (GTP-binding proteins), and nuclear regulatory proteins, such as myc (transcriptional activator proteins), are also abnormally expressed in oral cancers. Correlations have been identified between growth receptor overexpression and patient outcome.[51-53]

Critical cell adhesion proteins are altered in invasive oral cancer. Matrix-related proteins produced by tumor cells contribute to the breakdown of basement membrane and extracellular matrix proteins. Matrix metalloproteinases 1, 2, 3, 9, and 13 have also been demonstrated in invasive carcinomas and are believed to play a significant role in matrix degradation. In particular, matrix metalloproteinases 3 and 13 are associated with advanced head and neck carcinomas.[54,55] Beta-6 integrin, a keratinocyte adhesion molecule, and tenascin, an antiadhesion matrix molecule, may be of considerable significance in the early invasive process of oral cancers.[56] Matrix metalloproteinase expression has not been correlated with prognosis in oral cancer.

Angiogenesis is critical to permitting development of many tumors. Mechanisms for angiogenesis include induction or overexpression of angiogenic proteins (eg, vascular endothelial growth factor, and basic fibroblastic growth factor [FGF]), and/or through the suppression of proteins that inhibit angiogenesis are also important in the development of many human tumors. Vascular endothelial growth factor, FGF, and IL-8 (proinflammatory cytokine) have been identified in head and neck carcinomas and are believed to be responsible, at least in part, for the angiogenesis associated with the progression of these tumors.[57,58] Whether detection of these and related proteins in oral cancers will have predictive potential remains to be determined.

Finally, immunohistochemical detection of tumor-associated telomerase may have clinical applications. Cancer cells may produce telomerase, an enzyme that maintains telomere length and, thus, long-term viability. This mechanism is not present in normal adult cells. Most head and neck carcinomas have telomerase activity through neoexpression of this enzyme, giving the neoplastic cell extended life.[59,60] Its detection in premalignant mucosal lesions (leukoplakia) may also serve as a biomarker for high-risk lesions.

Table IV. Location and pattern of fluorescent antibody reactions in oral mucosal specimens from various mucocutaneous diseases*

	IgG	*IgA*	*IgM*	*C3*	*Fibrinogen*
Pemphigus	ECM	Neg	Neg	ECM†	Neg
Pemphigoid	BMZ	BMZ	BMZ	BMZ	Neg
Lupus (oral)	Granular BMZ	Neg	Granular BMZ	Granular BMZ	BMZ-SLP
Lichen planus‡	Neg	Neg	Neg	§,|	BMZ-SLP
Erythema multiforme‡	Neg	Neg	Neg	§	Neg
Linear IgA disease	Neg	BMZ	Neg	Neg	Neg
Epidermolysis bullosa acquisita	Wide BMZ	Wide BMZ	Neg	Wide BMZ|	Neg
Chronic ulcerative stomatitis	ANA	Neg	Neg	Neg	BMZ-SLP
Nonspecific chronic inflammation‡	Neg	Neg	Neg	§	Neg

ECM, Epithelial cell membrane fluorescence; *Neg,* negative or irrelevant fluorescence patterns (eg, fibrinogen deposition in a separated basement zone or cytoplasmic fluorescence in plasma cells); *BMZ,* linear homogeneous basement membrane zone fluorescence; *granular BMZ,* coarse granular basement membrane zone fluorescence; *BMZ-SLP,* fluorescence outlining the basement membrane zone with irregular extensions into the superficial lamina propria (shaggy appearance); *wide BMZ,* linear homogeneous basement membrane zone fluorescence, wider than MMP; *ANA,* speckled pattern nuclear fluorescence, in basal ⅓ of epithelium.

*In an individual case, these fluorescence patterns are often seen with only 1 or 2 reagents.

†With anti-C3, ECM is limited to basal and parabasal cells undergoing acantholysis.

‡"Cytoid" bodies may be seen in the epithelium or superficial connective tissue with anti-IgM, -C3, -IgA, or -IgG (listed in order of decreasing frequency of occurrence in LP).

§Fine-granular deposits of C3 in the BMZ are present in various types of chronic inflammation.

|C3 may also be seen in the wall of the blood vessels.

Knowing the principles and practice of applied immunohistochemistry makes the pathologist more competent and effective through greater understanding of tumor differentiation and classification. Immunohistochemistry adds an important tool in the microscopic diagnosis of difficult tumors and in tumor research.

CURRENT CONCEPTS OF DIRECT IMMUNOFLUORESCENCE IN DIAGNOSTIC PATHOLOGY

Use of direct immunofluorescence microscopy (DIF) for diagnosis in oral and maxillofacial pathology began about 30 years ago. Since then, applications have expanded and greatly improved through the use of newer reagents, offering greater specificity, stability, and variety, as well as enhanced imaging from improved microscopic filters, illumination, and optics. The principal application of DIF is in the identification of antibodies and other inflammatory proteins in tissue sections to confirm diagnostic impressions from routinely processed sections or to enhance the diagnosis of clinically suspected diseases exhibiting nonspecific inflammatory changes in routinely processed sections. DIF can be a valuable tool for the diagnosis of a group of diseases that are often difficult to separate clinically and in some cases are histologically similar (Table IV).

Specific examples for which DIF can be helpful include the following: the diagnoses of lichen planus (LP), lupus erythematosus (LE), pemphigus vulgaris (PV), and mucous membrane pemphigoid (MMP); in cases of "desquamative gingivitis"; to distinguish between the common diseases of erosive LP and MMP and less common oral diseases of PV; for the diagnosis of chronic ulcerative stomatitis (CUS) and linear IgA disease (LAD); to confirm diagnoses of MMP or PV; to focus diagnosis on the possibility of erythema multiforme (EM) by ruling out LP, LE, MMP, and PV; to distinguish between the clinically similar white lesions such as plaque type LP and hyperkeratosis; and to distinguish between lesions of LP and LE.

Principles and techniques of immunofluorescence

The fluorescent antibody technique (ie, immunofluorescence, or IF), introduced in 1941,[61] is based on chemically attaching a fluorescent label molecule (fluorochrome) to an antibody (fluorochrome conjugate or conjugated antibody) with known biologic binding properties. Since its earliest applications, the widest use of IF microscopy has been in the identification of microbial antigens. In the last few decades, many additional applications have been established for IF, making it useful in the practice of surgical pathology. DIF uses a conjugated antibody, applied to unfixed sections of a lesion to reveal the presence and location of an antigen specific to the antibody being used. DIF can be considered the earliest form of immunohistochemistry. Another form of IF, indirect immunofluorescence (IIF), has less applicability in the diagnosis of the oral manifestations of mucocutaneous diseases and uses serial dilutions of a patient's serum applied to sections of suitable tissue substrate (eg, monkey esophagus). Conjugated antihuman IgG is then applied to localize autoantibodies from the patient's serum that are bound in the tissue substrate.

Fluorochromes are molecules that have the capacity to absorb light of one wavelength and emit light of another (longer) wavelength. These agents are used to visualize the sites of *in situ* immunologic binding. The 2 fluorochromes most widely used in DIF are fluorescein isothiocyanate (called *FITC* or *fluorescein*) and tetramethyl-rhodamine isothiocyanate (called *TRITC* or *rhodamine*). DIF is made possible by light striking the specimen at wavelengths absorbed by the fluorochrome and by transmission of the wavelengths of light emitted by the fluorochrome.

Commercially available conjugated antibodies for DIF applications include a wide variety of specificities. In most diagnostic DIF applications, serial sections from the specimen are reacted with each of at least 5 different conjugated antibodies. Typically, these are fluorescein-conjugated, polyclonal goat or rabbit antisera against the following: human (1) IgG, (2) IgA, (3) IgM, (4) complement component 3 (C3), and (5) fibrinogen. The antisera against human immunoglobulins should be heavy chain–specific (ie, γ, α, and μ chains, respectively), and the goat or rabbit antisera should consist of the $F(ab)_2$ fragment.

Modern fluorescence microscopes work on the principle of epifluorescence (ie, illumination of the specimen from above the microscope stage). In such a microscope, a high-energy light source, usually from a mercury arc lamp, passes through an excitation filter, which transmits wavelengths of light corresponding to the optimal excitation wavelength for the fluorochrome conjugate used being 492 nm for fluorescein or 554 nm for rhodamine. The light then passes through a dichroic mirror into the microscope's objective lens and illuminates the subject. Any fluorochrome bound to the specimen is then seen by its unique emission wavelength: 515 nm (yellow-green color) for fluorescein or 573 nm (orange-red color) for rhodamine. Before the image passes through the eyepieces, a barrier filter improves the image contrast by transmitting light of wavelengths equal to or greater than the fluorochrome's emission wavelength and blocking the transmission of shorter wavelengths.

Tissue preparation for DIF

Tissue sampled for testing should not be ulcerated because the presence of epithelium and an intact basement membrane zone (BMZ) is essential for definitive DIF examination. When considering a vesiculobullous disorder, a biopsy specimen of predominantly erythematous and ulcerated mucosal lesions should be obtained a few millimeters away from an ulcer so that the specimen's epithelium and connective tissue will be intact. A biopsy specimen of lesions with a hyperkeratotic component (eg, LP or LE) should include that along with its adjacent erythematous mucosa.

Biopsy specimens to be examined by DIF can be immediately frozen or placed in a room-temperature transportation medium for shipment to the laboratory. Specimens must not be placed in conventional fixatives, such as formalin, because this will produce cross-linking of tissue proteins, including antigens, reducing their detectability by means of this method. It is important to note that false-negative DIF results may occur if the oral lesions have been treated with topical steroids before biopsy. Such treatment should be discontinued at least 1 month before obtaining a biopsy specimen. To snap-freeze a specimen, it should first be placed, epithelial side down, on a small piece of aluminum foil that is then enclosed in a suitable cryovial, immersed in liquid nitrogen, and stored at –70°C until cryosectioning.

Because rapid freezing of specimens requires special supplies and keeping them frozen during transportation is a packaging challenge, an excellent alternative is to place the specimen in a room-temperature transportation medium that permits convenient transport to a laboratory for processing. Michel's transportation medium[62] has become a standard for this purpose and is available commercially (Zeus Scientific, Inc, Raritan, NJ).

Studies have shown that Michel's medium is able to effectively maintain tissue-bound immunoglobulins and other inflammatory proteins for 1 or 2 weeks before DIF examination.[63,64] Because there is no tissue fixation produced by Michel's medium, progressive cytolysis of epithelial cells begins after only a few days. However, human skin BMZ components, tonofilaments, and basal epithelial cells are well preserved in this medium, even at the electron microscopic level, for up to 4 weeks. To best preserve tissue morphology, specimen transport in this medium should be done as expeditiously as possible. In general, Michel's medium is relatively unstable and must be stored appropriately and used before the manufacturer's expiration date.

Michel's medium contains a very high concentration of ammonium sulfate, which precipitates antibodies and other inflammatory proteins within the tissue; it also contains an enzyme inhibitor in citrate buffer. Therefore, when specimens are received in the laboratory in this medium, they must be washed in buffer to remove the high levels of salts and then embedded in an appropriate embedding medium (eg, OCT), frozen, and stored for later cryosectioning.

Applications of DIF microscopy

Currently, the primary applications of DIF microscopy in head and neck surgical pathology are in the identification of antibodies and other inflammatory proteins in sections of mucosa to diagnose or support the diag-

nosis of various immunologically mediated diseases. Usually, these proteins (antigens) are part of extracellular molecules such as immunoglobulins or inflammatory proteins, such as complement components or fibrinogen.

From the clinical perspective, DIF should be considered when diagnosing oral mucosal conditions characterized by chronic, multifocal, or diffuse blisters; erosions; ulcers; or combinations of those patterns. Application of DIF is indicated when the clinical differential diagnosis includes LP, LE, MMP, PV, or other less common diseases, as described in the next section.

An interpretation of DIF results is made on the basis of a combination of both the positive and negative observations from each of the 5 conjugated antisera generally used in this procedure: namely, antihuman IgG, IgA, IgM, C3, and fibrinogen (Table IV). Microscopic evaluation includes both the location and the pattern of fluorescence seen with each reagent.

Application of DIF to oral disease diagnosis

Pemphigus vulgaris. Pemphigus represents a group of autoantibody-mediated diseases in which the antibodies induce acantholysis in the skin or oral mucosa. Various forms of pemphigus produce skin lesions, but 2 of these, PV and paraneoplastic pemphigus, typically have oral lesions. Oral lesions of PV precede skin lesions in 75% of cases.[65] Oral lesions of paraneoplastic pemphigus can resemble oral PV, but the condition is rare.

An IgG autoantibody from the serum of patients with PV, which reacts with the surface of epidermal cells, was first discovered by using IIF in 1964.[66] Later, the in vivo deposition of those antibodies was observed by using DIF in lesional and perilesional biopsy specimens from patients. The PV antibody specifically binds to a 130-kd component of the desmosomes in stratified epithelia, called *desmoglein-3*.[67] More than 75% of patients with pemphigus have circulating antibodies against epithelial cell surface antigens that are detectable by IIF. Although circulating antibodies are used by some to monitor clinical response to therapy, a correlation between the titer of circulating antiepithelial cell surface immunoglobulin and the disease activity is inconsistent.[68]

In lesional and perilesional skin and mucosa of patients with PV, the autoantibody is demonstrated throughout the epithelium by DIF using conjugated anti-human IgG (Fig 9, *A*). This can be in the form of interepithelial cell fluorescence demonstrated by intact epithelium or membrane fluorescence of cells that have undergone partial or complete acantholysis (Tzanck cells). In all cases, C3 is also deposited in the same pattern, but it is limited to attached basal and parabasal cells and cells undergoing acantholysis (Fig 9, *B*). One study reported cell surface fluorescence with both IgG and C3 in more than 90% of cases, the remainder revealed C3 deposition only with none showing IgG deposition alone.[69] Essentially, all patients with active PV have a positive DIF test for IgG on the cell surface of epithelial cells in perilesional mucosa. Therefore, the diagnosis of PV should be questioned if such DIF test findings are negative.[70]

MMP. Pemphigoid refers to a group of clinically and histologically overlapping diseases in which similar subepithelial bullous lesions affect primarily the skin (bullous pemphigoid, BP), the conjunctiva (cicatricial pemphigoid, CP), and the oral mucosa (MMP). Oral lesions of MMP occur with or without accompanying conjunctival lesions, whereas ocular lesions of CP occur with or without oral lesions. Oral mucosal lesions are seen in approximately 20% of patients with cutaneous BP. All forms of pemphigoid are mediated by autoantibodies directed against different antigens in the epithelial BMZ.[71-73]

Through the use of IIF, IgG autoantibodies that bind to normal stratified squamous epithelial BMZ can be found in the serum of approximately 70% to 80% of patients with BP.[74] Most BP autoantibodies bind to the BP-230 antigen located in hemidesmosomes, and approximately 50% of these antibodies also bind to another hemidesmosomal antigen, designated as *BP-180*.[68] However, in CP and MMP, such circulating antibodies are seen in fewer patients than in BP and only at low titer. These bind to the BP-180 antigen, the α unit of laminin-5, located in the lamina lucida of the BMZ,[68] or to other antigens.[72] BP is relatively distinct, immunologically, from MMP and CP, but the latter 2 cannot be clearly distinguished from each other. There may be 5 or more subsets of MMP/CP based on clinical features and antigenic specificity of autoantibodies.[72]

Essentially all patients with MMP and CP have in vivo bound IgG, IgA, or C3, with DIF presenting as a homogeneous line in the BMZ of lesional and perilesional mucosa[69] (Fig 9, *C*). Deposition of C3 in the BMZ is detected in almost all patients; sometimes C3 is the sole immunologic reactant and is considered diagnostically significant. Siegel and Anhalt[75] noted that in DIF analysis of biopsy specimens of MMP with epithelium separation from underlying connective tissue, deposits of IgG appeared on the basal pole of the epithelial cells in an interrupted linear pattern (Fig 9, *D*).

LAD. LAD is an uncommon autoimmune disease of skin that shares many similarities with pemphigoid. Oral mucosal lesions may occur in a high proportion of patients with LAD, often appearing clinically as

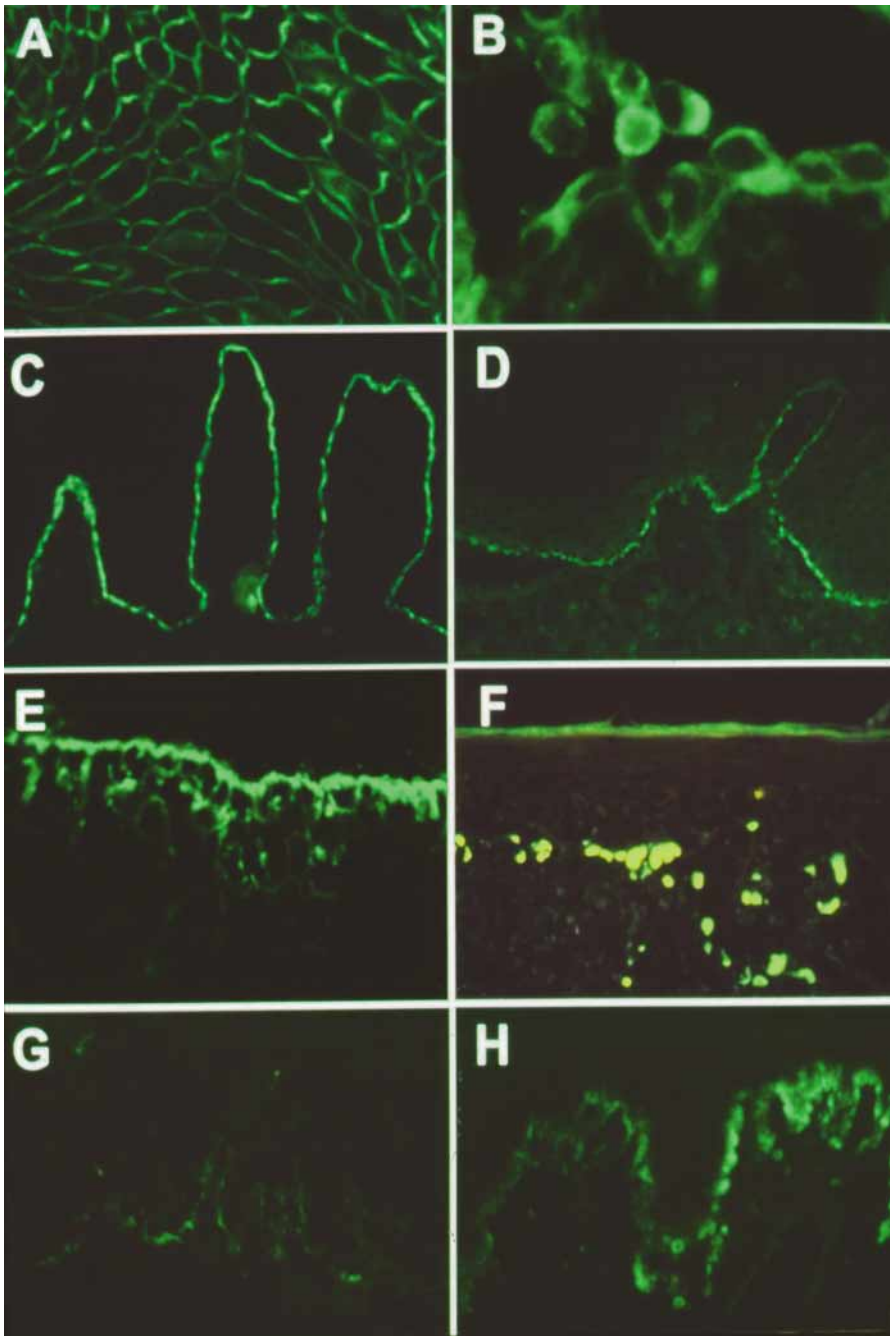

Fig 9. Direct immunofluorescence images using FITC-conjugated goat antihuman antibodies on sections of lesional oral mucosa: **A,** Pemphigus vulgaris—epithelial cell membrane fluorescence (intact epithelium) with antihuman IgG (×312). **B,** Pemphigus vulgaris—epithelial cell membrane fluorescence (area of acantholysis with Tzanck cells) with antihuman C3 (×625). **C,** Mucous membrane pemphigoid—basement membrane zone fluorescence (homogeneous, linear) with antihuman C3 (×156). **D,** Mucous membrane pemphigoid basement membrane zone fluorescence (note epithelial–connective tissue separation) with antihuman C3 (×156). **E,** Lichen planus—basement membrane zone–superficial lamina propria fluorescence with antihuman fibrinogen (×312). **F,** Lichen planus—"cytoid" bodies in superficial connective tissue with antihuman IgM (×156). **G,** Erythema multiforme—fine-granular basement membrane zone fluorescence (see Footnote § in Table IV) with antihuman C3 (×312). **H,** Lupus erythematosus—coarse-granular basement membrane zone fluorescence with antihuman IgM (×312).

gingival sloughing in the clinical pattern of desquamative gingivitis.[76,77] Unlike MMP, oral lesions of linear IgA disease are reported only rarely as the first or only lesions.[78]

There is considerable immunologic overlap between MMP/CP and LAD. A recent study observed that one third of MMP/CP patients and 20% of LAD patients had IgA antibodies in their serum that were reactive with pemphigoid antigen BP-180. This study also demonstrated that approximately 50% of patients with MMP/CP and 60% of patients with LAD had IgA antibodies in their serum that were reactive with the 97-kd LAD antigen.[79] Seventy-three percent of patients with MMP/CP showed both IgA and IgG tissue-bound antibodies in the BMZ as disclosed by DIF, whereas only IgA antibodies were observed by DIF in patients with LAD. The distinguishing immunopathologic feature of LAD in lesional and perilesional epithelium is BMZ deposition of a narrow, homogeneous band of IgA in the absence of other immunoglobulins.

LP. LP is a relatively common mucocutaneous disease with a chronic course and unknown cause. Immunologic mechanisms appear to be central to the lymphocyte-mediated damage to basal keratinocytes.[80] The oral mucosal lesions of LP have a variety of clinical appearances, including hyperkeratotic striae, mucosal erythema, erosions, and ulcers. Because the clinical appearance of oral mucosal LP often changes over time in individual patients and the clinical features of mucosal LP are variable and cannot always be considered pathognomic, DIF examination is often helpful. Studies of large series of patients have shown that DIF contributes significantly to the diagnosis of oral mucosal LP.[70,81,82]

Although there is no known antibody-mediated component in the pathogenesis of LP, there are T-lymphocyte–mediated changes revealed by DIF that are useful diagnostically. LP shows a characteristic pattern of fibrinogen deposition outlining the BMZ and extending irregularly into the superficial lamina propria, described as a "shaggy" or "fibrillar" pattern (Fig 9, *E*). This is present in all nonulcerated specimens of LP,[83] but it is also seen in LE[81,83] and CUS.[84,85] However, other tissue-bound antibodies or proteins distinguish these latter diseases (Table IV). Another finding in DIF typical of but less frequently observed in LP is the presence of IgM-, IgA-, IgG-, or C3-positive "cytoid," "colloid," or "apoptotic" bodies, located in the epithelium and superficial connective tissue[70] (Fig 9, *F*). We,[81] and others,[70] require either or both of these patterns, in the absence of other patterns, to render a DIF diagnosis of LP. A fine-granular deposition of C3 is frequently seen in the BMZ of LP specimens and other conditions but is not diagnostically useful (Fig 9, *G*).

LE. Oral mucosal lesions of LE may occur in patients with systemic LE, the skin disease chronic discoid LE, or they may be the only lesions present. Lesions of oral LE can clinically resemble LP (with irregular keratotic striae and erythema), skin lesions of chronic discoid LE, or have a nonspecific clinical presentation, such as an erythematous patch. The oral mucosal lesions of lupus do not correspond to the usual classification of systemic and cutaneous lupus. DIF reactions in specimens of oral mucosal LE exhibit coarse granular deposits of C3, IgM, IgA, and IgG in the BMZ (listed in order of decreasing frequency[83]; Fig 9, *H*). These deposits are a manifestation of the immune complexes that are found in other organs with systemic LE. These primary immunoglobulin and complement patterns are almost always seen along with the pattern of fibrinogen deposition described earlier in LP.

Epidermolysis bullosa acquisita. Lesions of epidermolysis bullosa acquisita are chronic subepithelial bullae that are clinically similar to those seen in the hereditary forms of epidermolysis bullosa, but which arise later in life with no apparent inheritance pattern.[68] Epidermolysis bullosa acquisita is an uncommon acquired autoimmune disease in which the patients develop antibodies directed against type VII collagen,[86] a protein forming the anchoring fibrils that join the BMZ lamina densa with the papillary layer of the lamina propria.[68] Oral lesions may occur at the same time as skin lesions[87] or months later.[88] Lesional and perilesional skin and mucosa examined by DIF exhibit deposition of IgG, IgA, and IgM in the BMZ that is more intense and broader than that seen in pemphigoid.[68]

CUS. This newly described, uncommon disease predominantly affects older women.[89,90] CUS is characterized by chronic erosions and ulcerations of the gingiva, often described clinically as desquamative gingivitis.[91,92] A unique feature of this disease is its resistance to local or systemic steroid treatment; however, its responsiveness to hydroxychloroquine provides long-lasting remission in many cases.[91]

CUS is associated with unusual antinuclear antibodies that bind exclusively to squamous epithelia.[92] Routine histopathologic findings are nondiagnostic, but this antibody can be identified in the serum by IIF or bound in vivo in lesional or perilesional biopsy specimens by DIF. The pathogenic potential of this antibody remains to be established. The DIF findings in perilesional epithelium include nuclear deposits of IgG in a speckled pattern, confined to the basal and parabasal cells.[84,85] Deposits of fibrinogen are seen in the BMZ and superficial lamina propria, with a pattern similar to that described earlier in LP.

EM. In many cases, the clinical lesions of oral mucosal EM and the histopathologic appearance of biopsy specimens are not distinctive. Although EM has no distinctive DIF findings, a negative result aids in ruling out immunologically mediated mucosal disorders that may show clinical resemblance to EM.

SUMMARY

Advances in the analysis of DNA, RNA, and proteins are currently revolutionizing the practice of surgical pathology. These changes have permitted improved insights into a range of disorders and led to improvements in the understanding and classification of many diseases. This article, the second of a 2-part series, has described the principles of immunohistochemical and immunofluorescent methods and their current and future applications to diagnostic pathology. In the future, the pathologist will continue to play a central role in diagnosis and the routine armamentarium of diagnostic tests will include many of the advances discussed here. Thus, these changes likely will improve the understanding of diseases that affect the head and neck and the ability of the pathologist to render a diagnosis.

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