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Special Techniques in Diagnostic Electron Microscopy

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The power of electron microscopy as a diagnostic tool can be amplified considerably by the application of ancillary preparative and analytic methods. Subcellular chemistry and structure can be examined by various forms of microprobe analysis and by special staining methods, including cytochemical, immunocytochemical, and negative staining. Qualitative ultrastructural examination can be augmented by morphometric analysis. Correlative microscopic survey methods can be used as a means of targeting ultrastructural investigations. This article provides an overview of the use of these special techniques in the diagnosis and classification of tumors and other selected pathologic processes. HUM PATHOL 29:1339-1346. Copyright © 1998 by W.B. Saunders Company

Key words: **diagnostic medicine, electron microscopy, microprobe analysis, immunoelectron microscopy, morphometry**

Abbreviations: **EM, electron microscopy; H&E, hematoxylin and eosin; LAMMA, laser microprobe mass analysis; SIMS, secondary ion mass spectrometry; NS, neurosecretory; IEM, immunoelectron microscopy; NK, natural killer; MF, mycosis fungoides.**

Electron microscopy (EM) plays a vital role in the diagnosis of tumors, particularly in the assessment of tumor cell lineage. Conventional EM techniques can often be augmented and rendered even more powerful by application of ancillary diagnostic methods. In many cases, the results of conventional EM examination can be extended by correlation with findings from other forms of microscopy performed in parallel ("correlative microscopy") or by novel methods of data analysis. In other instances, special EM preparative methods and/or imaging devices are employed.

Although conventional ultrastructural analysis often allows detection of lineage-specific features (eg, premelanosomes, Weibel-Palade bodies), it provides only indirect information about the biochemical makeup of the tissue under study. Valuable biochemical information can often be obtained by correlating the results of conventional EM with those of routine histochemistry, immunohistochemistry, and *in situ* hybridization. These staining techniques can also be adapted for direct ultrastructural observation. Molecular and elemental studies at the ultrastructural level can also be performed using methods such as laser microprobe mass analysis and electron probe x-ray microanalysis. The term "microtopochemistry," derived from the Greek word *topos,* meaning site or location, and chemistry, is often applied to these new techniques,[1] which provide important insights regarding the anatomic distribution, biochemistry, and physiology of a wide range of analytes, including ions and elements, genes and their products, molecular fragments, whole molecules, and macromolecular complexes such as ribosomes and viruses.

Routine examination of electron micrographs often overlooks quantitative information with diagnostic and prognostic significance. Such information can frequently be obtained by the electron microscopist armed with as little as a ruler and a calibration grid. Sophisticated computer morphometric techniques, however, have been used in several instances to provide important objective information about neoplasms.

Finally, constraints on the size of EM tissue specimens are an impediment to the analysis of tumors with focal features of interest (eg, small areas of differentiated or viable tumor admixed with larger expanses of undifferentiated or necrotic tissue). This problem has traditionally been addressed by light microscopic examination of survey sections produced from random EM embedding blocks. Although the value of this approach is unquestioned, new survey techniques using instruments such as the confocal laser scanning microscope hold promise for increasing the yield and accuracy of diagnostic EM.

In this article on special techniques, we review recent advances in electron microscopy, immunocytochemistry, microprobe analysis, and other ancillary methods as they are being used and are beginning to be used in diagnostic ultrastructural pathology. Whereas in the past these approaches were confined to research, they now are increasingly of value in diagnostic work. Much of the work in these areas has been developed in studies of nonneoplastic conditions. Accordingly, we will show these approaches with both neoplastic and nonneoplastic processes.

MICROTOPOCHEMISTRY

Microprobe Analysis

Electron Probe Analysis. Medical microprobe analysis is usually accomplished on an electron microscope equipped with an energy dispersive x-ray spectrometer. This type of analysis is commonly referred to as electron probe x-ray microanalysis. All elements with an atomic

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number equal to or greater than beryllium can be detected and usually quantitated.[2]

Although microprobe analysis was initially used primarily as a research tool, this is no longer the case. Microprobe findings now have diagnostic, therapeutic, and/or legal significance.[3] For example, the identification and characterization of intrapulmonary deposits is important not only in determining a patient's diagnosis, but also in some instances as a source of evidence for medicolegal situations. Accurate identification of the material involved may allow recognition of sources of exposure and lead to measures to reduce exposure and prevent the possibility of harm to others. Thus microprobe analysis can be an important tool in occupational medicine and public health. Currently the most commonly studied clinical conditions include the pneumoconioses, especially asbestosis and related conditions such as mesotheliomas (Fig 1), "hard metal" pulmonary fibrosis, and other mineral-induced pneumoconioses.[4,5]

Microprobe analysis can be used for the analysis of stones, particularly renal stones; in some situations, it can be more sensitive than x-ray diffraction or chemical techniques, particularly for the identification of small components of complex stones. Other applications include the identification of unexplained pigments or deposits and the study of unexplained granulomas. It also is particularly well suited for failure analysis of prosthetic devices such as metal joints[6] and ceramic implants[7] and has been used to detect silicone particles released systemically by failed hemodialysis tubing.[8]

One particular virtue of the energy dispersive x-ray spectrometer is that it can identify substances that were not suspected in advance. That is, it can answer unasked questions. For example, McDonald et al recently documented cerium and lanthanum in a lung biopsy. This finding had not been anticipated clinically or at the time of surgical pathology review of conventional hematoxylin and eosin (H&E) sections.[9] Edwardson et al have shown not only aluminum but also silicon in Alzheimer's disease.[10]

Future trends include the use of flash freezing to capture electrolytes *in situ* for subsequent analysis. This technique is the only way to define the subcellular anatomy of electrolyte concentrations, and should provide new understandings of pathophysiology; chemical microanatomy will augment structural microanatomy.

Laser and Ion Probe Microanalysis. Two other developing microprobe techniques should be mentioned: laser microprobe mass analysis (LAMMA) and secondary ion mass spectrometry (SIMS). These techniques have greater sensitivity than electron probe x-ray microanalysis. Whereas the sensitivity of electron probe x-ray microanalysis is generally on the order of 10 to 100 parts per million, the sensitivity of LAMMA and SIMS can be one or two orders of magnitude better (ie, parts per billion). Perhaps even more importantly, these techniques have capabilities for isotopic separation and the ability to detect and localize molecules and molecular fragments.

The LAMMA instrument provides a conventional

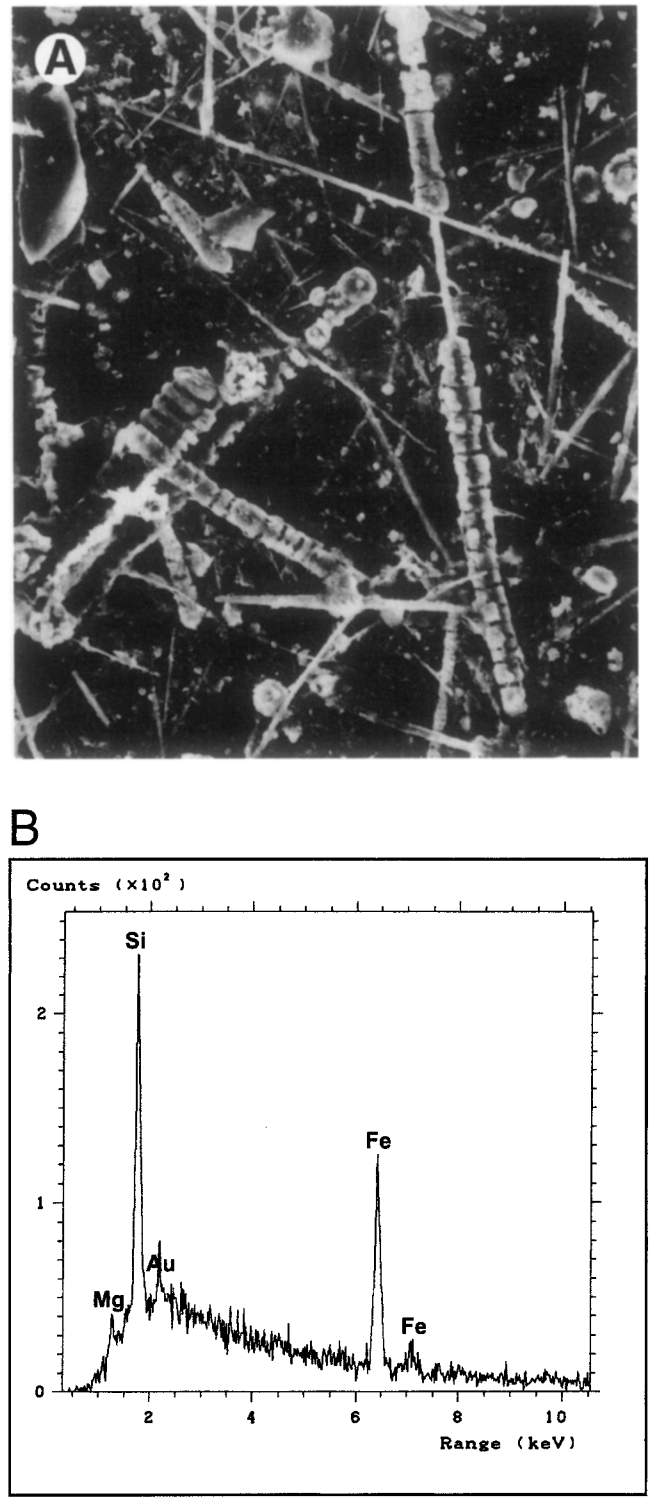

FIGURE 1. (A) Secondary electron image of a lung digest from a patient with asbestosis and mesothelioma. Note the numerous ferruginous bodies and uncoated asbestos fibers (magnification ×1,340). (B) Energy dispersive x-ray spectrum from the fibers shown in A. Peaks for magnesium, silicon, and iron are present in a pattern typical for amosite asbestos. (The small gold peak is caused by the coating.)

light microscopic image of a tissue section. Selected areas such as a nucleus or a cluster of lysosomes are vaporized with a laser beam coupled to a high precision time-of-flight mass spectrometer. Lateral resolutions less than one-half micron have been achieved. LAMMA has been used to detect and localize a variety of elements in histological sections, including aluminum in human dialysis-associated encephalopathy,[11] iron in iron-overload states,[12] gold in skin biopsies of patients with rheumatoid arthritis undergoing chrysotherapy,[13] and arsenic in a nerve biopsy from a patient with arsenic poisoning.[14]

SIMS is a microanalytic technique based on the energetic ion beam bombardment of solid samples, resulting in the desorption of ionized surface species. These so-called "secondary ions" are mass analyzed and detected with high sensitivity to provide elemental and molecular information on surface composition. Current imaging SIMS instruments have been developed by combining high performance time-of-flight mass analyzers with pulsed focused primary ion beams. Lateral resolutions less than one-twentieth micron have been achieved.

Special Staining Techniques

Cytochemistry. An example of the application of ultrastructural cytochemistry to tumor diagnosis is provided by the use of the uranaffin reaction in the examination of neuroendocrine neoplasms. Neuroendocrine cells are present throughout many of the epithelial layers and surfaces of the body and can form aggregates as well as distinct glands. All of these diverse types have been termed "paraneurons" because they share many biological properties with neurons.[15] Some of these properties include a receptosecretory function and the presence of neurosecretory (NS) granules.

The ultrastructural diagnosis of neuroendocrine neoplasms is, therefore, dependent on finding these characteristic NS granules, which have a distinct core surrounded by a clear halo.[16] Unfortunately, misdiagnoses can be made because NS-like granules can be seen in breast carcinomas, normal lactating breast, hepatocellular carcinomas, normal hepatocytes, thyroid carcinomas, normal human thyroid, and even lymphomas.[17,18] Argyrophilic stains, although widely used to identify neuroendocrine cells and their neoplasms, have been shown to be nonspecific and will stain the lactalbumin content of a significant number of breast carcinomas.[19] The uranaffin reaction, which was originally developed as a cytochemical technique for the localization of adenine nucleotides in organelles storing biogenic amines,[20] is able to identify true NS granules (Fig 2).[17] The specificity of the stain is such that lysosomes and exocrine granules from many different types of cells are not detected using uranyl salts at an acid pH.[17,21] The uranaffin reaction was even used to classify the fibrolamellar variant of hepatoma as a neuroendocrine neoplasm.[22]

Ultrastructural cytochemical techniques are also valuable in the diagnosis of nonneoplastic disorders such as platelet storage pool disease. The normal

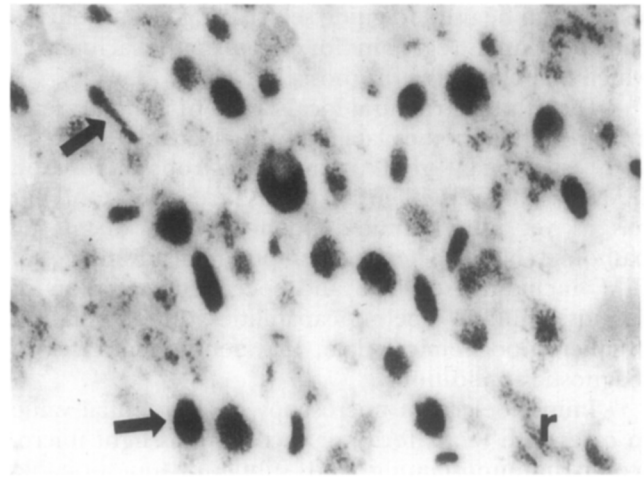

FIGURE 2. High-magnification electron micrograph of the cytoplasm of a pheochromocytoma cell showing uranaffin-positive neurosecretory (NS) granules (arrows). The reaction product is seen in the matrix of the NS granules where a high content of ADP is found, whereas the surrounding granule membrane does not stain and cannot be discerned in the micrograph. The ribosomes (r) stain positive with this reaction and serve as an internal positive control. (Uranaffin reaction; no osmication, no counterstain; magnification ×29,200.) Reprinted with permission.[21]

human platelet is ultrastructurally complex and contains several organelles, including alpha granules, dense bodies and mitochondria. The dense bodies store calcium, nonmetabolic nucleotides and biogenic amines, and are very similar to NS granules in their contents.[20,23] Abnormalities in platelet aggregation are frequently a result of primary platelet defects, including abnormalities in the contents of dense bodies (storage pool disease) or in the reaction that mediates their release. Because ultrastructural cytochemistry can be used to identify each of the major components of the dense body, a diagnosis of storage pool disease can be definitely rendered. Examination of three separate platelet preparations by the uranaffin reaction (stains the nucleotides [ADP/ATP]),[20,23,24] the chromaffin reaction (stains the amines [serotonin or 5-hydroxytryptamine])[25] and Weiss' fixation procedure (contains calcium chloride)[26] allows a comprehensive assessment of dense bodies.[27] In a routine clinical setting, one can choose to use the uranaffin reaction or Weiss' fixation procedure to assess the number of dense bodies. Techniques for proper sample collection and methodological details are provided in the references cited earlier.

Immunocytochemistry. Immunocytochemistry and *in situ* hybridization have in many ways revolutionized diagnostic surgical pathology, particularly in the area of tumor diagnosis.[28,29] In some instances, light microscopic immunocytochemical techniques have supplanted diagnostic EM in the field of tumor identification. The two techniques are best viewed as complementary rather than competitive, however.[30] In many cases, conventional transmission EM can provide a definitive diagnosis when reactivity of immunostains is weak or inconsistent. Conversely, subtle or inconclusive ultrastructural findings (eg, rare, poorly formed desmo-

somes) can be used to guide selection of immunohistochemical panels (eg, stains for cells of epithelial lineage) which may clinch the diagnosis.

Direct ultrastructural localization of immune reactants using antibodies labeled with electron-dense tags such as colloidal gold (immunoelectron microscopy, IEM) has enjoyed extensive use as a research tool, but has as yet found limited application in diagnostic pathology. Nevertheless, several potential advantages of IEM, including sensitive and specific localization of ligands, multiple labeling capabilities, and generation of microtopochemical data, may lend themselves to diagnostic applications.[31]

Immunoelectron microscopy is of potential value in cases with weak, focal, or incongruous light microscopic immunostaining and subtle or questionable ultrastructural findings. Examples include confirmation of the authenticity of NS granules in neuroendocrine tumors,[32-35] and identification of muscle-specific actin and desmin, respectively, in poorly differentiated tumor cells in myoepitheliomas[36] and rhabdomyosarcomas.[37] Immunoelectron microscopy also lends itself to a variety of multiple-labeling procedures that allow simultaneous detection of more than one ligand.[38-40]

Perhaps the most exciting diagnostic potential for IEM lies in the possibility that certain types of tumor cells may have differences or derangements in the ultrastructural distribution of constituent molecules. Examples include variations in the mitochondrial distribution of bcl-2 protein in thyroid tumors of different lineages[41]; expression of the CD15 antigen in different subcellular patterns by Reed-Sternberg cells in Hodgkin's disease and adenocarcinoma cells[42]; and expression of the CD3 antigen, a cell-surface antigen of T-lymphocytes, in an intracellular location in natural killer (NK) cells and NK-cell derived nasal lymphomas.[40]

Applications of IEM to the diagnosis of nonneoplastic diseases have also been described. Precise ultrastructural localization of autoantibodies within the epidermal basal lamina allows differentiation of several forms of autoimmune blistering disease.[43] Immunogold labeling for immunoglobulin light chains may be helpful in the diagnosis of early or equivocal cases of renal amyloidosis and light chain deposition disease.[44] Clumping or labeling of virus particles with specific antibodies or sera from convalescent patients are also used occasionally as adjuncts to viral diagnosis by EM (see below).

Negative Staining. Electron microscopy is a valuable tool for the diagnosis of infectious agents because of the catchall nature of ultrastructural analysis. Of particular importance in this diagnostic application is the technique of negative staining. Although not used to identify tumors per se, negative staining is particularly useful in diagnosis of opportunistic viral diseases resulting from immunosuppression during cancer therapy. Virus identification by EM has an advantage over biochemical testing in that it does not require specific reagents such as antibodies, nucleic acid probes, or protein standards, which necessitate prior knowledge of potential pathogens for selection of the proper reagent. Additionally, it does not require viable virions as does growth in tissue culture.

Pathology EM laboratories perform thin sectioning of tissues for virological investigations; however, negative staining can be an informative adjunct to this routine procedure. Negative staining is very rapid, requiring only a few minutes of specimen preparation time. It often reveals details of virus structure, such as surface capsomere patterns, that are difficult to visualize in thin sections. It also avoids the harsh chemicals necessary for processing tissue for thin sections. Negative staining is used for liquid samples, such as cerebrospinal fluid, urine, lavages and aspirates, blister fluids, or extracts of stool. These kinds of specimens are readily obtainable without invasive procedures.

Sample preparation involves clarifying the specimen by low speed centrifugation to remove cells, bacteria, and large debris, and then placing the sample onto a grid covered with a support film. Ultracentrifugation or antibody aggregation can be used to concentrate viruses present in low numbers. The staining procedure is simply to apply a drop of stain to the grid before the sample dries and then drain it with filter paper. The stain consists of a heavy metal salt to surround the virus particles, support them while drying, and darken the support film and the crevasses in the particles. The most commonly used stains are uranyl acetate and phosphotungstic acid. Others, along with recipes, methods, and use in virology have been described by Hayat and Miller[45] and Payne et al.[46]

The most frequently received specimen for negative staining is stool, most commonly submitted from infants and children with diarrhea and vomiting. Adult fecal samples are less common, because the disease is usually self-limiting in these patients. Human gastroenteritis is caused by a variety of viruses, all of which are fastidious and cannot be grown routinely in tissue culture. EM remains the single most effective laboratory technique available to detect these pathogens.[46-48]

Rotavirus is the most commonly diagnosed viral agent of acute gastroenteritis in childhood, accounting annually for an estimated 140 million infections, 1 million deaths in young children, and most hospital admissions for diarrhea in children under the age of two.[47] The classic rotavirus is 65 nm to 70 nm in diameter and has a characteristic double-shelled capsid with a wheel-like appearance with "spokes" and surface "holes" created by a circular arrangement of capsomeres (Fig 3). IEM has been used to aggregate viruses to increase the sensitivity of detection,[49,50] and to identify aberrant 30-nm to 54-nm single-shelled particles as rotaviruses.[51]

Another very frequent sample is urine from individuals who have received bone marrow transplants after cancer treatment. The agents most frequently seen here are polyomaviruses and occasionally adenoviruses or cytomegalovirus (a herpes virus). Negative staining and EM can also be used to examine skin lesions for the presence of pox or herpes viruses. Both agents are seen in individuals with AIDS, and herpes viruses (varicella zoster, herpes simplex) are frequent pathogens in cancer patients.

The morphological identification of viruses by EM

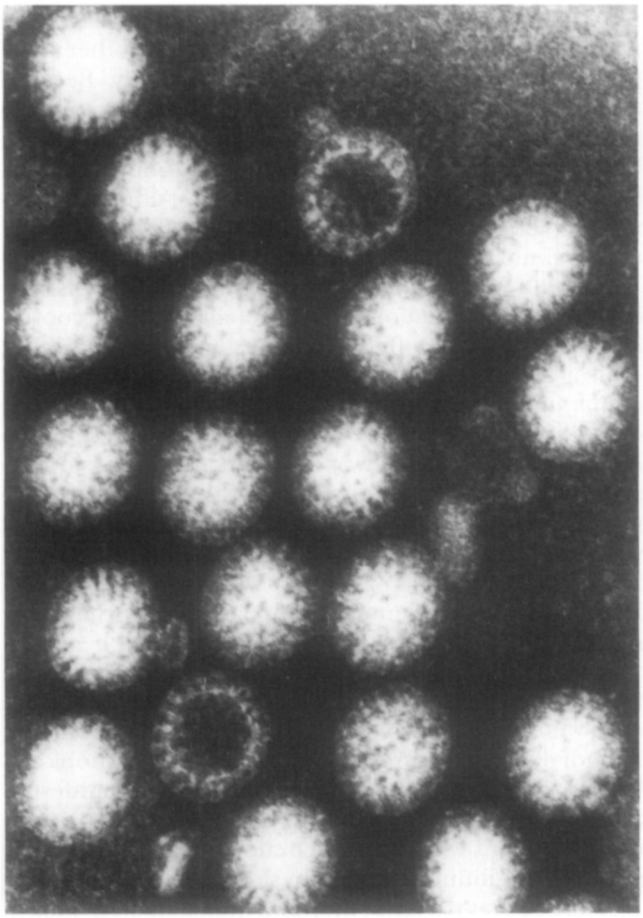

FIGURE 3. High-magnification electron micrograph of rotaviruses in a stool specimen from a child with gastroenteritis. The arrangement of the capsomeres resembles the spokes of a wheel, hence the name "rotavirus" (Greek derivation: rota, or "wheel-like"). The particles shown here are double-shelled; the "rim" of the wheel is formed by the second shell, giving the particle a smooth appearance. (Whole mount preparation; negative contrast with phosphotungstic acid; magnification ×233,000.) Reprinted with permission from Payne CM: Electron microscopy in the diagnosis of infectious diseases, in Connor DH, Chandler FH, Schwartz DA, et al (eds): Pathology of Infectious Diseases, Vol 1, Stamford, CT, Appleton & Lange, 1997, pp 9-34.[56]

has been described in detail.[49,52] Particular characteristics to note in differentiating viruses from each other and from cell components are whether the virion is naked or enveloped, its size, and the shape of its nucleocapsid. Three excellent atlases of viral morphology are available.[53-55] An extensive discussion of the role of EM in the diagnosis of specific viruses, parasites of the subkingdom protozoa, and bacterial infections, including ultrastructural cytochemistry for the identification of bacterial capsules, is provided in a recent book chapter.[56]

MORPHOMETRIC TECHNIQUES IN ULTRASTRUCTURAL PATHOLOGY

Tumor Diagnosis

Morphometry has proven valuable in the analysis of several paraneoplastic and neoplastic conditions. Measurement of fibril diameter in fibrillary glomerulopathies, for example, is useful in distinguishing fibril types often associated with plasma cell neoplasms (eg, amyloid), from other, larger forms of fibril without clear neoplastic associations.[57] A wide variety of tumors, including alveolar soft part sarcomas, neuroendocrine neoplasms, and others, contain crystalloids whose precise periodicity often provides an important diagnostic clue.[58]

A variety of morphometric techniques have also been applied to the diagnosis of mycosis fungoides (MF) and the Sézary syndrome. MF is conventionally diagnosed by light microscopy when a biopsy specimen is obtained from a late plaque or tumor stage of the disease process.[59] The diagnostic criteria include a prominent infiltrate of atypical mononuclear cells that invade the papillary dermis and epidermis with the formation of characteristic Pautrier microabscesses. Diagnostic difficulties are encountered at the premycotic erythemic stage and early plaque stage when little epidermal invasion occurs and the cellular infiltrate is indistinguishable at the light microscopic level from that of chronic dermatitis.

Electron microscopic examination of skin infiltrates in patients with MF revealed the consistent presence of atypical lymphocytes having highly convoluted cerebriform nuclei (Fig 4A). Because Sézary-like cells with a highly irregular nuclear contour (although not approaching that seen in Fig 4A) can be seen in benign disorders of skin,[60,61] the normal peripheral circulation,[62] and after antigen stimulation,[63] a subjective evaluation of lymphocytic populations as to their benign versus neoplastic nature can lead to misdiagnoses.

Ultrastructural morphometric methods, however, using computerized planimetry,[60-66] simple analytical shape factor analysis,[66] or the development of histograms (Fig 4B) that measure sharply-angled nuclear invaginations,[60,62] have been successfully used to distinguished benign from neoplastic lymphoid cell populations (Fig 4B).[67] Image analysis is easy to perform and can be used to evaluate randomly obtained low power electron micrographs of lymphocyte populations whose nuclei contain abundant heterochromatin.[60]

Quantitation of Ciliary Substructure and the Orientation of Cilia in the Diagnosis of Primary Ciliary Dyskinesia

EM is of proven diagnostic value in identifying ciliary disorders such as Kartagener's syndrome (situs inversus viscerum, chronic sinusitis, and bronchiectasis) and the immotile cilia syndrome (immotile spermatozoa and chronic airway infections).[68] Specifically, high resolution TEM often reveals the partial or complete absence of dynein with these conditions. Presumably, the absence of dynein arms, which contain an adenosine triphosphatase, causes the cilia to function poorly, resulting in decreased fertility as well as respiratory infections.

In addition to defects in the dynein arms (including the stubby arm variant), an absence of radial spokes,

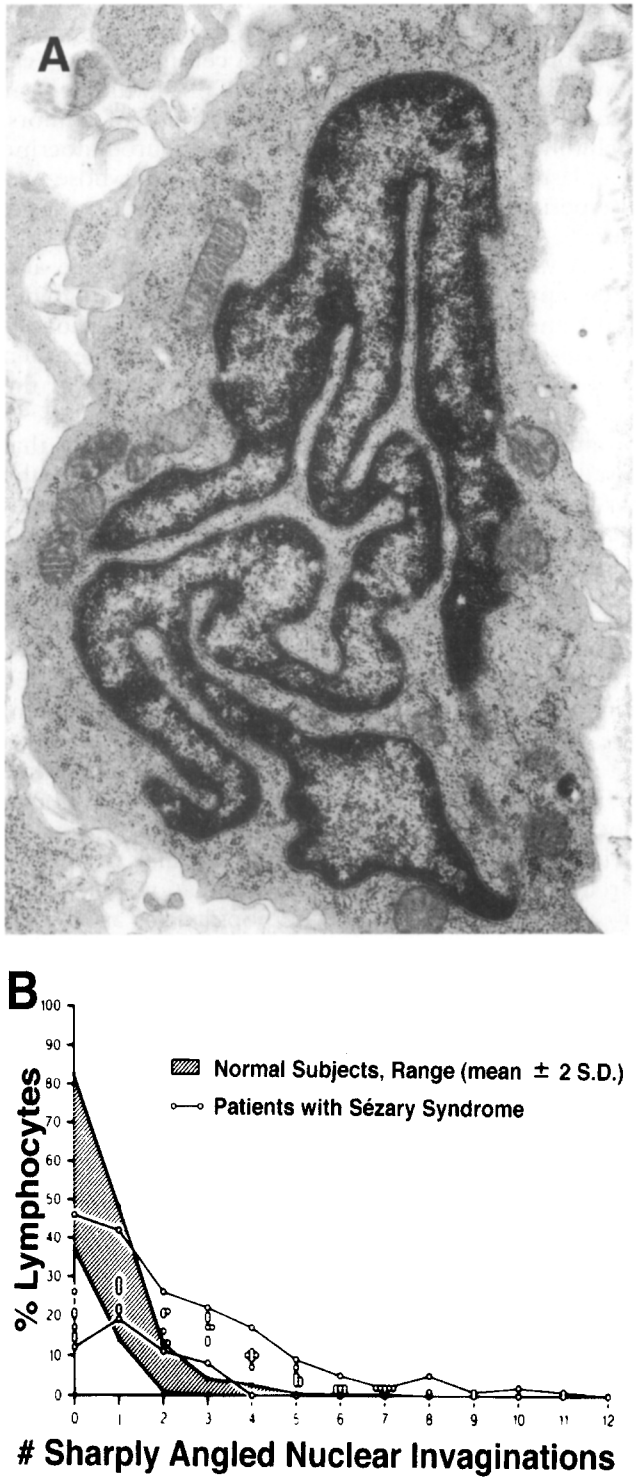

FIGURE 4. (A) High-power electron micrograph of a classic Sézary/mycosis cell with a serpentine nucleus. The nuclear contour is highly invaginated and the amount of heterochromatin is more characteristic of a mature lymphocyte as opposed to a lymphocyte undergoing blast transformation (magnification ×14,700). (B) Comparison of histogram values (percentage of lymphocytes having 0 to 12 sharply angled nuclear invaginations) from normal subjects with those from patients with Sézary syndrome. The shaded area represents the normal range of values (mean ± 2 SD) for the control group. Reprinted with permission from Payne CM, Glasser L: Ultrastructural morphometry in the diagnosis of Sézary syndrome. Arch Pathol Labs Med 114:661-671. Copyright 1990, American Medical Association.

nexin links, central microtubules, and sheath, and transposition of ciliary microtubules have been reported as causes of impaired ciliary motility. Because the genes that govern ciliary and flagellar structure are numerous, molecular biological techniques have not been used for their diagnosis in the routine clinical laboratory. EM is, therefore, a most cost-effective technique to evaluate whether the ciliary/flagellar substructure is abnormal.

Another cause of abnormal ciliary motility is random ciliary orientation.[69,70] If the cilia are not coordinated so that their effective strokes are oriented in the same general direction, microbes and other airborne contaminants will not be removed from the airways, even if all of the ciliary components are present. To diagnose this defect, low power electron micrographs are obtained and a line is drawn through the central microtubules and across the ciliary cross section.[69,70] Cilia will beat in a direction that is perpendicular to that line. A simple analysis of 10 low-power fields will reveal if abnormal ciliary orientation appears to be primary or secondary in nature.

In all cases, parallel evaluation of control samples from patients with upper respiratory disease but normal ciliary motility as assessed by light microscopy should be included, because chronic inflammatory conditions can lead to subtle abnormalities in ciliogenesis manifested as focal abnormalities in ciliary morphology or orientation. A patient with a true genetic defect in one of the parts of the cilium or flagellum should show a complete absence of that part, not merely a deficiency. To make a diagnosis of a genetic disorder, the defect should be shown in different anatomical locations and at several different points in time.[68]

SURVEY TECHNIQUES IN ULTRASTRUCTURAL PATHOLOGY

Conventional EM preparative techniques impose severe limitations on specimen size. As a result, ultrastructural studies generally provide high resolution at the expense of context. Accordingly, our laboratories have always emphasized the importance of correlative microscopy. In the case of electron microprobe analysis, we have published different regimens that allow the precise correlation of the chemical data obtained with an x-ray spectrometer with structural data seen by transmitted light microscopy.[2,71]

A special problem is posed by focal pathological processes, for which conventional (random) EM sampling methods may miss the areas of interest completely. Several survey methods for selecting areas of focal pathology for subsequent ultrastructural analysis have been devised.[72,73] We have recently described a technique in which confocal laser scanning microscopy is used to survey large slices of tissue produced with a vibrating microtome before embedment.[73,74] Focal areas of interest are excised, embedded, and examined by EM (Fig 5). Our initial application was in the area of viral diagnosis, where features such as tissue necrosis, multinucleate cells, and nuclear inclusion bodies can be

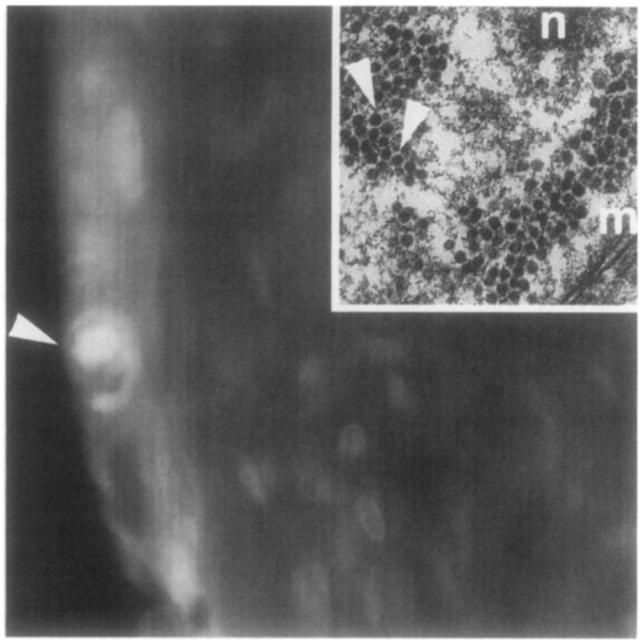

FIGURE 5. Confocal laser scanning micrograph of unembedded renal pelvic tissue from an immunocompromised child. The tissue slice was produced with a vibrating microtome and stained with propidium iodide. A urothelial cell containing a large intranuclear inclusion is present (arrowhead) (magnification ×550). The portion of the tissue containing the affected cell was excised, embedded, and examined by transmission EM, revealing clusters of 45-nm polyomavirus particles (arrowheads) within the nucleus (n); the nuclear membrane (m) is visible at one corner of the micrograph (inset, magnification ×45,400).

used to select areas of putative infection.[73] The method can also be applied to tumor diagnosis; we have recently used it to select viable areas of a largely necrotic pulmonary adenocarcinoma for EM, and to study focal areas of rosette-like differentiation in a malignant glial neoplasm (DN Howell, SE Miller, and JD Shelburne, unpublished observations).

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